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Perinatal Stem Cells

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This book is dedicated to my family—Katherine, Christopher and Zachary

A.A.

To my wife, Jess

S.V.M.

Preface

There has been increasing awareness and interest in the field of stem cell research from researchers, industry, and the general public. This is because of the remarkable potential of stem cells to develop into the many different cell types that are present in the body, providing a cell source for replacement functional tissues. Stem cells have become an essential tool for many other fields, including for developmental biology, development of cell therapies, drug discovery, disease models, and tissue engineering.

Some types of stem cells are known to reside in organ-specific niches and can become activated, proliferating and differentiating to maintain tissue homeostasis, or following injury to replace damaged cell types. These so-called adult or endogenous stem cells are capable of multipotent differentiation but are generally limited to cell types within their organ of origin. Endogenous stem cells have been widely studied to achieve a greater understanding of tissue turnover and responses to injury. Much research is focusing on how we can harness the power of endogenous stem cells as a source for regenerative medicine. While successful clinical application has been achieved for some organs, such as the hematopoietic system, difficulties in isolating and expanding many of these cell types ex vivo have limited their widespread application.

Embryonic stem cells have been an essential cell source for our current understanding of cellular developmental biology. These cells are derived from the inner cell mass of the blastocyst of an embryo. Embryonic stem cells have two important properties that make them attractive to researchers. The first is that they are pluripotent, capable of differentiating into all of the cell types of the three primary germ layers. Second, they are capable of expanding indefinitely in culture without losing their pluripotent differentiation capacity. The potential to generate large numbers of differentiated cell types in vitro has driven many groups to investigate the application of these cells to treat disease. Although many challenges remain, the potential of these cells to form tumors in vivo is a major concern, and the allogeneic nature of the cell source means that immune rejection of the cells is likely.

One of the major contributions of embryonic stem cell research to the field was the identification of "pluripotency factors," which are factors that promote properties and behavior common to pluripotent stem cells. This knowledge has been applied to generate what are known as "induced pluripotent stem cells," which are pluripotent embryonic stem cell-like cells generated by inducing the expression of pluripotency factors in mature somatic cells using virus, protein, or small molecule inducers. This technique has facilitated the generation of pluripotent stem cell lines without destroying human embryos and potentially allowing autologous applications. However, these cells still have a high risk of tumorigenicity, which has limited their current applications to in vitro studies such as disease modeling and drug discovery.

Perinatal stem cells are a group of cell types that can be derived from postembryonic, perinatal tissues, which includes tissues sourced at the time of birth, but also encompasses the time period from the 20th week of gestation through the neonatal period. These tissues are usually discarded at the time of birth and include the amniotic fluid, the placenta, placental membranes, umbilical cord, and blood. As a discarded tissue source, harvesting of stem cells from these sources represents a simple, noninvasive, and safe means for attaining therapeutic cell types. In addition to being easily accessible, perinatal stem cells can be isolated and expanded

in vitro, with some cell types capable of over 250 population doublings. Perinatal stem cells appear to have properties of both embryonic and adult stem cell types. Some have a highly multipotent differentiation potential, capable of forming functional cell phenotypes from all three lineages of the primary germ layers, but without the detrimental property of tumorigenicity associated with embryonic and induced pluripotent stem cells. The ability to transplant these cells safety, without any reported in vivo tumorigenicity, is a major advantage of these cell types.

During fetal development, perinatal tissues form a protective barrier between two immunologically distinct individuals. This function may confer perinatal stem cells with unique properties of immune privilege and immune suppression. Many studies have observed that perinatal stem cells can be delivered in allogeneic or xenogeneic setting without resulting in an immune response commonly seen with other cell types. Additionally, researchers have characterized the potent immunosuppressive properties of these cells, which are capable of influencing innate and adaptive immune responses in vitro and in vivo.

As described in this book, perinatal stem cells have found widespread application for the treatment of many diseases, injuries, and disorders. These cells have shown the capability to differentiate into functional organ-specific cell types and engraft in injured tissues to restore function following disease or injury. These cells have also found application in preventing or treating disease through modulation of the immune response. With inflammation playing an important role in disease and injury, regulating this response with cellular therapies could have a major impact on healing and tissue regeneration.

This book has been divided into four major sections, each dealing with commonly applied perinatal stem cell types as well as a final section on efforts supporting the clinical translation of these cells. Written by international experts in the field, the contributed chapters cover a wide range of topics, including efficacy, mechanisms of action, the application of perinatal stem cells for the treatment of disease or injury, and clinical translation. These parts are titled: Part I: Amniotic Fluid Stem Cells, Part II: Placental and Placental Membrane Stem Cells, Part III: Umbilical Cord Cells, and Part IV: Clinical Translation.

Part I: Amniotic Fluid Stem Cells

Part I focuses on applications of stem cells derived from the amniotic fluid, beginning with Sveva Bollini and coworkers describing the potential application of amniotic fluid stem cells for cardiac regeneration and discussing three different approaches, namely stem cell-based therapy, paracrine therapy, and cardiac tissue engineering.

Aleksander Skardal presents research demonstrating that delivery of amniotic fluid stem cells has the potential to be an effective cell therapy for facilitating wound healing. This chapter highlights the portfolio of potent growth factors secreted by amniotic fluid stem cells that are integral to skin regeneration and induction of angiogenesis in healing wounds.

Augusto Zani from Paolo DeCoppi's group has provided an excellent chapter describing the treatment of necrotizing enterocolitis with amniotic fluid stem cells. The chapter highlights the application of amniotic fluid stem cells in animal models of necrotizing enterocolitis, where this therapy significantly reduced gut damage and increased the survival of these animals.

Emily Moorefield provides an in-depth overview of the immunomodulatory properties of amniotic fluid cells and discusses the potential application of amniotic fluid stem cell therapy to selectively inhibit the immune response in graft versus host disease.

Margit Rosner from Markus Hengstschläger's group discusses the theory that amniotic fluid stem cells might be involved in fetal cell microchimerism during pregnancy. The authors discuss properties of amniotic fluid stem cells that support a role in fetal cell microchimerism as well as identify features that need to be tested to further support this theory.

Orquidea Garcia from the Children's Hospital Los Angeles has contributed an interesting chapter investigating the potential of amniotic fluid stem cell therapy for lung disease. In this chapter, the authors examine some of the challenges faced in treating respiratory disease, and how amniotic fluid stem cells have demonstrated the potential to address these challenges.

Koji Shido describes studies that aim to reprogram amniotic fluid stem cells into an endothelial cell phenotype and the application of these cells for injury repair and organ regeneration. This comprehensive review highlights recent advances in reprogramming of amniotic fluid stem cells, endothelial induction, and production of paracrine mediators to directly induce organ regeneration.

Andrea Preitschopf and Mario Mikula summarize developmental stages and factors involved in articular cartilage formation and degeneration. The chapter highlights recent advances in the application of amniotic fluid stem cells for the generation of cartilage tissue and how the endogenous cartilage formation process could be recapitulated during tissue engineering.

The chapter written by Simon Hoerstup and coworkers describes the potential application of amniotic fluid stem cells for cardiovascular tissue engineering. The authors comment on studies demonstrating that amniotic fluid-derived stem cells generate living autologous heart valve leaflets in vitro and the successful in vivo translation of amniotic fluid cell-based engineered heart valves into the ovine fetal model.

Jaehyun Kim from Wake Forest Institute for Regenerative Medicine discusses characteristics of amniotic fluid stem cells that make these cells appealing for osteogenic applications and reviews tissue-engineering approaches utilizing these cells for treating bone defects.

In their chapter, Weerapong Prasongchean and Patrizia Ferretti discuss therapeutic approaches for the treatment of birth defects in utero and perinatally. This review highlights the current experimental and clinical evidence of the potential of amniotic fluid stem cells for the treatment of birth defects either in utero of early postnatally.

Teodelinda Mirabella has provided an excellent chapter describing strategies to stimulate therapeutic angiogenesis using amniotic fluid stem cells. This chapter reviews studies that demonstrate the in vitro manipulation to direct amniotic fluid stem cells toward a vascular phenotype, stimulation of endogenous repair through recruitment of host progenitors, and the potential to use their pro-angiogenic secreted factors as a secretome-based therapy.

Part II: Placental and Placental Membrane Stem Cells

Part II is dedicated to chapters describing applications of stem cells derived from the placenta and placental membranes. These cell types include mesenchymal stromal cells from the placenta, amnion membrane, and chorion membrane as well as amnion epithelial cells and chorionic trophoblastic cells.

Ornella Parolini starts the section with a chapter with a brief description of the structure of the placenta and an in-depth description of the various types of placenta and placental membrane-derived cell types. This overview details the phenotypic and functional and immunological characterization that has been performed on many of these cell types.

The second chapter of this section, authored by Gi Jin Kim, describes the characterization of several kinds of placenta-derived stem cells and discusses recent investigations into the therapeutic potential of these cells for repair of liver injury and disease.

Alicia Bárcena from Susan Fisher's lab in the University of California San Francisco has provided an excellent review of the hematopoietic potential of the human placenta throughout gestation and speculates about the possible use of this tissue at birth for the harvest of hematopoietic stem cells and progenitors.

Shan-hui Hsu and coworkers discuss the potential of human placenta-derived mesenchymal stem cells as a candidate cell source for cartilage tissue engineering, describing studies highlighting the essential role of 3D scaffolds for induction of chondrogenic differentiation of these cells in vitro.

Clara Sanjurjo-Rodriguez described various cell types that can be derived from the amnion membrane, specifically human amniotic mesenchymal stem cells and human amniotic epithelial cells. The isolation and comparative characterization of these cell types is discussed.

In their chapter, Tomonori Minagawa and coworkers describe strategies for the use of the human amnion membrane for the reconstruction of functional bladder tissue. They indicate that biomaterials and cells derived from the human amnion membrane have a potential for the reconstruction of functional urinary bladders.

Euan Wallace from Monash Institute of Medical Research provides a comprehensive overview of the application of human amnion epithelial cells for the treatment of chronic and acute lung disease in both the adult and neonate. This chapter reviews the extensive preclinical and clinical studies that have been performed using these cells to treat lung disease and addresses likely mechanisms of action.

In the chapter titled "Potential Efficacy of Amnion Epithelial Cells to Treat Post-Stroke Inflammation," Christopher Sobey and coworkers review the current treatments and their limitations for treating ischemic stroke. This chapter describes the potential for amnion epithelial cells to improve stroke outcome given their unique properties, which include modulation of the immune response, differentiation into neural tissue, re-innervation of lost connections, and secretion of important factors to restore cellular function.

Courtney McDonald has written an interesting chapter investigating current evidence that human amnion epithelial cells are attractive candidates for the treatment of multiple sclerosis and other neurodegenerative disorders. Reviewed studies demonstrate that amnion epithelial cells suppress inflammation, migrate to inflamed sites within the central nervous system, engraft and differentiate toward neural lineages.

Sankar Venkatachalam expands on the previous chapter with a review of the current evidence of the beneficial aspects of amniotic epithelial cell transplantation for neurological conditions. Included in this review is the investigation of the application of amniotic epithelial cells for the treatment of contusive spinal cord injury.

The final chapter of this section is a review of the therapeutic potential of amnion epithelial cells for diabetes. This chapter, written by Chika Koike and coworkers, highlights the potential of amnion-derived cells to differentiate into insulin-producing cells in vitro and the transplantation of amnion-derived cells to normalize the blood glucose level in animal models of diabetes.

Part III: Umbilical Cord Cells

Part III focuses on cells derived from the umbilical cord blood and tissue and includes chapters describing applications of hematopoietic and mesenchymal-like stem cells. The first chapter, written by David Harris from the University of Arizona, focuses on the collection, processing, and banking of umbilical cord blood. The chapter provides an overview of umbilical cord blood collection, processing, and banking as well as providing an overview of clinical trials using cord blood.

Samberg, Eve, and Borlongan discuss the translational potential of cord blood-derived cells for treatment of a multitude of CNS disorders including Alzheimer's disease, amyotrophic lateral sclerosis, cerebral palsy, spinal cord injury, and stroke.

The third chapter of this section focuses on the application of umbilical cord blood for the treatment of cardiovascular disease. In this chapter, Santiago Roura Ferrer and coworkers describe umbilical cord blood as a rich reservoir of both hematopoietic and non-hematopoietic cells with great potential as a source for regenerative cell therapy for cardiovascular disease.

Kyoko Baba discusses the use of umbilical cord blood and Wharton's jelly mesenchymal stem cells, and provides an overview of the osteogenic potential of Wharton's jelly-derived cells for application in bone tissue regeneration.

Rita Anzalone from Giampiero La Rocca's group has presented an excellent overview on the application of umbilical cord blood and Wharton's jelly mesenchymal stem cells for the

treatment of Type I Diabetes. The authors analyze current literature regarding the features and potential of Wharton's jelly mesenchymal stem cells and propose that transplantation of these cells may be useful both to regenerate β-cells and also prevent the autoimmune destruction of remnant and neogenetic β-cells in patients.

The final chapter of this section, written by Benedikt Weber and coworkers, begins by describing the endothelial progenitor cells isolated from term human umbilical cord blood. The chapter details standardized chemically defined cell culture protocols for these cells and their applications in cardiovascular tissue-engineering purposes.

Part IV: Clinical Translation

The fourth and final section of this book deals with the important aspect of clinical manufacturing, commercialization, and patents for perinatal stem cells. This topic is of increasing importance as perinatal stem cell therapies are translated from the lab bench and animal studies into clinical trials, banking, and commercialization.

The first chapter in the section is written by Celena Heazlewood, Nina Iliac, and Kerry Atkinson and provides an in-depth description of the manufacturing of perinatal stem cells for clinical trials. This chapter covers the basic biology of placental-derived mesenchymal stem cells, the regulation and documentation involved in clinical manufacturing, and the personnel, infrastructure, and monitoring requirements for the manufacture of clinical grade MSCs using current Good Manufacturing Principles (cGMP).

Rouzbeh Taghizadeh and coworkers have contributed an excellent chapter exploring in-depth the potential clinical use and benefit of perinatal stem cell and analogous regenerative medicine therapies sourced from the umbilical cord. This chapter details the development of methods of umbilical cord tissue cell banking that maintain the full therapeutic benefit of each respective stem cell population, and goes on to highlight the clinical potential of these cells for the treatment of hematopoietic diseases and cancers, immune-related diseases, as well as autoimmune-related disorder, musculoskeletal injuries, neurodegenerative disorders, cardiovascular-related injuries, and wound repair.

The final chapter of this section is contributed by Tamara Yawno, Euan Wallace, and Rebecca Lim from Monash University. This chapter is dedicated to patents and commercializing of perinatal stem cells. The authors discuss the evolution of patent development for perinatal stem cells as well as highlighting recent patents on the collection, isolation, characterization, and application of stem cells derived from the placenta, placental membranes (amnion/chorion), amniotic fluid, umbilical cord tissue, and cord blood.

Perinatal stem cell research has been ongoing for decades, and the field has now matured to the stage where many groups are progressing through preclinical studies toward clinical application of these cells. Together with this work, many groups are supporting this endeavor by establishing clinical manufacturing techniques, banking facilities, and developing intellectual property for these cells and techniques. Previous books and journals have discussed the specific origins, phenotypes, and properties of various perinatal stem cell populations. This is the first book of its kind to discuss in-depth the current preclinical and clinical applications of these cells, as well as efforts to support the transition of perinatal stem cell therapies from the laboratory to the clinic.

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About the Editors

Dr. Anthony Atala is widely recognized as a true groundbreaker in stem cell research and regenerative medicine. He led the team that grew the first lab-grown organ to be implanted into a human. Aside from being involved in many of the field's top journals, he has devoted the last several decades of his career to the development of sustainable organs grown from a patient's own stem cells—technology that has the potential to solve the problem of patients dying while waiting for organs as well as the common organ transplant complication of rejection. Dr. Atala received a bachelor's degree in psychology from the University of Miami and his medical degree from the University of Louisville, where he completed his residency in urology. While a Fellow at Harvard Medical School, he trained with renowned pediatric urologic surgeons and eventually became Director of the Laboratory for Tissue Engineering and Cellular Therapeutics at Children's Hospital Boston, where his work involved the growing of human tissue and organs to replace those damaged through disease or defect. After that, Dr. Atala moved to Wake Forest, and it was there that he and his team developed the lab-grown bladder that was implanted in a human. A prolific author and editor of journal articles and books, Dr. Atala is the recipient of multiple awards and patents, presented an ovation-receiving TED lecture, and has been featured on *60 Minutes*. His accomplishments also include being ranked as the 56th most influential person of the year in a *Time Magazine* poll and being ranked by *Esquire Magazine* as one of the 75 most influential persons of the twenty-first century.

Dr. Sean V. Murphy received his Ph.D. from Monash University, Melbourne, Australia and is currently a Research Fellow at the Wake Forest Institute for Regenerative Medicine in North Carolina, USA. His research focuses on the clinical application of perinatal cells and tissues for the treatment of injury and disease. Dr. Murphy has received numerous awards and fellowships, most notably an American Lung Association Senior Research Training Fellowship, and an American Australian Association Sir Keith Murdoch Fellowship. Dr. Murphy is Director/Secretary and Founder of the International Perinatal Stem Cell Society, Chair of the Scientific & Professional Development Committee for the Tissue Engineering and Regenerative Medicine International Society (TERMIS) and serves on the editorial board of multiple international journals.

Part I

Amniotic Fluid Stem Cells

Amniotic Fluid Stem Cells for Cardiac Regeneration

1

Sveva Bollini, Michela Pozzobon, Nicola Smart, and Paolo De Coppi

Abstract

In recent years cardiac regenerative medicine has emerged as a fast-developing exploratory field with tremendous potential to treat end-stage heart disease. Different approaches have been investigated for the repair of cardiovascular ischemic injuries such as myocardial infarction, in order to improve heart performance in the long term. In this scenario, stem cell-based medicine has received a lot of attention, and several stem sources have been evaluated to identify the most suitable therapeutic approach. Cardiac regeneration has become a multi-disciplinary research area based primarily on different stem cell- and tissue engineering-based strategies, with the ultimate goal of preventing or reversing heart failure.

Amniotic fluid stem (AFS) cells are broadly multipotent and clonogenic cells which have emerged as a potent therapeutic agent in regenerative medicine and which can be easily obtained throughout pregnancy from surplus samples taken for prenatal diagnostic procedures. In this chapter we will discuss the most significant findings in the field of stem cell therapy for cardiac regeneration, focusing on the recent results using AFS cells.

Abbreviations

3D	Three dimensional
AF-MSC	Amniotic fluid-derived mesenchymal stem cells
AFS	Amniotic fluid stem
bFGF	Basic fibroblast growth factor
BM-MSC	Bone marrow-derived mesenchymal stem cells
CMTMR	(5-(and-6)-(((4-Chloromethyl)benzoyl) amino)tetramethylrhodamine)
CPC	Cardiac progenitor cells
cTnT	Cardiac troponin T
EC	Endothelial cell
EGM-2	Endothelial growth medium 2
ES	Embryonic stem
GFP	Green fluorescent protein
hAFS	Human amniotic fluid stem cells
hFGF	Human fibroblast growth factor
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor 1-alpha
HLA-DR	Human leucocyte antigen-DR
hptMyosin	Human-specific anti-platelet nonmuscle myosin
IGF-1	Insulin growth factor-1
IL-8	Interleukin 8

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iPS	Induced pluripotent stem cells
MCP-1	Monocyte chemoattractant protein-1
MI	Myocardial infarction
MMP9	Matrix metallopeptidase 9
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cells
NOD-SCID	Non-obese diabetic-severe combined immunodeficiency
PCR	Polymerase chain reaction
PDGF-AA/BB	Platelet-derived growth factor-AA/BB
PLGA	[Poly(D,L-lactic-co-glycolic acid)]
rAFS	Rat amniotic fluid stem cells
SDF-1	Stromal growth factor-1
SM	Smooth muscle
SMA	Alpha smooth muscle actin
SSEA4	Stage-specific embryonic antigen 4
TGFβ	Transforming growth factor beta
Tβ4	Thymosin beta 4
VEGF	Vascular endothelial growth factor
vWf	von Willebrand factor

1 Introduction: Regenerative Medicine for Cardiac Repair

Currently fewer people die from myocardial infarction compared to previous decades, but more of them survive with damaged hearts, following the pathological remodeling process which leads into heart failure. While pharmacological therapies and surgical interventions have reduced the mortality of patients experiencing myocardial ischemia, therapeutic strategies to improve the long-term conditions through the restoration of cardiac function are yet a major challenge; as such heart transplantation still represents the ultimate cure. Nevertheless, the recent advance of regenerative medicine has provided huge steps forwards, suggesting alternative therapeutic strategies to address the key aspects of cardiac repair, which involve modulating the inflammatory response, supporting neovascularization and implementing myocardial regeneration. The experimental strategies mostly described so far can be classified into three different approaches, namely stem cell-based therapy, paracrine therapy and cardiac tissue engineering, as represented in Fig. 1.1.

Until recently the stem cell regenerative paradigm for cardiac repair was based on the assumption that progenitor cells play a critical role in tissue repair mainly by means of their plasticity and transdifferentiation potential. In this scenario, various stem cells and progenitors have been tested and extensively analysed to assess their potential to generate cardiac lineages in vitro and in vivo. However, recent studies suggest that the mechanism underlying the benefits of stem cell transplantation might be due to paracrine modulatory effects, rather than replacement of affected cells at the site of injury. In the cardiovascular field several studies support this hypothesis, showing successful reduction of infarct size and improvement

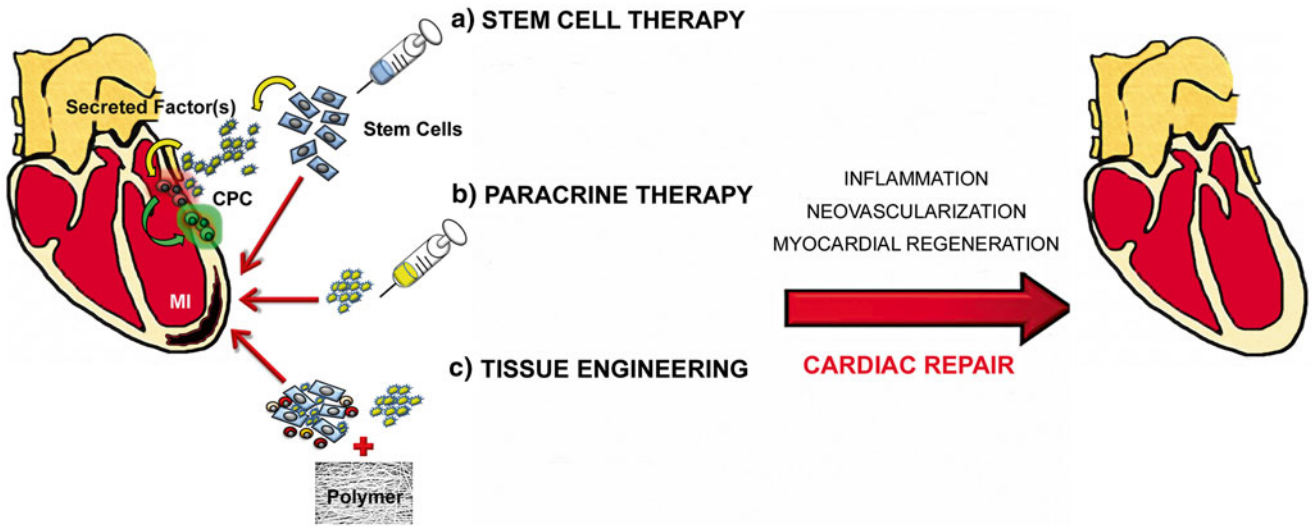


Fig. 1.1 Regenerative strategies for cardiac repair. To provide cardiac repair following ischemic injury such as myocardial infarct (MI), three different aspects should be addressed, that is (1) the modulation of the inflammatory response, (2) supporting neoangiogenesis and (3) providing new functional tissue through myocardial regeneration. Stem cell therapy (**a**) has been extensively investigated, suggesting a regenerative role for stem cells acting either via direct transdifferentiation into cardiovascular lineages or/and through the secretion of cardioactive soluble factors (cytokines, chemokines, etc. represented in the picture in *yellow*), which can mediate cardiac function improvement, decrease of the infarct size and local activation of the resident endogenous cardiac progenitor cells (CPC, represented here in *red*, before activation and in *green*, after stimulation). The beneficial effects achieved with the use of stem cell-conditioned medium and stem cell-derived soluble factors have suggested a new regenerative approach in paracrine therapy (**b**) based on the direct administration of these secreted mediators. Cardiac tissue engineering (**c**) has also been proposed as a therapeutic method to provide tissue grafts (valve replacements, blood vessels constructs and myocardial patches) for chronic stages of cardiovascular disease and for cardiac congenital defects by combining multidimensional biomaterials with stem cell culture

of cardiac output most likely attributable to the release of soluble pro-survival factors, rather than de novo cardiomyogenesis of the engrafted stem cells [1]. Significant discrepancy on the in vivo cardiac plasticity of transplanted stem cells has also been reported, with their efficacy in terms of survival and engraftment debatable [2]. Recent studies from the extensively investigated mesenchymal stem cells (MSC) to the more recently discovered induced pluripotent stem cells (iPS) have confirmed that the stem cell regenerative potential might be achieved either by generation of new tissue via transdifferentiation or by local release of paracrine factors/chemokines that act on the endogenous cells to improve the general cardiac outcome by mediating neoangiogenesis and attenuating fibrosis and scarring [3–8]. Hence, the quest for the ultimate treatment for myocardial repair via a stem/progenitor cell-based strategy seems to be based not merely on the identification/isolation of the most suitable stem cell candidate, but also on the paracrine-mediated repair via a new approach for cardiac regeneration.

While stem cell and paracrine therapy are meant to provide tissue repair in the acute phase following myocardial ischemic injury, cardiac tissue engineering has been suggested as a therapeutic strategy for the chronic stages of cardiovascular disease and for repair in case of congenital heart defects. Cardiac tissue engineering is based on the combination of multidimensional biomaterials with cultured stem cells in order to create tissue grafts, such as valve replacements, blood vessels constructs and myocardial patches, and it has recently heralded a new promising and exciting horizon in cardiac regeneration. Significant advances have recently been achieved by creating biomaterials and biocompatible matrices/scaffolds which can provide 3D systems to culture and deliver progenitor cells, improving the intercellular crosstalk with the host tissue, thus sustaining their in situ potential for repair and/or regeneration [9–11].

In the broad scheme of stem cells, amniotic fluid is an appealing source with significant potential for cardiac therapy. Amniotic fluid contains multiple cell types derived from the developing foetus and may represent a therapeutic agent which can be easily collected during amniocentesis. In the last few years, several groups have reported the presence of different progenitors in the amniotic fluid, mainly with mesenchymal characteristics [12, 13]. Amniotic fluid stem (AFS) cells expressing stem cell-specific markers such as c-kit, SSEA4 and OCT4 were shown to possess an "immature" phenotype, intermediate in their properties between embryonic and adult stem cells, making them particularly attractive for cellular regeneration. These cells have been demonstrated to be clonogenic, to possess remarkable self-renewal potential while maintaining a stable karyotype and to be pluripotent, giving rise to derivatives of all the three germ layers [14]. Amniotic fluid stem cells can also be cryopreserved and banked for use in future cell-based therapy, due to their peculiar properties, such as survival at lower oxygen tension and their ability to withstand protracted cryopreservation with unaltered self-renewal potential. Their use may also be envisioned to be used for allogeneic therapies, as they have been shown to express similar immunomodulatory properties as adult mesenchymal stem cells [15]. In this chapter we will broadly discuss the most significant findings and the different methods that have been suggested for the use of amniotic fluid stem (AFS) cells for cardiac regeneration.

2 The Stem Cell-Based Therapy

2.1 Stem Cell Transplantation: Let the Right One In

Within cardiac regenerative medicine, stem cell transplantation has been the most widely investigated approach for the treatment of the ischemic myocardium and to prevent/cure heart failure. Consequently, attention has turned to the identification of the most suitable cell source. Many stem cell populations have been proposed and extensively analysed, from the pluripotent embryonic stem (ES) cells to the multipotent adult stem cells [16, 17]. Significant advances in this field have also arisen via the recent derivation of iPS cells by genetic reprogramming of somatic adult cells [18, 19] and with the direct reprogramming of adult cardiac fibroblast into cardiomyocytes [20, 21]. Furthermore, the possibility of deriving multipotent and pluripotent stem cells from foetal tissues, with properties intermediate between embryonic and adult stem cells, has also been described by many groups. Hence, the final aim is to identify the perfect cell candidate that fulfils numerous requirements: the ideal stem source should be safe, neither immunogenic nor tumorigenic; it should improve cardiac function and mediate tissue repair by modulating the inflammation, sustaining the neovascularization process and also generating new cardiomyocytes that functionally couple with host tissue; it should be easily delivered in vivo and be amenable to safe in vitro expansion while, at the same time, circumventing ethical objections. Numerous studies have highlighted the relative merits of specific stem cell types, according to these different criteria.

Foetal tissue represents a potentially viable source of stem progenitors for cell therapy because of its pluripotency, proliferative ability and lack of immunogenicity; such tissue can be obtained from a direct biopsy of the foetus during gestation or from cord blood, term placenta, villi or amniotic fluid [22–25]. Several foetal mesenchymal stem cells with a therapeutic potential for cardiovascular disease have indeed been identified [26–30]; in addition, trafficking of foetal cells to injured maternal myocardium has recently been reported, confirming their homing potential and plasticity once integrated into the infarcted heart [31]. Though these

cells have shown a remarkable cardiovascular potential in vitro and capacity to support cardiac repair and regeneration in vivo, their ability to transdifferentiate directly into mature cardiomyocytes has not been definitively demonstrated and it remains debatable [32, 33].

2.2 The Cardiovascular and Cardiomyogenic Potential of AFS Cells

2.2.1 In Vitro Cardiovascular Differentiation of AFS Cells

Amniotic fluid stem cells have been demonstrated to possess remarkable endothelial and smooth muscle plasticity in several in vitro studies. Endothelial differentiation of human c-kit+ AFS and amniotic fluid-derived mesenchymal stem cells with upregulation of CD31 was observed following treatment using EGM-2 differentiating medium, supplemented with recombinant human bFGF, whereas addition of VEGF and hFGF also resulted in the expression of von Willebrand factor (vWf) [13, 14]. More recently, mesenchymal cells isolated from amniotic fluid were demonstrated to acquire endothelial features when stimulated by shear force in addition to growth factors, producing angiogenic mediators such as VEGF and HGF in response to hypoxia [34]. The potential of AFS and AF-derived mesenchymal stem cells towards phenotypic conversion into the smooth muscle lineage has been supported by different studies, both through in vitro co-culture with smooth muscle cells and by applying specific media [35, 36].

Moreover, undifferentiated c-kit+ AFS cells from human and rat demonstrated expression of smooth muscle and endothelial markers, such as smoothelin, angiopoietin1, CD146 and of the "angioblast-endothelial" lineage, like Flk-1 and vWf, together with the antigens of smooth muscle commitment α smooth muscle actin (SMA), smooth muscle 22α and calponin, both at mRNA and protein levels. Their propensity to acquire endothelial and smooth muscle fate was then enhanced by the use of induction culture media [37]. Similarly, c-kit+ GFP+ rat AFS cells were shown to possess smooth muscle and endothelial features, such as in vitro expression of α smooth muscle actin and Flk-1 [38]. These data seem to suggest that AFS cells may contain a subpopulation of cardiovascular progenitors identified by the expression of endothelial and smooth muscle mRNA and proteins in their undifferentiated state; therefore they possess a cardiovascular plasticity *in fieri*, which needs to be triggered and enhanced by specific culture conditions.

While the acquisition of a vascular phenotype seems to readily occur in the c-kit+ AFS cells and in the AF-derived mesenchymal stem cells, their in vitro induction into the cardiomyocyte lineage has always been more difficult to prove. Although undifferentiated human AFS and AF-mesenchymal stem cells (AF-MSC) showed expression of cardiac transcription factors, such as mef2, GATA4 and Nkx2.5 in their transcriptome, the acquisition of a mature and functional myocyte phenotype seems to need a stimulation more effective than the use of growth factors or induction media [29, 37, 39]. Myocardial differentiation of human and rat AFS cells has been reported via co-culture with neonatal rat cardiomyocytes, with cardiomyocyte sarcomeric features of 3.5–5 % of treated AFS cells after a few days, increasing to almost 16 % after 9 days [37, 38]. Along with the expression of structural cardiac proteins, such as troponin I and T and sarcomeric α-actinin with myofibrillar organization, c-kit+ rat AFS cells also showed functional acquisition of a more mature phenotype, by expression of synchronous contractile activity and electrical excitability, with detection of action potentials and pace-making activity similar to the surrounding neonatal cardiomyocytes [38]; moreover, other independent studies using AFS cells (with or without c-kit sorting) supported the co-culture method as a tool to induce these stem cells to acquire a cardiogenic fate and develop functional gap junctions, with expression of connexin 43 and *N*-cadherin proteins between the two cell types [40]. Interestingly, these results were obtained only when AFS cells were in direct contact with the neonatal cardiomyocytes, with no evidence of transdifferentiation in co-culture experiments either using inserts to separate the two cell sources or when using conditioned media [38]. Although the contribution of a fusion process cannot be ruled out, these results seem to suggest that specific features of the neonatal cardiomyocytes, such as direct physical contact or short-range chemical signals, may induce the AFS cells to acquire a cardiogenic phenotype while in co-cultures.

Cardiac differentiation of AFS cells has also been reported by treatment with the demethylating agent 5-aza-2´-deoxycytidine, though despite an initial upregulation of cardiac structural markers in the treated cells, no mature structural and functional phenotype was observed in the long term [39].

A comparison of the cardiomyogenic properties of c-kit+ AFS cells versus the c-kit– counterpart has also been reported, showing that the myocardial differentiation capacity was enhanced in the c-kit+ population, with detection of GATA-4, cTnT, α-actin, connexin 43 mRNA and proteins after myocardial induction, compared to the c-kit– counterpart in which only GATA-4 mRNA and protein were detected [41]. More recently, first trimester human AFS cells were demonstrated to be reprogrammed to complete pluripotency without use of ectopic factors or viral vectors, but only via specific culture conditions, such as medium supplemented with valproic acid, a histone deacetylase inhibitor. This treatment resulted in the formation of embryoid bodies in vitro with rhythmically contracting activity, suggesting

transition to a primitive cardiogenic phenotype [42]. Despite all the encouraging results achieved with the techniques mentioned here, some important considerations remain to be addressed, such as the direct transdifferentiation potential of the AFS cells versus cell fusion and the efficiency of the cardiovascular differentiation acquired in vitro, which needs to be tested for the long term, in order to assess the stability and the safety of the phenotypic conversion.

2.2.2 In Vivo Cardiogenic Potential of AFS Cells

In recent years different studies have been proposed to evaluate the therapeutic potential of AFS cells as an allograftable stem cell source for in vivo cellular cardiomyoplasty. Transplantation in preclinical animal models of myocardial infarction (MI) has been broadly analysed in order to validate the cardiomyogenic and cardiovascular potential of the AFS cells.

Rat mesenchymal stem cells isolated from amniotic fluid were compared to bone marrow-derived stem cells (BM-MSC) for cellular cardioplasty by intramyocardial injection in the acute necrotizing ischemic area of syngeneic and athymic rat hearts in a preclinical model of cardiac cryoinjury. AF-MSC showed lower engraftment than BM-MSC in the short term, but after 30 days both cell types were detected in similar numbers in the host tissue. AF-MSC were shown to possess a greater proliferative potential in the long term. About 34.6 % of the injected BM-MSC and 49.6 % of the transplanted AF-MSC acquired a cardiovascular phenotype in vivo with expression of cardiac troponin T, vWF or α smooth muscle actin. Notably, while BM-MSC possess a broad cardiovascular potential, giving rise to cells of myocardial, smooth muscle and endothelial lineages, forming both capillaries and small arterioles, whereas AF-MSC demonstrated a more restricted myocardial and endothelial fate in vivo [36].

Human c-kit+ AFS cells xenotransplantated into a rat model of myocardial infarction via intramyocardial injection (with or without cyclosporine treatment as well as in nude rats) were found in the host tissue as traces of the original cell inoculum after 15 and 30 days. Some AFS cells expressing both the human-specific anti-platelet nonmuscle myosin (hptMyosin) and cardiovascular antigens, such as cardiac troponin T, were found as tissue/cell fragments in the transplanted heart. These hptMyosin+ AFS cells still expressed the stem cell marker SSEA4 suggesting an incomplete acquisition of a cardiogenic phenotype, while easily forming smooth muscle and endothelial cells, as confirmed by expression of α smooth muscle actin or vWf [37]. Despite the negative expression of HLA-DR antigens in the human c-kit+ AFS cells, all the animal recipients in this study—immunocompetent, immunodeficient or immunosuppressed—showed a significant infiltration of inflammatory cells, indicating that these cells struggled to survive and engraft, due to the host xeno-immune response. In a more recent study, human AFS cells were analysed for their therapeutic potential for cardiac repair in a similar immune-suppressed rat model with MI, via intramyocardial injection into peri-infarct areas. After 4 weeks the treated animals showed attenuation of the left ventricle remodelling with higher vascular density and an overall improvement in cardiac function, when compared with the saline injected control group. Furthermore the engrafted human AFS cells showed expression of cardiac marker such as Nkx2.5, α-actinin, cardiac troponin T together with connexin 43, suggesting structural coupling with the host tissue and supporting their therapeutic potential for cellular cardiomyoplasty [40]. The in vivo potential of rat GFP+ ckit+ AFS cells for myocardial repair was further evaluated by transplantation in the heart of animals subjected to cardiac ischemia/reperfusion injury and monitored by magnetic resonance imaging (MRI), following the labelling of cells with a super paramagnetic iron oxide particles solution. Three weeks after transplantation a small proportion of rat AFS cells acquired a cardiovascular phenotype with expression of endothelial and smooth muscle antigens and, to a lesser extent, cardiomyocyte markers, including cardiac troponin I. Despite the low number of rat GFP+ ckit+ AFS cells engrafted in the heart, there was still an improvement of the ejection fraction as measured by MRI, with a trend towards physiological values [38]. In light of these results, AFS cells have been shown to possess a remarkable propensity to acquire a cardiomyogenic phenotype in vitro while preserving cardiac function and differentiating into cardiovascular lineages in vivo, although their potential seems to be limited by the poor survival and low engraftment in an allogeneic setting in the long term.

To overcome one of the major limitations in cardiac cell therapy, i.e. the significant loss of transplanted cells and their low long-term engraftment and survival, alternative approaches to the injection of a single cell suspension have also been tested. Injection of spheric cell bodies obtained with AFS cells and extracellular matrix structures has been shown to enhance cell retention and functional benefits, following myocardial infarction in a preclinical rat model; bioluminescence imaging, real-time polymerase chain reaction (PCR), echocardiography and MRI revealed that the human AFS cells "bodies" remarkably enhanced cell retention and engraftment, both in the short- and in the long-term, limiting the progression of heart failure and improving cardiac function. Furthermore, expression of HGF, bFGF and VEGF were upregulated in the ischemic area, along with direct differentiation of the transplanted human cellular bodies into angiogenic and cardiomyogenic lineages [43]. The aforementioned results demonstrate that enriching the human AFS cells injectable preparation with extracellular matrix components can support their in vivo retention, improving functional engraftment with host tissue.

Despite these encouraging preclinical results, further validation of the in vivo cardiogenic potential of the AFS cells for cellular cardiomyoplasty is still required. Indeed, animal models present several limitations and clinical trials in highly selected patients are required to fully test their therapeutic applicability. Furthermore, a better understanding of the immunomodulatory and immunogenic properties of AFS cells, along with detailed mechanisms of their cardiovascular regenerative potential, is required to translate these preliminary results into the clinical arena.

3 Moving Forward from Stem Cell-Based Therapy into Stem Cell Factor-Based Therapy: The Paracrine Hypothesis

Initially the regenerative paradigm in stem cell therapy was based on the assumption that progenitors could play a critical role in cardiac repair by means of their plasticity in giving rise to new cardiovascular cells. The results of a large number of studies have revealed that the mechanism underlying the benefit achieved by transplanting stem/progenitor cells in the heart appears to relate primarily to their paracrine modulatory effect, rather than direct replacement of lost cells through direct transdifferentiation. Indeed considering the limited long-term engraftment of the transplanted cells, together with their general low transdifferentiation propensity, while significantly improving the cardiac function, it is indeed more plausible that stem cell-derived regenerative effects may act through local release of soluble factors and their effect on host cells, rather than from differentiation of exogenous transplanted cells. Considering the stem cell populations mostly investigated thus far, such as bone marrow-derived stem cells, several studies report a temporal improvement in cardiac function and neovascularization with a decrease in infarct size, following transplantation. Since permanent engraftment and direct cardiac transdifferentiation of the injected stem cells have been refuted, current hypotheses to explain their beneficial effect include their ability to alter the local microenvironment by secreting bioactive trophic factors, acting to reduce cardiomyocyte apoptosis and ventricular remodelling and support neovascularization, thereby stimulating cardiac regeneration [5]. Thus, mounting evidence that stem cell-mediated repair relies more on the paracrine stimulation, rather than their plasticity, has led to a paradigm change in regenerative medicine, shifting the attention from the stem cell genome to the stem cell "secretome", represented by the modulatory cytokines, chemokines and growth factors that they can release to influence the surrounding tissue. MSC have been demonstrated to secrete a wide array of cytokines and soluble factors involved in cardioprotection, neoangiogenesis and cardiac regeneration, such as FGF, bFGF, VEGF, TGFβ, IGF-1 and SDF-1 [1]. Moreover, the administration of stem cell-conditioned medium was shown to reduce apoptosis and improve cardioprotection in preclinical experimental models of myocardial infarction, reproducing beneficial effects of cell transplantation [44]. In light of these results, there has been a shift in emphasis from cell-based therapy to protein-based therapy, establishing a new therapeutic approach based on modifying stem cells, inducing them to express higher levels of specific paracrine factors. Impressive results have been reported with stem cells over-expressing pro-survival agents, such as bone marrow-derived MSC over-expressing Akt or HIF-1α, which led to reduced apoptosis and ventricular remodelling and restoration of cardiac function in vivo [1, 45].

Another mechanism accounting for the improved outcome of the injured myocardium, following stem cell transplantation, has been suggested in the synergistic crosstalk between the injected exogenous stem cells and the endogenous resident cardiac progenitor cells (CPC). Indeed, the exogenous transplanted cells may provide crucial factors via paracrine signalling that can boost the in situ activation, proliferation and mobilization of the endogenous CPC, as represented in Fig. 1.1 [46]. This "teamwork" hypothesis seems to be confirmed now by several studies. Transplantation of MSC into infarcted hearts led to the formation of cardiomyocytes co-expressing the CPC marker c-kit in a pig model, whereas endothelial progenitor cells triggered proliferation of c-kit+ CPC via secretion of VEGF, IGF-1 and SDF-1 [47, 48]. More recently, an elegant study analysed the effect of bone marrow-derived stem cell therapy on the cardiac endogenous regenerative response after myocardial infarct, confirming that c-kit+ bone marrow-derived stem cells activated endogenous cardiac progenitors, resulting in their differentiation into cardiomyocytes and a general improvement of ventricular function. This effect was suggested to be dependent on paracrine communication between the exogenous transplanted cells and the host cells [49]. Hence identifying the detailed mechanism and the key soluble factor(s) through which the transplanted stem cells "communicate" and support/recruit resident CPC for cardiac repair may facilitate progress in regenerative medicine, establishing the basis for the development of protein-based therapies and refining the current state of stem cell-based strategies.

3.1 A Role for the AFS Cell Secretome

Growing interest has been recently reported towards the analysis of the AFS cell secretome, as a source of therapeutic paracrine factors responsible for the strong angiogenic effect and regenerative properties that these cells exert in vivo. The in vitro analysis of human c-kit+ AFS cell-conditioned medium revealed the presence of cytoprotective, pro-differentiative and

chemoattractive soluble mediators, such as MCP-1, IL-8, SDF-1 and VEGF, which can provide in vivo tissue regeneration in a mouse preclinical model of hind-limb ischemic injury, both by recruiting the host endothelial cells and supporting neo-arteriogenesis and also via the remodelling of pre-existing collateral arteries [50]. Similarly, the regenerative potential of the AFS cells secretome was tested using a rat model of ischemic full-thickness skin flap in the epigastric region with topical delivery of the c-kit+ AFS cell-conditioned medium, embedded in a gelatin membrane. After 7 days of treatment, the ischemic area showed a better histological profile, together with improved reperfusion and repopulation of the area by host-derived endothelial precursors, confirming the beneficial therapeutic effect of the AFS cell-derived chemokines and growth factors on the ischemic lesion [51]. Furthermore, spindle-shaped amniotic fluid-derived mesenchymal stem cells were also shown to enhance new vessel formation both in vitro and in vivo, via the modulation of migration, proliferation and vascular tubule formation of human umbilical cord blood endothelial colony forming cell, due to their paracrine potential in secreting soluble factors such as IL-8, PDGF-AB/BB and MMP9. Notably these cells also supported increased angiogenesis/vasculogenesis in an NOD-SCID murine model more effectively than bone marrow-derived stromal cells [52].

The in vivo regenerative effect of human c-kit+ AFS cells for cardiac repair has been recently evaluated in a rat model of acute myocardial infarction by ischemia/reperfusion injury, to assess whether their therapeutic potential may be mediated by secretion of critical paracrine factors. In this study, systemic administration of AFS cells during the acute phase of reperfusion provided a remarkable decrease of 14 % in the infarct size, compared to control animals, together with a reduced number of apoptotic cardiomyocytes. Thus, these data confirmed the hypothesis of the cardioprotective role of the ckit+ AFS cells in vivo [53]. Considering the reduced amount of human c-kit+ AFS cells engrafted in the cardiac tissue over the limited time of the study (2 h) and the rapidity of the therapeutic effect exerted on the host, the authors suggested that the results obtained were achieved via a local paracrine effect. The human c-kit+ AFS cell-conditioned medium was then analysed, revealing a significant enrichment for thymosin beta 4 (Tβ4), a paracrine key factor for coronary angiogenesis during development, as well as a remarkable stimulator of cardiomyocyte survival and epicardial progenitor cells reactivation and cardiac differentiation following injury, as recently demonstrated [54–58]. The cardioprotective paracrine potential of human AFS cells was subsequently confirmed by repeating the set of experiments using the cell-conditioned medium and showing similar results, in terms of reducing the necrotic infarcted area and supporting the cardiomyocyte survival within the reperfusion time. These results suggested that, in the setting of acute myocardial infarction c-kit+ AFS cells possess a significant cytoprotective regenerative potential by paracrine effect with a potential therapeutic candidate in the small peptide Tβ4, which can activate the resident endogenous CPC to provide cardiac repair.

In light of these results, AFS cells may represent an appealing source for cardiac repair with an indirect regenerative role through a paracrine mechanism of action, rather than by direct cardiomyogenic transdifferentiation. Hence, a new stem cell-based strategy for cardiac regeneration may be based on culturing stem cells, such as AFS cells, to isolate therapeutic bioactive molecules produced in their secretome. These regenerative factors could then be administrated to the injured myocardium, in order to exert a paracrine modulation of the inflammation process, activation of cardiomyocyte pro-survival pathways while supporting neo-angiogenesis and stimulating the activation of the endogenous CPC to influence cardiac repair and regeneration in the acute phase following an injury.

Despite the significant effort that has been invested in stem cell-based therapy for cardiovascular regeneration and repair, much scepticism still surrounds cell transplantation methods, due to limitations associated with the safety, immunogenicity and limited efficacy of the transplanted cells, therefore paracrine therapy is envisioned to be a more straightforward translational approach and the isolation and administration of specific stem cell-derived regenerative factors for protein therapy may represent an extremely promising strategy. This approach offers a more clinically feasible mechanism to obtain stem cell-mediated regenerative effects in vivo, without the inherent complications associated with stem cell delivery. If translated successfully, curing cardiovascular diseases and congenital defects by administrating a cocktail of proteins in the form of a pill or of an injection may soon become a clinical reality.

Furthermore, paracrine therapy offers specific advantages for the clinical read out. Cardiovascular disease patients need prompt therapeutic intervention. Therefore it would be ideal to have access to "off-the-shelf" products for simple administration. Stem cells can be utilized as "drug-stores" to produce specific soluble factors, which can ultimately be administered orally as a pill or systemically as a suspension. This strategy could replace the invasive or additional surgical procedures generally required by cell therapy or tissue engineering methods.

In this scenario the ideal stem cell source should be selected upon consideration of their paracrine potential and the feasibility of their isolation together with their in vitro self-renewal properties. AFS cells unambiguously fulfil both criteria, as previous results showed that they possess an appealing cardiomyogenic and cardiovascular potential, being at the same time an attractive reservoir of regenerative factors with angiogenic and cardioprotective effects. These features make them an attractive source of paracrine factors for protein therapy.

4 Mending a Broken Heart: The Tissue Engineering Approach

Along with stem cell-based therapy approaches, another promising therapeutic strategy that has rapidly evolved is represented by cardiac tissue engineering. Tissue engineering represents "the restoration of function through the delivery of living elements which becomes integrated into the patient", as first reported by Vacanti in 1999 [59] and it has developed with the aim of restore and restoring and improving the diseased or damaged heart, through the application of a living graft, cardiac patch, vascular and valve constructs. New cardiac tissue engineering approaches combine the application of nanotechnology and chemical engineering with medicine, using smart biocompatible polymeric biomaterials and a broad range of cell types, including stem or stem cell-derived cardiac cells [11, 60, 61]. The main function of the biomaterial is to mimic the biological function and mechanical support of the extracellular matrix providing mechanical stimuli and an instructive scaffold for both the in vitro and/or in vivo stem cell growth, differentiation and maturation and/or a way for their delivery into the host tissue improving retention, engraftment and function. Compared to the traditional approach based on stem cell therapy, cardiac tissue engineering offers some important advantages, overcoming particular problems related with cardiac cell-based therapy and stem cell transplantation. Combining the use of bioactive matrices with stem cell biology provides a better cardiac-instructive microenvironment, via inter-cell and cell–matrix interactions, to support the in vitro and in vivo stem cells maturation, therefore improving their differentiation potential and the secretion of paracrine and trophic factors involved in cardiac regeneration [9, 10, 62]. Different biomaterials have been studied and engineered in several diverse modalities, attempting to faithfully reproduce the morphological and physiological properties of the heart. Scaffolds may be natural biomaterials, derived from matrix proteins (e.g., collagen or alginate, or synthetic polymer substances such as poly-lactic acid or poly-lactic glycolic acid), that can be produced in form of meshes, fibres, porous, solids or hydrogels [63] or, alternatively, may be the extracellular matrix produced by the cells themselves, injected as "sheets" or "patches" (also known as "scaffold-free" approach [64]. Acellular matrices, obtained by removing cellular components from specific tissues have been also used to produce in vitro functional bioartificial cardiac tissue; for example, by reseeding an acellular, perfusable vascular architecture of the mouse heart with endothelial and cardiomyocyte cells [65] or as injectable scaffold with the capacity to form a nanofibrous structure in vivo and to increase arteriole formation post-injection [66]. A more novel method has recently been developed based on innovative formulations like hydrogels used to entrap cells to improve their delivery and engraftment in the host tissue. In addition to the necessary requirements (biocompatible, biodegradable and biomimetic), hydrogels have also been shown to offer an improved alternative, as they represent a versatile biomaterial that can be injected in vivo without distorting the heart geometry and acting as a cell-carrier, delivering them directly into the infarcted wall to increase their survival, engraftment and functional integration [67, 68]. Hydrogels also provide the encapsulated stem cells and the necessary extracellular matrix signals so as to enhance their survival following engraftment. Promising results have also been achieved by establishing a tissue engineering platform for the delivery of human mesenchymal progenitor cells based on a composite matrix, obtained by applying fibrin hydrogel onto decellularized sheets of human myocardium. Once implanted into infarcted nude rat hearts, the transplanted human cells migrated to the ischemic area and supported a paracrine-mediated neovascularization response, resulting in the recovery of baseline levels of ventricular dimensions and contractility [69].

4.1 Re-building the Heart by Engineering AFS Cells

One of the fundamental challenges in tissue engineering is ensuring proper vascularization of the transplantable graft. Substantial efforts have been made to integrate vascular networks within engineered constructs in order to ensure functional incorporation in the host. This is particularly relevant when considering tissue engineered grafts to be implanted and integrated in complex biological structures such as the heart. In this scenario, amniotic fluid stem cells offer several advantages over embryonic and adult stem cells, given their powerful angiogenic potential. The AFS cells potential to support the recruitment of endothelial cells in vivo has also been assessed by tissue engineering. Ectopic implants obtained with biomaterials, such as polyurethane and matrigel, seeded with human c-kit+ AFS cells were shown to elicit a strong angiogenic response in a mouse model after 20 days, with collateral vessel networks infiltrating the graft and significant blood perfusion [50].

Tissue engineering has also been combined with cellular cardiomyoplasty, in order to improve the graft retention and cardiomyogenic potential of the transplanted stem cells, following myocardial infarction. A methylcellulose hydrogel system has been recently tested to culture human amniotic-fluid stem cells in order to "engineer" cell sheet fragments for direct intramyocardial injection. The ability of human AFS cell sheet fragments to preserve both the endogenous extracellular matrix components and cell phenotype was shown; following xenogenic transplantation into the border area of an ischemic injury of an immune-suppressed rat

model, they resulted in significant improvement in heart function with an increase in neovascularization, in situ proliferation with expression of cardiovascular markers and improved engraftment, compared to animals treated with saline or dissociated human AFS cells [70]. Another method to ensure good retention of the engrafted AFS cells was achieved using spherical porous beads of PLGA [poly(D,L-lactic-co-glycolic acid)] as a platform for in vivo cell delivery. These porous beads were used to engineer human AFS cells on microscaffolds forming a composite cellularized 3D structure while preserving endogenously secreted extracellular matrix components such as collagen III and the adhesion molecules fibronectin and laminin. Following intramyocardial transplantation into a rat model of myocardial infarction, the cellularized beads showed engraftment at the site of injection with prevention of pathological ventricular remodelling and improvement of cardiac function. Histological analysis supported induction of the delivered human AFS cells into cardiovascular and cardiomyogenic lineages with expression of vWf, Nkx2.5, sarcomeric actin and cardiac troponin I [71].

In the context of pediatric cardiology congenital heart malformations often required surgical treatment shortly after birth and it would be advantageous to identify an autologous source of progenitors from the foetus to engineer in vitro living heart tissues in parallel to pregnancy, ready for implantation at birth. Again, in this scenario, amniotic fluid stem cells may represent an ideal source with suitable potential for cardiac tissue engineering applications, which can be easily isolated prenatally during gestation and engineered to obtain a graft to be implanted at birth. Several studies have been carried out using amniotic fluid-derived stem cells to obtained tissue engineered heart valve replacements for cardiac repair, as broadly discussed elsewhere in this book.

Despite the valuable contributions that cardiac tissue engineering has demonstrated in stem cell-based cardiomyoplasty, additional studies are still needed to examine whetter the AFS cell grafts can functionally integrate with host cardiac tissue and terminally differentiate into cardiovascular lineages in the long term.

5 The Cardiac Regenerative Potential of AFS Cells: New Perspectives for the Pediatric Patient

Another crucial clinical aspect in cardiac repair is represented by the therapeutic options for prenatal and perinatal congenital heart defects. Prenatal diagnosis has improved dramatically leading to the early detection and management of a variety of foetal congenital diseases. The concept of foetal therapy has lately emerged as an innovative strategy for many disorders diagnosed before birth but several practical obstacles for its complete translation into clinical practices have yet to be overcome.

Foetal cardiac surgery (i.e. valvuloplasty, septostomy) remains limited to specific malformative syndromes, whereas prenatal stem cell and gene therapy can be applied to a larger number of foetal diseases [72]. In utero stem cell transplantation indeed offers some therapeutical advantages over the more traditional postnatal cell therapy: the foetus represents an immunologically immature environment that can support stem cells engraftment and integration of cardiac tissue engineered constructs during growth [73, 74]; moreover, the small size of the foetus allows the transplantation of much larger cell doses per kilogram than in postnatal age [75]. In this scenario, foetal stem and progenitor cells, and in particular AFS cells, may represent the most tractable cell type for in utero stem cell therapy and cardiac tissue engineering, being an autologous source, easy to isolate, accessible in large numbers at the time of prenatal diagnosis, such that they can be engineered to provide cardiovascular constructs to be implanted before birth. As a proof of principle, amniotic fluid-derived stem cells were isolated in early gestation pregnant sheep to explore the feasibility for autologous in utero stem cell/gene therapy [76]. The progenitor cells were transduced with a viral vector to express GFP and subsequently injected into the peritoneal cavity of each donor foetal sheep under ultrasound guidance. PCR, Western blot and histological analysis confirmed GFP-positive cells in foetal tissues including liver, heart, placenta, membrane, umbilical cord, adrenal gland and muscle, at 12 days following injection, demonstrating that autologous amniotic fluid-derived stem cells have the potential for widespread organ migration and may offer an alternative therapeutic approach for prenatal congenital disease, including cardiac malformations.

6 AFS Cells for Cardiac Repair: Limits and Challenges to Overcome

AFS cells might represent a new therapeutic agent for cardiac repair. They possess potent cardiovascular and cardiomyogenic potential in vitro and mediate cardioprotection with inhibition of fibrosis and of the pathological remodelling in vivo, also being a source of regenerative soluble factors with a possible paracrine mechanism of action and a potential molecular candidate via Tβ4 release, as summarized in Fig. 1.2 and Table 1.1.

Despite all the encouraging results obtained so far, many barriers still need to be overcome for translating these preliminary findings into clinical reality.

- Safety is one of the most important aspects that have to be addressed with care. It is mandatory to develop methodologies to ensure the most appropriate delivery of the cell products to patients, according to good manufacturing

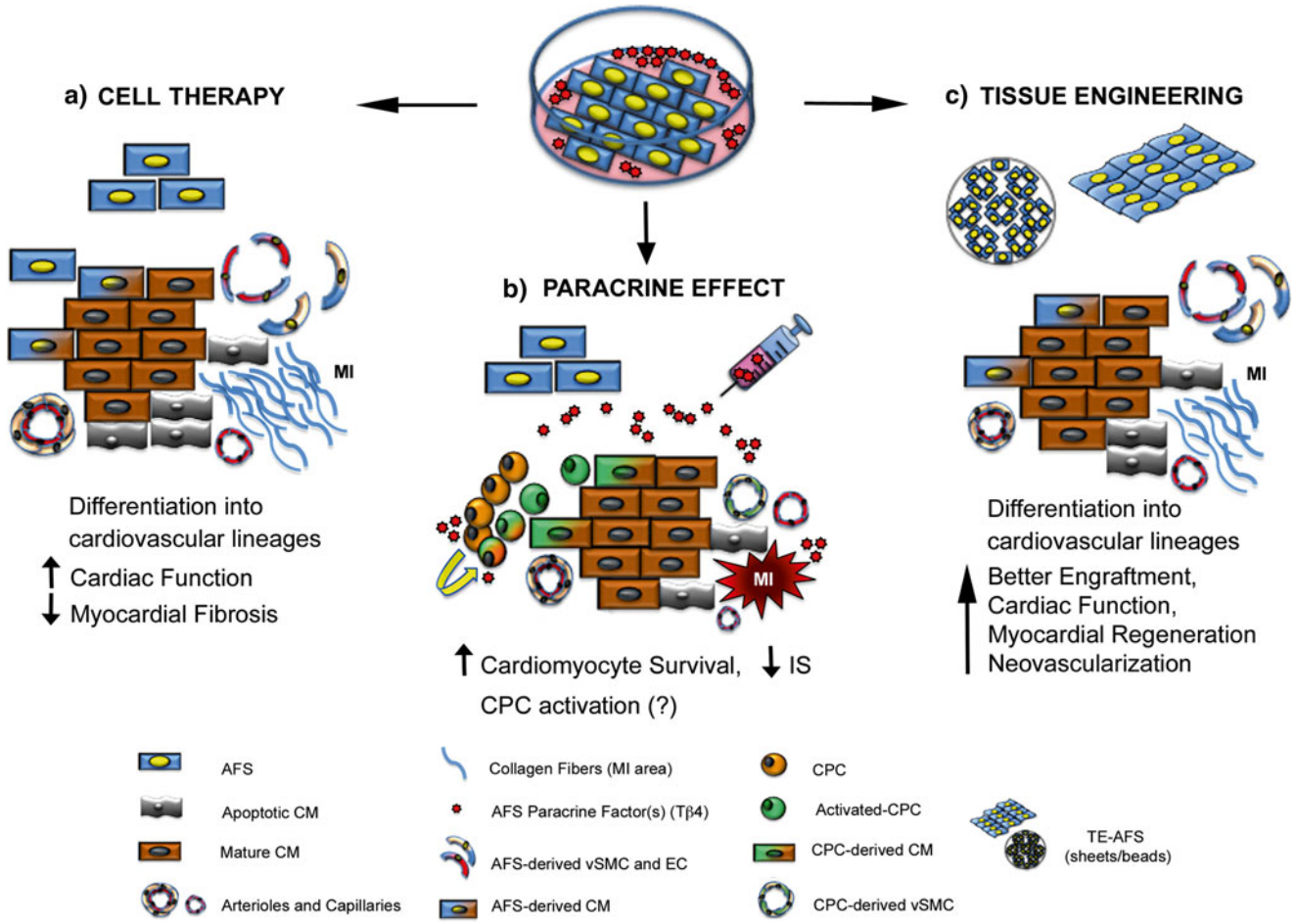


Fig. 1.2 Amniotic fluid stem cells for cardiac regeneration. Amniotic fluid stem cells (AFS) have been shown to acquire a cardiovascular phenotype, by expressing cardiomyocyte, endothelial and smooth muscle cell markers after in vivo transplantation in several cell therapy studies, as reported in the schematic in (**a**). AFS cells were demonstrated to exert a therapeutic effect by improving cardiomyocyte survival and decreasing infarct size (IS) 2 h after injection into an acute ischemia/reperfusion model in rat, as represented in (**b**). These results were confirmed using cell-conditioned medium (represented here by the *syringe*), suggesting a paracrine effect possibly mediated by the cardioprotective peptide thymosin beta 4 (Tβ4), which has been shown to mediate endogenous cardiac progenitor cell activation and differentiation into cardiovascular lineages and functional cardiomyocytes. AFS cells have also been processed to obtain cellular sheets or combined with biocompatible porous beads to improve their engraftment and survival in the host tissue in vivo, via a tissue engineering approach, as illustrated in (**c**). This strategy resulted in better engraftment, improvement of cardiac function, increased neovascularization in addition to differentiation into cardiovascular lineages. *AFS* amniotic fluid stem cells (ckit+ AFS and/or AF-MSC), *CM* cardiomyocyte, *MI* myocardial infarction, *vSMC* vascular smooth muscle cells, *EC* endothelial cells, *CPC* cardiac progenitor cells, *TE-AFS* tissue-engineered AFS

practice rules. Hence it's essential to consider some crucial factors in translation from the laboratory bench to the patient bedside, such as the materials, reagents and the cell products used in AFS cell isolation and processing, which must be well characterized, reliable, sterile and genetically stable [77].

- Immunomodulatory and immunogenic properties. This aspect is still much debated, due to controversial results obtained in studies where human c-kit+ AFS cells showed low engraftment and acute rejection, following injection into the infarcted region of rats, despite the use of immunosuppressant [37]. While highly contentious, this particular study was based on xenogenic transplantation; therefore it need not completely preclude the prospect of using AFS cells in an allogeneic approach in the future, in addition to a prospective autologous use for congenital defects.
- The functional efficacy of the AFS cell's cardiovascular potential has to be defined in detail, as a crucial question for their therapeutic translation is whether they can terminally differentiate into the required tissue-specific lineage in the long term; to date functionally mature contracting cardiomyocytes have not been obtained in vivo from this stem cell source with significant efficiency and efficacy.
- Finally, further detailed studies are needed to elucidate the precise mechanisms underlying the beneficial effects they

Table 1.1 In vivo strategies for cardiac repair with AFS cells

	Cell source	Model	Delivery	Results	Reference
Cell therapy	2×10^6 rat CMTMR+ AF-MSC	Left anterior ventricular cryoinjury in syngeneic Lewis and athymic rats	i.c. in cryoinjury area at 7d post-injury	At 30d few AF-MSC showed cTnT and vWf markers and capillary structure	[36]
	2×10^6 human ckit+ hAFS	Permanent left coronary artery ligation in Sprague-Dawley and athymic nude rats	i.c. in peri-ischemic area 20m after MI	Trace of hAFS cells found after 30d expressing SM and EC phenotype. Marked inflammatory response	[37]
	3×10^6 human AF-MSC	Permanent left coronary artery ligation in Lewis rat	i.c. in MI peri-ischemic area at 7d post-injury	After 28d less myocardial fibrosis. Some cells positive for Nkx2.5, a-actinin, or cTnT	[40]
	3×10^6 human AF-MSC transfected to express firefly luciferase and forming bodies enriched with ECM	Permanent left coronary artery ligation in immunosuppressed Lewis rats	i.c. in MI peri-ischemic area	After 4 weeks attenuation of heart failure, improvement of cardiac function. Differentiation into angiogenic and cardiomyogenic lineages	[43]
	5×10^6 GFP+ ckit+ rat AFS	30 min occlusion of left coronary artery with ischemia/reperfusion injury in Sprague-Dawley rats	i.c. in MI peri-ischemic area	After 3 weeks trend in cardiac function improvement. Few rAFS cells expressing SMA, vWf and cTnT proteins	[38]
Paracrine effect	5×10^6 ckit+ human AFS	30 min occlusion of left coronary artery with ischemia and reperfusion injury in Sprague-Dawley rats	i.v. through jugular vein at reperfusion	After smaller infarct size, inhibition of cardiomyocyte apoptosis; similar results with conditioned medium	[53]
Cardiac tissue engineering	3×10^6 human AF-MSC on methylcellulose hydrogel as cell sheet fragments	Permanent occlusion of left coronary artery in immunosuppressed Lewis rat	i.c.in peri-ischemic area 6d after MI	After 4 weeks improvement in heart function, increase in neovascularization, expression of cardiovascular markers, better engraftment than with dissociated AFS cells	[70]
	3×10^6 human AF-MSC + PLGA porous beads	Permanent occlusion of left coronary artery in immunosuppressed Lewis rats	i.c. peri-ischemic area 7d after MI	After 4 weeks prevention of remodelling, improvement of cardiac function. Expression of vWf, Nkx2.5, cTnT	[71]

mediate in vivo, namely transdifferentiation versus fusion and/or the regenerative paracrine capacity of their secretome, with possible stimulation of resident cardiac progenitors by the local release of specific cardioactive factor(s), as seen in some ischemia/reperfusion models [53].

Overall, in cell-based regenerative medicine autologous cells may represent the best therapeutic options as allogenic cells are generally immunogenic (except maybe some mesenchymal stem cells) and require immunomodulatory therapy. However, the use of patient-specific cells, presents its own limitations, as they frequently derive from an unhealthy donor. The idea of using these cells may be experimentally feasible, but practically and clinically still controversial [78].

In summary, AFS cells may represent a real alternative as a therapeutic option for cardiac repair since they are a tractable source of autologous cells with wider potential than adult postnatal stem cells and less immunogenic. In addition they may be harvested during pregnancy and stored in a cell bank, or processed in parallel to gestation, to be used in the pediatric patient pre- or perinatally in order to effect heart regeneration for congenital cardiac defects.

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Amniotic Fluid Stem Cells for Wound Healing

2

Aleksander Skardal

Abstract

Amniotic fluid-derived stem (AFS) cells are an attractive cell source for applications in regenerative medicine due to their proliferation capacity, multipotency, immunomodulatory activity, and lack of significant immunogenicity. In addition, they have the ability to modulate inflammatory responses and secrete therapeutic cytokines. Because of these characteristics, AFS cells have been explored for treatments in wound healing and skin regeneration. Studies show that AFS cells are effective in accelerating wound healing in skin in fetal environments, and more recently in adult wounds. Evidence indicates that delivered cells are often transient, not permanently integrating into the final skin tissue. Instead, they secrete a portfolio of potent growth factors that are integral to skin regeneration and angiogenesis, suggesting a trophic mechanism of augmenting wound healing. These initial works of research suggest that delivery of AFS cells has potential to be an effective cell therapy for facilitating wound healing and should be further considered for clinical use in excessive skin wounds in human patients.

1 Introduction

Extensive burns and full thickness skin wounds can be devastating to patients, even when treated. There are an estimated 500,000 burns treated in the United States each year [1, 2]. The overall mortality rate for burn injury was 4.9 % between 1998 and 2007 and medical costs for burn treatments approach $2 billion per year [3]. Globally, this statistic increases to 11 million injuries per year [4]. In addition to burns, full-thickness chronic wounds constitute a large patient base, and despite technological advancement of treatments, healing rates remain below 50 % successful [5]. These non-healing chronic wounds are estimated to effect 7 million people per year in the United States, with yearly costs approaching $25 billion [6]. Patient survival is inversely proportional to the amount of time required to cover and stabilize a wound. Patients with burns greater than 15–20 % of total body surface area are likely to go into shock without rapid treatment. Furthermore, without sufficient and rapid fluid resuscitation, patient conditions deteriorate and mortality rates increase rapidly [7]. Inadequate treatment regimes result in long-term complications for patients, including open wounds, pain, problems with temperature sensation, loss of feeling, prominent scars, and itching [8].

Patients who suffer from either of these types of injuries benefit from rapid treatments that result in complete closure and protection of the wounds. In particular, burn patients who receive delayed treatments often are subject to extensive scarring that can result in negative long-term physiological effects.

Recent advances have been made in treatments for wound healing; however, the gold standard still employed in the clinic, is an autologous split-thickness skin graft. This involves removing a piece of skin from a secondary surgical site for the patient, stretching the skin, and re-applying the graft on the wound or burn. While this treatment yields a reasonable clinical outcome, if the wound is extensive,

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then the number and size of donor sites are limited, making autografts unusable in cases that require prompt and aggressive measures to maintain the lives of wounded patients. Allografts are an additional option, but suffer from the need of immunosuppressive drugs to prevent immune rejection of the graft. These limitations have thus led to the development of noncellular dermal substitutes, which most often comprises a polymeric scaffold. Examples include INTEGRA® Dermal Regeneration Template (Integra LifeSciences) and Biobrane® (UDL Laboratories), and although such materials result in improved wound healing over untreated controls [9, 10], they are costly to produce and result in relatively poor cosmetic outcomes.

Recent advances in tissue engineering have led to more complex biological skin equivalents that may yield more suitable wound treatment options for patients. Examples include cellularized graft-like products, such as Dermagraft® (Shire), Apligraf® (Organogenesis), and TransCyte® (Advanced BioHealing). These products generally comprise a polymer scaffold patch that is seeded with human fibroblasts and cultured in vitro prior to application. Unfortunately, these grafts are also expensive to produce, and as allografts, can suffer from the same immunological drawbacks discussed above.

Commercially available hydrogel dressings have advantages in that they are often immunologically inert, regulate fluid exchange from the wound surface, and are available as gels, sheets, and impregnated in ordinary cotton gauze pads [10, 11]. For shallow, flat wounds, hydrogel sheets are applied as a primary dressing and remain in place for approximately 4–7 days. For irregular and/or deep wounds, amorphous hydrogels can be used to fill the wound and are held in place with a secondary dressing that is generally changed at least once daily. While these applications have advantages over traditional wound care regimes, the potential for hydrogels to promote wound healing is not limited to inert wound dressings. Other hydrogel materials have been developed that (1) possess crosslinking methods that facilitate deposition and immobilization in situ, and (2) comprise bioactive materials that can improve integration in the wound, can release potent biological signals, and even deliver therapeutic cells to the wound. In order to deliver cells, cell spraying and bioprinting technologies have recently been developed for wound treatment. In these approaches, cells are deposited over the wound, generally mixed in a hydrogel carrier vehicle, via a spray nozzle or print-head. Indeed, in our laboratory, we used a bioprinting device [12] to deposit amniotic fluid-derived stem cells in a full thickness wound healing model with great success [13]. New approaches such as these that permit immediate burn wound stabilization and support functional recovery are necessary. Figure 2.1 summarizes the various wound healing products and technologies discussed above.

Category	Product	Source/Material	Manufacturer
Donor-Provided	Autograft	Human skin tissue	N/A
	Allograft	Human skin tissue	N/A
Non-Cellular Dermal Substitutes	Integra™ Meshed Bilayer Wound Matrix	Collagen – Bovine Tendon	Integra LifeSciences
	Biobrane	Silicone film, nylon fabric and porcine collagen	UDL Laboratories
	Restylane	Hyaluronic acid – Bacterial (*Streptococci)*	Q-Med
Cellular Skin Equivalents	Dermagraft	Polyglactin mesh, human fibroblasts and fibroblast-secreted ECM	Shire
	Apligraf	Type I collagen, human dermal fibroblasts and keratinocytes	Organogenesis
	TransCyte	Nylon mesh and human fibroblasts	Advanced BioHealing
Hydrogels Approved for Use in Humans	Tegagel™ Hydrogel Wound Filler	Calcium alginate Seaweed/bacterial	3M
	Phytacare Alginate Hydrogel wound dressing	Calcium alginate Seaweed/bacterial	CuraPharm
	Intrasite gel	Carboxymethyl cellulose and polyethylene glycol	A Smith & Nephew
Examples of Materials Currently used for Research	Extracel Hydrogel	Crosslinked hyaluronic acid and gelatin	Glycosan Biosystems
	Fibrin-collagen gel blends	Bovine-derived fibrinogen and thrombin, and rat-tail collagen	Various
	Silk-based films	Silk fibroin from silkworm cocoons	N/A

Fig. 2.1 Examples of materials used clinically to treat wounds and experimental materials currently in research stages

Rather than a passive gel dressing that will have a minimal role in the healing process, technologies are needed that will actively promote wound healing by acting as a substrate for endogenous cell migration and proliferation. Cell-based therapies, such as the application of stem cells, may meet these needs.

2 A Brief Introduction to Stem Cells Derived from the Amniotic Fluid

The cell source used in cellular therapies for wound healing is an important consideration that has implications in the cost, speed, and outcome of the treatments. Human keratinocytes are perhaps the optimal cell type to employ as they make up the epidermal zone of skin tissue. However, autologous and

allogeneic keratinocytes suffer from the same drawbacks as their autologous and allogeneic skin graft counterparts, i.e., secondary surgical sites and potential for rejection, respectively. This begs the question: Can we employ cells that are beneficial to wound healing, but may be immunoprivileged despite originating from an allogeneic source?

Mesenchymal stem cells (MSCs) have shown therapeutic potential for repair and regeneration of tissues damaged by injury or disease. In particular, MSC treatment of acute and chronic wounds result in accelerated wound closure, increased epithelialization, formation of granulation tissue and angiogenesis [14]. Amniotic fluid-derived stem (AFS) cells are a cell type that shares some characteristics with MSCs, but are perhaps even more potent as they are sourced from an earlier point along the developmental timeline, namely the gestational period. As such, they are an attractive cell source for applications in regenerative medicine due to their high proliferation capacity, multipotency, immunomodulatory activity, and the lack of significant immunogenicity [15, 16]. Unlike embryonic stem cells (ESCs), AFS cells do not form teratomas when injected into immune-deficient mice. Furthermore, AFS cells remain stable and show no signs of transformation in culture. The isolation of AFS cells is a simpler process than that for isolation of MSCs and large numbers of AFS cells can be isolated and expanded from as little as 2 mL of amniotic fluid. These cells proliferate rapidly with doubling times of 30–36 h, and do not require supportive feeder layers [15]. The immunomodulatory and high proliferation properties of AFS cells suggest that they may be appropriate as an effective "off the shelf" cell therapy product for wound healing.

3 Flexibility of Amniotic Fluid-Derived Stem Cells in Regenerative Medicine

In recent years AFS cell-based research for applications in regenerative medicine has become more prevalent in the literature. As with other stem cell types that were established earlier such as MSCs, adipose-derived stem cells (ADSCs), and ESCs, AFS cells have been explored for force induced differentiation into cells of various tissue types, for repairing and replacing damaged or diseased tissues, and antitumor treatments. Additionally, the secretory profiles of AFS cells suggest they may be effective as deliverable "drug factories," efficiently secreting cytokines and growth factors at target sites such as MSCs have been shown to do [17].

For differentiation purposes, AFS cells have been force induced into a number of lineages, including those of the liver [18], pancreas [19], cartilage [20], fat, muscle, bone, neuronal, and endothelial lineages [15, 21]. In addition to a level of multipotency, AFS cells share several other characteristics with MSCs, including the ability secrete cytokines, many of which are pro-regenerative or immunomodulatory. The ability to secrete biological molecules such as these makes AFS cells, like MSCs, useful for various reparation therapies as well as antitumor treatments. The immunomodulatory activity of AFS cells has been implemented to treat chronic allograft vasculopathy, by decreasing inflammation, thus preventing intimal wall thickening and loss of the graft [22]. This immunomodulatory activity has been further shown in controlled in vitro immune reaction studies, in which AFS cells as well as cell-free supernatants from AFS cell cultures inhibit lymphocyte activation, suggesting the responsible mechanism is through secreted soluble immunomodulatory factors [16]. These characteristics, secretory profiles and low immunogenicity, have also served as motivation to explore the use of AFS cells in anticancer treatments. Like MSCs, AFS cells show a propensity to home to tumor sites with administered intravenously [23]. In one such example, AFS cells expressing interferon beta were administered systemically to mice in a bladder tumor model, where they homed to the tumors and succeeded in inhibiting tumor growth and prolonging survival [24].

This ability to secrete growth factors, immunomodulatory factors, anti-inflammatory molecules, and to migrate to sites of injury has suggested another use in cell therapy—namely can AFS cells be implemented in wound healing treatments in skin?

4 Perinatal Wound Healing

There is a vast amount of literature that argues towards the potential that AFS cells have in wound healing applications, which stems from the phenomenon of perinatal, or fetal, wound healing, in which injuries to a fetus can heal completely scar free [25]. During development the fetus is surrounded by amniotic fluid (AF) within the amniotic sac, the same environment from which AFS cells are isolated. Components of the AF may be responsible for its regenerative properties, and also be able to imbue the regenerative properties inherent in AFS cells. A number of studies have determined that the amniotic fluid has a unique composition that changes over time, supporting scar-free healing at the early stages of gestation, and then transitions to more traditional healing processes at the end of pregnancy. For example, human amniotic fluid was assessed in vitro for its ability to regulate several major proteases involved in wound healing. It was found that amniotic fluid enhanced collagenase, thereby inhibiting collagen formation, a large component of scarring. Conversely, activities of hyaluronidase were inhibited, thereby allowing hyaluronic acid (HA), an anti-inflammatory glycosaminoglycan common to the extracellular matrix (ECM), to be present in high levels [25]. Furthermore, it has been demonstrated that fibrotic healing that occurs in late

gestational and adult wounds is correlated with increased hyaluronidase activity, which decreases HA levels [26]. HA has often been associated with regeneration and anti-inflammation, and is now commonly used in applications demanding such characteristics [27].

Amniotic fluid-based fetal wound healing studies have yielded a knowledgebase consisting of growth factors, cytokines, and extracellular matrix components that have been further explored to minimize scarring in adult wounds. Indeed, it has been shown that amniotic fluid, containing high levels of basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF), was a potent stimulant of both fetal and adult skin fibroblast growth [28]. A number of clinical trials have been instigated, but none have reached the level of FDA approval [29]. However, the concept of using the fetal developmental environment to "fool" the body into scar-fee healing holds merit.

5 Amniotic Fluid-Derived Stem Cells in Wound Healing

Secretory profiles of AFS cell-conditioned media contain a number of cytokines known to be important in wound healing, such as vascular endothelial growth factor (VEGF), epithelial growth factor (EGF), and transforming growth factor beta (TGF-ß) [30]. This suggests that AFS cells delivered to wound sites may be able to secrete these factors in situ, contributing to wound healing in cell therapy administrations. MSCs have been shown to have a paracrine role in wound healing, secreting similar factors [31]. Interestingly, MSCs have also been documented as being able to integrate permanently by differentiating into cells of myofibroblast phenotype in vivo [32] and acting as vasculature-supporting pericytes in vitro [33], another potential mechanism by which AFS cells could potentially impact wound healing.

As discussed above, the environment within the amniotic sac itself may be the main component in effective wound healing, but until recently it was unknown whether a cellular component of the environment was important. In a fetal lamb wound model, fetuses receive an infusion of autologous AFS cells into the amniotic sac. Access to wounds was either not impeded, or made impermeable to cells using exterior titanium chambers. AFS-impermeable wounds showed significantly slower healing rates. Furthermore elastin content in those wounds were significantly decreased compared to wounds that were accessible to the AFS cells, further indicating the effectiveness of the cells themselves in wound healing, albeit in a fetal environment [34].

Our laboratory has a track record in pioneering isolation, characterization, and applications of AFS cells [15, 19, 21, 35, 36]. However, until recently no one had explored the use of human AFS cells in healing of severe wounds in adults. We recently demonstrated the use of AFS cells using a full thickness wound model in mice [13]. We employed a bioprinting device previously developed in house [12] to deliver AFS cells to wounds within a fibrin-collagen gel vehicle, and compared this treatment to MSCs or no cells delivered in the same gel delivery vehicle. AFS cells and MSCs showed similar potency in topographical wound closure (Fig. 2.2), significantly accelerating wound closure compared to the cell-free control. However, upon histological inspection, we observed that AFS cells induced significantly greater vascularization than both MSC and cell-free treatments (Fig. 2.3a–h) during the short 2-week time course. Additionally, vasculature in AFS-treated wounds also appeared more mature, displaying robust smooth muscle actin surrounding larger vessels and a lack of extravasated red blood cells (Fig. 2.3i–n). Extravasated

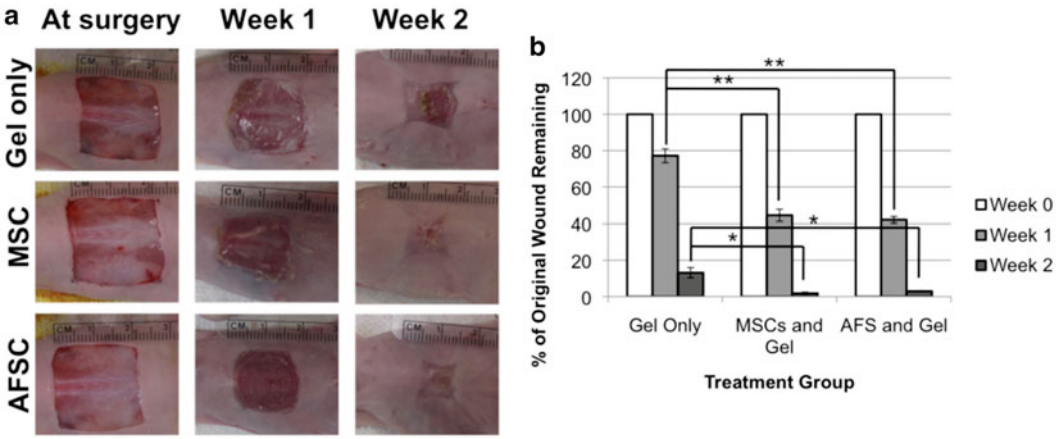


Fig. 2.2 Wound closure rates are increased in AFS- and MSC-treated mice. (**a**) Gross histology images illustrating wound closure in Gel-only, MSC, and AFS treatments. (**b**) Percentage of unclosed wound remaining at surgery, 1 week, and 2 weeks. Significance: $*p<0.05$; $**p<0.01$

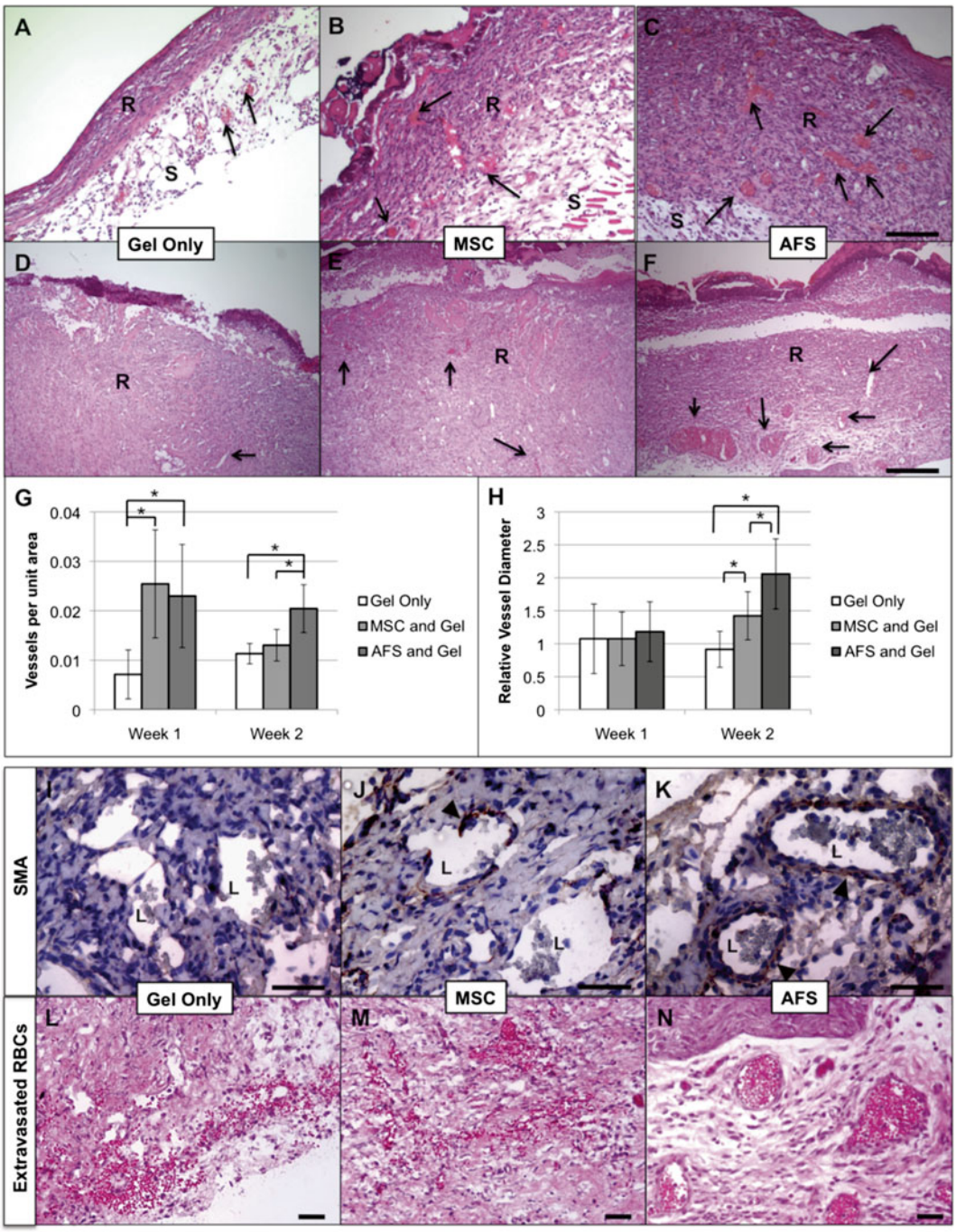


Fig. 2.3 AFS cells induce neovascularization and blood vessel maturation in vivo. H&E staining revealed thicker regenerating tissue w/more blood vessels in AFS and MSC group compared to the gel-only group. (**a**–**c**) Week 1; (**d**–**f**) Week 2; (**a** and **d**) Gel-only; (**b** and **e**) MSC; (**c** and **f**) AFS. *Arrows*—vessels. AFS cells increase the number of newly formed vessels and induce formation of larger vessels. (**g**) Microvessel density (MVD) and (**h**) vessel diameter were quantified using histology images and ImageJ software. Significance: $*p<0.05$. AFS cells induce formation of mature blood vessels. SMA staining around blood vessels in regenerated tissues of gel only (**i**), MSC (**j**), and AFS (**k**) groups. *Arrows*: Cells expressing SMA. Extravasated RBCs were present in gel only (**l**) and MSC (**m**) groups, but not in the AFS (**n**) group

red blood cells were observed in the other groups, indicating leaky or less mature blood vessel structures. Interestingly, both AFS cells and MSCs were transient in our model as evidenced by tracking of the cells after transfection with green fluorescent protein (GFP). Cells were present immediately after treatment, 4 days after treatment, and 7 days after treatment—albeit in fewer numbers. However, no evidence of either cell type was found after day 7 (Fig. 2.4).

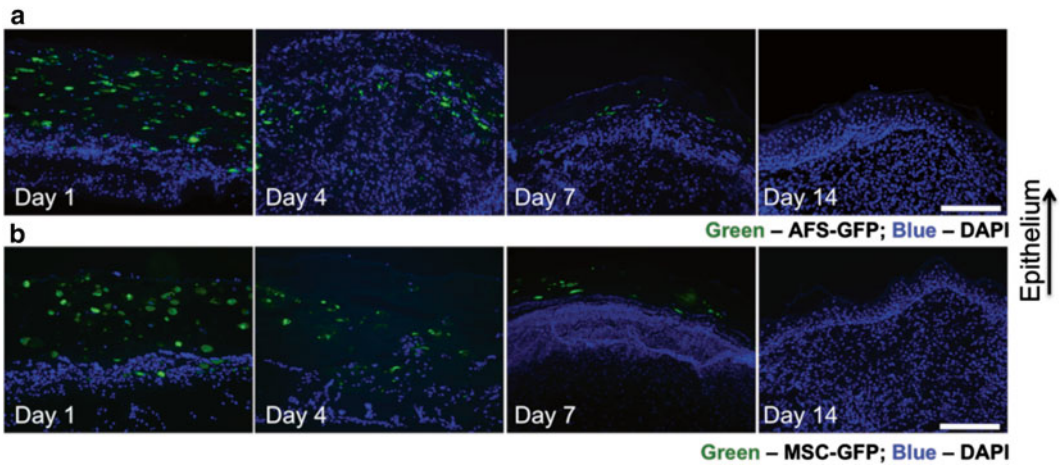


Fig. 2.4 AFS cells and MSCs are transient in the regenerating wound and do not permanently integrate into the tissue. Regenerating skin was harvested at day 1, day 4, day 7, and day 14 in order to determine the presence of labeled cells. GFP-labeled AFS cells (**a**) and MSCs (**b**) are visible in decreasing numbers of the time course of the experiment. *Green*: GFP-expressing AFS or MSC; *Blue*: Nuclear staining, DAPI

Nevertheless, these results suggested that AFS cells have the potential to be an effective therapy for treating skin wounds. Based the transient nature of the cells, we hypothesized that the augmentation of skin regeneration was due to trophic delivery of cytokines. To further investigate this concept, we first generated conditioned media from these particular populations of AFS cells and MSCs, and analyzed it for a panel of potent growth factors using a proteomics array. As expected, samples tested positive for cytokines such as bFGF, VEGF, hepatocyte growth factor (HGF), and members of the insulin growth factor-binding protein (IGFBP) superfamily (Fig. 2.5). In general, AFS-conditioned media tested positive for more of these cytokines and at higher concentrations. Based on the increased angiogenic activity noted above, we then hypothesized that AFS cells were likely responsible for recruiting endothelial cells via secretion of these factors. We next performed a Transwell migration assay using human umbilical vein endothelial cells as the migrating population. In order to simulate the mechanically soft environment of the in vivo wound bed we employed a 2 kPa stiff substrate versus much stiffer traditional plastic substrates on which the AFS cells were seeded as the recruiting population. In addition, a cell-free control was used. A greater number of HUVECs migrated when exposed to the AFS cultures compared to the cell-free control. Interestingly, AFS cells cultured on the softer 2 kPa substrate were significantly more adept at HUVEC recruitment, suggesting that the environment in which they exist plays an important role in their therapeutic effectiveness [13].

The transient nature of this cell therapy combined with the experiments described above certainly argue strongly that the primary mechanism by which AFS cells aid wound healing biology is through secretion of trophic factors. In particular, we demonstrated the ability of AFS cells to recruit endothelial cells, behavior that was conserved in vivo in that a significantly increased number of blood vessels formed after AFS cell treatment. Indeed, other groups have found that AFS cells themselves and factors from conditioned media derived from AFS cells can induce neovascularization in ischemic skin flap models, restoring viability to the skin [37–39].

Other recruitment mechanisms may be at play as well, as suggested by the high levels of IGFBPs observed in AFS-conditioned media. Members of the IGFBP superfamily have

Growth Factor Analysis of Conditioned Media
-
(units: pg/mL)

Growth Factor	AFS	MSC
bFGF	459	-
FGF-7	1507	3106
GDF-15	95	50
GDNF	21	-
HGF	218	-
IGFBP-2	1490	4167
IGFBP-3	8852	-
IGFBP-4	65249	987
IGFBP-6	17470	-
NT-3	530	-
OPG	1882	7852
PDGF-AA	-	176
PIGF	-	18
VEGF	5006	10953

Fig. 2.5 Quantification of growth factors secreted by AFS cells and MSCs in vitro. AFS cells secrete more growth factors than MSCs. Presence of growth factor concentrations in AFS- and MSC-conditioned media were determined by proteomic arrays. *Dashes* indicate that growth factors were undetectable

been shown to modulate survival, migration, and proliferation of cells in the dermis and epidermis [40]. In fact, IGFBP-7 was believed to be responsible for increased migration of both fibroblast and keratinocytes in in vitro scratch assays [41]. In a separate study, IGFBP was shown to promote high levels of survival and proliferation of both HaCaT (immortalized keratinocytes) and primary human keratinocytes [42].

6 Maximizing the Effectiveness of AFS Cells in Wound Healing

The potential for AFS cells to be effective as a wound healing treatment is evident from the data presented above. However, the transient behavior observed limits that effectiveness, since when the AFS cells are no longer present, no additional GFs can be secreted. We have been recently exploring alternative hydrogel delivery vehicles to improve the logistics of cell delivery and extend the period of potency of the AFS cells and the cytokines they secrete in the wound.

The fibrin-collagen gel employed above performed adequately, but was perhaps not the most optimal material to be used for bioprinting and wound healing. First, it required two components to be deposited in situ, relying on diffusion to mix them together in order for gelation to occur. This resulted in relatively slow gelation that caused some of the gel to pool in lower regions of the wound, and the loss of some gel solution from dripping out of the wound. Additionally, collagen is known to contract during gelation and is the primary component in scarring, potential drawbacks in skin regeneration.

Hydrogel biomaterials have been widely explored for bioprinting applications. We previously developed a series of hyaluronic acid (HA)-based hydrogels with different crosslinking techniques to facilitate deposition from bioprinting devices. The base materials of the HA-based hydrogels we implemented consist of thiolated HA, thiolated gelatin, and a polyethylene glycol diacrylate (PEGDA) crosslinker [43, 44]. This hydrogel has been implemented in numerous regenerative medicine applications, including 3-D cell culture [45], postsurgical adhesion prevention [46], tumor xenografts [47, 48], and wound healing [49]. However, in their native form, these materials require 15–30 min to crosslink at physiological pH, which is unsuitable for the fast deposition nature of bioprinting. To overcome that limitation, we employed variations of the hydrogel and different crosslinking approaches to improve bioprinting procedures. First, we developed a 4-arm PEG-based crosslinker which resulted in a stiffer hydrogel, allowing stacking of cellular and noncellular extruded rods in an increased aspect ratio into a tubular structure [50]. Second, we implemented methacrylate-based photopolymerization to employ a 2-step photocrosslinking protocol to print a cellular tube [51]. Next, we employed the thiophilic nature of gold nanoparticles and used them in place of the PEGDA crosslinker to make slow-forming gels. This property allowed extrusion of partially gelled materials into 3-D orientations that would later fuse together in a single seamless structure [52]. Recently, we discovered that by adding the Irgacure 2959 photoinitiator a solution comprises the HA and gelatin base materials mentioned above, we could use UV irradiation to achieve nearly instantaneous photopolymerization. We tested this hydrogel against a panel of other commonly used materials for characteristics such as gelation times, ease of use, biocompatibility, and immunogenicity and found that it was suitable for use in our bioprinting devices and likely a good choice for implementation as a cell delivery vehicle for a wound healing treatment [53].

Preliminary studies have demonstrated that the properties of the photocrosslinkable HA hydrogel, including the ability to incorporate heparin for growth factor release, pair well with the pro-angiogenic and secretory properties of AFS. In vitro testing confirmed that encapsulation of AFS cells in the heparinized HA hydrogel (Extracel-HP, commercially available from ESI-BIO) supported daily release of AFS-secreted protein for 2 weeks. Furthermore, AFS-secreted VEGF release was maintained for same length of time. Notably, in fibrin-collagen gels, VEGF release was not detectable after day 7. We tested the combination of AFS cells delivered in Extracel-HP in a similar mouse model of full thickness wound healing as described above in which we observed similar wound closing and angiogenic activity. However, we also noted an improvement in the extracellular matrix composition. Specifically, there was a lower ratio of fibrotic collagen Type I to collagen Type III, which is associated with healthy healing. Additionally, there was greater staining for elastin, proteoglycans, and GAGs in HA-AFS tissues than other groups. These in vitro and in vivo data indicate that the choice of delivery vehicle for cell therapy matters greatly and can effectively improve cell therapies. By employing a less inflammatory and contractile hydrogel with dynamic growth factor release capabilities, we believe we maintained growth factor activity in the wound for a longer period of time and improved the ECM in the regenerated skin.

7 Limitations and Future Directions

In the work in our laboratory, the primary limitation we have come across in our results is the inability to limit contraction during the wound healing process. In both of our studies described above, contraction contributed significantly to the overall wound closure. However, we believe that by incorporating the optimal delivery vehicle we can minimize healing by contraction, and rather induce re-epithelialization to be primarily responsible. Interestingly, when we employed HA above, the overall level of contraction did not decrease dramatically. However, the aspect ratio of the healing wound changed significantly. Specifically, wounds treated with HA

healed uniformly resulting in roughly a square-shaped area of re-epithelialized skin. In contrast, control wounds and fibrin-collagen-treated wounds healed with a high aspect ratio, contracting more in the medial-lateral direction. This difference may be significant for limiting contraction and scar formation, but will require further investigation.

More general limitations, but limitations nonetheless, are those of regulatory hurdles associated with bringing any cell-based therapy or product to market and to patients in the clinic. The time and financial cost required to generate a simple drug molecule that is commercially available is staggering. Products that incorporate living cells are inherently more complex, resulting in a regulatory pathway that is therefore more complex, and not fully developed at this time. To that end, we have been exploring cell-free, but bioactive biomaterials that can mimic the biological behavior of AFS cell therapy in wounds. By using a cell-free, but AFS-like, approach we hope to address these issues.

8 Conclusions

Extensive skin wounds including severe burns can be devastating to patients, even when treated, and have a huge financial burden on the medical establishment. These patients require a treatment that results in protection of the wound during the healing process and closure of the wound in as short a time as possible. Although many improvements have been made in treatments for such wounds, the autologous split-thickness skin graft is still the best clinically available treatment despite its limitations. The studies presented here suggest that AFS cells may have the potential to address the clinical need for more effective treatment of burns and skin wounds. AFS cells seem to be able to induce accelerated wound healing through mechanisms associated with secreted cytokines, rather than integrating in the regenerating tissue. Because of this transient nature, the delivery vehicle in which the cells are deposited is important for maximizing their effect. Combinations of effective and versatile cell populations such as AFS cells in tandem with easy-to-use biomaterials complete with their own beneficial characteristics may lead to cell therapy products that will make a substantial impact in medicine.

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Treatment of Necrotizing Enterocolitis (NEC) with Amniotic Fluid Stem Cells

3

Augusto Zani, Mara Cananzi, Simon Eaton, and Paolo De Coppi

Abstract

Necrotizing enterocolitis (NEC) is a devastating disease of newborn infants, characterized by necrosis of one or more portions of the small and/or large bowel.

Despite extensive research and advancement in medical and surgical treatment over the last six decades, NEC still represents a challenging condition with a mortality rate as high as 40 % especially in very low birth weight infants.

Stem cell therapy has become an option for other intestinal diseases, such as refractory Crohn's disease, which share some features with NEC.

We first attempted administration of amniotic fluid stem (AFS) cells in a well-established neonatal rat model of NEC. In this study, AFS cells integrated in the bowel wall and improved rat survival and clinical conditions, decreased NEC incidence and macroscopic gut damage, improved intestinal function, decreased bowel inflammation, increased enterocyte proliferation and reduced apoptosis. The beneficial effect was achieved via modulation of stromal cells expressing cyclo-oxygenase 2 in the lamina propria. Moreover, AFS cells differentially expressed genes of the Wnt/β-catenin pathway, which regulate intestinal epithelial stem cell function and cell migration and growth factors known to maintain gut epithelial integrity and reduce mucosal injury.

The same beneficial effects of the amniotic fluid have been later confirmed by other authors using different experimental models of NEC.

Stem cell therapy may represent a new therapeutic option for children with NEC. Understanding the mechanism of action of AFS cells in experimental NEC may help the development of new cellular or pharmacological therapies for human infants with NEC.

1 Introduction

Necrotizing enterocolitis (NEC) is one of the primary causes of morbidity and mortality in neonates. Despite extensive research, there are still no medical therapies that have proven to be of clinical benefit for the cure of affected neonates and surgery remains the treatment of choice in case of necrotic bowel. There is a growing body of evidence that stem cells could play a therapeutic role in inflammatory bowel diseases and other intestinal pathologies that share physiopathological features with NEC. It has been recently (2013) demonstrated that perinatal stem cells (i.e. amniotic fluid stem cells) may improve survival, clinical status, gut structure and function in animal models of NEC.

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2 Necrotizing Enterocolitis

NEC is an acute ischemic-inflammatory disease of the neonatal intestine characterized by variable degrees of intestinal inflammation and necrosis of one or more portions of the

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Box 3.1 Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC)

It is the most common gastrointestinal surgical emergency occurring in neonates, especially premature.

The etiology of NEC remains elusive but is likely to be multifactorial: perinatal insults (e.g. formula feeding, abnormal bacterial intestinal colonization, hypoxia, hypoperfusion) trigger an exaggerated and inappropriate inflammatory response in the immature gut leading to intestinal injury.

Medical treatment is only supportive and there are still no etiologic therapies that have proven to be of clinical benefit. Surgical resection of the bowel is unavoidable in case of intestinal necrosis.

Mortality rates range from 15 to 30 %. Up to 30 % of survivors experience significant morbidity including neurodevelopmental impairment and short bowel syndrome.

small and/or large bowel. It is a devastating disease of infants and a leading cause of morbidity and mortality in neonatal intensive care units around the world [1] (Box 3.1).

2.1 Epidemiology

Population-based studies have shown that NEC occurs in 1–3 infants per 1,000 live births. The disease mainly affects preterm newborns: >90 % of affected babies are born prior to 36 weeks of gestation and/or weigh less than 2,000 g [1]. NEC, however, can also occur in full term infants with an incidence of 0.05 per 1,000 live births, especially if predisposing risk factors, such as perinatal asphyxia, polycythemia, respiratory distress and congenital cardiac diseases, are present [2]. As in the last decades advances in neonatology have improved the survival of smaller more premature infants, the incidence of NEC has progressively increased [3].

2.2 Pathophysiology

The etiology of NEC remains elusive but is likely to be multifactorial (Box 3.1). Since premature infants are at higher risk for NEC, immaturity of the intestinal tract has been implicated as the crucial element for the development of the disease. Current theories suggest that NEC is initiated by perinatal insults (e.g. formula feeding, abnormal bacterial colonization of the immature gut, lack of appropriate gut colonization with commensal organisms, hypoxia and hypoperfusion) in response to which the immature intestine mounts an exaggerated and inappropriate inflammatory response leading to intestinal injury. In the term infant, the intestine adapts to luminal microbes by downregulating the immune system, whereas, in the preterm infant, it does not. Intestinal epithelial cell toll-like receptor (TLR) 4 responses appear to be much more active in the preterm intestine than in the term intestine [4]. Intestinal epithelial TLR4 activation, driven by exposure to luminal Gram-negative bacteria, leads to increased mucosal injury through accelerated enterocyte apoptosis as well as reduced healing through impaired intestinal restitution and proliferation [5]. Mice deficient in TLR4 show reduced severity in models of NEC [6]. Genetic factors have also been recently advocated as predisposing factors involved in the pathogenesis of NEC [2,3, 7]. In particular, polymorphisms of genes involved in inflammatory signalling (e.g. Interleukin 12) and circulatory regulation (e.g. Vascular Endothelial Growth Factor, Carbamoyl Phosphate Synthetase 1) have been recognized as risk factors for the development of NEC [8, 9, 10].

2.3 Clinical Picture, Diagnosis and Management

Infants might either have a rather benign disease limited to gastrointestinal symptoms (e.g. feeding intolerance, abdominal distention, blood in the stool) or a catastrophic illness characterized by gross intestinal bleeding, intestinal necrosis and perforation, disseminated intravascular coagulopathy, multiorgan failure and profound shock [1] (Fig. 3.1a) (Box 3.1). Laboratory tests reveal non-specific indicators of an inflammatory process, thus no single examination is diagnostic of NEC [2]. Depending on the illness severity, abdominal radiographs may demonstrate the presence of intestinal distension, ileum, pneumatosis intestinalis and pneumoperitoneum [11) (Fig. 3.1b). The combination of physical signs, laboratory tests, and radiographic findings is used to suspect, diagnose and assess the severity of NEC. The medical management of NEC is only supportive and includes bowel rest, administration of intravenous fluids and treatment with broad-spectrum antibiotics. Approximately one third of infants with NEC require surgical intervention. The resection of necrotic bowel loops (Fig. 3.1c) and the creation of ostomies are the standard of care for the surgical treatment of NEC [2,3, 1, 12].

2.4 Outcome

Despite advances in neonatal intensive care, the mortality from NEC remains high, ranging from 10 to 30 %. Up to one third of surviving children experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, bowel obstruction and short bowel syndrome [13]. NEC remains the main

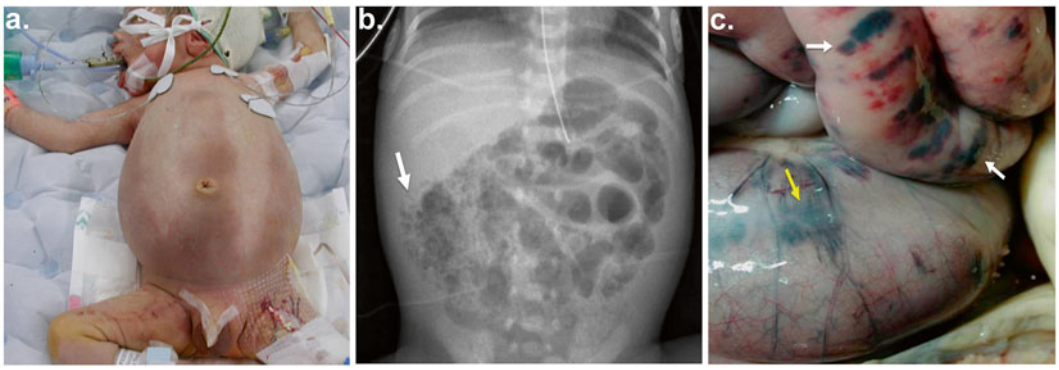


Fig. 3.1 (**a**) Infant admitted in a Neonatal Intensive Care Unit for a severe form of NEC with multiorgan failure. (**b**) Abdominal radiograph of a neonate with NEC showing diffuse bowel dilatation and extensive intramural gas in the right lower quadrant (*arrow*). (**c**) Intestinal segments affected by NEC: patchy areas of necrosis (*white arrows*), edematous and dilated bowel loop (*yellow arrow*) with questionable tissue viability

cause of short bowel syndrome in patients born with a normal gastrointestinal tract and one of the principal causes of intestinal failure in childhood.

2.5 Current Demands

Despite the high clinical impact of NEC in the pediatric population, many questions remain to be addressed concerning its etiology, diagnosis and treatment. In clinical practice, new medical approaches able to prevent the progression of the disease and its long-term complications are of absolute need. Current research efforts are mainly focused on the one hand on unravelling the pathogenesis of NEC and on the other on developing new therapeutic strategies.

2.6 Animal Models of NEC

There are a number of accepted animal models to study NEC. They all aim to create in animals a necrotic bowel resembling that of human newborns affected by NEC. Many models induce intestinal damage through administration of pro-inflammatory cytokines (e.g. PAF and TNF-alpha); others are based on the supply of an ischemic stress to the intestine (e.g. superior mesenteric artery clamping, systemic hypoxia) [14, 15]. Very few models combine a hypoxic and inflammatory stress (e.g. increasing gut intraluminal pressure and injecting *E. coli* into the intestinal lumen) [16]. The majority of available models do not contain the aspect of prematurity and the multiple pathogenetic elements that are present in NEC.

Among existing experimental models, the one proposed by Barlow in 1974 is the most reliable and the one that most closely resembles NEC [17, 13] as it employs all the main contributory factors involved in the development of human disease. This model mainly consists in the administration of bacteria (i.e. Klebsiella spp.), hyperosmolar formula and hypoxic stress to newborn rats, delivered by Caesarean section, during their first 4 days of life [18]. NEC reproducibility using this model, however, varies among different studies with a prevalence ranging from 35 to 71 % [19, 20]; this is mainly due to different grades of intestinal bacterial colonization depending on microbiological variations among animal facilities. To overcome this source of variability, an LPS-modified gavage model of NEC has been established [21, 22], in which the oral administration of bacteria is substituted with that of bacterial lipopolysaccharide (LPS). This LPS-modified gavage model of NEC is consistent and reproducible with a constant NEC incidence, estimated on gut histology, of 70 % at the end of the treatment [22] (Fig. 3.2). Moreover, in this variant of the Barlow's model two novel parameters (i.e. gut macroscopic appearance and animal clinical status) well correlate with intestinal histological damage, thus permitting to evaluate NEC severity with no need to process gut specimens for histology [22]. As intensive care support and operative treatment normally provided to human neonates with NEC (e.g. ventilation, cardiovascular support, intravenous fluids, antibiotics, parenteral nutrition) cannot be given to pup rats, this model is not compatible with long-term survival [23]. At present only inhibition of nuclear factor-kB [24] and administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) [21] have been shown, in this model, to decrease animal mortality at 72 and 96 h, respectively.

3 Stem Cell Therapy as a New Strategy for the Treatment of NEC

NEC is a devastating disease of newborns for which no specific treatment is currently available. Thus, there is the need of new therapeutic strategies capable either to repair the functioning intestinal surface or to avoid its massive destruction. Ever-increasing experimental evidences support the hypothesis that stem cells may promote tissue repair/regeneration in NEC as well as in other intestinal diseases with similar physiopathological features.

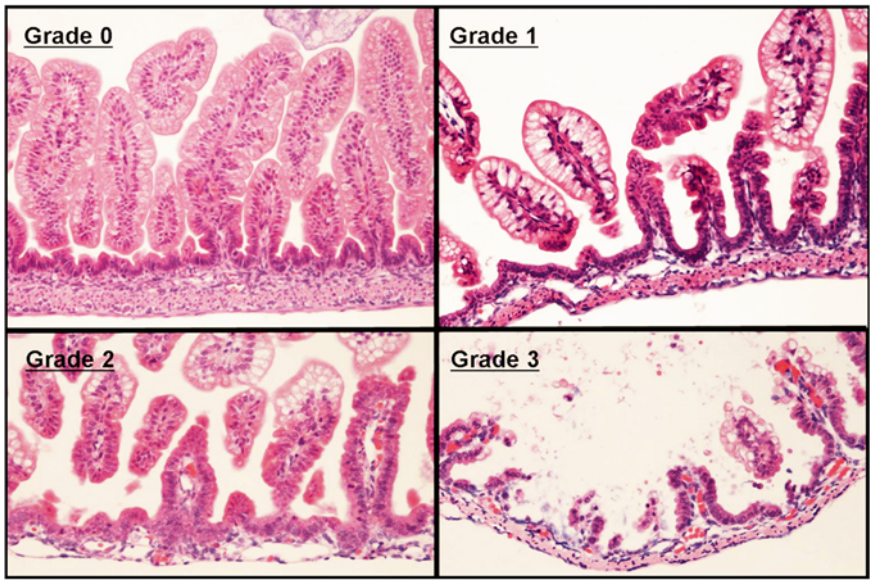


Fig. 3.2 Histological changes of the ileum in rats with NEC induced employing the LPS-modified Barlow's model. Representative sections for each grading score (H & E staining; original magnification 200×): *grade 0*: normal intestine; *grade 1*: disarrangement of villus enterocytes and mild villus core separation; *grade 2*: disarrangement of villus enterocytes and severe villus core separation; *grade 3*: epithelial sloughing of the villi. NEC is defined as grade 2 or above

3.1 Potential of Stem Cells in Gut Damage

Due to the complexity of the gut as an organ and the variety of its cell populations, regenerative medicine for intestinal diseases is still at an early stage of development. In 1993, for the first time a case of Crohn's disease regression was reported following haematopoietic stem cell transplantation for haematopoietic malignancy [25]. This beneficial effect was thought to be determined by the radical ablation of an aberrant immune system followed by reconstitution of a naive, non-aberrant immune compartment. By in situ hybridization of intestinal specimens of women who had received bone marrow transplant from male donors, in 2002 it was demonstrated that donor-derived cells do also integrate in the recipient intestinal tract persisting long-life after bone marrow transplantation [26].

After these first reports, the therapeutic potential of stem cells was tested in animal models of intestinal damage. On the one hand, several groups reported that bone marrow-derived mesenchymal stem cells could: integrate in the gut [27]; differentiate into intestinal epithelial cells [28, 26]; and contribute to the repair and formation of intestinal blood vessels via differentiation into myofibroblasts, endothelial cells, vascular smooth muscle cells and pericytes [29, 30]. On the other hand, different authors demonstrated that bone marrow-derived mesenchymal stem cells could reduce intestinal inflammation in experimental colitis via paracrine mechanisms (e.g. decrease of inflammatory mediators such as TNF-α, IL-1β) [31,32].

The means by which mesenchymal stem cells provide clinical benefit in animal models of intestinal damage is still under investigation. Available evidences support the hypothesis that stem cells could act both by regenerative and immunoregulatory mechanisms: stem cells can directly repopulate the intestine via differentiation into committed cells, and can reduce inflammation via paracrine mechanisms [33].

3.2 Stem Cells as a Treatment of Sepsis

There is a growing body of evidence that stem cells may offer therapeutic benefit in sepsis. In 2007, Xu et al. demonstrated that mesenchymal stem cell administration in mice subjected to intraperitoneal administration of lipopolysaccharide (LPS) resulted in a significant decrease of pro-inflammatory cytokines such as IFN-γ, IL-1β and IL-6 [34]. Gupta et al. showed that the intrapulmonary delivery of mesenchymal stem cells in LPS-inoculated mice resulted in less pulmonary oedema, decreased inflammatory cytokines in plasma and bronchoalveolar lavage, and increased survival [35]. Nemeth et al. demonstrated that administration of mesenchymal stem cells in mice before or shortly after inducing sepsis by coecal ligation and puncture reduced mortality and improved organ function [36]. The authors found that mesenchymal stem cells, activated by LPS or TNF-α, act via prostaglandin E2-dependent reprogramming of host macrophages to increase their IL-10 production. Gonzalez-Rey et al. demonstrated that human adult stem cells derived from the adipose tissue are able to protect mice with dextran sulphate sodium-induced colitis from both intestinal damage and sepsis [37].

3.3 Stem Cells as a Treatment for NEC

NEC is characterized by the combination of intestinal and systemic inflammation, which in severe cases lead, respectively, to intestinal necrosis and generalized sepsis. The results obtained with mesenchymal stem cells in experimental colitis

and in animal models of sepsis, together with the limited therapeutic options currently available, prompt the evaluation of the therapeutic potential of stem cells also in NEC.

To date, two groups investigated the efficacy of bone marrow-derived mesenchymal stem cells in a neonatal rat model of NEC. Notwithstanding a reduction in the severity of intestinal damage, in both studies adult bone marrow-derived mesenchymal stem cells failed to provide any significant benefit on animal survival [38,39]. The different therapeutic effect of adult mesenchymal stem cells obtained in experimental NEC vs. animal models of inflammatory bowel disease (IBD) could be linked to pathogenetic differences. In NEC, the primary causes of enterocolitis are bowel immaturity, bacterial infection and ischemia [2,3], whereas in IBDs, the pathological changes are primarily related to a dysregulation of the immune system [40]. Similarly the NEC model, comprises several pathogenic factors (i.e. ischemia, feeding with hyperosmolar formula, administration of LPS) that are directly implicated in the human disease [13], while models of IBD are usually obtained administering either dextran sodium sulphate [41] or peptidoglycan-polysaccharide [42] to animals that, in the vast majority of cases, carry genetic alterations of the immune system (e.g. $IL10^{-/-}$ mice) [43].

The lack of effect of adult bone marrow-derived mesenchymal stem cells in experimental NEC prompted the evaluation of a population of perinatal stem cells with higher regenerative potential and greater potency: amniotic fluid stem (AFS) cells.

AFS cells represent a unique population of stem cells of foetal origin with intermediate characteristics between embryonic stem cells and adult mesenchymal stem cells; on the one hand, they express embryonic markers, harbour a great proliferative and clonogenic potential, differentiate into multiple lineages and generate embryoid bodies and, on the other hand, possess mesenchymal markers and do not form teratomas after implantation in vivo [44,45]. Since their first isolation in 2007 [46] succeeding studies have provided evidence concerning their broad therapeutic potential in animal models of disease both via direct tissue regeneration [47, 48, 49, 50] and paracrine stimulation of tissue repair [51,52, 53, 54]. Moreover, the gut homing capacity of AFS cells when directly injected into the peritoneal cavity, even in the absence of organ damage, was recently reported along with their safety [55]. The authors injected 2×10^6 AFS cells transfected to express the green fluorescent protein (GFP) into the peritoneum of healthy newborn rats born at term via vaginal delivery and followed up the animals: none of the animals died or showed adverse effects related to the procedure. AFS cell systemic diffusion and tissue integration were evaluated by immunofluorescence (i.e. detection of GFP) and molecular biology (i.e. amplification of the *gfp* gene). Both techniques demonstrated that AFS cells mainly distribute in organs of the abdominal compartment, preferentially localizing in the intestine, which resulted positive for GFP in more than 60 % of treated animals. This could be determined by direct diffusion of the cells from the peritoneal surface into the intestinal wall as well as by cell spreading via lymphatic drainage or local blood circulation. Contrarily, intravenously administered AFS cells home primarily to the lung, subsequently colonize kidneys, spleen and liver, but do not distribute into the gut [52].

The potential utility of AFS cells in NEC was also supported by the publication of Good et al, who demonstrated that mouse amniotic fluid injected into the gut wall attenuates the severity of intestinal damage in experimental NEC. The protective agent in amniotic fluid appears to be the epidermal growth factor (EGF), which has an inhibitory effect on TLR4 signalling through the peroxisome proliferator-activated receptor. Inhibition of the EGF receptor with cetuximab or depletion of EGF in the amniotic fluid blocks the inhibitory effects of amniotic fluid on TLR4. Similarly, the amniotic fluid does not prevent TLR4 signalling in EGF receptor-deficient mice or peroxisome proliferator-activated receptor γ-deficient enterocytes [56].

All these data encouraged the evaluation of AFS cell therapeutic role in experimental NEC.

4 Treatment of Experimental NEC with Amniotic Fluid Stem Cells

The potential of AFS cells for the treatment NEC has been recently evaluated in a neonatal rat model of disease [39]. The effects of AFS cells on animal survival and clinical status, as well as on gut damage were primarily investigated along with stem cell mechanism of action.

4.1 Experimental Design

According to the UK Home Office regulations for Animals, a neonatal rat model of disease based on gavage feeding with hyperosmolar formula, hypoxia and oral administration of LPS was employed [22,39]. After 24 h of life, NEC rats were randomized to receive, at 24 and 48 h of life and via i.p. injection, either AFS cells or placebo (i.e. phosphate buffered saline, PBS). Further groups of animals were used as controls: breastfed (BF) rats and NEC rats injected either with adult stem cells (i.e. bone marrow-derived mesenchymal stem cells, BM-MSCs) or with fully differentiated cells (i.e. myoblasts). Animals were followed up until natural death occurred to assess survival or analysed at 96 h of life to investigate AFS cell effect on the intestine as well as post-transplantation localization and trans-differentiation (Fig. 3.3).

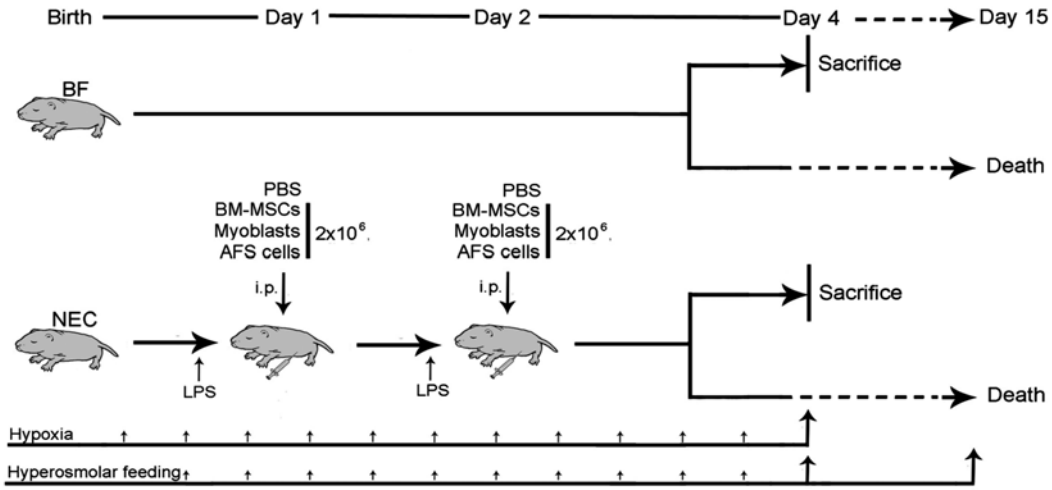


Fig. 3.3 Experimental design. Rats were divided in two main groups: breastfed (BF) and NEC animals. The BF group consisted of rats born at term (E21–22) via vaginal delivery from time-mated pregnant Sprague-Dawley rats; pup rats were housed with their mothers, breastfed ad libitum and not subjected to any stress. The NEC group consisted of rats delivered by Caesarean section at E21, forcedly fed by gavage with artificial milk and exposed to stress (i.e. hypoxia and LPS administration) during their first 4 days of life. At 24 h of life, NEC rats were randomized to receive, at 24 and 48 h of life and via i.p. injection, either cells (2×10^6 AFS cells, BM-MSCs or myoblasts in 50 µl of phosphate buffered saline, PBS) or placebo (i.e. 50 µl of PBS). In a first set of experiments all rats were analysed at 96 h of life, while in a second set of experiments (survival studies) rats were followed up from birth until natural death occurred

AFS cells were obtained from green fluorescent protein (GFP+) transgenic Sprague-Dawley rats at E14; clonal cell lines generated by limited dilution [46] were administered to the animals. BM-MSCs were obtained from the femurs of adult Sprague-Dawley rats following standard protocols [47]. Commercially available rat skeletal muscle myoblasts were employed.

4.2 AFS Cell Effect on Animal Mortality and Morbidity

NEC rats injected with AFS cells survive significantly longer than rats injected with PBS ($p<0.0001$), myoblasts ($p<0.0001$), or BM-MSCs ($p=0.024$). There are no differences in terms of mortality between PBS, myoblast and BM-MSC groups (p=n.s.). As expected BF rats survive significantly longer (94 % survival) than all other groups ($p<0.0001$), confirming that NEC induction is a source of mortality (Fig. 3.4a). The beneficial effect of AFS cells is robust and reproducible, even when tested on a large number of animals (Fig. 3.4b). The ability of AFS cells to lengthen survival is particularly important, as intensive care support and operative treatment given to human neonates with NEC cannot be given to pup rats and this model is therefore not compatible with long-term survival. Moreover, the specificity of this effect to AFS cells is in contrast with other animal models of bowel disease, in which BM-MSCs are effective [31, 29, 32].

At 96 h of life, AFS cells also improve the clinical status of NEC rats as assessed with a validated clinical sickness score, which in this animal model is also a representative marker of gut damage [22] (Fig. 3.4c).

4.3 AFS Cell Effect on Intestinal Morphology and Function

The pronounced and consistent effect of AFS cells on animal morbidity and mortality is determined by decrease of intestinal damage and preservation of gut function, as proven by different indicators.

In situ bowel assessment with high-resolution µMRI shows that, while NEC rats treated with PBS have abundant ascites, thinned gut walls and dilated bowel loops, animals treated with AFS cells are indistinguishable from BF rats (Fig. 3.5a). The radiological appearance of the gut agrees with the better macroscopical and histological appearance of the intestine of AFS cell treated rats as assessed at 96 h of life. Gut appearance of AFS rats is similar to BF rats, with significantly less damage and necrosis than PBS rats (Fig. 3.5b). At histology, AFS cells reduce the incidence of NEC from 75 % (i.e. PBS rats) to 42 % ($p=0.0009$). Villus sloughing, venous congestion and villus core separation are classic histological features of NEC [22]; AFS cell-treated animals have a normal intestinal architecture with decreased incidence of all of these hallmarks (Fig. 3.5c).

AFS cells also have a protective effect on intestinal function. Intestinal motility, measured with carmine red transit, is severely decreased in NEC rats injected with PBS in comparison with BF rats, but it is preserved in the group

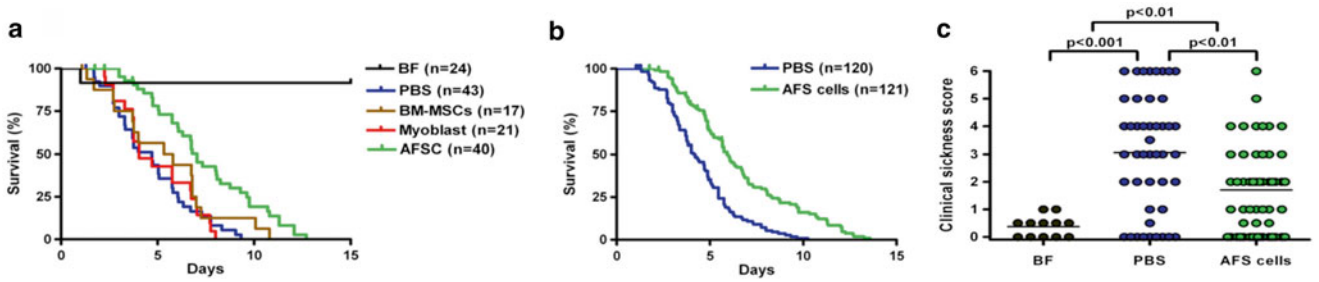


Fig. 3.4 AFS cell effect on animal mortality and morbidity. (**a**) When treated with AFS cells, NEC rats prove to survive significantly longer than NEC rats treated with BM-MSCs ($p=0.024$), PBS ($p<0.0001$) and myoblasts ($p<0.0001$). (**b**) This effect of AFS cells is extremely reproducible, as cumulative results of several experiments show a consistent survival benefit of NEC rats injected with AFS cells compared with those receiving PBS alone ($p<0.0001$). (**c**) Morbidity, evaluated using a validated clinical sickness score, also shows a significant benefit of AFS cell treatment in comparison with PBS ($p<0.001$), although AFS cell-treated rats are still sick compared with BF rats ($p<0.01$)

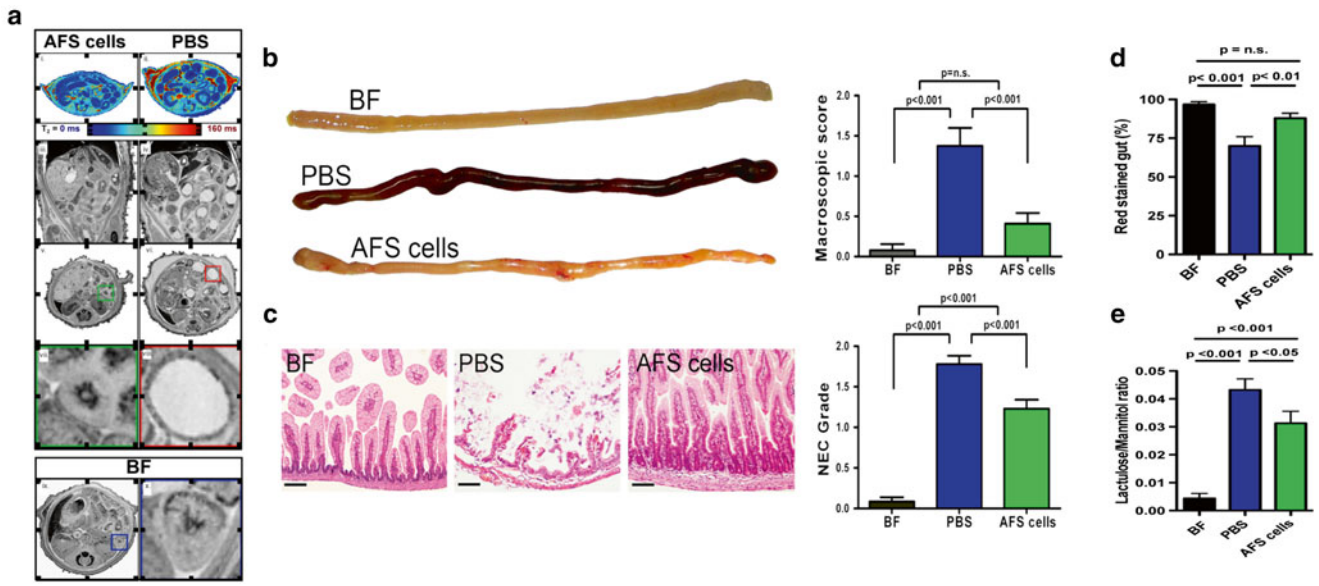


Fig. 3.5 AFS cell effect on intestinal morphology and function. (**a**) MRI of AFS cell-treated NEC rats (*left column* of images) and untreated rats (*right column* of images). Row 1 (*i* and *ii*): degree of ascites measured using T2 maps: the total number of voxels with T2 > 160 ms identified as dark red regions which indicates areas of fluid accumulation are different between the PBS (1682 ± 453) and AFS (224 ± 135, $p<0.05$) groups which do not differ from the BF group (278 ± 27). Row 2 (*iii* and *iv*): bowel wall thickness using MRI images: marked structural changes are observed in the untreated rats. Row 3 (*v* and *vi*): representative axial slices demonstrate a similar pattern. Row 4 (*vii* and *viii*): magnified images of bowel loops from the respective axial slices highlight the loss of bowel wall integrity in the untreated rats. Row 5 (*ix* and *x*): representative axial slices of BF rats and magnified image of bowel loop showing normal intestinal architecture (**b**, **c**). AFS cells decrease macroscopic and microscopic intestinal damage. (**b**) Examples of the macroscopic gut appearance of BF rats and NEC rats injected with PBS or AFS cells. Gut damage of NEC rats injected with AFS cells (0.4 ± 0.7, $n=27$) is significantly smaller than in animals injected with PBS (1.4 ± 1.1 $n=24$, $p<0.001$), but shows no difference with BF rats (0.1 ± 0.3; $n=12$, $p=$n.s.). (**c**) NEC rats treated with AFS cells reveal significantly less histological damage (0.23 ± 0.8, $n=48$, $p<0.001$) in comparison with NEC rats treated with PBS (1.78 ± 0.7, $n=50$); no damage is observed in BF rats (0.08 ± 0.2, $n=12$). (**d**) Carmine red solution administration reveals that motility is decreased in NEC rats injected with PBS ($p<0.001$) but it is normal in rats injected with AFS cells ($p=$n.s.) when compared with BF. (**e**) Intestinal permeability, measured as plasma lactulose/mannitol ratio: in comparison with BF rats (0.004 ± 0.002, $n=9$), PBS rats have a significant increase in intestinal permeability (0.043 ± 0.004; $n=21$, $p<0.001$), which is restored in AFS-treated rats (0.031 ± 0.004; $n=25$, $p<0.05$)

receiving AFS cells (Fig. 3.5d). Although gut weight and length do not differ among the groups, carmine red completes gut transit in 75 % of BF rats, 19 % of PBS rats and 47 % of those receiving AFS cells. This is confirmed by organ bath studies in which only the intestine from AFS rats shows spontaneous contractions, resembling peristalsis. Similarly, while intestinal permeability (measured by plasma lactulose/mannitol ratio) is significantly higher in PBS rats compared with BF rats, AFS cell injection in NEC rats partially prevents this increase (Fig. 3.5e).

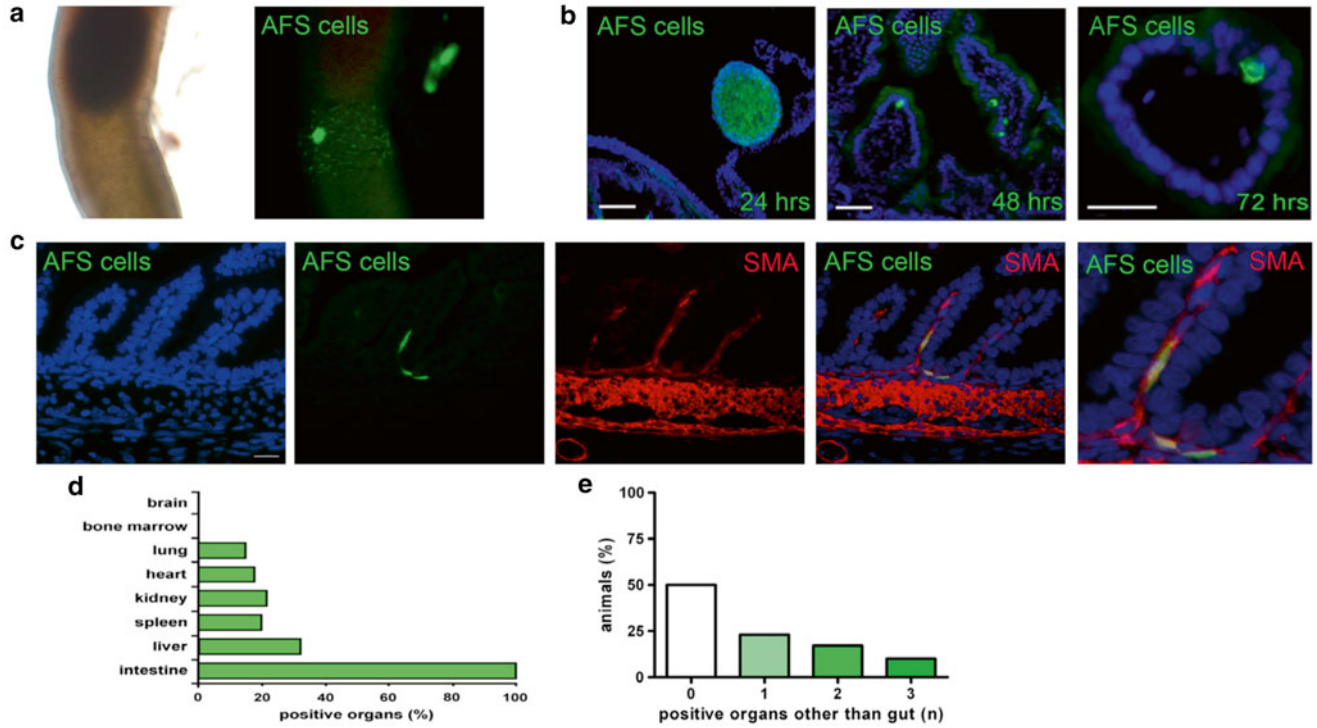


Fig. 3.6 Amniotic fluid stem (AFS) post-transplantation localization and differentiation. (**a**) Macroscopic appearance of bundles of AFS cells injected i.p. on the mesentery and on the gut wall. (**b**) GFP epifluorescence from AFS cells: 24 h after injection cells are adherent to the serosa of the intestine. At 48 h and 72 h rare AFS cells are found within the villus structure in close junction to the epithelial layer. (**c**) Sections of rat ileum stained with anti-GFP antibodies to localize AFS cells (*green*) and anti-smooth muscle actin (SMA) antibodies to label smooth muscle cells (*red*) (scale bar 20 μm). AFS cells co-expressing GFP and SMA are found integrated in the mucosal layer. (**d**, **e**). PCR analysis of gfp DNA in organs of animals injected with GFP+AFS cells; AFS always migrate into the intestine (**d**) and in 50 % also to various other abdominal and thoracic organs (**e**)

4.4 AFS Cell Post-Transplantation Localization and Differentiation

After i.p. injection AFS cells migrate into the damaged intestine (Fig. 3.6a), integrate in the tissue and differentiate into committed cells. AFS cells exhibit various degrees of distribution mainly depending on the time occurring from their i.p. injection: after 24 h, cell bundles are adherent to the mesentery (Fig. 3.6b); at 48 h, AFS cells localize into gut wall (Fig. 3.6b); at 72 h few AFS cells migrate in the mucosa where they can express markers of mesenchymal differentiation (Fig. 3.6b, c) but not of epithelial or neuronal commitment. As demonstrated by PCR amplification of the *gfp* gene, the intestine is always positive for GFP (i.e. 100 % of the animals injected with AFS cells), while liver is positive in 32 % of animals, kidneys in 21 %, spleen in 20 %, heart in 17 % and lungs in 15 %; no GFP signal is ever detectable either in the brain or bones/bone marrow (Fig. 3.6d, e). Thus, while AFS cells administered intravenously home primarily in the lung and do not distribute to the gut [52], and AFS cells injected i.p. into healthy newborn rats colonize the gut in less than 70 % of the animals [55], when injected i.p. into pup rats affected by NEC AFS cells localize in the gut in 100 % of animals, homing to the mesentery or to the gut wall.

4.5 AFS Cell Mechanism of Action

As suggested by the above reported data, the beneficial role of AFS cells on animal outcome and intestinal damage is closely related to their presence in the gut. However, as the improvements in morbidity and mortality occur within hours after cell administration, at which time relatively small numbers of AFS cells can be found in the bowel, their direct contribution to tissue regeneration is unlikely to be the major mechanism for AFS cell beneficial effect on the intestine. This is also in accordance with the low engraftment rate of stem cells in the intestinal wall observed at 96 h and with the restricted differentiation of engrafted cells towards the epithelial, mesenchymal and neuronal lineage at that time.

The aforementioned observations suggest that AFS cells could act by releasing one or more substance(s) harbouring a trophic effect on intestinal resident progenitor cells. This paracrine mechanism of action is demonstrated by a survival study directly comparing AFS cells vs. AFS cell-conditioned medium (CM): AFS cells and AFS cell-CM equally improve rat survival in comparison to both PBS ($p<0.01$) and not conditioned medium (i.e. α-MEM; $p<0.0001$) (Fig. 3.7). Moreover, by hierarchical cluster analysis of intestinal cDNA arrays, the intestine of NEC rats receiving placebo (i.e. PBS

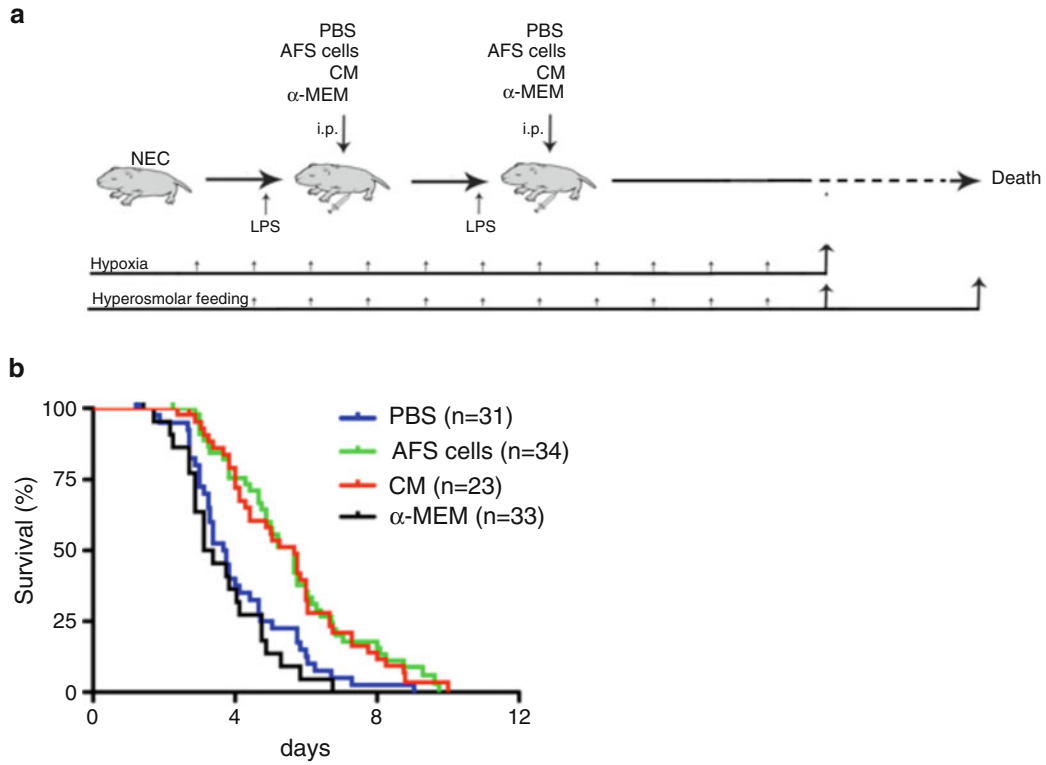


Fig. 3.7 Survival study comparing the effect of AFS cells vs. AFS cell-conditioned medium. (**a**) Experimental design. At 24 and 48 h of life NEC rats received an i.p. injection of either: (1) 2×10^6 AFS cells in 50 µl of PBS (AFS cells group), (2) 50 µl of serum-free medium conditioned by AFS cells (CM group), (3) 50 µl of serum-free medium not conditioned by AFS cells (α-MEM group) and (4) 50 µl of PBS alone (PBS group). (**b**) The survival of rats injected with AFS cell-conditioned medium is similar to that of rats treated with AFS cells (p = n.s.), while is significantly superior to that of NEC rats injected with PBS ($p<0.01$) or with α-MEM ($p<0.0001$). No differences are observed among rats treated with PBS or α-MEM p = n.s.)

or α-MEM) is clearly distinguishable from that of animals treated with AFS cells or AFS-CM (Fig. 3.8a). Unsurprisingly, the genes with the largest expression differences are mainly involved in inflammation and tissue repair (e.g. Aoc3, Itgb6), cell cycle regulation (e.g. Atf2, Dusp16, Gpx4, Mxd1) and enterocyte differentiation processes (e.g. Acsl5, Rab8a, Thra) suggesting, in accordance to other authors [57], that damage resolution in NEC is achieved via activation of multiple pathways acting on tissue inflammation, cell apoptosis and proliferation. These gene expression differences are confirmed by biochemical and immunohistochemical assays evaluating general parameters of inflammation, epithelial proliferation and apoptosis. In NEC rats, AFS cells: reduce lipid peroxidation (MDA level; Fig. 3.8b) and neutrophil infiltration (MPO activity; Fig. 3.8c); decrease apoptosis (cleaved caspase 3 immunohistochemistry; Fig. 3.8d), particularly in the crypts (positive cells in 45 % of PBS rats with NEC vs 12 % of AFS rats, $p<0.05$; Fig. 3.8e); and promote enterocyte migration from crypts to villus tips (EdU staining; Fig. 3.8f, g).

Intriguingly, inducible cyclooxygenase 2 (COX-2), normally expressed at low levels in the gut [58], has been reported to have effects similar to those of AFS cells: it decreases enterocyte apoptosis [59, 60], diminishes granulocyte infiltration [61 62] and promotes epithelial proliferation [63]. Moreover, it has been demonstrated that stromal cells expressing COX-2 present in the lamina propria of the intestinal mucosa are necessary to maintain the proliferation of colonic epithelial progenitors after damage [41].

Based on these evidences, the presence of stromal cells expressing COX-2 in the lamina propria has been also investigated in experimental NEC [39]. COX-2+ cells are detectable in the lamina propria of newborn healthy rats (i.e. BF rats) as well as of animals affected by NEC either receiving placebo (i.e. PBS) or AFS cells (Fig. 3.9a); however, substantial differences are present among these groups of animals concerning COX-2+ cell number and location. COX-2+ cells are markedly diminished in PBS rats in comparison to BF rats and AFS rats (Fig. 3.9b). While the number of COX-2+ cells in the villus axis is similar in AFS rats and BF rats (Fig. 3.9c), cryptal COX-2+ cells are increased in AFS rats compared with BF rats and PBS rats (Fig. 3.9d). Moreover, the number of COX-2+ cells per villus unit (Fig. 3.9e) and the number of cryptal COX-2+ cells (Fig. 3.9f) inversely correlate with the degree of intestinal damage. A survival study using COX-1 and COX-2 inhibitors confirms that the beneficial effects of AFS cells are COX-2 dependent. BF rats and NEC rats receiving PBS or AFS cells were randomized to receive: (1) vehicle; (2) sc-560 (COX-1 inhibitor);

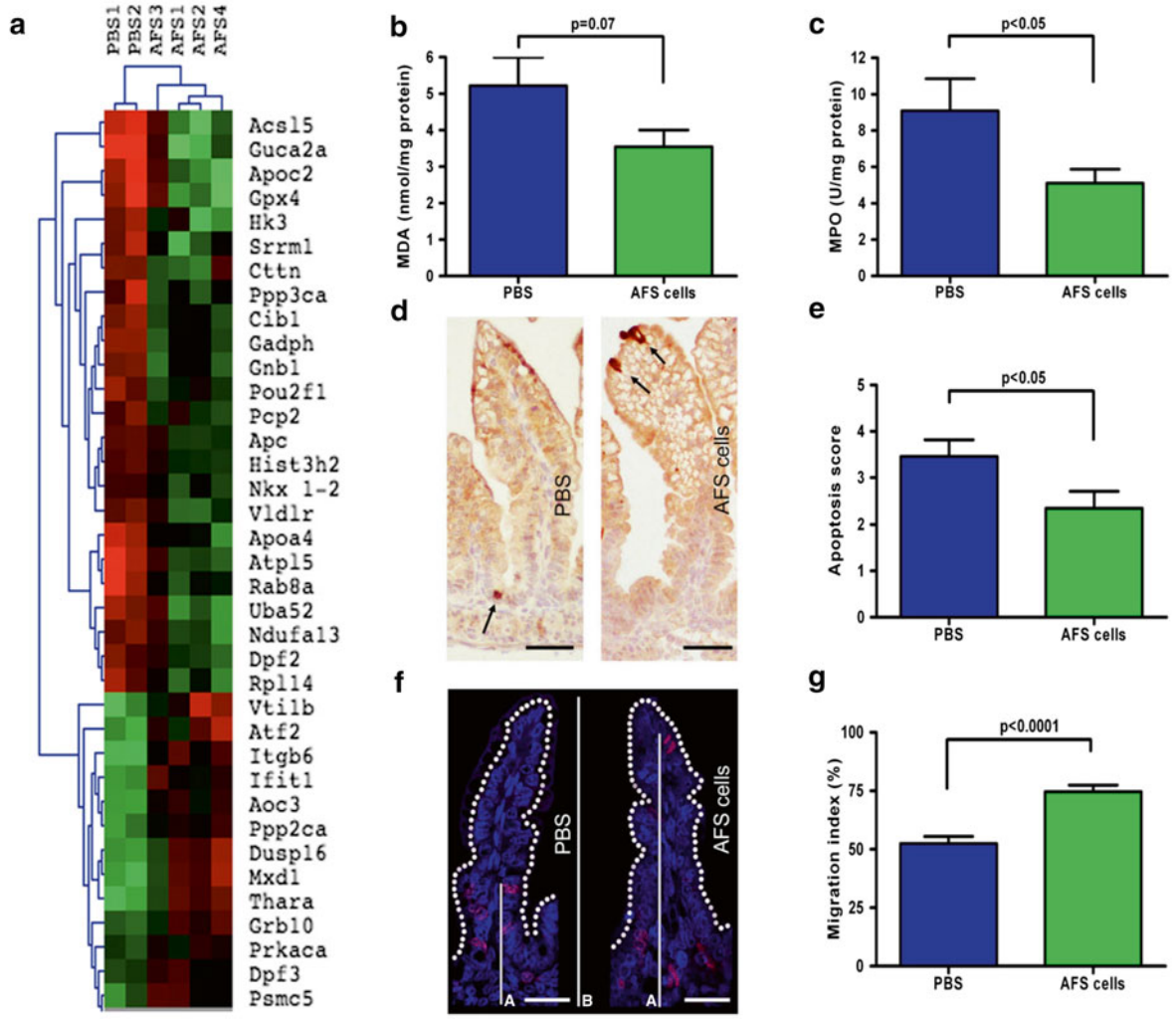


Fig. 3.8 Amniotic fluid stem (AFS) cells decrease gut inflammation and enterocyte apoptosis and promote enterocyte proliferation/migration. (**a**) Cluster analysis of cDNA microarray data. The intestinal gene expression profile of NEC rats receiving phosphate buffered saline (PBS) is significantly different from that of NEC rats treated with AFS cells as revealed with a two-dimensional hierarchical clustering by the Euclidean distance. *Rows* correspond to genes, and *columns* to NEC rats treated groups (PBS: PBS1, PBS2; AFS cells: AFS1, AFS2, AFS3, AFS4); *red* and *green*, respectively, indicate gene upregulation and downregulation relative to a reference sample consisting of a pool of intestinal RNA from five different breastfed rats. Thirty-seven genes, mainly involving inflammation, tissue repair, cell differentiation and cell cycle regulation, result able to differentiate the two groups of animals at a molecular level. (**b**) NEC rats injected with AFS cells show lower degree of intestinal lipid peroxidation (3.6±2.7 nmol/mg protein), measured as malondialdehyde (MDA) level, when compared with NEC rats injected with PBS (5.2±4.6; p=n.s.). (**c**) Gut neutrophil infiltration, measured as myeloperoxidase (MPO) activity, is significantly lower in NEC rats treated with AFS cells than in those injected with PBS (AFS cells 5.1±0.8 U/mg protein; PBS 9.1±1.7; $p<0.05$). (**d**) Cleaved caspase 3 immunostaining of terminal ileum reveals apoptotic cells (*arrows*) in the villus crypts of NEC rats injected with PBS and at the villus tips of NEC rats injected with AFS cells. (**e**) The apoptotic index, scored blindly as expression of cleaved caspase 3, is significantly lower in NEC rats injected with AFS cells than in those injected with PBS (p=0.04). (**f**) Fluorescence detection of dividing cells stained with EdU for cell migration/proliferation (EdU, *purple*, counterstained with DAPI, *blue*) of terminal ileum of NEC rats injected with PBS or AFS cells. Enterocyte migration/proliferation is calculated as percentage of the foremost EdU-labelled enterocyte (A) over the total mucosal thickness (B). (**g**) The enterocyte migration/proliferation index assessed by EdU is significantly increased in NEC rats treated with AFS cells than in those treated with PBS (75 % vs. 53 %, $p<0.0001$)

(3) ibuprofen (COX-1+2 inhibitor); and (4) celecoxib (COX-2 inhibitor). As expected, NEC rats treated with AFS cells + vehicle survive significantly longer (Fig. 3.9g) than NEC rats treated with PBS+ vehicle. The survival effect of AFS cells is abolished by the selective COX-2 and the non-selective COX-1+2 inhibitors, but is unaffected by the selective COX-1 inhibitor (Fig. 3.9g). Similarly, the improved clinical status in AFS rats is annulled by COX-2 inhibition, reduced by COX-1+2 inhibition and unaffected by COX-1 inhibition (Fig. 3.9h). None of the COX inhibitors modifies survival (Fig. 3.9g) or clinical status (data not shown) of PBS or BF rats [39].

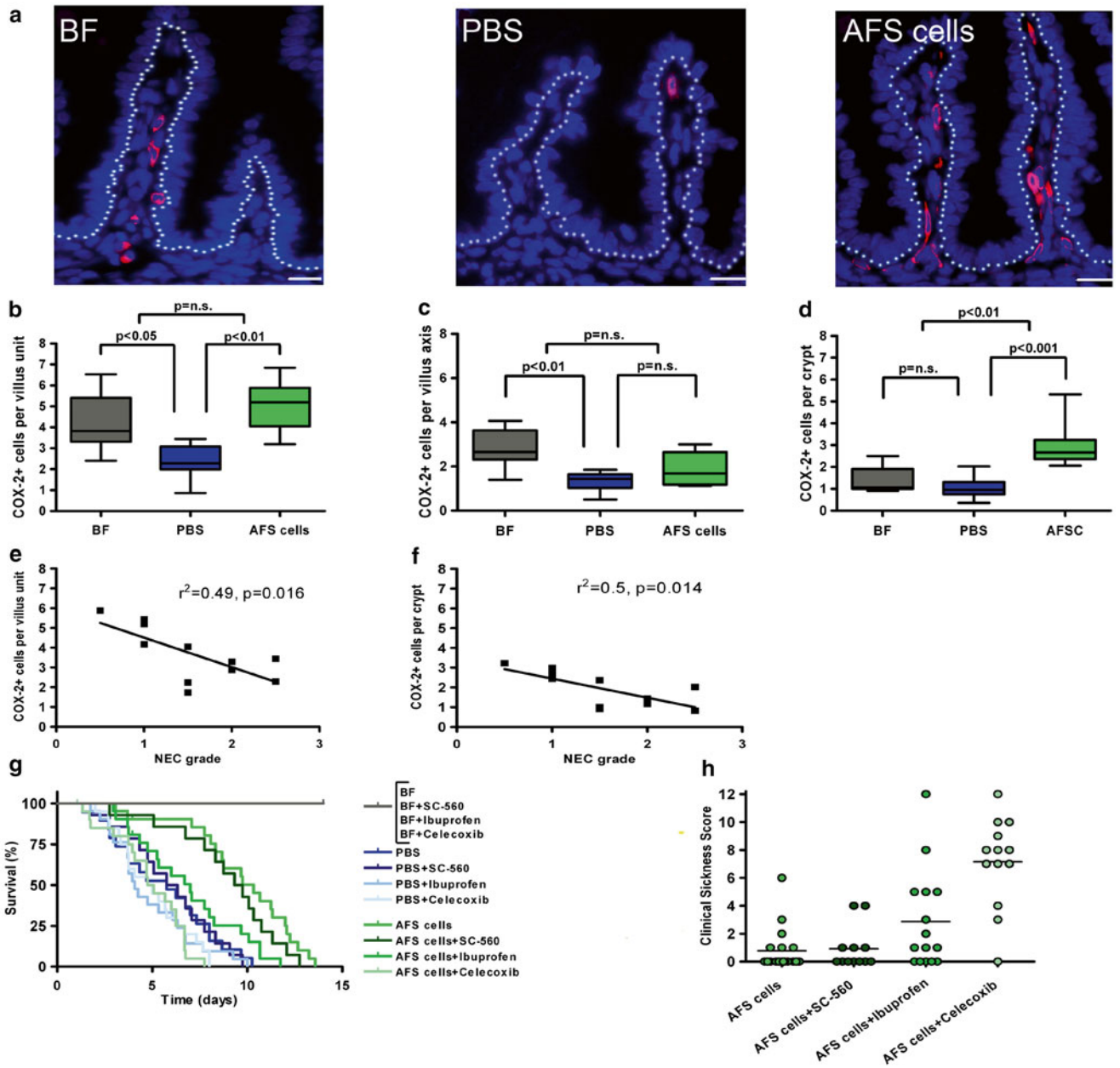


Fig. 3.9 Amniotic fluid stem (AFS) cells modulate stromal cells expressing COX-2 in the lamina propria. (**a**) Representative 3 µm sections of the terminal ileum from BF rats and NEC rats receiving PBS and AFS cells stained with anti-COX-2 Ig (*red*) and DAPI (*blue*). Scale bars: 20 µm. (**b**) In NEC rats treated with AFS cells, the number of COX-2+ cells per villus unit is similar to that of BF rats but higher compared to NEC rats treated with PBS. This difference is not determined by the quantity of COX-2+ cells in the villi, which is similar between the groups (**c**), but by their number underlying the cryptae which is higher in NEC rats treated with AFS cells (**d**). (**e**, **f**) The number of COX-2+ cells per villus unit and in the cryptae inversely correlates with the histological grade of NEC by linear regression. (**g**) A survival study employing selective and non-selective COX inhibitors shows that: (1) COX inhibitors do not modify the survival of BF rats (BF + vehicle vs. BF + sc-560, BF + ibuprofen, BF + celecoxib: $p = $ n.s.) and NEC rats receiving PBS (PBS + vehicle vs. PBS + sc-560, PBS + ibuprofen, PBS + celecoxib: p = n.s.); (2) the improved survival of NEC rats receiving AFS cells is annulled by COX-2 (AFS cells + celecoxib vs. AFS cells + vehicle: $p < 0.0001$; AFS cells + celecoxib vs. PBS + vehicle: $p = $ n.s.) and COX-1 + 2 inhibitors (AFS cells + ibuprofen vs. AFS cells + vehicle: $p < 0.01$; AFS cells + ibuprofen vs. PBS + vehicle: $p = $ n.s.), but conserved in rats receiving COX-1 inhibitor (AFS cells + sc-560 vs. AFS cells + vehicle: $p = $ n.s.; AFS cells + sc-560 vs. PBS + vehicle: $p = 0.001$). (**h**) The clinical sickness score improvement observed in NEC rats treated with AFS cells (0.77 ± 0.36) is abolished by COX-2 inhibitor (7.15 ± 0.89; AFS cells + celecoxib vs. AFS cells + vehicle: $p < 0.001$), diminished by COX-1 + 2 inhibitor (2.86 ± 0.91; AFS cells + ibuprofen vs. AFS cells + vehicle: $p = $ n.s.) and unaltered by COX-1 inhibitor (0.92 ± 0.39; AFS cells + sc-560 vs. AFS cells + vehicle: $p = $ n.s.)

Conflicting results exist concerning the role of COX-2 in NEC. On the one hand, the perinatal and postnatal exposure to glucocorticoids and non-steroidal anti-inflammatory drugs, which inhibit COX-2 activity, increase the risk of developing NEC in neonates; additionally, both in human and experimental NEC, high levels of COX-2 expression may contribute to disease development through pro-inflammatory effects [64, 65, 66]. On the other hand, systemic COX-2 inhibitors worsen intestinal inflammation and increase mortality in experimental NEC, while COX-2 protects against the apoptosis of intestinal epithelial cells associated with NEC [19, 67]. In contrast to the study of Zani et al., in previous reports COX-2 expression was evaluated throughout the whole intestinal wall (i.e. not restricted to the mucosal layer) and no correlation was found between COX-2 expression and the severity of intestinal injury [19]. These different findings can be reconciled when the localization of COX-2+ cells is taken into account: the proliferation of COX-2+ cells present in the lamina propria and their migration of COX-2+ cells towards the pericryptal area promotes epithelial proliferation while preventing apoptosis. This is in agreement with the demonstration that the repositioning of COX-2+ cells underneath the crypts is necessary to maintain the proliferation of epithelial progenitors after colonic injury with dextran sodium sulphate [41]. Migration of COX-2-expressing stromal cells from the lamina propria of the villus to the lamina propria adjacent to crypt epithelial cells has also been described in response to oral administration of lactobacillus GG, or intraperitoneal administration of hyaluronic acid in models of intestinal radiation-induced injury [68, 69]. Both oral lactobacillus GG and intraperitoneal hyaluronic acid are radioprotective in the small intestine; their administration is associated with decreased radiation-induced apoptosis and increased survival of crypt epithelial stem cells.

It is known that COX-2 expression can be enhanced by different pathways (e.g. TLR-Myd88, cytokine and growth factor receptors) [41, 68, 69, 70] and it has been recently demonstrated that COX-2+ cells in the lamina propria are stimulated by FGF9 [71]. Concerning the mechanism by which COX-2+ cells are specifically activated by AFS cells, in vitro experiments show that, after LPS stimulation and compared with BM-MSCs cells and myoblasts, AFS cells respond increasing the expression of VEGFα, FGF-2, TGFβ1, TGFα, PDGF β (Fig. 3.10a). These growth factors are able to induce COX-2 directly (i.e. FGF-2, PDGFβ, TGFβ1, VEGFα) or via activation of the epidermal growth factor receptor (EGFR) (i.e. TGFα, which is itself a ligand for the EGFR) [39]. In fact, COX-2 products (i.e. PGE2) activate the EGFR but also the EGFR plays an important role in the induction of COX-2 expression [72]. AFS cells do also express the primary EGFR ligand (i.e. EGF) in basal conditions, but, differently from the other growth factors tested; they do not modify its expression after LPS stimulation [39]. Notably, as well as COX-2, EGFR activation in enterocytes stimulates mucosal repair after injury, promotes cell survival, reduces intestinal inflammation and protects against experimental NEC [73, 72, 74].

The different expression profile of AFS cells vs. BM-MSCs is also confirmed by cluster analysis of cDNA microarray data; in comparison with BM-MSCs, AFS cells differentially express genes involved in the wnt-β catenin pathway which regulate intestinal epithelial stem cell function (e.g., AXIN, APC and CTNNA1) and cell migration (CXCL12), and growth factors known to maintain gut epithelial integrity and to reduce mucosal injury in experimental inflammatory bowel disease (e.g. IGF-1, FGF-1, FGF-3 and FGF-4, fibroblast growth receptor-1; Fig. 3.10b) 39].

4.6 Open Questions

The results provided by the study of Zani et al. [39], raise the possibility that AFS cells or some component(s) of AFS cell-conditioned medium might be effective in treating NEC. A new mechanism for NEC treatment is suggested: migration of COX-2+ stromal cells present in the lamina propria from the villus axis to the pericryptal area, where resident intestinal stem cells reside [75]. These data raise a number of questions both concerning the mechanism of action of activated COX-2+ cells and the definition of the factors able to activate COX-2+ cells. (1) As the beneficial effects of lactobacillus GG and hyaluronic acid in models of intestinal radiation-induced injury are similarly COX-2 dependent but also, respectively, TLR2 and TLR4 dependent [68, 69], whether the protective effects of AFS cells in experimental NEC model are dependent on TLR signalling needs to be investigated. Moreover, since the protective effect of EGF in NEC is determined by the EGF receptor through the peroxisome proliferator-activated receptor, the effect of AFS cells (or AFS cell-conditioned media) in EGF receptor-deficient animals should be directly addressed. Finally, the capacity of misoprostol, a stable PGE2 analogue, as well as lactobacillus GG and hyaluronic acid to prevent/treat experimental NEC ought to be tested. (2) Experiments should be performed to recognize, among the factors secreted by AFS cells and known to potentially induce COX-2, those able to able to specifically activate COX-2+ cells in the lamina propria of the intestine. This could possibly permit to identify novel therapeutic agents for the treatment of NEC.

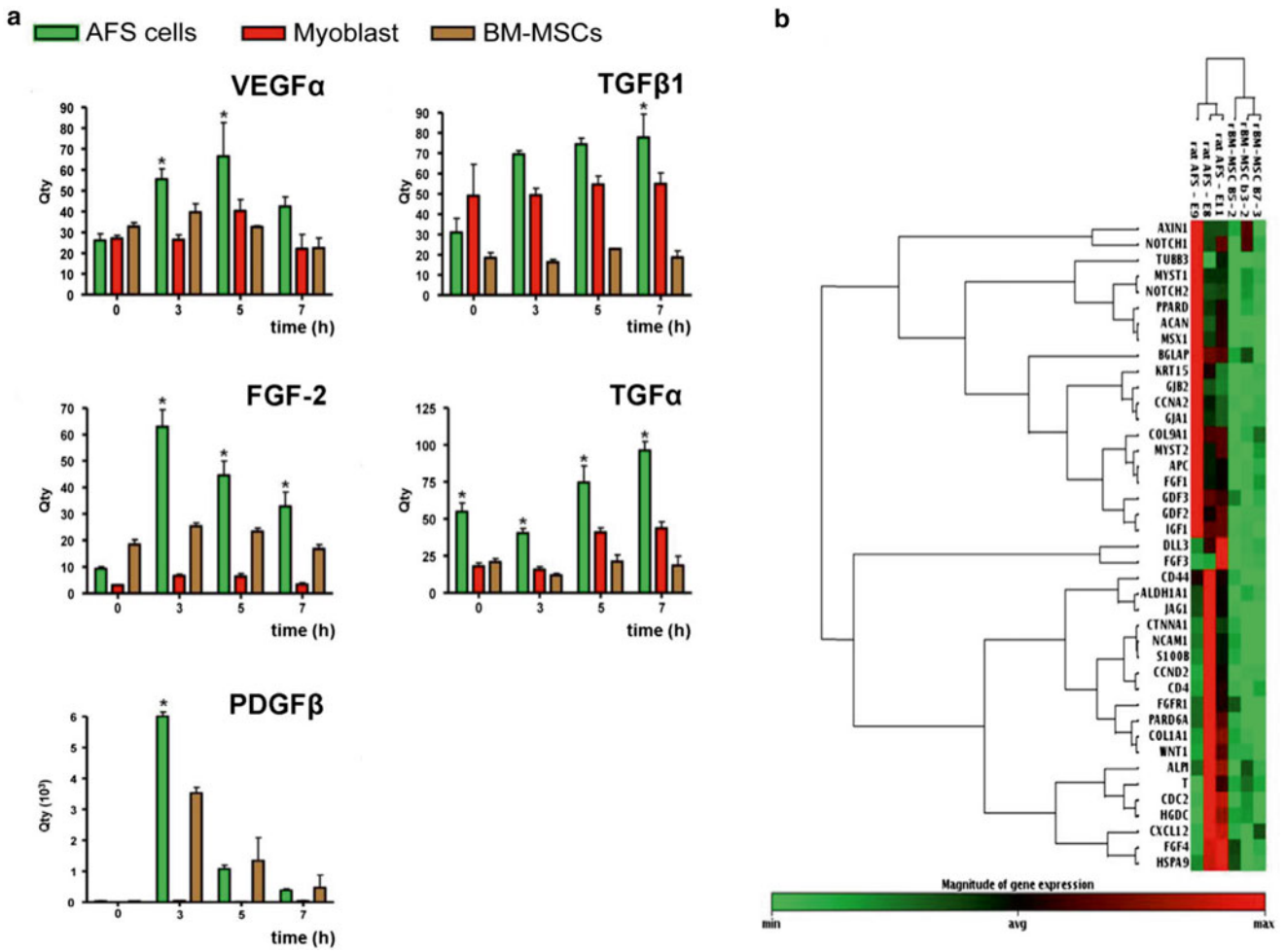


Fig. 3.10 (**a**) Amniotic sluid stem (AFS) cells increase the expression of several growth factors in response to lipopolysaccharide (LPS) exposure. In order to evaluate specific factors, which may be induced by the NEC environment, AFS cells, myoblasts and BM-MSCs were cultured in the presence of 1 μg/ml LPS. RNA was extracted from the different cells at basal level (time 0) and at 3 h from LPS stimulation and retrotranscribed into cDNA. Levels of transcription factors were measured by real time PCR (Sybr *green*) after normalization with three different housekeeping genes (gadph, β2m, βact). AFS cells respond to LPS stimulation progressively increasing the expression of VEGFα (b), Tgfβ1 (c), Fgf2 (d), TGFα (e) and PDGFβ (f). In the myoblast lines and BM-MSCs, the relative yield of these transcripts remains substantially unchanged ($*p<0.05$). (**b**) RT2 profiler PCR array system comparing three clonal cell lines of AFS cells (E8, E9, E11) with three from bone marrow mesenchymal stem cells (BM-MSCs) preparations (B3-2, B5-2, B7-3) used in the experiments: there is an upregulation of AFS cells expressed genes involved in the wnt-β catenin pathway, cell migration and several growth factors

5 Conclusions

When injected in a widely used model of experimental NEC, AFS cells improve survival and clinical status of the animals and significant reduce gut damage. These beneficial effects do not appear to be due to repopulation of damaged intestine by AFS cells, but instead are probably related to their paracrine effects. These latter include a decrease in inflammation and apoptosis and a concomitant increase in enterocyte proliferation and migration, thus aiding epithelial restitution. COX-2+ cells may mediate these effects, as on the one hand their presence in the cryptae is significantly enhanced by AFS cell injection, and on the other hand COX-2 inhibitors completely abolish AFS cell beneficial effects (Fig. 3.11; Box 3.2).

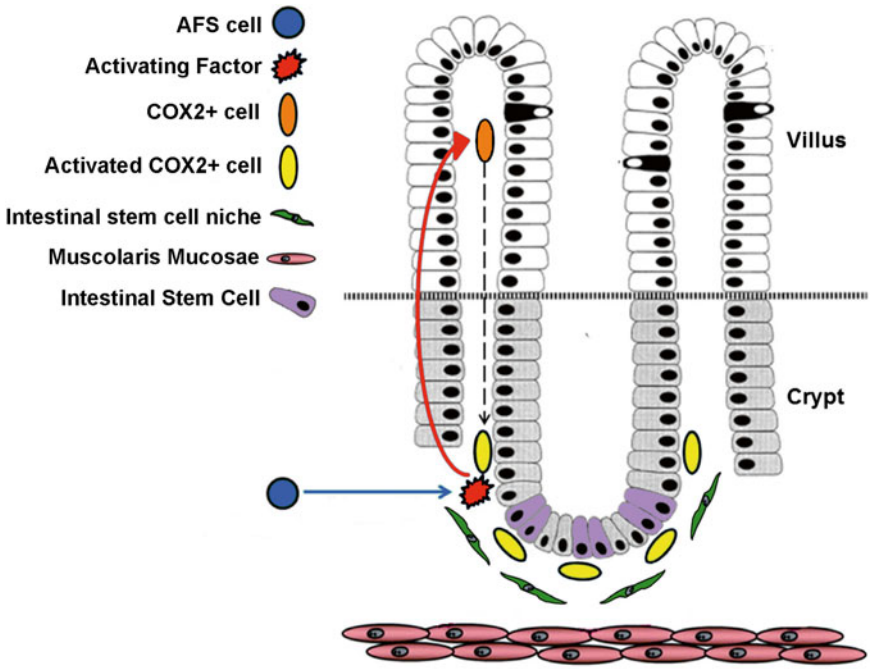


Fig. 3.11 Proposed mechanism of action of AFS cells in NEC. AFS cells secrete one or more activating factor(s) that directly or indirectly (e.g. via pericryptal macrophages, myofibroblasts or perhaps crypt epithelial cells) activate stromal cells expressing COX-2 present in the lamina propria. Once activated COX-2+ cells increase in number and migrate from the villus axis to the pericryptal lamina propria, where they respectively stimulate the proliferation and reduce the apoptosis of intestinal epithelial cells

Box 3.2 Therapeutic Potential of AFS cells in Experimental NEC

In a well-established neonatal rat model of NEC, AFS cells

Improve animal morbidity and mortality, reduce NEC incidence and recuperate intestinal function.

Engraft into the damaged intestine and migrate systemically.

Decrease gut inflammation and enterocyte apoptosis and promote enterocyte proliferation.

Act by modulating stromal cells expressing COX-2 in the lamina propria through a paracrine mechanism.

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Therapeutic Application of Amniotic Fluid Stem Cells for Graft-Versus-Host Disease

4

Emily C. Moorefield, Mark E. Furth, and Colin E. Bishop

Abstract

Amniotic fluid stem (AFS) cells are a renewable cell source possessing immunomodulatory properties, making them a prime candidate for the treatment of a variety of inflammatory and immune disorders, including graft-versus-host disease (GVHD). GVHD is a complication of allogeneic transplantation that occurs when immune cells of the donor graft recognize host tissues as foreign. The immune-mediated attack that ensues threatens graft survival and damages host tissues and organs. The high incidence and high transplant-associated mortality rate associated with GVHD makes prevention and treatment key for successful transplantation. Current GVHD treatment includes high doses of broad-acting corticosteroids used to suppress the immune response. While effective in some patients, corticosteroid therapy has harsh side effects and a subset of patients is unresponsive. Stem cell-based therapy may offer a more targeted approach to suppress immune function in the treatment of GVHD. Beyond their ability to mediate the immune response, AFS cells can be expanded indefinitely in culture and can be fully characterized and banked for future use, making them a superior cell source for the treatment of GVHD.

1 Introduction

With the rapid advancement of the cell and tissue transplantation fields comes a great need for improved ability to mediate the immune response. Graft-versus-host disease (GVHD) is a common complication of allogeneic transplantation and is the result of immune cells from the donor source (graft) reacting against the tissues of the recipient (host). Pharmacological therapies including high-dose corticosteroids are most often used in response to GVHD to suppress the donor immune response [1, 2]. These harsh, broad-spectrum immunosuppressive regimens target T cells and carry with them the risks of severe side effects including infection and malignancy. In addition, up to half of GVHD sufferers are unable to respond to corticosteroid therapy [1]. These steroid-refractory patients have an extremely poor prognosis, making the identification of alternative therapeutics critical. Current efforts are focused on identification of new and improved therapies that more selectively mediate the immune response and have fewer side effects.

Cell-based immunomodulation offers a targeted and preventative approach to altering the immune cells and immune response of the donor. Stem cells possessing immunomodulatory capabilities offer an exciting opportunity as a renewable source of GVHD treatment without the negative side effects associated with pharmacologics. Mesenchymal stromal cells derived from the bone marrow (BM-MSCs) have

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shown promising results in clinical trials for the treatment of GVHD and other inflammatory disorders. However, there are several drawbacks involved with using BM-MSCs clinically and other cell sources are currently being examined.

The amniotic fluid comprises multiple fetal-derived cell types including cells from the fetal skin, respiratory system, urinary and gastrointestinal tracts, and populations of mesenchymal stromal cell (MSCs) [3–6]. Stem cells have been isolated from amniotic fluid based on expression of the cell-surface receptor for stem cell factor (SCF), c-kit (CD117) [7]. Amniotic fluid stem (AFS) cells are a renewable source of stem cells with immunosuppressive capabilities that are ideal for GVHD therapeutics.

2 Allogeneic Transplantation and the Immune Response

2.1 The Immune Response and Recognition of Nonself

The immune system is made up of a complex array of protective cell types and processes that aim to recognize and eliminate invading pathogens. The immune response also plays an important role in defending against cancer by recognizing and destroying transformed cells. The adaptive immune response takes several days to mount and is made up of antigen-specific reactions by T cells and B cells. Immune cells secrete cytokines, soluble factors that are important in mediating the adaptive immune response. Discrimination between self and nonself is a critical factor in effective immune response and is primarily based on recognition of receptors or antigens present on pathogens or atypical cells but not present on host cells.

Major histocompatibility complex (MHC) molecules, termed human leukocyte antigen (HLA) in humans, are displayed on the surface of every cell and are responsible for presenting antigens to T cells. Individuals have distinct HLA expression profiles as a result of the complex genetic organization and inheritance of these molecules [8]. In the MHC class I region the classical HLA-A, -B and -C and nonclassical HLA-D, -E, -F, and -G genes are encoded. In the MHC class II region, HLA-DR, -DQ and -DP molecules are encoded [8]. MHC class I molecules are constitutively expressed on most cell types and bind intracellular peptides for presentation to cytotoxic T cells (Tc) [6]. While MHC Class II molecules are constitutively expressed primarily by professional antigen-presenting cells (APCs) including macrophages, B cells and dendritic cells (DCs) and bind and present peptides to helper T (Th) cells [9].

Allorecognition occurs when donor T cells recognize and respond to the foreign host. Direct allorecognition occurs when donor T cells discover and react to the foreign MHC molecule while indirect allorecognition is dependent on foreign antigen presentation for donor T cell detection [10]. In GVHD, both direct and indirect allorecognition occur, making matching of donor and recipient HLA-I and -II genes a critical requirement for transplantation engraftment and success [11]. The risk of transplantation treatment failure is significantly reduced when HLA-A, -B, -C, -DRB1, and -DRBQ are allele-matched between donor and recipient [12, 13]. However, even with careful matching, genetic polymorphisms in minor histocompatibility (H) genes may still produce alloantigens recognized by the host immune system [14].

2.2 Allogeneic Hematopoietic Stem Cell Transplantation

Allogeneic transplantation involves the transfer of cells or tissues from a donor source to a recipient patient for disease treatment. Allogeneic hematopoietic stem cell transplantation (HSCT) is an established procedure commonly used in the treatment of several hematological conditions including cancer and more recently autoimmune conditions [15]. The goal of HSCT is to ablate defective patient bone marrow and reconstitute with hematopoietic stem cells (HSCs) from a normal donor. In this procedure, HSCs are extracted directly from the donor bone marrow or from the blood following mobilization [16]. The cells are then transplanted into a recipient patient who has undergone a conditioning procedure to eliminate their own bone marrow and allow for donor engraftment. The patient bone marrow can then be reconstituted with the donor HSCs. In HSCT, the donor HSC graft contains a mixture of cells made up of stem cells that have the ability to repopulate the bone marrow and mature immune cells including T cells.

3 Graft-Versus-Host Disease

The major complication and cause of death in cases of allogeneic HSCT is GVHD [17, 18]. GVHD is a life threatening condition in which immune cells from the donor identify the host as foreign based on MHC recognition and mount an uncontrolled response against host tissues. The immunologically competent donor cells recognize the host tissue as foreign upon transplantation and proceed to attack epithelial cells and mucous membranes in the skin, GI tract and liver [18]. Depending on the type of donor, conditioning regimen of the recipient and the degree of MHC matching GVHD can occur in up to 85 % of allogeneic HSCT recipients [19]. Diagnosis of GVHD is primarily based on clinical observations in conjunction with distinct pathology of affected organs. The severity of GVHD is scored based on the clinical assessment of the most affected organs and ranges from mild to severe.

Comparison	Acute GVHD	Chronic GVHD
Timing	Less than 100 days post transplantation	More than 100 days post transplantation
Organs most often affected	Skin, GI tract, Liver	Skin, GI tract, Liver, Nails, Mouth Eyes, Muscles, Joints, Genetalia
Current treatment	Corticosteroid	Corticosteroid

Fig. 4.1 Comparison of acute and chronic graft-versus-host disease (GVHD)

GVHD can be characterized as acute or chronic based on the etiology and timing of disease induction (Fig. 4.1). Acute GVHD occurs within 100 days of transplantation and primarily affects skin mucosa, liver, and the gastrointestinal tract [20]. Acute GVHD begins when allogeneic donor T cells mount cytotoxic responses mediated by cell-surface proteins and secreted cytokines against tissues and organs of the recipient [21]. Chronic GVHD develops later than 100 days after transplantation and can take up to 5 years to manifest clinically [22]. Chronic GVHD is one of the most common complications in allogeneic HSCT recipients surviving more than 3 months and is characterized by inflammation, development of fibrosis and potential functional disability [23]. Most often, cases of chronic GVHD follow acute GVHD, making the prevention of acute GVHD a critical goal [23].

3.1 Pathophysiology of GVHD

Physiologic causes and effects of acute GVHD have been well characterized (Fig. 4.2) [18, 21, 24]. The complete understanding of this multistage process allows for ease in identification of novel therapeutic targets.

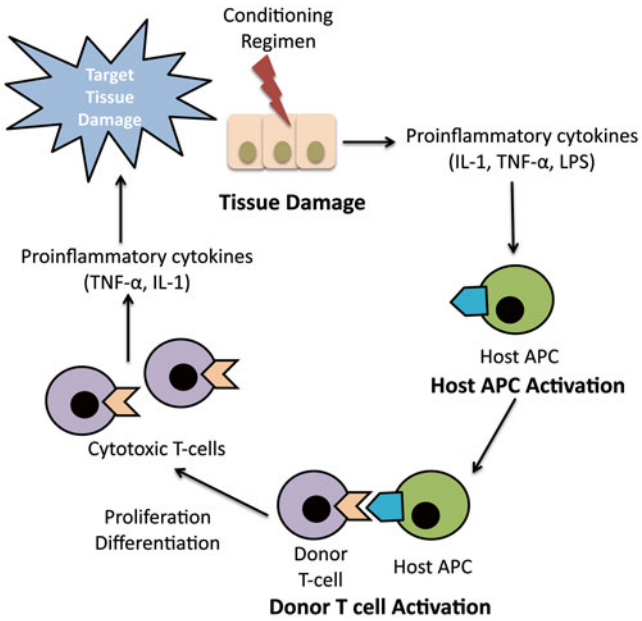


Fig. 4.2 Pathophysiology of GVHD. Phase I. Prior to transplantation the patient undergoes conditioning (radiation and/or chemotherapy) which leads to damage and activation of host tissue. Release of proinflammatory cytokines from activated host tissues increases HLA expression on host cells and enhances their antigen-presenting ability. Phase II. Transplanted donor T cells are activated upon interaction with host antigen resulting in proliferation and differentiation. Cytotoxic T cells produce additional inflammatory cytokines. Phase III. Cytotoxic T cells and cytokines act to damage host tissue

3.2 Stage 1: Host Tissue Damage and APC Activation

To prepare for transplantation, recipients must undergo a conditioning regimen involving immunosuppressive therapy to ablate the host immune response and allow for donor HSC engraftment. This high-dose regimen most often consists of chemotherapy and radiation therapy that causes tissue injury and results in inflammatory cytokine release including tumor necrosis factor alpha (TNF-α) and interleukin (IL)-12 [25–27]. Although critical to achieve successful engraftment, the creation of an inflammatory environment by the conditioning regimen has the potential to start the GVHD process. It is this initial release of proinflammatory cytokines brought about by tissue damage from the conditioning regimen in the recipient that is thought to underlie the initiation of acute GVHD [28].

The inflammatory cytokine release initiates an immune response cascade and results in recipient antigen presenting cell (APC) activation, upregulated MHC and costimulatory molecule expression and enhanced antigen presentation [20].

3.3 Stage 2: Donor T Cell Activation

After transplantation, donor T cells enter the inflammatory environment and recognize alloantigens presented by APCs. This recognition activates donor T cells causing them to expand, produce additional inflammatory cytokines including interferon gamma (IFN-γ) and recruit more immune cells

to mount an attack against the host [27]. Donor T cell inflammatory cytokine release triggers a proinflammatory cytokine cascade, which further drives donor T cell proliferation. Tissue damage caused by donor T cells creates a cytotoxic environment and leads to recruitment of additional effector cells including NK cells and neutrophils, which leads to additional damage and immune cell recruitment, creating a cycle that is difficult to break.

3.4 Stage 3: Cytokine and Cell Mediated Tissue Damage

Finally, activated immune cells mediate the destruction of host tissues and result in multiple organ GVHD. Inflammatory cytokines enhance the cytotoxic effect of T cells in GVHD by attracting additional immune cells and by directly damaging the target tissue [29]. GVHD associated tissue damage is brought on by an influx of graft immune cells that includes T cells and NK cells and results in a dramatic increase of inflammatory cytokines including TNF-α, IFN-γ and reactive oxygen species [30]. Cytotoxic T cells and NK cells respond and stimulate monocytes to produce IL-1 and TNF-α, directly resulting in tissue damage. TNF-α acts by activating host APCs, which leads to enhanced antigen presentation to donor T cells [31]. Active tissue damage causes release of additional proinflammatory cytokines and perpetuates the cascade.

It is clear that proinflammatory cytokines play a critical role in initiation and perpetuation of the GVHD. However, anti-inflammatory cytokines including IL-10, IL-1 receptor antagonist (IL-1ra) and transforming growth factor beta (TGF-β) may also play a role in GVHD pathogenesis. Evidence suggests that increased expression of IL-10 leads to a decreased risk of GVHD development following allogeneic transplantation [32]. TGF-β has also been shown to mediate the immune response and has been shown to diminish acute GVHD [33] but may lead to chronic GVHD [34].

3.5 Current Therapeutics for GVHD

Current treatment for GVHD is based on high-dose corticosteroids for global immunosuppression. However, this therapy is not without significant drawbacks. Although it works to decrease GVHD, corticosteroid treatment also increases the risk of infection and relapse, major causes of death following HSCT [1]. In addition, a subset of GVHD termed steroid-refractory GVHD does not respond to conventional corticosteroid therapy [35]. The relatively complete understanding of the initiation and perpetuation of GVHD allows for identification of new pathways that may be exploited to prevent or treat GVHD.

4 Cell-Based Immunomodulation for Prevention and Treatment of GVHD

Cell-based therapeutics are of particular interest in the treatment of GVHD. As described, the initiation and perpetuation of GVHD is a very involved process and includes many different cells types and mediators at various stages. It is the complexity of this system that makes the development of specific pharmacological interventions challenging. Cells have the unique ability to respond to the environment around them and can produce varying types and amounts of trophic factors depending on the circumstance. The ability to adjust based on the situation makes cells an excellent therapeutic option for intricate processes such as GVHD.

4.1 Bone Marrow Mesenchymal Stromal Cells

A promising cell-based immunomodulatory source that is currently being examined in clinical trials of GVHD is MSCs [36, 37]. MSCs are most often derived from bone marrow (BM-MSC) or adipose tissue and can give rise to differentiated cells of the mesodermal lineage including bone, fat, cartilage, tendon, and muscle [38–40] MSCs have a proven ability to evade detection by the immune system after transplantation and suppress the immune response [41, 42]. MSCs have become an increasingly promising form of cellular therapy for a range of inflammatory and immune disorders including GVHD due to their ability to produce significant amounts of trophic factors, ability to modulate the activity of immune cells, and predisposition to home to sites of injury [42, 43].

The immunophenotype of MSCs has been well characterized and they exhibit low levels of HLA-I along with complete absence of HLA-II and costimulatory molecules, a profile that suggests potential immune privilege [44]. BM-MSCs are able to suppress T cell proliferation and activation in response to allogeneic activation or chemical stimulation in vitro and in vivo [5, 45, 46]. Immunoregulation by BM-MSCs is thought to result from both direct interactions between the stromal and immune cells [47–49] and the actions of anti-inflammatory soluble factors released by the stromal cells [50, 51]. The secretion of these factors occurs in response to proinflammatory signals from the local environment, including IFN-γ, TNF-α, IL-1α, and IL-1β [52–54].

Clinical applications for which the trophic action of MSCs may prove valuable include support of hematopoietic transplantation and the treatment of GVHD [55–58]. BM-MSCs display remarkable immunomodulatory properties and are able to mediate function of the major populations of immune cells. MSCs modulate the activation,

proliferation, and function of effector and regulatory T cells [59]. In this capacity, MSCs are able to suppress the formation of cytotoxic T cells and to inhibit the expansion and inflammatory cytokine production of activated helper T cells [49, 51, 60, 61]. At the same time, MSCs have the ability to induce expansion of protective regulatory T cells [62]. MSCs inhibit B cell proliferation, differentiation toward plasma cells and antibody production [63]. In addition, MSCs suppress cytokine driven NK cell proliferation and inhibit their ability to produce the proinflammatory cytokine IFN-γ [64]. Finally, MSCs affect APC populations by reducing the formation and maturation of DCs [65, 66], reducing costimulatory molecule expression [67] on DCs and altering cytokine production [68].

The first trial to show the therapeutic effect of immunomodulatory BM-MSCs was in the accelerated recovery in patients with acute GVHD [58]. In addition, MSCs have proven to be effective in enhancing engraftment and reducing steroid-resistant GVHD after HSCT [58, 69, 70]. Possible mechanisms of MSC-mediated immunomodulation in the successful treatment of GVHD include reducing generation and maturation of DCs increasing regulatory T cell production by producing TGF-β, promoting generation of regulatory DCs which produce IL-10 and suppressing effector T cells by release of growth factors, inducible nitric oxide synthase (iNOS), heme oxygenase (HO)-1, prostaglandin or IDO [68]. Soluble factors are able to suppress the activity of immune cells by inhibiting their proliferation [71], decreasing their proinflammatory cytokine production [63], and conversion toward an anti-inflammatory phenotype [72]. MSCs may also act by downregulating immunoglobulin production by B cells and by inhibiting natural killer (NK) cell cytotoxicity and proliferation [18, 63].

The combination of regenerative and immunomodulatory properties in MSCs may prove beneficial in tissue and organ transplantation. While these results show great promise in the treatment of GVHD, MSCs have several considerable downfalls that must be addressed prior to widespread clinical use including significant amounts of donor-to-donor heterogeneity in immunomodulatory function and their relatively limited proliferation ability under standard culture conditions.

5 AFS Cells for Treatment of GVHD

Human amniotic fluid stem (AFS) cells are a population of well-characterized, broadly multipotent fetal-derived stem cells [7]. AFS cells share many characteristics with MSCs including surface marker profile and lineages to which they most easily differentiate. However, AFS cells have several distinct advantages over MSCs for clinical use.

c-kit-selected AFS cells are a particularly attractive therapeutic cell source because of their extensive capacity for self-renewal, their broad differentiation capabilities, immunomodulatory potential, and lack of teratoma formation [7]. Clonal lines of AFS cells have been shown to expand extensively in culture (greater than 80 population doublings) and without a change in phenotype [7]. In addition, AFS cells have been shown to express markers associated with pluripotency (Oct4 and SSEA4) as well as markers of adult stem cells (CD29, CD44, CD73, CD90, and CD105) [7]. AFS cells also have the ability to differentiate into cell types representative of each of the three germ layers including adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic cells [7]. AFS cells have been shown to possess immunomodulatory capabilities both in vitro and in vivo [73–75]. The combination of these properties makes AFS cells and ideally suited cell source to be used in the prevention and treatment of GVHD.

5.1 AFS Cells Evade Immune Recognition

The immunomodulatory capabilities of AFS cells are of great interest because these cells are derived from an immunoprivileged site and are able to evade recognition and destruction by the maternal immune system [76].The fetus represents a site of immune privilege where there is a complete lack of maternal immune response against fetal tissues expressing paternally inherited alloantigens. Fetal-derived AFS cells do not express surface markers associated with rejection and therefore are likely able to evade immune recognition [73]. The HLA molecules are a critical part of the immune response and while AFS cells demonstrate HLA-I expression they show a complete lack of expression of HLA-II and costimulatory molecules (CD80, CD86, CD40) [7, 73]. Stimulation with IFN-γ upregulated expression of MHC Class I and induced expression MHC Class II [73]. The immunophenotype of AFS cells is very similar to that of BM-MSCs and indicates that they may not strongly activate immune responses in allogeneic hosts. The ability to evade the allogeneic immune recognition is critical for a cell-based therapeutic to be used in GVHD.

5.2 AFS Cells and AFS Derived Cell-Free Supernatant Suppress T cell Activation

In allogeneic transplantation resulting in GVHD, T cells are activated upon exposure to nonself HLA molecules and to foreign antigens [66]. In turn, T cell activation results in proliferation, maturation, and cytokine secretion, which induces effector cell function and leads to host tissue destruction [65]. One of the goals of GVHD therapeutics is to inhibit T cell activation and block effector cell function, which would result in decreased tissue destruction. Activated

T cells produce the proinflammatory cytokine IFN-γ, among others [68]. In vitro culture systems can stimulate T cells and use IFN-γ production as a measure for the amount of T cell activation [67]. AFS cells are able to inhibit in vitro T cell activation in a dose-dependent manner to levels comparable to that of BM-MSCs [73]. These studies prove that cell-to-cell contact between AFS cells and immune cells results in inhibition of the in vitro immune response as measured by IFN-γ production of activated T cells [73]. Suppression of T cell activation is a critical factor for the treatment of GVHD because it is the activated, cytotoxic T cells that induce injury. Blocking this activation process may lead to reduced inflammatory cytokine release and a reduction in host tissue damage.

In addition, AFS cells have been shown to secrete cytokines that are able to inhibit T cell activation in vitro. Cell-free supernatants derived from AFS cells under normal expansion conditions are unable to significantly inhibit T cell activation. However, it is known that immunomodulatory cell sources, including BM-MSCs, must be exposed to pro-inflammatory stimuli to evoke production of protective, anti-inflammatory cytokines [52]. AFS cells activated by co-culture with either total peripheral blood mononuclear cells (PBMCs), meant to mimic exposure to the immune system or IL-1β, a proinflammatory cytokine released by immune cells, release immunomodulatory factors that reduce T cell activation and IFN-γ production [73]. AFS cells respond to the inflammatory environment by producing immunomodulatory mediators capable of inhibiting the immune response. This is a factor critical in the treatment for GVHD because initiation and perpetuation of GVHD is dependent on proinflammatory cytokines including IL-1.

5.3 Cytokine Secretion of Activated AFS Cells

Cytokines are critical mediators of the immune response and elucidating the factors being produced by AFS cells is critical for their future clinical use in treatment of GVHD. Studies examining cytokines, chemokines, and growth factors reveal that proliferating AFS cells produce low levels of very few cytokines. However, upon activation with immune cells or proinflammatory cytokine IL-1, AFS cells produced highly elevated levels of GRO, GRO-α, monocyte chemotactic protein-1 (MCP-1), IL-2, IL-6, and granulocyte macrophage colony stimulating factor (GM-CSF), and lower levels of MIP-3α, IL-2, regulated upon activation, normal T cell expressed, and secreted (RANTES) and epithelial neutrophil-activating protein-78 (ENA-78). Importantly, these factors were also upregulated in stimulated BM-MSCs that had proven immunomodulatory capabilities [73]. However, BM-MSCs produced several additional cytokines that were not generated by AFS cells including monocyte chemotactic protein-2 (MCP-2), macrophage inflammatory protein 1-alpha and 3-alpha (MIP-1α, MIP-3α) and Activin revealing the potential for alternative molecular mechanisms of AFS cell immune response modulation.

The mechanisms involved in the inhibitory effect of AFS cells have not yet been completely elucidated. In response to activation, the most highly upregulated protein in AFS cells was interleukin 6 (IL-6). IL-6 is a broad-acting cytokine involved in the control of the immune response as well as stem cell development and regulation [55, 77]. Mesenchymal stem cells derived from cord blood (CB-MSCs) and BM-MSCs have also been reported to secrete high amounts of IL-6 when activated with IL-1β [78]. The biological relevance of this IL-6 response likely lies in both local and systemic protection against inflammation [69, 70]. AFS cells also released high levels of growth related oncogene family members GRO and GRO-α as well as MCP-1 upon IL-1β activation. These chemokines have well-known effects on cells of the immune system and are important in inflammation and wound healing. GRO acts on neutrophils and MCP-1 acts mainly on macrophages, recruiting them to sites of inflammation [79].

In conclusion, the mechanism of immunosuppression by AFS cells involves both direct cell–cell contact between the stem cells and the immune cells and indirect interaction through immunosuppressive factors secreted by the stem cells in response to proinflammatory cytokines [73]. A proposed model showing the immunosuppressive capabilities of AFS cells is shown in Fig. 4.3. These AFS-mediated immunomodulatory actions may prove beneficial in the treatment of GVHD.

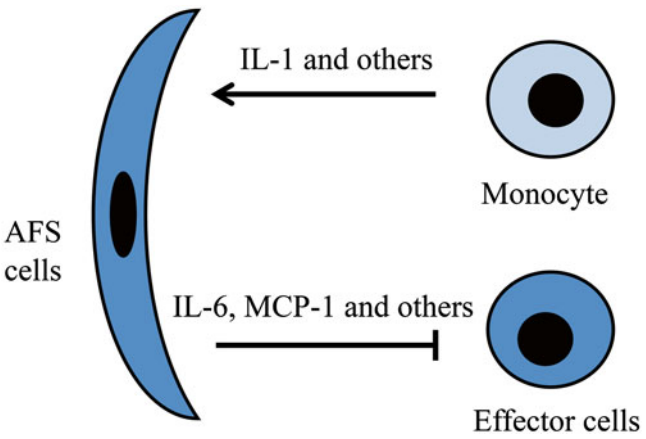


Fig. 4.3 Model of proposed mechanism by which human amniotic fluid stem (AFS) cells interact within the confines of the immune response. Secretion of IL-1β and other cytokines from monocytes causes an activation of the immunodulatory properties in AFS cells. In response, AFS cells secrete a battery of soluble factors including IL-6, MCP-1 and others which inhibit T cell activation

5.4 In Vivo Evidence Suggesting a Role for AFS Cells in GVHD

AFS cells also possess immunomodulatory capabilities in experimental in vivo models of inflammation. Recent studies prove that rat AFS (rAFS) cells significantly improve survival and enhance repair of the damaged intestine in a rat experimental model of necrotizing enterocolitis (NEC) [80]. Similar results have been achieved using human AFS cells to repair the kidney and restore its function in an immunodeficient mouse model of acute tubular necrosis (ATN) [74]. In this model AFS cells appear to possess immunomodulatory function, initiating the release of several murine anti-inflammatory cytokines and reducing the release of proinflammatory cytokines [74].

Several studies have attributed favorable outcomes observed after transplantation of fetal-derived stem cells to the release of protective or reparative trophic factors by the stem cells. Indeed, introduction of cell-free conditioned medium from amniotic fluid has proven beneficial in restoring blood flow in injury models [81] and in stimulation of repair in wound models [82]. The mechanisms behind these effects remain under investigation but immunomodulatory capabilities of these cells may be involved in limiting damage and stimulating repair of injured tissue. Based on these studies we have developed a proposed model demonstrating the potential mechanism of action of AFS cells in treating GVHD (Fig. 4.4).

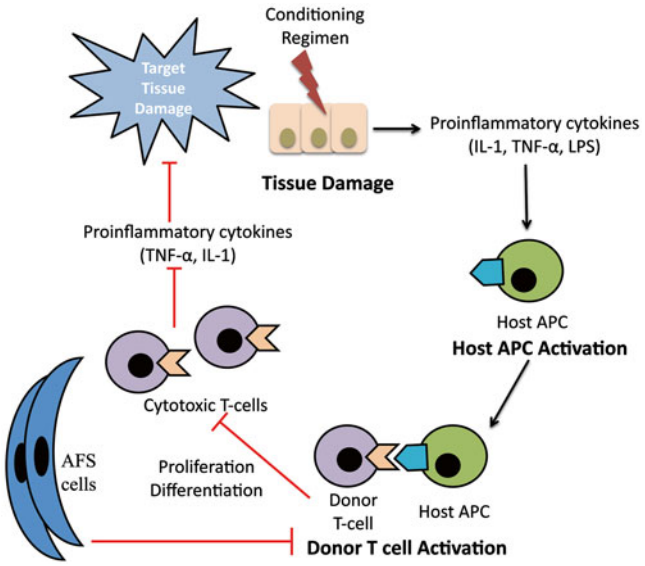


Fig. 4.4 Proposed mechanism of human AFS cells in the treatment of GVHD. Human AFS cells may act to inhibit T cell activation in Phase II of GVHD. Prevention of T cell activation may decrease the proliferation, differentiation, and inflammatory cytokine release of T cells, leading to a decrease in host tissue damage

6 Advantages of AFS Cells in the Treatment of GVHD

The main characteristic of AFS cells that makes them an ideal cell type for therapeutic applications in the treatment of GVHD is their proven immunomodulatory capacity [73, 74, 80]. However, AFS cells have additional features that make them an attractive therapeutic cell source including their ability to expand extensively in culture and their inability to form tumors when implanted into immune deficient mice.

AFS cells are able to proliferate extensively in culture without acquiring chromosomal abnormalities while maintaining their initial properties [7]. This is in stark contrast to MSCs which have such limited in vitro expansion capabilities that cells from several donors must be pooled to achieve one therapeutic dose [83]. The capacity for uninhibited growth in vitro is critical to allow for the extensive expansion and full characterization of clonally derived AFS cell lines with immunomodulatory capabilities. Not surprisingly, donor-to-donor variability has been revealed in the ability of MSC extracts to mediate the immune response [84]. The ability to fully define the potentially unique immunomodulatory characteristics of individual AFS cell lines is a huge benefit in GVHD treatment. Following expansion and characterization, each AFS cell line can be cryopreserved and banked for future use as a reproducible "off the shelf" cell-based therapy for the treatment of GVHD. A summary of the advantages of using AFS cells for the treatment of GVHD is found in Fig. 4.5.

7 Future Considerations

The immunomodulatory effects of AFS cells that have been documented thus far are promising and suggest a role for these fetal-derived cells in the treatment and prevention of GVHD. However, it is critical to consider issues such as

Advantages AFS cell treatment of GVHD
Proven immunomodulatory properties
Indefinite *in vitro* expansion
Ability to be fully characterized
Clonally derived
Avalibility for biobanking
Do not form teratomas *in vivo*

Fig. 4.5 Advantages of using human AFS cells for prevention and treatment of GVHD

dosing of AFS cells and timing of the AFS cell transplantation before their clinical use is considered.

One important issue to address is the dose of AFS cell that will be required for optimal reduction of GVHD. In vitro assays demonstrated a dose-dependent T cell response to AFS cells and showed that even at the lowest dose tested (1 AFS cell to 32 T cells), AFS cells were able to inhibit T cell activation [73]. However, performing a similar dosing experiment in an in vivo model of GVHD to prove safety and efficacy of AFS cells for GVHD treatment is critical. Timing of AFS cell administration is another important consideration in their potential treatment of GVHD. It is likely that AFS cells would be infused at the time of allogeneic transplantation to prevent initiation of acute GVHD. However, another option to consider is waiting until the observation of GVHD symptoms before administration of AFS cells.

AFS cells also have the potential to serve as a tool for identification and isolation of soluble factors involved in immunomodulation that may be used in cell-free therapeutics for GVHD. Cell-free supernatant derived from IL-1β stimulated AFS cells has the ability to suppress T cell activation. In vitro characterization of the soluble factors secreted by AFS cells in response to stimulation with IL-1β was performed by protein array. Application of the factors identified including IL-6, MCP-1, GRO and GRO-α, individually or in combination, to systems of inflammation including GVHD would advance knowledge on the effects of these morphogens. Further characterization of the complex profile of soluble molecules secreted by AFS cells is critical and may lead to the development of novel strategies for direct application of these factors for therapeutic approaches.

In the future, it is critical to make additional efforts to classify the immune response to AFS cells in vitro and in vivo. It is known that AFS cells or cell-free supernatant are able to inhibit T cell activation as measured by IFN-γ release. However, T cells represent only one part of the immune response; many additional cell types and responses make up the complete immune system. Dendritic cells (DCs) are an APC type critical in the immune response. It is critical to examine the ability of AFS cells to inhibit of upregulation of costimulatory molecules and chemokine receptors and their ability to decrease in the formation of dendrites. B cells and natural killer (NK)-cells are also important in the immune response and it is important to examine the ability of AFS cells to inhibit their proliferation [63, 85]. Identification of immune cell/stem cell interactions and further characterizing the mechanisms of AFS-mediated immunomodulation is essential in determining their ideal therapeutic use.

8 Conclusions

GVHD is a major complication of allogeneic transplantation and primary cause of transplant-related mortality. Discovery and development of novel GVHD therapeutics is critical, especially for cases of steroid-refractory GVHD. Human AFS cells are an immunomodulatory cell source derived from an immunoprivileged site that can be expanded indefinitely in culture. AFS cell therapy may selectively inhibit the immune response in GVHD as opposed to the current global immunosuppressive medications. These properties make AFS cells an ideally suited cell source to be used in efforts to prevent and treat GVHD.

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Amniotic Fluid Stem Cell Features Supporting Their Putative Role in Fetal Cell Microchimerism

5

Margit Rosner, Katharina Schipany, and Markus Hengstschläger

Abstract

Since the discovery in 2003 that fetal stem cells float in the amniotic fluid, a major question still remains unanswered. So far, the in vivo biological relevance of amniotic fluid stem cells (AFSCs) is unclear. Also already since many years, it is evident that fetal stem cells with putative regenerative potential exist in the circulation of pregnant women. To support the hypothesis that these two stem cell entities share a common origin, AFSCs would have to harbour a variety of specific features. Several of these properties, including anchorage-independent growth, high differentiation potential, fine-tuned apoptotic disposal and the absence of a malignant transformation potential, have already been demonstrated for AFSCs. Here we discuss that additional features, such as migration, invasion, extravasation or the potential to translocate from the amniotic fluid to the maternal circulation, should be tested to further support the theory that AFSCs might be involved in fetal cell microchimerism during pregnancy.

1 Fetal Cell Microchimerism

Microchimerism is the presence of a small amount of foreign cells or DNA within the tissues or circulation of an individual. In nature that occurs when cells (or DNA) are exchanged between the foetus and the mother and vice versa [17]. Both together, cell-free, fetal DNA in mother's blood and fetal cells in mother's tissues represent the well-established phenomenon of fetal cell microchimerism. An interesting, but still unclear aspect hereby is, whether transplacental trafficking only occurs for fetal cells, which then later undergo apoptosis in the maternal organism leading to cell-free, fetal DNA, or whether fetal DNA also translocates through the placenta [3, 44, 65].

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The discovery of fetal, microchimeric cells was the consequence of ongoing efforts to improve non-invasive prenatal diagnosis. The analysis of cell-free, fetal DNA for non-invasive prenatal diagnosis of numerical chromosomal aberrations is already routinely offered to pregnant women. In future, it will routinely be possible to sequence the entire fetal genome in the course of non-invasive prenatal diagnosis, what already initiated an accompanied ethical discussion [2, 9, 21, 38, 47].

Until now, the exact role of fetal cell microchimerism remains an unsolved topic. It is important to mention that currently there exists an active discussion within the scientific community, to which extent microchimerism is beneficial, deleterious or without biological relevance. Favouring the deleterious side of microchimerism, it is argued that it could be involved in typical pregnancy diseases, such as, e.g. pre-eclampsia, or in autoimmune diseases or even in the development of specific cancers. The "beneficial hypothesis" suggests that the fetal cells in the mother could fulfil healing, reparative and perhaps regenerative functions in specific maternal tissues and could support the mother's defence against cancer or infections. Sometimes in the literature it is even speculated that fetal cell microchimerism might at least

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be partly associated with the longer life expectancy of females. Last but not least, some argue that the entire phenomenon is simply an incidental by-product of pregnancy without any biological relevance [6, 15, 19, 27, 37, 67].

For a better understanding of the biological role of fetal cell microchimerism and its relevance for maternal health, it is essential to identify the cellular origin of these fetal cells. Many different studies demonstrated that, when finally detected in maternal tissues, the fetal cell population is phenotypically diverse [13, 16, 55, 72]. However, it is not clear, whether these microchimeric fetal cells represent a population of different cells with different origins, or whether all the different fetal cells detected in the mother originate from one specific cell type. Independently of this question, it is widely accepted that these microchimeric fetal cells are stem cells with characteristics and potentials between those of embryonic and adult stem cells. Accordingly, they have been named "pregnancy-associated progenitor cells (PAPCs)". Many different observations support the stemness of PAPCs. PAPCs detected in the mother have diverse phenotypes, a high plasticity of morphology and have been found to integrate into various tissues including blood, lung, liver, spleen, heart, skin and even brain. For example, it has also been shown that fetal cells in the maternal bone marrow harbour the potential to differentiate into different lineages [20, 24, 31, 34, 37, 41, 54]. A variety of studies in mouse models further revealed that fetal, microchimeric cells can be involved in many different tissue regeneration processes in the mother and are detectable, e.g. upon hepatic or spinal cord injury [32, 78], in breast cancer [12], in lung and bone marrow [71], in the brain [73, 77], upon kidney injury [5], in skin wound healing [43], in muscle regeneration [68] or upon cardiac injury [28]. And still, the cellular origin of these fetal microchimeric stem cells, named PAPCs, remains elusive.

2 Amniotic Fluid Stem Cells

Besides ultrasound investigations and maternal serum screening, the isolation of fetal cells via the invasive procedure of amniocentesis is a widespread diagnostic tool in prenatal diagnosis. A family history for a certain genetic disease, specific ultrasound signs or maternal age are classical indications to analyse the genetic status of the foetus via the investigation of cells obtained from the amniotic fluid [45, 46, 59]. Despite their wide and well-established usage in routine diagnosis, the knowledge about the cells contained in amniotic fluid was very limited [18, 23, 30, 56].

In 2003 we reported the detection of a highly proliferative cell type expressing the pluripotent stem cell marker Oct-4 in human amniotic fluid, what was the first description of a new stem cell entity, named amniotic fluid stem cells (AFSCs) [57]. Thereafter, the field of AFSC research was developed further step by step [62]. Both, the confirmation of Oct-4 positive stem cells in amniotic fluid and the differentiation of these cells into various different cell types, were reported by many groups [4, 7, 10, 22, 25, 26, 29, 33, 35, 40, 48–53, 58, 60, 69, 70, 74].

In 2006, the first suggestion that AFSCs could be pluripotent was published. Descending from one single Oct-4- and NANOG-positive AFSC, adipogenic, osteogenic and neurogenic differentiation could be induced [75]. In 2007, isolation of clonal AFSCs via immunoselection, which expressed the markers Oct-4 and CD117 (c-kit), allowed to demonstrate the induction of differentiation upon adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic cell lineages. Furthermore, in this publication it was shown that injection of human AFSCs into SCID mice does not induce teratoma development [8]. As a consequence of this report, it became widely accepted that AFSCs fulfil the loose criteria for the usage of the term "pluripotent", namely self-renewal and the differentiation into cells of the mesodermal, endodermal and ectodermal lineage. But, since AFSCs are not tumorigenic in themselves and do not form teratomas in mice, they do not fulfil the stringent criteria for pluripotency. AFSCs only can form teratomas in mice upon stem cell preconditioning, e.g. with valproic acid [42]. This absence of inherent tumour-inducing properties might be the big advantage of these cells when it once comes to therapeutic usage [63]. Further support for their pluripotent status came from experiments showing that clonal human AFSCs can form embryoid bodies (what is known to be the principal step in the differentiation of pluripotent stem cells) when cultured without anti-differentiation factors under conditions in which they are unable to attach to the surface of culture dishes and without contact to feeder cells. The formation of such three-dimensional multicellular aggregates is accompanied by a decrease of stem cell marker expression and by the induction of differentiation upon various cell lineages [76].

A very promising and fast growing stem cell research field has been established supporting the notion that AFSCs could once indeed be used for regenerative stem cell therapies. Furthermore, AFSCs characteristics, such as their high proliferative potential, their phenotypical and genomic stability, and the high transfection efficiencies one can achieve with these cells, also highlighted them to be an optimal cellular model for basic molecular science [61, 66]. And still, the biological relevance of the existence of AFSCs in vivo remains unknown so far [1, 11, 64].

3 Proven Features of Amniotic Fluid Stem Cells Supporting a Role in Fetal Cell Microchimerism

As already mentioned above, the cellular origin of the fetal stem cells involved in fetal cell microchimerism remains elusive so far. The most important features a fetal stem cell type must harbour to be considered as a putative candidate for a

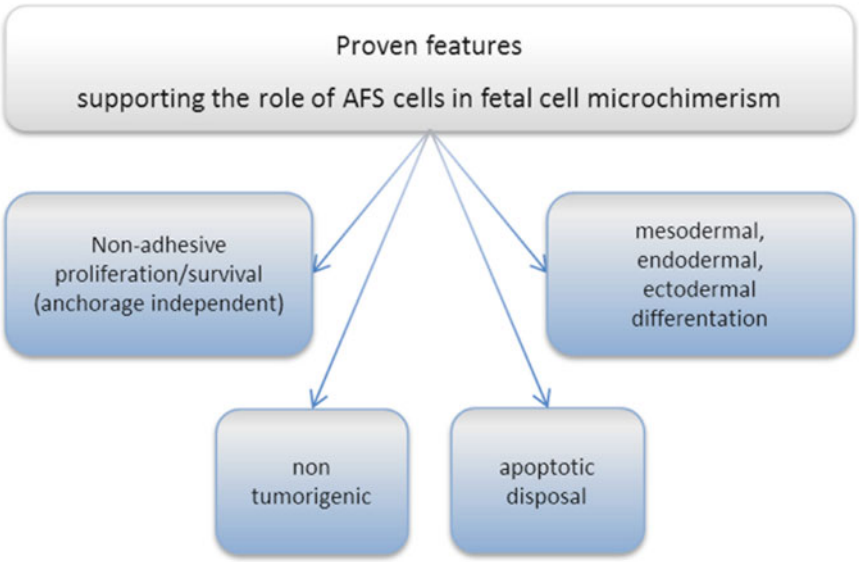


Fig. 5.1 Proven features of amniotic fluid stem cells (AFSCs) supporting a role in fetal cell microchimerism

PAPC include anchorage-independent growth, a certain potential for differentiation, probably even pluripotent potential, and the absence of a malignant transformation potential [15, 17, 24, 31, 34, 44, 54] (Fig. 5.1). AFSCs float in the amniotic fluid and have been shown to harbour proliferative potential and to express markers for cell cycle prolongation in vivo [57]. Since starting from one cell AFSCs can be differentiated upon the mesodermal, endodermal and ectodermal lineages [8, 75] and can form embryoid bodies [76], this fetal stem cell type fulfils the loose pluripotent criteria. And finally, as described above, AFSCs do not have inherent tumour-inducing properties [8, 42] (Fig. 5.1).

Fetal cell microchimerism is also represented by the presence of fetal DNA in the maternal circulation [2, 3, 21, 38, 47]. Recently, it has been reported that AFSCs underlie a strictly regulated survival control. The capacity of AFSCs to maintain homeostasis via fine-tuned, apoptotic disposal of excess or defective cells was demonstrated by the observation that slight deregulation immediately triggers AFSCs to go into apoptosis [14]. Assuming that AFSCs could be candidates for PAPCs, such a stringent survival control in AFSCs could give rise to cell-free DNA in vivo (Fig. 5.1).

Taking all that into account, AFSCs perfectly fulfil the major features, which must be designated to a stem cell type to be considered as a candidate for the origin of PAPCs. Furthermore, one could also assume AFSCs to be the origin of cell-free, fetal DNA in the mother's blood, which is already routinely used for non-invasive prenatal diagnosis. Accordingly, we have recently suggested a model in which AFSCs give rise to what we currently know as PAPCs and cell-free, fetal DNA [65].

4 Features, Which Need to be Tested to Further Support a Role of Amniotic Fluid Stem Cells in Fetal Cell Microchimerism

Migration is normally used to describe any directed cell movement within the body, whereas invasion is defined as the penetration of tissue barriers and infiltration into tissues [36]. Extravasation is used to describe the process by which a circulating cell passes through the blood vessel wall into various body areas [39]. Considering everything what is known about PAPCs today, one would assume that these fetal, microchimeric cells harbour additional potentials, such as migration, extravasation and invasion [2, 6, 15, 17, 19, 34, 44, 54] (Fig. 5.2). To our best knowledge, explicit experimental approaches to investigate in detail whether the here described c-kit-positive, monoclonal AFSCs harbour the potential for migration, extravasation or invasion, have not been reported so far.

Last but not least, ultimate support of the hypothesis that AFSCs could function as PAPCs would be to prove that AFSCs floating in the amniotic fluid could finally end as cells in maternal tissues or as cell-free, fetal DNA in the maternal circulation during pregnancy (Fig. 5.2).

Unravelling the identity of PAPCs in support of AFSCs might give answers to two very important questions in stem cell biology: What is the origin of PAPCs? What is the biological function of AFSCs? Accordingly, we think it is absolutely warranted to design experimental settings and approaches to investigate these features in AFSCs.

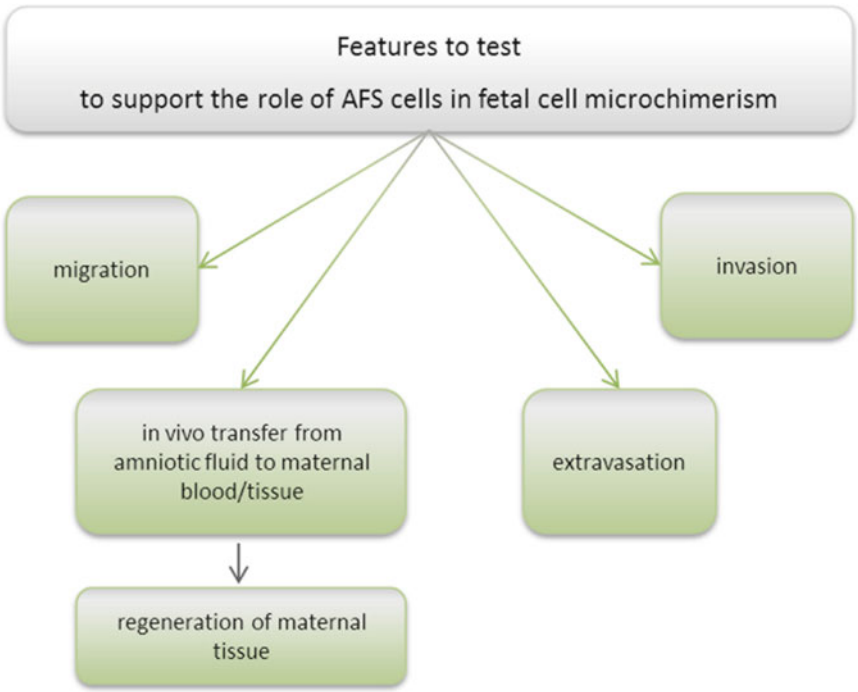


Fig. 5.2 Features, which need to be tested to further support a role of AFSCs in fetal cell microchimerism

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Amniotic Fluid Stem Cell Therapy for Lung Disease

6

Orquidea Garcia and David Warburton

Abstract

Amniotic fluid stem cells (AFSC), a unique multipotent stem cell population, have demonstrated significant experimental promise for the treatment of lung injury and disease as well as for de novo tissue engineering. Furthermore, the translation of experimental models, to clinical endpoints in the lung, present a litany of distinct challenges that AFSC have demonstrated the potential to address. With a minimum of 40 phenotypically distinct cell populations, limited regenerative capacity, 23 architecturally and physiologically diverse functional regions and the etiology of many lung diseases and disorders poorly understood, the respiratory system itself is perhaps one of the most challenging systems to treat. This chapter examines some of the challenges posed by the respiratory system, AFSC-based therapy for the treatment lung disease, and the future prospects of this stem cell population within the context of the lung.

1 The Lung

1.1 Introduction

Before considering or developing any cell-based therapy for any organ or organ system, a fundamental understanding of the target organ is crucial. In order to effectively design and deliver a cellular therapy, the development, anatomy, physiology, regenerative targets and diseases affecting the lung and current treatments must first be considered. Because of the plasticity demonstrated by AFSC, and other stem cell therapies, in response to the environment in which they are placed a cursory understanding of the genetic, developmental, inflammatory, and injurious milieus that these cells will eventually be placed in is necessary to attempt to predict their therapeutic response, properly manipulate stem cells prior to treatment and prevent potentially deleterious responses. For example, a patient suffering from a genetic mutation affecting proper surfactant protein C (SP-C) folding may benefit from stem cell-based therapy, delivered directly to the alveolus, in which cells have been differentiated prior to administration to elicit SP-C expression. On the other hand, a patient suffering from idiopathic pulmonary fibrosis (IPF) where pro-inflammatory cytokine activity is constitutively expressed may benefit from intravenous stem cells administered during key inflammatory events in order to capitalize on the known anti-inflammatory properties of these cells. Without a basic understanding of lung disease and treatment targets, efficiently designing a stem cell-based therapy becomes impossible. Thus, a brief overview of these topics will first be discussed prior to discussion of the development and use of AFSC within the context of lung disease.

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1.2 Development

The developing lung has been divided into four distinct histological stages (Fig. 6.1): the pseudoglandular stage (5–17 weeks of gestation in humans), the canalicular stage

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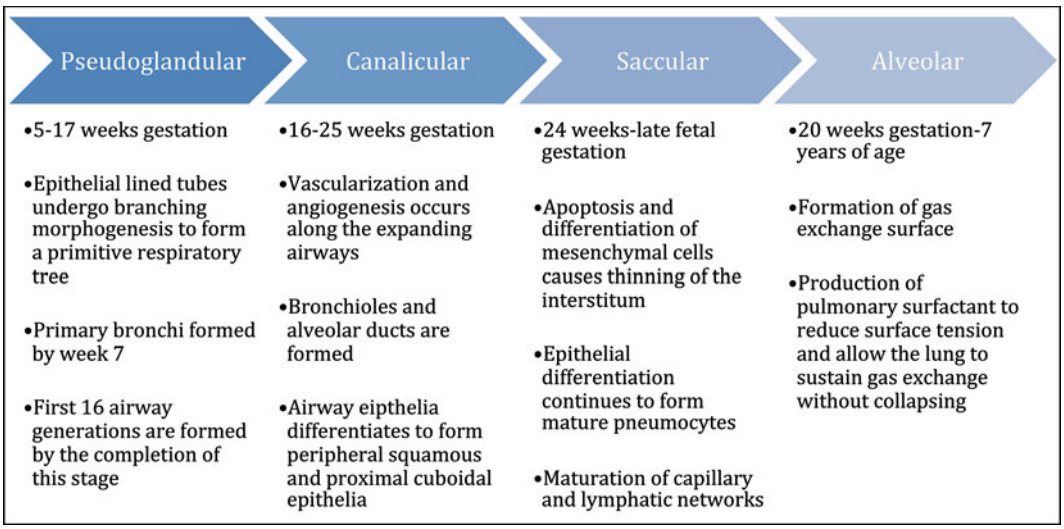


Fig. 6.1 Human lung development

Table 6.1 Functional regions of the human lung

Anatomical region	Airway generation	Cell populations contained in region	Function
Trachea	0	• Ciliated columnar cells	• Warm and humidify inspired air
Main bronchi	1	• Goblet cells	• Remove inhaled particulates
Bronchi	2–8	• Secretory cells • Serous cells • Brush cells • Neuroendocrine cells • Basal cells	• Turbulent air conduction
Bronchioles Terminal bronchioles	9–14 15	• Ciliated cells • Clara cells	• Distribute and deliver inspired air further down respiratory tree
Respiratory bronchioles	16–18	• Clara cells • Few ciliated cells	• Gas exchange • Inhaled particulate clearance • Air distribution
Alveolar ducts Alveolar sacs	19–23	• Bronchoalveolar stem cells • Type II alveolar epithelial cells • Type I alveolar epithelial cells	• Gas exchange

(16–25 weeks in humans), the terminal saccular stage (24 weeks–late fetal gestation in humans), and the alveolar stage (20 weeks–age 7 in humans) [1]. Because the development of the lung encompasses a sequential series of steps and involves the careful coordination between the endothelium, mesenchyme, and epithelium any disruption in the normal course of development can have severe functional consequences [2]. Furthermore, because the lung is one of the last organs to fully develop, as alveolarization continues well into childhood, additional factors such as premature birth can have a profound impact on the future of lung function and health [3].

Development provides the first context where AFSC-based therapies could prove useful. Many genetic and developmental deficiencies, such as pulmonary hypoplasia, could potentially be addressed in utero, perhaps with the patient's own AFSC. Furthermore, knowledge of which developmental stage, and what potential genes and pathways are affected are important when attempting to rescue developmental abnormalities later in life.

1.3 Anatomy and Physiology

1.3.1 Macro

Distilled down to its basic function, the lung is designed to serve as a gas exchange system: warming, humidifying and distributing inspired oxygen and eliminating carbon dioxide. The fully developed lung extends from the trachea and terminates in the alveoli. Within the lung there are 23 distinct generations of airways, which serve as distinct functional regions, each of which has evolved to meet specific physiological functions as well as withstand the physical stretch and pressure involved in the process of respiration (Table 6.1) [4].

Table 6.2 Putative stem/progenitor cell niches within the lung

Cellular niche	Anatomical region	Regenerative capacity
Airway submucosal gland cells (SMG) Tracheal basal cells (keratin 14 expressing)	Trachea and proximal airways	• Regeneration of tracheal basement membrane and epithelium in a heterotopic syngeneic tracheal transplant mouse model [9] • Repopulation of tracheal epithelia including ciliated cells and columnar secretory cells following Clara cell specific naphthalene injury [10]
Variant Clara cells–neuroendocrine body (NEB) associated	Distal airways	• Regeneration of airway epithelia following Clara cell depletion [11, 12]
Variant Clara cells–bronchoalveolar duct junction (BADJ) associated	Terminal bronchi	• Regeneration of terminal bronchiolar epithelium following Clara cell depletion [13]
Type II alveolar epithelia (AECII)	Alveolus	• Lineage tracing elucidated self renewal capabilities, ablation of AECII demonstrated clonal expansion and differentiation [14, 15]

1.3.2 Micro

Although the exact number of distinct cellular phenotypes within the lung is presently unknown, characterizations of populations within the mouse lung have revealed the existence of 40 cell lineages at present [5]. Furthermore, these 40+ lineages are surmised to arise from at least 5 stem/progenitor cellular niches that have been characterized primarily through the use of injury/regeneration models within the adult mouse lung [6]. These niches, outlined in Table 6.2, are the regenerative target in the treatment of lung injury and disease [7]. Recent studies, however, have suggested the involvement of additional stem/progenitor cell populations and mechanisms employed during homeostatic maintenance of the lung [8], such as low density AECII precursors.

Critical to the development of regenerative strategies is an understanding of the regenerative capacity of the lung. Numerous studies have demonstrated the low rate of cell turnover in the lung [16]. Furthermore, the rate of cellular turnover is dependent on architectural region of the lung, with rapid cell turnover observed in the proximal lung and slower turnover in the more distal regions [17]. Consequently, the inherently low regenerative capacity of the lung compounded with the manner of disease or injury in the lung affecting any one of these niches can prove overwhelming for the homeostatic regenerative capacity of the lung.

1.4 Disease and Injury

Disease and injury in the lung can be attributable to genetic abnormalities, such as in the case of patients suffering from cystic fibrosis, or acute or chronic injury and disease, such as disorders resulting from inhalation exposure. As the etiology of many lung diseases, such as idiopathic pulmonary fibrosis, are often poorly understood, determining and treating the root cause of lung dysfunction often becomes secondary to managing and inhibiting disease progression.

Whatever the cause, the first hurdle when treating the lung is understanding the geographical complexity of the affected region. Consequently, this means that multiple cell lineages and niches are likely responsible for repopulating and driving homeostasis in the injured or diseased lung. Compounded with the fact that many of the stem and progenitor cellular niches within the human lung are still not fully characterized, designing effective stem cell-based treatments and therapies to target or repopulate specific cell lineages is quite challenging.

1.5 Current Treatment Strategies

In the case of acute pulmonary injury, such as in acute respiratory distress syndrome, immediate medical and surgical intervention is often necessary. Patients are often monitored closely throughout recovery and respiratory function is often aided through the use of mechanical ventilation or oxygen, which itself can induce injury. In the case of chronic disease or injury, immediate medical intervention does not always occur. The ability for the lung to compensate for loss of function due to injury or disease is tremendous [18, 19]; unfortunately, in the case of many degenerative diseases, once a patient has lost the majority of their lung function, and a diagnosis is finally made, treatment options become quite limited. Palliation of symptoms for patients who are less than ideal candidates for transplantation has become the standard course of treatment. Medications such as corticosteroids, oxygen therapy, and attempts at pulmonary rehabilitation are strategies often employed to further halt the progression of chronic disease. For patients who do receive lung transplants, pharmacologically based anti-rejection therapies must be employed for the remainder of the patient's life.

1.6 Lung Specific Considerations for Developing Stem Cell-Based Therapies

The disparity of treatment options for lung injury and disease provides ample opportunity for the development of cellular therapies. Furthermore, the magnitude and diversity of injury and disease affecting the lung suggests that cellular therapies for the treatment of respiratory disease will most likely need to encompass a multitude of cell types and approaches to effectively treat specific diseases and specific stages within those diseases [20, 21]. In the lung, both endogenous and exogenous stem cell-based therapies have been investigated with varied success, as summarized in Table 6.3 [14].

Once a suitable stem cell-based treatment strategy has been devised, clinicians and scientists must determine appropriate delivery strategies. Cell-based therapies pose an interesting challenge in that, unlike pharmaceuticals, they are dynamic and can be responsive to the environments and conditions to which they are exposed [24–30]. Thus, careful consideration must be given to the desired outcome of the stem cell-based treatment, the clinical status of the patient, as well any secondary mechanisms that affect or are employed by the stem cell to ensure appropriate delivery to target locations (Table 6.4).

Thus, taking into account the aforementioned topics of development, anatomy and physiology and variety of diseases affecting the lung, one can begin to understand the complexity of designing any type of cellular therapy for the lung. The varied architecture and variety of cell types and litany of functions performed by these cells suggests that multiple cell types are perhaps required to treat the lung. For example, developmental and genetic abnormalities, such as in cystic fibrosis, resulting in deleterious respiratory phenotypes may require manipulation of stem cells, to overexpress and deliver functional CFTR protein, prior to treatment in order to compensate for endogenous abnormalities. In cases where injury or disease results in the minimal loss of organ

Table 6.3 Potential cell populations for the treatment of lung disease

Endogenous		Exogenous	
Cell type	Consideration	Cell type	Considerations
Niche specific cells (SMG, tracheal basal cells, Clara^{v}, AECII)	• Difficult to culture • Low isolation yield • Low capacity to differentiate • Difficult to isolate from patient without invasive procedure	Embryonic stem cells	• Form teratomas • Require differentiation prior to transplant in lung • Immunomodulatory potential • Must be obtained from donor
Lung stem cells [22]	• Multipotent • Self renew • Proliferate long term • Requires further validation	Mesenchymal stem cells	• Easy to isolate from patient • Easy to culture • Immunomodulatory potential • Can differentiate into various lineages • Through to contribute to fibrotic disease
Multipotent lung stem cells [23]	• Obtained from homogenized lung • Niche and functionality still undetermined in vivo • Self renew • Differentiate into specific cell types (AECII and Clara cells)	Amniotic fluid stem cells	• Do not form teratomas • Are in contact with the developing fetal lung • Immunomodulatory • Can differentiate into various lineages • Respond to injured lung milieu [24, 25] • Obtained from donor

Table 6.4 Delivery strategies for AFSC into the lung

Intravenous	Aerosol	Intraperitoneal
Must travel through circulation to reach lungs	Deposited directly into lungs	Injected systemically
Minimally invasive	Risk of barotrauma	Minimally invasive
Can be administered without interfering with ventilation machinery	May potentially interfere with ventilation machinery	Can be administered without interfering with ventilation machinery
Minimal propulsory forces exerted on cells	Possibility of shearing upon aerosolization	Minimal propulsory forces exerted on cells
Need for culture prior to injection	Need for culture prior to aerosolization	Need for culture prior to injection
Cell homing to diseased region is possible	Cell homing to diseased region is possible	Cell homing to diseased region is improbable

function, such as in inhalation exposure, treatment strategies may focus on using stem cells to stimulate endogenous repair mechanisms to improve and promote wound healing. In cases of catastrophic loss of organ function (such as in acute respiratory distress syndrome, ARDS), or progressive disease (as in the case of pulmonary fibrosis), stem cell-based therapies may be useful to inhibit the progression of disease. Finally, in cases where total loss of organ function is inevitable, tissue and organ engineering using stem cells may be a logical treatment strategy. Interestingly, exogenous stem cells have been found within the amniotic fluid that has demonstrated varying levels of success within each of the aforementioned arenas.

Various stem and progenitor cell populations have been found and characterized in amniotic fluid and have demonstrated multiple developmental origins [31]. Thus, it is plausible that stem and progenitor cells, including those of lung origin, can be found in the amniotic fluid may serve as a heterogeneous pool of cells, at various stages of differentiation, which, shed by the developing fetus, may be useful in a treatment capacity. Furthermore, as developing lungs are filled with amniotic liquid, cell populations found within amniotic fluid have already been exposed to the lung and its secretions. One population in particular, amniotic fluid stem cells (AFSC), selected for expression of c-kit, a stem cell growth factor receptor, seems to be a promising choice for a cell-based therapy, as will be demonstrated in the next few sections.

2 AFSC as a Therapeutic in the Lung

2.1 Composition and Development of AFSC

As previously mentioned, the characterization of the composition of AFSC and other cell populations within the amniotic fluid has been extensively studied [32–34]. Not surprisingly, gestational age plays an important role in the composition of cell populations derived from the amniotic fluid [27]. Thus, when investigating the therapeutic potential of AFSC, the potential changes in inherent characteristics of AFSC, at their time of harvest, must be considered when determining their therapeutic or regenerative potential [31]. For example, AFSC derived from a gestationally older donor may express lineage markers of terminally differentiated cell populations, which may be prove useful for repopulation of adult lung tissue or the replacement of specific injured populations, while primitive uncommitted gestationally younger AFSCs may be useful for de novo tissue engineering applications. While the field of AFSC derived treatment strategies for the lung is still quite nascent, there are a few key areas where these cells have demonstrated great promise.

2.2 The Use of AFSC to Treat the Developing Lung

The potential for AFSC to integrate and differentiate into lung specific lineages was first described by Carraro et al. in 2008 [26]. Through the use of in vitro organ culture, murine E11.5 and 12.5 lungs were microinjected with human AFSC (hAFSC) and culture for 7 days. The injected hAFSC, which had been previously characterized, did not express a critical lineage marker present in developing lung epithelia, thyroid transcription factor-1 (TTF-1). Following culture however, the hAFSC not only expressed human TTF-1, they had integrated into the developing epithelium and mesenchyme of the cultured lungs.

Further supporting the potential of AFSC to treat developing lungs, in a nitrofen induced pulmonary hypoplasia rodent model, AFSC improved both in vitro and in vivo embryonic lung hypoplastic phenotypes [35]. In particular, lung surface area and terminal bud numbers and peristaltic activity increased following AFSC rescue. Additionally, AFSC treatment rescued levels of PGP 9.5, a pan neuronal marker utilized to identify ganglionic precursors, in nitrofen treated lungs to that comparable with vehicle treated controls. Although AFSC reportedly demonstrated minimal indications of engraftment and expression of lung specific markers in this model, it was observed and hypothesized that AFSC were exerting a paracrine effect on developing lungs as increases in FGF-10 and VEGFα were observed following AFSC treatment.

These studies demonstrate two very different, yet critical mechanisms for treating developmental abnormalities. First, Carraro et al. demonstrated that AFSC could integrate and differentiate into the correct phenotypes in the developing lung demonstrating the ability of this cell population to correctly respond to the microenvironment and differentiate into the appropriate lineage showing the potential for AFSC to serve as surrogate lung epithelia in the developing lung. Secondly, Pedervia et al. demonstrate the ability of AFSC to sense the microenvironment into which they are placed and respond, by yet unknown mechanisms, to rescue a deleterious phenotype during development.

2.3 The Use of AFSC to Treat Acute Lung Injury

Also investigated in the 2008 Carraro et al. paper was the ability of AFSC to respond to acute lung injury, within the adult lung, in two separate injury models: hyperoxia and naphthalene injury. In both acute injury models, hAFSC retention within the lung was increased when compared to controls. Following hyperoxic lung injury, tail vein injected hAFSC,

demonstrated increased engraftment within injured lungs when compared to non-injured lungs. Furthermore, expression of TTF-1 and SP-C (both lung epithelial lineage markers) was demonstrated in hAFSC following injection into hyperoxic mice, again demonstrating the ability of hAFSC to differentiate into lung specific lineages following epithelial injury. In a second acute injury model, naphthalene injured mice receiving hAFSC again demonstrated increased reported engraftment of hAFSC following injury. Interestingly, within the context of the naphthalene injury model, Carraro and colleagues were the first to compare delivery strategies and their effect on AFSC efficacy. Following intravenous hAFSC injection, CM-Dil labeled hAFSC were observed to localize adjacent to airway tissue, but did not demonstrate integration. In contrast, intratracheal administration of hAFSC demonstrated significantly increased cellular retention in the upper airway epithelium. Furthermore, as naphthalene injury preferentially ablates CC10 expression Clara cells, intratracheally injected hAFSC were observed to express CC10 in naphthalene injured lungs. Finally, Carraro et al. investigated a potential homing mechanism for acute cell retention within injured lungs by demonstrating CXCR4 expression on the surface of hAFSC and increased SDF-1 (the ligand for CXCR4) expression following naphthalene injury.

Buckley et al. further demonstrated the ability of hAFSC to respond to injured lung milieu and differentiate into the appropriate phenotypes in 2011 [24]. Utilizing two models of acute AECII injury, Buckley and colleagues harvested bronchoalveolar lavage (BAL) from hyperoxic rats, as well as conditioned media from in vitro scratch injured AECII. Following hyperoxic lung injury, and in vitro AECII scratch injury, hAFSC were preferentially chemotactically attracted to both injured BAL and conditioned media milieus. Following in vitro scratch damage, co-cultured hAFSC were observed to attach within damaged areas, demonstrating more efficient wound healing in scratch damaged AECII as well. When analyzed proteomically, hAFSC expressed MIF and PAI-1 in the presence of AECII damage, both cytokines which demonstrated beneficial wound healing effects in the AECII scratch injury models described.

Most recently, Grisafi et al. further demonstrated the utility of AFSC in an adult rat hyperoxic injury model [36]. Rats exposed to hyperoxia for a period of 14 days and treated intratracheally with AFSC demonstrated low levels of AFSC retention, however the paracrine effects of AFSC on injured lungs increasing secondary crest numbers while decreasing mean linear intercept and alveolar size measurements. Measured VEGF gene expression along with CD31 positive cell populations increased in AFSC treated lungs suggesting an effect of AFSC treatment on microvessel density. Finally, analysis of IL-6, IL-1β, IF-γ and TGF-1β indicated that following injury, these cytokines increased yet decreased in AFSC treated lungs.

These acute injury studies demonstrated the utility of AFSC in acute injury models, both in vivo and in vitro, further elucidating the plasticity of AFSC in response to their environment. In addition, these studies demonstrated both the ability of AFSC to differentiate into lung phenotypes in response to being placed in injured lung milieu, although this mechanism does not appear to be the primary mechanism of action employed by AFSC following injury. Most importantly however, the paracrine immunomodulatory effects of AFSC were explored in these studies.

2.4 Chronic Injury

Surprisingly, investigation of AFSC within a chronic injury model was not investigated until recently [25]. In an intratracheal bleomycin injury model, which induces inflammation followed by fibrotic lesion formation, the use of AFSC was investigated to determine whether disease progression could be inhibited. Bleomycin injured cohorts were treated with AFSC at either an acute intervention period (2 h post injury) or a chronic intervention period (14 days post injury). Because of the well-documented evidence of the immunomodulatory potential of AFSC, pro-inflammatory and profibrotic cytokines were proteomically analyzed to determine the impact of AFSC on the injurious milieu. Garcia et al. demonstrated that the pro-fibrotic cytokine CCL2 was significantly increased following bleomycin injury and significantly decreased following AFSC treatment in both acutely and chronically treated cohorts. This reduction in CCL2 was correlated with decreased collagen deposition (fibrosis) following injury and inhibition of the loss of pulmonary function. Furthermore, Garcia and colleagues were first to demonstrate a potential mechanism for the reduction of CCL2 in AFSC treated mice, the secretion of MMP-2 by AFSC, which resulted in the proteolytic cleavage of CCL2 rendering it a putative receptor antagonist. Finally, a chemotactic mechanism dependent on CCR2 expression in AFSC and CCL2 secretion by injured AECII was demonstrated elucidating the potential for chemotactic targeting of AFSC to injured lesions.

These experiments were the first to demonstrate a direct response by AFSC to a specific pro-fibrotic pro-inflammatory signaling cascade within the context of fibrotic disease. While regeneration of tissue was not observed, this study introduces the possibility of using AFSC-based therapy to inhibit the progression of fibrotic disease, at lesion specific sites, based on known deleterious biomarkers. Again, like the acute and developmental studies, this study demonstrated that without prior manipulation, other than normal selection and culture conditions, AFSC had the potential to respond to the environment they were placed within in a beneficial manner.

2.5 Whole Organ Regeneration

In cases where lung disease or injury has progressed to the point where transplantation is the only treatment option, clinicians and researchers are investigating the possibility of whole organ replacement via tissue-engineered lung. Tissue-engineered lung can be derived from either endogenous or exogenous stem or progenitor cell populations seeded on allogeneic or xenogeneic lung matrix. Seeded matrices would then, in theory, be cultured to repopulate the organ with functional organ specific tissue. Such a strategy reflects a novel approach to regenerative medicine which could result in a decrease in transplant waiting time limited only by organ regeneration time, improved patient survival rates and a reduction in transplant rejection as exact donor matches would be unnecessary. As the American Lung Association estimates that lung disease is the third leading cause of death in USA, responsible for one in six deaths, the potential impact, scope and scalability of this translational treatment strategy is enormous. The successful creation of tissue-engineered lung involves a twofold approach: selection of an appropriate candidate stem cell population and the use of appropriate scaffolding for repopulation. Currently, groups are investigating the potential of AFSC via this innovative approach.

3 Future Prospects

The lung is a complex organ that comprises a large surface area that interfaces with the outside environment as well as the circulatory system, making it particularly vulnerable to injury and disease. To address growing public health concerns in the lung, novel therapies that stimulate and protect endogenous lung stem or progenitor cells or supply exogenous cells for regenerative or immunomodulatory purposes have become a therapeutic target. These novel and groundbreaking approaches may have the potential to contribute to repair processes, slow the decline in lung function, or even perhaps to regenerate functioning lung.

As the field of AFSC-based therapy moves forward, the identification and characterization of the cytokines, growth factors, and other biomarkers that dictate the response to disease is key to understanding, diagnosing, treating, and determining the trajectory of corrective AFSC-based therapies. Critical to the further development of AFSC as a treatment strategy is the development of bold and innovative techniques and experimental systems that more effectively mimic the clinical etiology of the disease so that therapeutic strategies can be rigorously tested.

The prospect of using cell-based therapies, specifically AFSC, as a treatment strategy for lung disease is exciting in that this versatile cell population has demonstrated the ability to serve as a useful therapeutic tool at multiple intervention periods, in multiple developmental, injury and disease models. The further development of this therapeutic cell population, and the investigation of their mechanisms of action, will surely continue to elucidate the potential for AFSC in this and numerous other injury and disease states.

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Direct Reprogramming of Amniotic Cells into Endothelial Cells

7

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Abstract

Organ regeneration promises unlimited access to replacement tissues. The current paradigm of organ regeneration requires transplantation of adult tissue-restricted stem and progenitor cells to repair the damaged organ. However, healing injured organs often leads to fibrosis with little recovery of function. An alternative approach is to harness the regenerative activity of microvascular endothelial cells (ECs) to support endogenous organ repair. Recent work demonstrates that organ regeneration can be directed by paracrine mediators, called "Angiocrine Factors," elaborated by tissue-specific ECs to support stem and progenitor cells to directly induce organ regeneration without maladaptive fibrosis. Yet, the regenerative function and the repertoire of angiocrine factors elaborated by ECs depend upon the organ from which they originate.

New technologies have emerged to transcriptionally reprogram amniotic fluid cells (ACs) into generic "unspecified" ECs that acquire tissue-specific function promises a ready source of transplantable ECs to be used for organ regeneration. Generic AC-derived ECs can be induced to acquire organ-specific functions by a process of "in vivo education" wherein extravascular cues trigger transcriptional programs within engrafted ECs enabling them to acquire tissue-specific functions and to deploy angiocrine growth factors that drive organ repair without aberrant pro-fibrotic remodeling. Identifying tissue-specific transcription factors regulating tissue specification of EC is at the frontier of this new approach for organ regeneration.

The chapter is expected to overturn the scientific conceptualization of a monofunctional, inert, microvasculature by revealing a dynamic, tissue-specified role for ECs in organ repair that will enable therapeutic use of "educated," tissue-specified ECs that home to their native injured organs and supply tissue-specific angiocrine signals to orchestrate organ regeneration.

1 Introduction

Organ regeneration promises unlimited access to replacement tissues to treat a wide variety of human diseases. However, clinical scale production of cells with regenerative function is difficult. Reprogramming human amniotic fluid cells (AFCs) offers a new and potentially better way to promote organ regeneration via cell-based therapies. Vascular endothelial cells (ECs) function not just as passive blood conduits but also provide regenerative cues to damaged tissues, but ECs are difficult to propagate in vitro from primary cell cultures. Advanced technologies allow, for the first time,

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efficient generation of ECs by "reprogramming" differentiated cells such as human AFCs. AFCs appear to have unique attributes that permits stable and efficient reprogramming and promise to make AFC-derived grafts available to promote regeneration and potentially to directly treat vascular diseases. AFCs can be directly reprogrammed into fully functional ECs without passing through a pluripotent state thereby avoiding the potential for teratoma formation. Derivation of patient-specific ECs derived from banked human AFCs is expected to facilitate development of customizable, functional grafts on-demand, matched to the immune profile of a recipient receiving a new class of personalized, reconstructive therapy for tissue damage resulting from disease or trauma. The selection of donor cell sources, derivation technologies, delivery approaches, and definition of the end points used to assess effectiveness are all areas of active investigation. Ultimately, success will require the availability of high-quality regenerative cells produced from well-defined sources on a clinically relevant scale with standardized functional characterization.

This chapter highlights recent advances in reprogramming assays of human AFCs that have lead to a new generation of fully viable, functional, three-dimensional endothelial cells. "Reprogramming" human AFCs to generate clinically scaled, functional ECs is now possible by enforced expression of vascular directing ETS genes. This new technology appears to more efficiently, reliably, and stably yield functional ECs thereby circumventing the limitations of strategies that rely upon directed differentiation of pluripotent stem cells.

1.1 Endothelial Cells and Amniotic Cells

Stretched out, the circulatory system of each person extends over 100,000 miles [1]. Thus, each human has enough endothelial cells (ECs) lining the inner surface of the circulatory system to wrap around the earth two to four times (equator: 24,901.5 miles). We think of the endothelium as the passive lining of blood and lymphatic vessels but recent studies clearly show that ECs also provide instructive cues to host tissues. ECs within each organ system are highly tuned to their resident tissues. As a result, ECs within the body are heterogeneic with each organ hosting unique tissue-specific (organotypic) ECs.

Regenerative cell therapy requires large numbers of engraftable cells. It has been difficult to produce enough progenitors in vitro despite the development of highly refined cell culture conditions and cytokines. Tremendous efforts have so far failed to capitalize on the promise of pluripotent stem cells (PSCs)—such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)—to produce cells with regenerative function. This approach is hampered by the low efficiency with which grafts can be produced and the general instability of the differentiated cell progeny. Adult stem and progenitor cells, with more restricted potential such as mesenchymal stem cells (MSCs), hematopoietic stem and progenitor cells (HSPCs), and endothelial progenitor cells (EPCs), can be useful if sufficient numbers of cells can be obtained. However, expansion of these cells in vitro typically leads to divergent differentiation yielding highly heterogeneous cell populations. A new approach is to instead, produce cells that can direct host-resident progenitors towards their regenerative potential. ECs have this capacity via production of instructive, angiocrine, factors that spur-resident tissue stem and progenitor cells to repair tissue damage. This approach promises that EC donor grafts could be used to promote organ regeneration. For this strategy to work, one needs access to a sufficient number engraftable ECs with organ-specific angiocrine function.

The precise definition of differentiated ECs remains a matter of debate. Nevertheless, to date ECs are widely defined as plastic- or extracellular matrix (ECM) proteins (collagen, fibronectin) [2, 3] adherent cells that, under closely controlled conditions, express EC-specific lineage markers (VE-cadherin, CD31, KDR, vWF). Cultured ECs can uptake acetylated LDL, bind to *Ulex europaeus* lectin, and respond to exogenous growth factors [4, 5] and environmental cues such as shear stress [6]. Both genotypic and phenotypic characteristics of differentiated endothelial-like cells fully depend upon formulation of the media composition that determines efficacy and functional potential. As yet, few if any endothelial-like cells produced from pluripotent or adult stem cells express all characteristics of genuine ECs relevant to human cell therapy.

Recently, "direct reprogramming" of terminal somatic cell from various tissues that trans-differentiate into linage committed ECs has emerged as an attractive new approach. Human dermal fibroblasts have been trans-differentiated towards ECs during in vitro culture on gelatin scaffolds in endothelial growth medium (EGM) [7, 8]. Direct reprogramming of functional vascular ECs from AFCs by forced ectopic expression of endothelial-specific transcription factors has also been shown to reliably yield naive ECs that acquire tissue-specific functions after transplantation into regenerating organs [9, 10].

Human AFCs are an ideal population of nonvascular cells amenable to reprogramming into authentic vascular ECs. Amniotic fluids are routinely cultured from the amniocentesis of mid-gestation human fetus for diagnostic purposes and have received significant attention for their potential to differentiate into various cell types [11, 12]. Now, standardized methods enable reliable derivation of functional vascular ECs that circumvent limitations of PSC-based approaches and are suitably scalable for the development of regenerative cellular therapy. Such engineered endothelial grafts for organ

regeneration are also well suited to promote revascularization of the regenerating organ thereby restoring blood flow and maintaining the graft after implantation. Direct reprogramming of human AFCs using defined ETS genes to generate generic ECs offers the potential for high-throughput large-scale production of endothelial grafts for use in regenerative medicine applications.

1.2 Vascular Beds Instruct Organ Regeneration via Angiocrine Factors

Endothelium is a distinct inner layer of cells that form highly branched tubular vascular networks connecting tissues to their environment. ECs are well positioned to regulate respond to changes in the microenvironment by altering gene-encoded metabolic pathways. Capillary bed ECs have numerous surface projections to form the smallest vascular systems, called microvessels or microcirculation. Capillary walls are composed of single layer of EC closely associated with pericytes via microvilli and filopodia. Sinusoidal ECs are a discontinuous endothelium anastomosed to capillary beds that can acquire organ specification in response to tissue-specific microenvironment-derived signals that influence the morphogenesis of endothelial heterogeneity.

The ECs in direct contact with blood are called vascular endothelial cells and form arteries and veins, whereas ECs are in contact with lymph fluid are knows as lymphatic EC. Vascular and lymphatic ECs line the entire circulatory system in the body. The lymphatic ECs allow the lymphatic vasculature to drain interstitial fluid. Vascular and lymphatic ECs are distinguished by their distinct expression of molecular signatures that define and likely contribute to their functional and morphological differences. Understanding the physiological role of ECs has been mainly directed by studies of their role in tissue homeostasis as vascular conduits, that is their role in blood and lymphatic plumbing. Yet the lining of these structural conduits have a myriad of functional roles in blood circulation: modulating blood coagulation and fibrinolysis; setting vascular tone; mediating blood–tissue exchange and fluid filtration; responding to biomechanical factors such as shear and tensile stress; altering the metabolome and hormone trafficking; and as gatekeepers selecting or refusing migration of blood and bone marrow (BM) cells.

More recently, ECs have also been shown to support and direct adult stem and progenitor cells during homeostasis and organ regeneration by elaborating juxtacrine/paracrine molecules referred to as "angiocrine factors." ECs with such function comprise an "endothelial instructive niche" that orchestrates organ regeneration/repair [13]. Angiocrine factors central to niche function include juxtacrine signaling via the Notch pathway (Notch-1, Notch-2) and cytokines with trophic paracrine activity on neighboring cells [14]. Angiocrine factors provide local instructive cues within damaged tissues to direct regeneration mediated by resident stem and progenitor cells. ECs deploy distinct angiocrine factors in response to particular microenvironmental cues and these adaptive functions provide fine control over stem cell proliferation and differentiation. In pathologic conditions, tumor cells appear capable of co-opting ECs to provide pro-tumorigenic angiocrine/niche functions [15].

The processes of repair, remodeling, and regeneration generally restores tissue continuity by replacement of lost cells and synthesis of the ECM proteins that bind them. Specialized cells are recruited to the site of regeneration, and undergo differentiation, replication, re-specialization, and go on to replace the missing tissue. Host tissue stem cells are induced to proliferate and differentiate as necessary to regenerate the lost tissue. An instructive role of sinusoidal ECs in organ regeneration was shown in mouse liver and lung resulting from the interplay of cytokines, growth factors, and metabolic pathways at the injury site of injury. During regeneration, cell traffic dynamics are altered at the original anatomical site via modulated expression of chemokines expressed by ECs to augment recovery of the missing organ mass. For example, liver sinusoidal ECs—immunophenotypically defined as $VEGFR3^{+}/VEGFR2^{+}/VE\text{-}cadherin^{+}/FactorVIII^{+}/CD34^{-}/CD45^{-}$ cells—releases angiocrine factors that initiate and sustain liver regeneration induced by 70 % partial hepatectomy [16]. Priming of the vascular niche by organ injury prepares the way for new tissue formation by providing a vascular network employing essential instructive cues at the anastomosed sites to promote repair and regeneration of the damaged tissue. These regenerative functions of ECs are normally dormant, adaptively revealed during recovery from tissue damage. The nature and extent of endothelial plasticity is poorly understood and its centrality to regeneration is only starting to be appreciated.

1.3 Endothelial Cell Plasticity, Ontogeny, and Reprogramming

Much has been learned about cell fate plasticity from recent efforts to directly reprogram terminally differentiated somatic cells to other cell types. Such transdifferentiation captures nascent cellular plasticity—typically by enforced expression of lineage-specifying transcription factors—to drive epigenomic remodeling towards other potential cell fates. Normally, "plasticity" permits a cell to cope with environmental/conditional variations. A clear example of reprogramming occurs within the zygote

following fertilization. In vitro, somatic cell nuclear transfer (SCNT) enables the reprogramming factors in the ooplasm of an enucleated egg to faithfully reprogram the epigenome of a donor nucleus to other cell types, so called "nuclear reprogramming" [17–19]. In addition, differentiated cell types can be reprogrammed by treatment with pluripotent cell extracts [20–22]. The largest impact has resulted from the observation that enforced expression of a small number of transcription factors can reprogram terminally differentiated cells to iPSCs [23].

It is now recognized that not all methods of reprogramming are suitable for all cell types. A major determinant of cellular plasticity is the cell's intrinsic epigenetic state that defines its ability to switch cell fate. DNA methylation plays a significant role in reprogramming because agents that demethylate DNA methylation can enhance the efficiency and effectiveness of cellular reprogramming [24]. It is clear that reprogramming requires both a permissive epigenetic "ground state" amenable to complete reprogramming and the correct "cocktail" of reprogramming factors to direct the transition.

Some cell types are normally plastic. Plasticity is a predominant feature of adult stem cell types permitting them to differentiate towards multiple lineages.

Plasticity of endothelial progenitors was first observed during embryonic development in primordial ECs and hematopoietic cells of the yolk sac [25–27]. Emerging from these studies is the concept of a hemangioblast, the common mesodermal precursor of vascular and hematopoietic cells [28]. This ontogeny persists in the definitive embryo and critical fate decisions rely upon the differential expression of particular transcription factors. Hemogenic endothelium—endothelial precursors that can yield either definitive endothelium or hematopoietic cells—is transiently generated and appears dependent upon expression of Scl/Tal1 and perhaps ETV2 [29–31]. The transcription factor Runx1 is then required to generate definitive hematopoietic cells from hemogenic endothelium [32], suggesting this master regulator directs endothelial precursors to hematopoietic fates. The central role of epigenetic regulation is demonstrated by the inappropriate expression of neural genes in hemangioblasts when components of the polycomb repressive complexes 1 (PRC1) were deleted [33]. Thus, hemogenic endothelium must be epigenetically poised for both endothelial and hematopoietic futures but not other possible fates.

The hierarchy of vascular identity is initiated early during embryogenesis. Endothelial progenitors are modeled towards arterial or venous fates before endothelial tubes form in the chick embryo [25, 26]. Yet embryonic ECs also retain arterial–venous plasticity until late in embryonic development [13, 34]. Adult ECs also retain remarkable plasticity and a large body of work suggests that plastic, EPCs persist throughout life. It is perhaps from such endothelial progenitors that the tissue-specific characteristics of microvascular ECs emerge after being engrafted into host tissues [35]. Indeed, heterogeneity of the angiocrine signals deployed by ECs in different tissues is only now emerging [14, 16, 36, 37]. This heterogeneity means that regenerative therapies attempting to replicate the angiocrine function of organ-resident endothelium must recapitulate the organ-specific angiocrine regenerative functions provided by endothelial grafts.

One approach is to generate large numbers of naive endothelium that engraft damaged tissues where they acquire the necessary angiocrine functions to promote regeneration. That EPCs can only be purified in small numbers is a significant hurdle to their clinical application. So other approaches are being taken. PSCs can be directed to endothelial fates but current methods yield only small numbers of cells with endothelial features (Daylon papers). An ongoing challenge of grafts generated by in vitro directed differentiation of pluripotent cells is residual plasticity that allows the progeny to drift to other fates or yield teratomas. It is now clear that differentiation from a pluripotent state is unnecessary and perhaps detrimental.

Mature somatic cells harbor residual plasticity that can be captured by the enforced expression of transcription factors that induce their conversion to other cell types. Such cellular interconversions elicited experimentally often mimic aspects of developmental cell fate transitions and it is possible that the two processes are fundamentally similar. As early as 1987, MyoD1 was shown to convert fibroblast to skeletal myocytes [38, 39]. Additional examples of such direct reprogramming of mature somatic cells provide important insights into the core transcriptional networks and cellular signaling pathways required for intra- and inter-lineage fate transitions [40, 41]. It remains an open question whether direct reprogramming of mature cells will circumvent the challenges identified in regenerative grafts derived by directed differentiation of pluripotent cells.

2 Transcriptional Regulation of Stage-Specific Endothelial Differentiation: *Mini Review*

A dynamic morphogenic process that all cells come from pre-existing cells is the mean to measure "different" cell identity, and the morphological adaptation steps in relation to cellular function are called "differentiation." Differentiation is determined by a cell fate and by spatiotemporal specification. A cell fate is identified either by autonomously (committed), conditionally (reversible), or syncytially "specified"

or by "determined (irreversible)" to corresponding progeny,[1] and relies on cellular morphogenesis property. Major advances in molecular tools and microscopy technology have been transformational in understanding the mechanisms that drive the specification for a cell fate by tracing cell lineages during differentiation steps. Cell lineage tracing (or "fate mapping") provide information about a differentiation to learn about the actual changes in biochemical and structural status, functional morphometric characterization, biointerface communications, and phenotypic adaptations in an incessant signaling feedback that governs cellular fate.

Experimental findings on in vitro directed differentiation of stem cells suggests that PSCs can unlimitedly differentiate into ECs. Embryonic stem (ES) and iPSCs represent PSCs, and have been an in vitro models to analyze molecular mechanisms underlying differentiation steps to generate ECs through sequential differentiation steps through embryoid body (EB) formation that mimics the in vivo vascular morphogenic process, followed functional development into arterial, venous, capillary, and lymphatic ECs, as well as smooth muscle cells [42]. In vitro culture condition for PSCs can be highly defined, xeno free and/or serum free for accurate profiling in molecular pathways governing development of ECs and assembly of complex vascular networks occur in vasculogenesis, angiogenesis, lymphangiogenesis, and vascular remodeling. Nonetheless, the PSC culture platform can identify populations of reprogrammed ECs derived from various cells including PSCs, mesenchymal stroma cells (MSCs), and AFCs from amniotic tissues, as well as to a variation of signaling pathway attribute governing the maintenance of different PSC types, including leukaemia inhibitory factor (LIF) and bone morphogenetic protein (BMP) for mouse ESCs, a nodal factors, activin/inhibin/transforming growth factor beta (TGFβ), and fibroblast growth factor (FGF) for human embryonic stem cells (hESCs).

Recent advances in direct reprogramming of somatic cells to stem cells or to other type of somatic cells via epigenetic reprogramming by forced expression of specific transcription factors of a different cell fate enriched in the target cell suggest that the erection of a cell fate switch could be a key event in induced differentiation. This approach has been successful in generating functional ECs. The process in general requires an epigenetic reprogramming achieved by the transcription factors that can directly interact to chromatin for epigenetic modification, followed by exposure to specific developmental cues to generate cell populations of a given fate. This forced lineage conversion has attracted increasing attention as a potential alternative to the directed differentiation of PSCs to obtain cells of a given lineage. Importantly, although a biochemistry description of cellular genetics and epigenetics is taken significant credit for differentiation, additional players have recently reported, which may provide better understanding for the differentiation which one day will lead to more facile and efficient manipulation of cell fate for regenerative medicine. As such, specific noncoding RNAs and micro RNAs can be used to mark to track and predict for cell fate switch that can be used to directly manipulate cell fates.

The question remains as to which development stage represents the best candidate for the strategy to generate such ECs with the best functional benefits which account with enhanced angiogenesis, reduced vascular remodeling, or to cytokine-mediated effects that promote the survival of endogenous cells. Pluripotency-associated factors and lineage specifiers have generally been considered to determine the identities of pluripotent and differentiated cells, respectively [43, 44]. The pleiotropic expression patterns of embryonic genes that are usually suppressed in definitive developmental stages, and are persistently present in the PSC cultures in developmental differentiation process is governed by dynamic interchangeable regulation of heterogeneity. A hallmark of PSCs, contributing to plasticity to lineage specification to EC is shown driven by reduction of the transcribed portion of the genome [45]. Different stages of developmental differentiation may be determined by the different set of signals by the targeted activation of stage-specific genes or of lineage-specific genes or by selective silencing of genome regions. Stage-specific transcriptional expression of the targeted cells may sporadically enhance or suppress tissue-specific genes, is called cell plasticity that is accompanied by disproportionate expression of set of chromatin-remodeling genes and the transcription machinery. This transcriptional hyperactivity landscape in the target cells can be profiled using whole-genome tiling arrays, i.e., high-throughput RNA sequencing.

2.1 Endothelial Cell Identity by Transcriptional Regulation

Understanding the cellular identity that can be grafted to give rise to large number of functional ECs without developing malignant outputs is a major goal of directed reprogramming strategies for clinical therapy. During early commitment to a mesodermal lineage, the complex transcriptional and epigenetic changes are associated to EC lineage commitment

[1] A cell specified autonomously will develop into a specific fate based upon cell-intrinsic properties that are cytoplasmic determinants with no regard to the environment the cell is in.

A cell specified conditionally will develop into a specific fate based upon cell-extrinsic process that relies on cues and other surrounding cells or morphogen gradients.

A cell specified syncytially will develop into a specific fate based upon a hybrid of the autonomous and conditional method involves the action of morphogen gradients within the syncytium that can influence nuclei in a concentration-dependent manner.

A cell determined will develop into a specific cell types followed by differentiation.

process, and tens of transcription factors have been identified. ETS family factors specify cell autonomous function of de novo vasculogenesis and myelogenesis by direct interaction with promoters that can switch on vascular-specific genes by efficient transcription of signaling in angiogenic repertoire evident in adult vasculature. ETS binds to endothelial promoter/enhancer and directly regulates expression of VE-cadherin [46, 47], Tie/Tek [48], Flk-1 [49, 50].

In the developing endothelium, the ETS proteins function redundantly with the exception of non-redundant role of Etv2/ER71 as a primary player in specification and differentiation of the endothelial lineage [51]. The endothelial-specific zebrafish Ets1-related protein (Etsrp) is functional orthologs of human Etv2/ER71 (zebrafish: Etsrp, mouse: ER71, human: Etv2/ER71) that assume regulation of endothelial gene expressions. Etsrp, which expression is restricted to endothelial precursors and differentiated ECs in the zebrafish embryos [52], has been identified as a primary player in specification and differentiation for vascular reprogramming that activates other ETS transcription factors. Overexpression of mouse ER71 and zebrafish Etsrp caused strong expansion of hemangioblast and vascular endothelial lineages [53]. Analysis of one of ETS family of transcription factor, fli-1 in the zebrafish embryo has identified that the initial expression of fli-1 in the posterior lateral mesoderm is the earliest indicators of hemangioblast formation [54].

Using microarray profiling and genetic tracking for the directed differentiation of hESC into embryonic ECs, ER71, Fli1, and Erg isoform 2 (Erg-2) have been reported as key ETS transcription factors that are expressed during differentiation of hESCs into ECs [10]. More recently, high-throughput RNA-sequence analysis revealed that adult vascular ECs constitutively express Etv2/Er71, Fli1, Erg, Ets1, Ets2, Elf1, Elk1, Vezf, and Etv6, while Etv2/ER71 is only transiently expressed during the early phases of fetal development specifying vasculogenic differentiation of hESC-derived mesodermal precursor cells, and its expression is shut off in adult vascular ECs [9]. Whereas, other ETS transcription factors expressed in committed ECs, such as Fli1, VEZF, and ETV6, as well as various Erg and Elf isoforms are constitutively expressed during fetal development and adult ECs.

In addition to the ETS family, several transcription factors are known to induce endothelial-specific gene expression. In adult EPC precursor cells, the endothelial nitric oxide synthase (eNOS) promoter was shown silenced by DNA methylation and prominent repressive histone H3K27me3 marks, and silencing of histone demethylase, Jmjd3, indicating that hypoxia plays a role in recruiting EPC precursor cells in ischemic tissue that facilitates eNOS expression via activation of Jmjd3 that can turn on the epigenetic modifications [55]. An involvement of FBW7 in regulation of endothelial function in migration, angiogenesis, inflammation, and barrier integrity has been shown that FBW7 targets the zinc finger transcription factor Krüppel-like factor 2 (KLF2) in ECs upon activation of KLF2 by glycogen synthase kinase-3 (GSK3) at two conserved phosphodegrons [56, 57].

Implications for gene regulation during development are more complex involved in the case for co-activator and it's binding to enhancer of the activating genes. In hemogenic endothelium of mouse ESC, a hemogenic endothelial development regulator, Sox17 has been known expressed. Sox17 is suggested that it plays a pivotal role in the development and/or expansion of hemogenic endothelium through the Notch signaling pathway [58]. During the differentiation of hESC to EC, forkhead box protein A2 (FOXA2), also known as hepatocyte nuclear factor 3-beta (HNF-3B), or as transcription factor 3B (TCF-3B), has been identified differentially expressed transcription factor specific to early mesoderm commitment, which is bivalently marked with histone modifications for both gene activation (H3K4me3) and repression (H3K27me3) in early stage of differentiation of hESCs to mesoderm [59]. Cooperation between KLF2 with ERG was suggested that synergistically activate transcription of Flk1 while KLF2-binding site is located on the Flk1 enhancer region [60]. The mitogen-activated protein kinase (MAPK) family of transcription factor, Foxo1 (FKHR) has been shown to interact with Ets-1 and functions as a coactivator for Ets-1 on Flk-1 promoter in bovine carotid artery ECs [50]. Myocyte Enhancer Factor 2 (MEF2) family of transcription factors that is expressed very early in the development of the endothelium, has been identified for the enhancer gene with four consensus Ets transcription factor-binding sites that is upstream of MEF2-binding site [61]. Zebrafish hemangioblast gene Cloche was identified to act upstream of the homeobox gene, Hhex (Flk-1 homologue), in blood island [62]. A zebrafish homolog of Etv2/ER71, Estrp, was identified as an upstream regulator for Cloche and SCL in the pathway from mesodermal precursor to hemangioblast. F-box and WD repeat domain-containing 7 (FBW7), the substrate-binding subunit of E3 ubiquitin ligase SCF (FBW7) (a complex of SKP1, cullin-1 and FBW7) is required for vascular development.

The lymphatic vasculature forms during embryogenesis through vasculogenesis via predominant means of vessel formation after arterial–venous EC specification within hemangioblast mass [63] via transcription regulation switch into a thin-walled, blind-ended lymphatic vessels with valves to transport fluid to the lymph node comprised a single, non-fenestrated EC layer [64]. The lymphatic network forms a second vascular system lined with vascular ECs, which complements the vascular network by modulating tissue fluid balance, allowing interstitial protein transport, and initiating the immune response [65].

VEGF-C/VEGFR-3 signaling is well known for lymphatic EC sprouting after lymphatic EC specification by Prox1 that can rescue lymphatic EC sprouting in whole-mount explants of this mutant LYVE-1 and podoplanin [66]. The sequence, organization, and variation of lymphatic ECs are regulated by

the VEGFR3 gene which proximal promoter is TATA-less that contains stretches of sequences homologous with the mouse Vegfr3 promoter region, and control EC-specific transcription of downstream reporter genes, suggesting that EC-specific elements occur in the proximal promoter, although further enhancer elements are probably located elsewhere [67]. Several transcriptional factors regulate VEGFR3 expression have been reported, Spred/Sprouty family proteins, negative regulator for growth factor-induced ERK activation, Spred-1/2 has been shown involved in lymphangiogenesis by negatively regulating VEGF-C/VEGFR-3 signaling [68]. Induction of VEGFR-3 expression by VEGFR-3 promoter binding is also possible by the Notch-1 complexed with DNA-binding protein CBF-1/suppressor of hairless/Lag1 (CSL) [69].

More recently, mutation in transcription factor coding gene sex determining region Y [SRY]-box 18 (Sox18) was shown selectively impaired lymphatic sprouting from the cardinal vein and resulted in defective lymphatic thoracic duct formation, suggesting that a cross talk between the growth factor and transcription factor pathways regulate lymphangiogenesis [70]. In mouse dorsolateral sector of the cardinal vein at E9, a subpopulation of venous ECs has been identified to acquire to express the transcription factors, Sox18 approximately 1.5 day prior to the onset of prospero-related homeobox 1, a homeobox transcription factor (Prox1) and podoplanin [71]. In human, COUP transcription factor 2 (COUP-TFII), also known as nuclear receptor subfamily 2, group F, member 2 (NR2F2) has been shown required to activate Prox1 through direct binding to the Prox1 promoter region [72], suggesting that an acquisition of Sox18/COUP-TFII is essential for venous ECs to switch into lymphatic fate, and upregulation in Prox1 is associated with induction of lymphangiogenesis. In mouse ESC-derived Sox18+ lymphatic vessels, forced expression of Prox1 induces suppression of Sox18 expression, and concomitant upregulation of FoxC2, angiopoietin-2 and HoxD8. Furthermore, HoxD8 was found upregulate Prox1 expression, indicating a positive-feedback-loop mechanism in which Prox1 expression specified by HoxD8 [73]. This suggests that Sox18-Prox1-HoxD8 axis may play a pivotal role in the regulation of lymphangiogenesis. It is, however, not clear yet what molecular mechanisms are responsible for the restriction of its expression to the lymphatic endothelium during development.

2.2 Genetic Regulator and TGFβ for Endothelial Function

The TGFβ signal inhibition has been shown essential for a reprogramming of human AFCs into EC with transient expression of Etv2/ER71 and constitutive Fli1 expression [9, 74]. The vascular growth requires a bifunctional regulator, TGFβ signals that are activated by contact between EC and mesenchymal cell, modulates vascular growth [75, 76] by a punctuated pattern of TGFβ expression that is set aside a ventral mesodermal population. As such, low dose upregulate angiocrine factors, whereas high doses appear to inhibit EC growth [76–78], which low dose expression is required in the de novo synthesis of primordial vasculature. As such, suppression of nonvascular genes attenuated by TGFβ signal inhibition is essential for the assembly of VEGFR2 signaling pathway and remodeling, which results in VEGF-A-induced expansion of large numbers of reprogrammed ECs via interaction with pericytes or vascular smooth muscle cells [74].

TGFβ has been known its bifunctional role for vascular cell growth and differentiation in vascular formation via induction of mural cell development, activation and inhibition of EC proliferation and differentiation, augmenting EC migration via alterations in EC matrix deposition [79–81]. TGFβ-deficient mice die in mid-gestation from defective yolk sac vasculogenesis ranging from delayed vasculogenesis to the total absence of vessels, thus implicated important in hereditary hemorrhagic telangiectasia (HHT), atherosclerosis, tumorigenesis, and immunomodulation [82–84]. The TGFβ family members, TGFβ1, TGFβ2, TGFβ3, BMPs, activins, inhibins, and endoglin, bind to two types of a serine threonine kinase receptors, type I (Alk1, Alk5), type II (TGFβRII), and type III (Endoglin) that are also expressed on ECs [85]. Although TGFβ signaling is not required for differentiation of extraembryonic mesoderm into ECs, it is necessary for their subsequent organization into robust vessels. A major function of TGFβ in yolk sac mesoderm is to regulate production and deposition of fibronectin in the ECM that maintains yolk sac integrity, which TGFβ signals are tightly regulated for the differentiation of hemangioblasts [86]. With targeted deletion of TGFβ1, half of the mutant embryos die at E9.5-E10.5 due to defective yolk sac vasculogenesis while half survive several weeks before succumbing to inflammation [83, 86, 87]. Deletion of TGFβRII [88, 89] as well as ablation of Alk1 or Alk5 [84] causes in embryonic lethality due to defective vasculogenesis in the yolk sac, suggesting that these TGFβ signaling pathways are not redundant. Alk1 phosphorylation activates smad1, smad5, and smad8 whereas Alk5 activates smad2 and smad3, which then complex with smad4 to activate transcription in the nucleus.

TGFβ induces a number of genes associated with the ECM on ECs, including fibronectin and collagens I, IV, and V [85]. Mice lacking one of TGFβ family, endoglin, also known as CD105, END, FLJ41744, HHT1, ORW, and ORW1, die from defective vascular development by gestational day 11.5 caused poor vascular smooth muscle development [90–92], by arresting endothelial remodeling with vascular malformations [83, 93]. ETS factors regulate matrix turnover by activating transcription of several metalloproteinases (MMPs), that was shown in the study that Ets1 undergo acetylation in response to TGFβ signaling [94]. TGFβ was shown to affect the transactivation activity of Ets-1. TGFβ augments the transactivation activity of Ets-1

by inducing a protein that interferes with the binding of Ets-1 to the DNA-binding site. This study also showed that Ets-1 expression to the level equivalent to FGF2 enhances DNA-Ets complex formation, whereas TGFβ attenuates basal and FGF2-enhanced DNA-Ets complex formation via the promoter activity driven by Ets-1 [95].

A beneficial effect of TGFβ inhibition attenuated on the expansion and preservation of vascular phenotype in ECs derived from hESC that are differentiated to embryoid body (EB) culture on matrigel-coated plates in serum-free media supplemented with VEGF-A [10]. The proliferation of vascular cells resulting in a net 36-fold expansion into EC-like cells exhibited a transcriptional profile of Id1(high) VEGFR2(high)VE-cadherin(+) ephrinB2(+) that Id1 is required for increased proliferation and preservation of EC lineage commitment [10]. The contribution of TGFβ signal inhibition to the ETS transcription factor mediated reprogramming into ECs was also shown a significant decrease in total VEGFR2 expression, which confirmed that TGFβ signaling regulates VEGFR2 expression in EBs [96]. More recently, inhibition of TGFβ was shown essential for transcriptional direct reprogramming into function ECs with forced expression of transient Etv2/ER71 with concomitant Fli1/Erg1 derived from human amniotic fluid-derived cells (AFCs) of second trimester amniocentesis. These data suggest that in combination with overexpression of ER71 and Fli1, complete reprogramming into ECs requires TGFβ inhibition to induce and maintain the expression of functional VEGFR2 signaling apparatus [9].

2.3 The Production and Directed Differentiation of Embryonic Stem Cells into Endothelial Cells

The prospects for use of hESCs derivatives in regenerative medicine are significant, and there is much optimism for their potential contributions to human regenerative medicine. The methods for deriving hESCs are well established from chromosomally euploid, aneuploid, and mutant human embryos that are available from in vitro fertilization (IVF) clinics treating patients for infertility or preimplantation genetic diagnosis (PGD) from four- to eight-cell morula and from blastocysts and from isolated inner cell mass (ICM) cell clusters. The ESCs can be formed and maintained on human somatic cells in xeno-free, serum-free culture system that can spontaneously differentiate when grown in the absence of the feeder cells, when overgrown in culture, or when removed from the maintenance colony. All three major embryonic lineages are produced in differentiating flat attachment cultures and unattached embryoid bodies (EBs). Stage-specific differentiation can be identified by stage-specific surface markers, or by expression of reporter genes, as well as by characteristic morphology. Directed differentiation methods are well documented for ectodermal lineage (neural cells) and mesendodermal lineage (cardiomyocytes, hematopoietic cells, endothelial cells), but few for endodermal lineage, which may be due to limited arrays of stage-specific markers available for endodermal lineage [97].

A derivation of mouse and rat ESCs from the late epiblast layer of post-implantation embryos and long-term maintenance demonstrated using chemically defined, activin-containing culture medium that a direct differentiation of ESCs into specific adult cell types is regulated by endoderm organogenesis via a nodal signal, activin/TGFβ, that promotes the expression of a conserved transcription factor network; a mix-like homeobox genes Foxa2, Sox17, Eomesodermin, and Gata4-6 [98]. This network of transcription factors can activate a cascade of gene expression that functions to commitment to an endodermal fate, segregate the endoderm and mesoderm lineages, and integrate signaling events that regionalize the nascent endoderm [99, 100], suggesting an evolutionarily conserved role in the derivation and the maintenance of pluripotency in stem cell. The precise roles of these factors vary between species, thus distinguishing human progenitors requires purification schemes based on additional information.

Reported in 2002, the function of BMP4 was identified that BMP4-induced emergence of trophoblasts from hESCs [99, 101]. Conversely, BMP4-treated hESCs may endogenously expressing trophoblast gene via induction of mesoderm by cooperating with FGF2 that act through BRA and CDX2 [102]. Similarly, directed hES differentiation into the definitive endoderm lineage in the presence of a nodal signal, activin A, and low serum culture condition was shown possible for up to 80 % definitive endoderm cells, which both early endoderm and mesoderm genes were co-detected when enriched using CXCR4 [103], which was confirmed in visceral endoderm of mouse embryos [104]. Mouse ESCs emerge in response to 3-day treatments with BMP4 or retinoic acid that promotes development of posterior epiblast precursors, which give rise to primitive streak mesoderm, extra embryonic mesoderm tissues and primordial germ cells [105, 106]. Whereas retinoic acid has been shown to involve in the emergence of precursors that give rise to neuroectoderm and neural crest [107]. This selective culture condition deriving mixed meso-endoderm lineages using markers to monitor the differentiation using CD25, goosecoid (Gsc)+/Sox17+ definitive endoderm, and Gsc−/Sox17+ visceral endoderm, followed by the gene expression transcriptional profiling analysis of developmental genes [108]. BMP4-induced subsets of ESC culture was also shown to exhibit gene signatures of mesoderm, trophoblast, and vascular endothelium, suggesting correspondence to gastrulation-stage primitive streak, chorion and allantois precursors, respectively [104]. Same group identified that intermediate subset of cells positive to a receptor-related 2 (ROR2) produce mesoderm progeny, cells positive to aminopeptidase-A

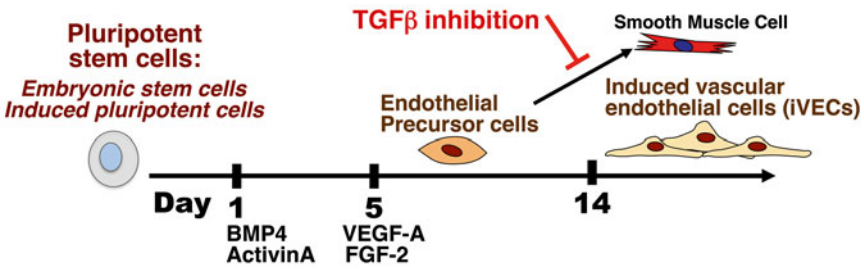


Fig. 7.1 Differentiation of pluripotent stem cells into vascular endothelial cells (ECs). Human or mouse embryonic stem cell (ESCs), or induced pluripotent stem cell (iPSCs) are incubated with growth factors to generate ECs. Inhibition of TGFb is essential to block the drift of ECs towards the nonvascular cells

(APA) generate syncytiotrophoblasts, and cells positive to urokinase receptor (CD87) give rise to vasculature. ROR2, also known as tyrosine-protein kinase transmembrane receptor, or neurotrophic tyrosine kinase, is responsible for aspects of bone and cartilage growth. CD87, a GPI-linked receptor for urokinase plasminogen activator (uPA), is involved in the conversion of plasminogen to plasmin on the leading edge of migrating cells. Of all studies reviewing with a focus on early stages of endoderm formation how developmental mechanisms regulate endoderm organogenesis using PSCs, has emergence to a concept, epithelial-to-mesenchymal transition (EMT), in attention to a dynamic gene expression profile may be reminiscent of vertebrate gastrulation. However, whether endoderm cells emerge in whole or in part from mesendoderm progenitors have never been shown.

2.4 Lineage-Specific Differentiation of Pluripotent Stem Cells into Endothelial Cells

Lineage-specific differentiation, also known as "directed differentiation," is referred to manipulating stem cell culture conditions to induce differentiation into a particular cell type. Because of the huge expectation to the potential of hESCs and iPSCs, lineage-specific differentiation protocols experts in the field present a comprehensive collection of protocols designed for labs around the world. Techniques used for maintenance of ESCs and iPSCs in either small or large scale, for directing lineage specification, for enhancing the maturity of differentiated cells, for generating patient-specific cells, and for translating into new therapies.

Efficient directed lineage-specific differentiation of hESCs into functional ECs was reported to augment differentiation of embryoid body (EB) culture on matrigel-coated plates in serum-free media supplemented with VEGF-A and inhibition of TGFβ by SB431542 [10]. Cloned hESCs transduced with a reporter vector in which EC-specific Vascular Endothelial Cadherin (VE-cadherin) promoter drives the expression of GFP (VPr-GFP hESCs) to track the emergence of ECs (Fig. 7.1). hESC H9-derived EBs express EC-specific markers including CD31, VE-cadherin, and CD34. Sorted CD31-expressing cells by flow cytometry were cultured on a 1 % gelatin-coated plates in EGM-2 medium for several passages induced expression of several adhesion molecules, including N-cadherin and VE-cadherin, that formed capillary-like structures in matrigel-coated plates for several days [42, 109, 110]. Directed differentiation platform for vascular cells in EB expanded 36-fold into EC-like cells exhibiting a transcriptional profile of Id1, VEGFR2, VE-cadherin, and ephrinB2. However, these differentiated ECs from PSCs are unstable and drift towards nonvascular cell fates, and could not generate fully functional ECs, and have limited proliferative potential. In addition, current approaches to derive ECs from hESC results in generation of ECs that are epigenetically unstable and have low reprogramming efficiency and kinetics that derives ECs with low expansion potential. In addition, directed differentiation approaches to derive ECs from hESC results in generation of ECs that are epigenetically unstable and have limited expansion potential [10].

Interestingly, VEGFR2^{+}/CD31^{neg}/VPr-GFP^{neg} EC-like cells were further transduced with lentiviral ER71, Erg-2, or Fli1, following five additional days of culture in VEGF-A and FGF-2, and the TGFβ inhibitor SB431542 [10], the percentage of derivatives in which the EC-specific transgene VPr-GFP or CD31 was significantly increased in response to overexpression of all three ETS family members [9]. The emergent VPr-GFP^{+} EC populations derived from hESCs displayed profiles consistent with EC identity and showed localization of VE-cadherin and CD31 proteins at intercellular junctions. Notably, a graded increase in VEGFR2 and VE-cadherin in response to an increasing multiplicity of lentiviral transduction of ER71, Fli1, and Erg-2 were noted when measured for relative transcript abundance, indicating the shift in molecular signature of the population toward a genuine EC phenotype [9].

Although a large expectation is brought to iPSC differentiation to establish patient-specific customized generic therapy, a novel high-resolution RNA-sequencing analysis for the construction of a detailed reprogramming route map demonstrate that reprogramming is not simply the reversal of

the normal developmental processes. Devised methods to differentiate ECs from iPSCs have been controversial indicating this might be a challenge. It may be hindered in nature of iPSC derivation, which biological mechanisms essential for successful iPSC generation require both accurate capture of cells undergoing the reprogramming process and identification of the associated global gene expression changes. Comparison in the OP9 differentiation system to differentiate hESCs and iPSCs obtained both hematopoietic progenitors and ECs with no significant difference, that all differentiated ECs expressing VE-cadherin, vWF, VEGFR-2, CD31, CD49d, and CD105 [111]. When compared in a 2D culture system, the morphologies and characteristics of hESCs and iPSCs, it was shown also there were no difference and both are equally differentiated into ECs detected with VE-cadherin expression and other EC markers including FLK-1, CD31, and CD34 [112], suggesting no morphological differences between ECs derived from human ES and iPSCs, and both cell types show identical gene expression patterns. Whereas, iPSCs derived with a reduction of reprogramming factors reduced not only reprogramming efficiency but may also worsen subsequent differentiation of EC marker expressing cells [113].

Hindered to these lineage-specific differentiation systems for both hESCs and hiPSCs exhibited poor terminal differentiation, suggesting that refined modes of directed differentiation to ECs may be required to fulfill fully interpretable results. Directed differentiation of iPSCs into ECs awaits for the improved understanding of the reprogramming process of iPSC derivation and to new reprogramming and differentiation strategies into ECs.

2.5 Lineage-Specific Differentiation from Fetal and Adult Tissues into Endothelial Cells

Directed differentiation into ECs of human hematopoietic cells from bone marrow (BM), umbilical cord blood (UCB), and mobilized peripheral blood (PB) has been well studied for a resource to give rise graftable ECs as EPC-based therapies to isolating high proliferative and clonogenic potential of stem and progenitor cells (HSPCs) that are cultured with multiple growth factors.

Mesenchymal stromal cells (MSCs) are advantageous in that can be easily obtained than other PSCs, and possess immune privilege that can both boost the immune system and suppress it [114, 115]. MSCs are a wide cluster of highly plastic non-hematopoietic cells from bone marrow (BM), adipose, amniotic fluid, and blood vessels of mesodermal origin. Much evidence from tremendous amount of preclinical studies supporting that MSCs from BM and other tissues can be differentiated into ECs [12, 116–119], epithelial cells [120, 121], and neural cells [122]. Administration of ex vivo expanded isolated MSCs that home to and engraft to injured tissues modulates the inflammatory response through synergistic downregulation of proinflammatory cytokines, upregulation of prosurvival and anti-inflammatory factors, and suppression of T-cell, NK cell functions, and modulating dendritic cell activities [123–131].

Although it is believed that there are common gene expression repertoires among various types of MSCs probably a stemness keeping genes, MSC markers are loosely defined with undefined "stemness" markers due to MSC proliferation retain their multipotency that are reflective of their propensity to differentiate into different cell lineages [132–135]. As such, ex vivo-expanded MSCs without imprinting of stemness have been shown efficient differentiation with comparable expansion potential in numerous studies [9, 136–138], suggesting the alternative set of regulators that are non-stemness gene profiles can define differentiation potentials, thus, molecular signature and in vivo distribution status of MSCs remain unknown. The central question would be whether they might be differentiated into cells without stemness markers.

Remarkable feature of MSC property have been identified from the co-transplantation studies that provided insight into unregulated differentiation deciphering the molecular machineries that might be used to define the different roles of MSCs in switching cell fate. As such, MSCs co-transplanted with hematopoietic stem cells (HSCs) differentiate into lung, gut, skin [139], neuroectodermal cells [140, 141], skeletal myoblast [142, 143], liver and biliary epithelium [139, 144–146], and endothelium [147–149], showing highly plastic property in response to extrinsic cues and to interacting with neighboring cells. Differentiation of MSC may be involved in functional properties in the physiological control of acute inflammation in various tissues as modulators of extrinsic cues, stemness may not be a required feature. Better understanding the molecular mechanisms underlying the maintenance of cellular identities and determination of cell lineages is critical for the future clinical use of MSCs.

Atala and colleagues showed that AFCs are highly plastic to in vitro manipulation of directed differentiation into a range of cell types, such as nestin-positive neural stem cells, dopaminergic and glutamate-responsive neurons, osteoblasts, putative hepatocyte-like cells expressing a-fetoprotein, albumin, hepatocyte nuclear factor and growth factor, and of secreting high levels of urea [12]. Human AFCs of mid-gestation human fetuses represent an attractive donor cell population based on the thousands of amniocenteses for diagnostic purposes. Freshly isolated AFCs are routinely cultured that are performed each year worldwide, generating a sufficient quantity from individuals with broad genetic and ethnic backgrounds. They display high proliferative poten-

tial and can be HLA typed, cryopreserved, and publicly banked for clinical use. This opened the new avenue of research to demonstrate the cell's utility in the regenerative medicine for the potential benefits and the obvious advantages of AFCs.

Both the origin of AFCs and its unique multilineage differentiation potential similar to PSCs have not yet been fully elucidated [150]. Alkaline phosphatase (ALP) expressing stem cell like line derived from human amniotic membranes by the colonial cloning technique using aMEM supplemented with epidermal growth factor (EGF), lymphocyte inhibitory factor (LIF), and 10 % fetal bovine serum (FBS) was shown contributed to the formation of chimeric mouse embryoid bodies and gave rise to cells of all germ layers in vitro, demonstrating that human amniotic membranes derived stem cells [151]. Amniotic epithelial cells isolated from human placenta was shown to express PSC surface markers transcription factors, octamer-binding protein 4 (Oct-4) and Nanog, and differentiated to three germ layers in vitro [152].

Ectoderm-derived epithelial cells that are devoid of significant population of precursor cells could augment tissue regeneration and repair via production of numerous pro-regenerative substances. Mesoderm-derived amniotic cells are known to display MSC-like signature similar to that of adult bone marrow-derived MSCs, and have been shown to differentiate into ECs [12, 116–119]. It is likely that they derive from the amnion, a membrane surrounding the fetus, arises from the epiblast. The amniotic cavity forms between the embryonic ectoderm and mesoderm in the second week of human development, and persists throughout gestation [153–155]. Human fetal tissues, fetal membranes (amnion and chorion), and amniotic fluid, that consist of two cell types of different embryonic origin: ectoderm and mesoderm, have been shown to harbor cells with multipotent MSC-like properties of pro-angiogenic, anti-fibrotic, anti-scarring, and low immunogenicity [151, 156–158].

Human AFCs have been classified on the basis of their morphological, biochemical, and growth characteristics, with three subgroups of lineage-committed amnion-derived cells identified: epithelial-like (E-type), amniotic fluid-specific (AF-type), and fibroblast-like (F-type) cells [159, 160]. E-type amnion is believed to originate from fetal skin, while F-type cells are derived from connective tissue. At the beginning of the amniotic cell culture E-type cells are dominantly found [159, 161].

Phenotypic markers that are characteristic of AFCs exhibit stem cell markers (ALP, Oct4, Sox2, Nanog, SSEA3/4, Klf4, CD90, CD117), pro-angiogenic factors (laminin, fibronectin, collagen type I, III, IV, V, Tie2, Ang1, VEGF, VEGFR, vWF, KDR, Flt4, CD105), and extracellular membrane molecules (CD29, CD44, CD49, CD73, CD166), and typically negative for CD14, CD34, CD45 [118, 151, 162–164].

3 Direct Reprogramming of Amniotic Cells into Endothelial Cells

Recently, AFCs have been identified amenable to direct reprogramming into abundant functional endothelial cells (ECs) via transient expression of Etv2 and co-expression of Fli1/Erg1 and TGFβ inhibition [9]. Forced ectopic expression of transient Etv2/ER71 with concomitant Fli1/Erg1 derived from human AFCs from second trimester amniocentesis [9] (see Appendix). In the USA each year, approximately 200,000 amniocenteses are performed, ensuring that sufficient genetically matched AFCs will be available for reprogramming into plentiful ECs for therapeutic transplantation into a large cross section of the population.

Although the precise cellular makeup of the AFC population remains vague, in vivo engraftment of the naïve ECs derived from reprogrammed AFCs (rAC-VEC) has been successful into functional vascular beds in regenerating livers. Molecular pathways regulating the tissue specification of EC function can be achieved by rAC-VECs, showing that the engrafted rAC-VECs are endowed with morphological profiles in size, shape, and angiogenic repertoire, and highly proliferative and functional adult ECs matching to immature vascular ECs that are found in postnatal vasculogenesis. Assessment to functionality of rAC-VECs for angiogenic indexes showed strong tubulogenesis on a matrigel tube formation assay, significant accumulation of acetylated-LDL (Ac-LDL) uptake, numerous functional vessels anastomosed with host vasculature in Matrigel plugs in NOD-SCIDIL2Rg$^{-/-}$ mice within 2 weeks. The bona fide functionally viable VEGF-A-dependent proliferative vessels have been generated in the regenerating liver (Fig. 7.2).

Notably, transduction of AFCs with ER71/Fli1 not only resulted in complete induction of vascular signature, it also turned off the expression of the nonvascular program in AFCs, indicating that AFCs provide the status of their chromatin state allows for complete reprogramming of these cells into authentic vascular ECs with the potential for forming long-lasting durable highly proliferative ECs. This technology allows for the generation of large quantities of bona fide ECs that could be used in a wide array of clinical applications.

Most published studies involving direct reprogramming of adult murine fibroblasts have not studied whether their use of various organ-specific inductive transcription factors has resulted in silencing of the original genetic repertoire upon completion of reprogramming to neuronal cells or cardiac cells [165–167]. The "loose" criteria was used in previously reported EC-differentiation protocols using VPr-GFP vector transfected EB stimulated by BMP2 and VEGF-A and TGFβ inhibition for 10 days. As such these direct differentiated ECs express markers similar to vascular precursor markers as VEGFR2^{+}/CD31^{neg}/VPr-GFP^{neg} [10], and simple EC function

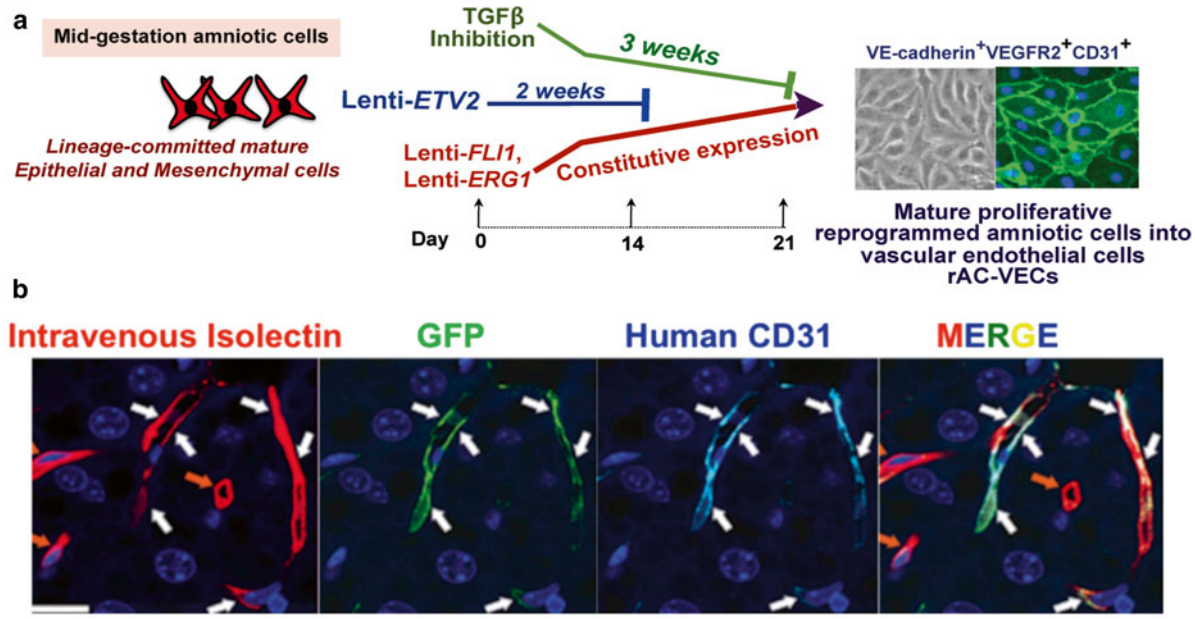


Fig. 7.2 (**a**) Schematic reprogramming of amniotic fluid cells (AFCs) into vascular endothelial cells (ECs): mid-gestation AFCs comprise mature mesenchymal and fibroblasts cells. Transient conditional lentiviral transduction of AFCs using tetracycline-on cassette expressing ETV2 is sufficient to induce expression of vascular genes. Constitutive expression of Fli1 and Erg1 is critical for induction of vascular genes that endow nascent ECs with long-term stability and identity. TGFb inhibition is essential to induce expression of VEGFR2 and other vascular genes concomitant with Fli1 and ERG1 generated abundant and stable ECs within 21 days. (**b**) Histology of transplanted reprogrammed ECs: three million human GFP+ ECs (*green*) were transplanted via intrasplenic route into immunocompromised NSG mice undergoing liver regeneration. After 3 months the mice were injected intravitally with Isolectin (*red*) to identify patent vessels. The engrafted GFP+ engrafted human ECs were identified with an antibody to human CD31 (*blue*) (adapted from [9])

like Matrigel tube formation. However, more robust function such as regenerative angiocrine function, engraftment into host tissue and RNA-seq/PCA plot was not convincing.

It is conceivable that subsets of these cells are not completely reprogrammed; rather, they may have maintained some of their original fibroblastic or mesenchymal signature. Here, we show that ER71/Fli1 transduction not only results in a full complement of activated EC-specific genes, but also that these two transcription factors switched off nonvascular AFC-expressed genes, such as Calponin-1, Kertain-7, and Teneurin. This is an important and therapeutically relevant finding, since any persistent expression of nonvascular genes in reprogrammed AFCs may result in malfunction of ECs. For example, sustained expression of AC-expressed genes, including Calponin-1 or Keratin-7 in iVECs may predispose in vivo engrafted ECs to vascular deformity and thrombus formation. Therefore, reprogramming of any nonvascular cells into ECs has to guarantee silencing of pro-coagulation and inflammatory mediators to allow for the generation of long-lasting and functional vessels.

It has been shown that the mechanism by which ER71 switches on EC-specific genes in embryonic tissues is mediated through interaction with specific Mef2C enhancer regions [168]. Binding of ER71 and FoxC2 to the specific enhancer FOX:ETS regions dramatically enhances the expression of the VEGFR2, VE-cadherin, and other EC specification genes. Notably, FoxC2 is expressed constitutively in AFCs and its expression is maintained during ER71/Fli1 mediated reprogramming into ECs. These data suggest that the remarkable capacity of ER71 to switch on the majority of the EC-specific genes within 4 days of transduction into AFCs may be due to the fact that complimentary transcription factors, such as FoxC2, are expressed in AFCs. Alternatively, ER71 and/or Fli1 may have the capacity to turn on other unknown complimentary transcription factors that are necessary to enhance the expression of EC-specific genes and switch off AC-expressing genes.

ER71 and Fli1 as well as Erg have been implicated in the specification of hemogenic as well as hematopoietic cells. However, under the inductive conditions established in our reprogramming protocol, ER71/Fli1 in conjunction with FGF-2, VEGF-A, and TGFβ inhibition failed to induce any hematopoietic-specific genes in ECs, including CD45 (PTPRC), Pu.1, thrombopoietin receptor (c-Mpl), Flt3 receptor, or Lhx2. However, Tal/Scl, and Vav3, as well as LMO2 genes that are also expressed in certain adult ECs were also induced by ER71/Fli1. These findings indicate that transient expression of ER71 is essential for reprogramming AFCs to ECs, without inducing genes that support hematopoietic cell generation.

Transduction of ER71 alone was insufficient to switch on all EC-specific genes. However, the majority of genes turned on by ER71 were factors that supported the specification of AFCs into ECs. These include VEGFR2, VE-cadherin, Tie2, Edge-1, VEGFR1, and Notch signaling pathway genes. Fli1, meanwhile, was essential to turn on more mature EC factors, such as CD31 and ECM components including Laminin-a4 that are essential for conferring ECs with their full angiogenic potential. Thus, ER71 specifies AFCs into immature EPCs but not hematopoietic cells, while Fli1 induces genes that render these progenitors into fully mature functional ECs. Indeed, both the pattern of growth as well as size and shape of ECs transduced with both ER71 and Fli1 were more similar to de novo isolated HUVECs. In this regard, although Fli1 could induce hematopoietic genes, coexpression of Fli1 in conjunction with ER71 results in specific induction of ECs but not hematopoietic cells. Therefore, in human cells transient ER71 expression favors induction of the vascular but not long-lasting hematopoietic cells.

Most adult ECs do not express ER71 but constitutively express Fli1, as well as specific isoforms of Erg and many other ETS members including Vezf, ETV6, Elk, and Elf to name a few. Notably, transient ER71 expression was sufficient to switch on each of these downstream EC-specific ETS transcription factors except Fli1 and Erg-2. These data suggest that similar to zebrafish and murine vasculogenesis, ER71 is the master regulator of vasculogenic specification resulting in full induction of vasculogenic genes. Indeed, enforced expression of ER71 in zebrafish results in induction of the key genes that specify ECs. The epigenetic mechanism by which transient expression of ER71 could result in permanent specification of EC-specific genes in AFCs is intriguing and has not been reported by any other reprogramming approaches of adult fibroblasts. It is plausible that upon silencing of ER71, constitutive expression of Fli1, Erg, and other EC-specific ETS transcription factors maintain the long-term expression of the EC-specific genes.

Transduction of ER71 and Fli1 into AFCs without TGFβ inhibition failed to generate functional proliferative ECs. This is because, despite the capacity of ER71 and Fli1 to switch on EC-specific genes, functionally active VEGFR2 by VEGF-A was impaired in the absence of TGFβ inhibition. In mice lacking functional VEGFR2, there are severe defects in vasculogenesis [169]. This remarkable finding suggests that reprogramming into ECs not only requires transduction of AFCs with ER71 and Fil1, but also is dependent on restoring the functionality of VEGFR2 that is essential for sustenance of vasculogenic and angiogenic potential of ECs. Whether biomechanical stimulation of ER71/Fli1 transduced AFCs could also functionalize other as yet unrecognized EC-specific receptors to enhance the number of reprogrammed ECs is unknown and will be the subject of future studies.

In adults, organ-specific ECs are endowed with a unique molecular and phenotypic signature. Although microenvironmental cues may play a major role to confer organ-specificity to ECs, transcription factors may also play a role in this process. Reprogrammed ECs generated from AFCs have the molecular profile of generic mature adult ECs. We could not detect selective expression of EphrinB2, or EphB4 that could distinguish between arterial or venous ECs. The genetic repertoire of reprogrammed ECs approximates that of generic in vitro cultured ECs such as HUVECs. Indeed, cultivation of any organ-specific ECs results in immediate erasure of the endogenous organ-specific phenotype. As such, similar to other adult ECs, the reprogrammed ECs from AFCs may undergo further tissue-specific specialization once reintroduced into the microenvironment of a given organ.

4 Discussion

Organ regeneration promises unlimited access to replacement tissues. There is currently difficulty to promote organ regeneration in humans, and prevailing paradigm of organ regeneration is dependent on transplantation of adult tissue-restricted stem and progenitor cells to repair damaged organ. This has been challenged by recent findings that blood vessels are active participants in organ function that tissue-specific vascular endothelial cells (ECs) produce defined set of paracrine mediators, called “Angiocrine Factors” to directly induce organ regeneration.

Injury repair is typically associated with scarring and abnormal function. During embryonic development, endothelial cells, the cells that line the blood vessels, can direct organogenesis independent of their function as the lining surface of blood conduits. Regeneration could be directed by tissue-specific vascular ECs functioning as an instructive niche to promote organ regeneration and repair without provoking maladaptive fibrosis. Yet, the regenerative function and repertoire of instructive angiocrine factors elaborated by mature ECs depends upon the organ from which they originate, that are still awaiting for precise mechanism insight into the molecular determinants of EC and angiocrine heterogeneity.

The vast proliferative capacity of immature, naive ECs that can be efficiently derived from AFCs permits large numbers of cells to be produced and their naive characteristics allow them to be specified for organotypic function. These naïve ECs can acquire tissue-specific function by a process of “education” wherein microenvironmental cues trigger transcriptional programs that enable EC specification. The tissue-specific function of ECs can be harnessed to promote health by supporting organ regeneration that these immature ECs attain their specialized functions and unique growth factors to stimulate organ repair. The reprogrammed naive ECs can be programmed to target particular vascular beds to promote tissue-specific organ

regeneration, targeted to the molecular pathways regulating the tissue specification of EC function, once microenvironmental cues that specify ECs for organotypic regenerative functions and transcription factors that mediate tissue-type EC specification for organ repair are identified.

The potential of ETS transcription factors can induce vasculogenic specification in more lineage-committed nonvascular cells. Direct reprogram of human nonvascular adult cells derived from human bone marrow fibroblasts and mesenchymal cells were not successful. After screening a large number of nonvascular cells from human fetal and adult tissues, midgestation-derived human AFCs are more amenable to reprogramming into vascular cells. This phenomenon could be due to the possibility that subsets of AFCs, such as c-Kit$^+$ cells, which compose 0.2 % of the AFCs, represent multipotent stem cells that readily differentiate to ECs in appropriate conditions. However, there is no evident detection of the canonical pluripotency marker Oct4 in AFCs. The overwhelming majority of the AFC population was negative for Oct4 protein, confirming similar results from other groups [170].

Sample-to-sample variability and gestational origin (i.e., fetal age at the time of amniocentesis) embody two parameters that are significant in determining the specific subpopulations of epithelioid vs. mesenchymal cell types [171]. Nevertheless, a growing body of research has begun to resolve this ambiguity, primarily through the identification of specific cell markers via flow cytometric analysis [163, 170, 172]. Through such scrutiny, several groups have recently identified AC subpopulations that, under proper growth conditions, can be driven toward specific cell fates [12, 172]. Nonetheless, the lack of pluripotency genes such as Oct4, Nanog, and Sox2 in the majority of AFCs suggests that the predominant population of AFCs is composed of epithelial and mesenchymal committed cells. Additionally, the number of c-Kit$^+$ multipotent cells is less than 1 %. Notably, within 4 days of transduction of AFCs with ER71/Fli1 and TGFβ inhibition, close to 40 % of the AFCs expressed VE-cadherin, all of which ultimately transition into fully functional ECs. This finding suggests that the majority of the AFCs, which predominantly comprised lineage-committed cells, are amenable to reprogramming into ECs.

Methods to directed differentiation of ECs are an important tool for regenerative medicine that would be used to regenerate and repair damaged tissues and organs. Differentiation of vascular ECs in clinically relevant numbers for injection into ischemic areas could offer therapeutic potential in the treatment of cardiovascular conditions, including myocardial infarction, peripheral vascular disease, and stroke. However, attempts in direct reprogramming of adult human nonvascular cells into abundant functional vascular ECs sustaining vascular identity have confronted major technical and conceptual hurdles. The generation and expansion of vascular ECs from readily obtainable nonvascular cell sources are shown to differentiate from PSCs, including iPSCs and hESC, that often proliferate poorly and drift into nonvascular lineages [173].

A comparison between the tissue-resident EC and the delivering reprogrammed EC is important for understanding the most key transcriptional regulatory pathways that establish tissue-specific EC niches in various organs. Sophisticated strategies of high proliferative reprogrammed ECs in xenobiotically transplanted into immunocompromised mice are tools to identify tissue-specific vascular transcription factors. The time frame for the EC to be reprogrammed may represent the generic "acute" phase of regeneration, not the long-term tissue-specific EC within a few days. Specifically, lack of knowledge of specific transcription factors and permissive culture conditions that are optimal for induction of human vasculogenesis has hindered studies for in vitro generation of vascular ECs that could be exploited for regeneration of injured tissues.

Generic ECs transcriptionally reprogrammed from AFCs is expected to overturn the scientific conceptualization of a monofunctional, relatively inert, microvasculature by revealing a dynamic, organ-specified role for ECs in injury repair and regeneration. Successful completion of these studies will enable therapeutic use of "educated," tissue-specified ECs that home to their native injured organs and elaborate tissue-specific angiocrine signals to orchestrate organ regeneration. This transformative approach will open up new therapeutic avenues of research to stimulate organ repair without aberrant fibrosis in clinical setting, as well as for treating a wide spectrum of human diseases: ultimately, organ damage is responsible for virtually the majority of human "sicknesses."

5 Appendix: Material and Methods

5.1 Cell Culture

Culture Condition for Amniotic Cells (AFCs):

- Amniotic Media (AM): Amnio-Max + Supplement (GIBCO) with 1X Pen/Strep (Invitrogen)

Differentiation of AFCs:

- Transfected AFCs with lentiviral vectors
- Culture media supplemented with Endothelial Growth Media (EM) in Medium 199 (Thermo Scientific), 15 % Fetal Bovine Serum (Omega Scientific), 20 mg/mL endothelial cell supplement (Hallway), 1 % antibiotics (Hallway), 20 units/mL heparin (Sigma), and where indicated, 5 mM SB431542 (Tocris)
- Gelatin powder (J.T. Baker, Phillipsburg, NJ), 0.2 % in sterile water and stored at 4 °C
- HEPES: 1 M HEPES made in sterile water, pH adjusted to 7.55 at room temperature
- Bovine serum albumin
- 500 mL filter system

5.2 Lentiviral Vectors and Transduction

Human amniotic fluid cells (AFCs) from second trimester amniocentesis are forced ectopic expression of transient Etv2/ER71 with concomitant Fli1/Erg1 using cloned human ER71, Erg-2, and Fli1 cDNAs into lentiviral vectors and TGFβ inhibitor, SB431542 [10]. Successful transduction specifies AFCs directly reprogrammed into naïve endothelial cells (ECs) [9].

Functionality of reprogrammed ECs assessed in vitro and in vivo models whether these ECs have acquired full angiogenic potential. A matrigel tube formation assay performed on 21-day-old ER71/Fli1 transduced ECs that were concurrently under TGFβ inhibition. Notably, ECs expressing ER71 and ER71/Fli1, but not naïve AFCs, were capable of forming tubes in vitro comparable to tubulogenesis observed by HUVECs. A second in vitro assay was then performed on ECs to demonstrate another EC attribute—Acetylated-LDL (Ac-LDL) uptake. Incubation of 21-day-old ECs with Ac-LDL showed significant accumulation of this lipoprotein similar to Ac-LDL uptake seen in HUVECs. GFP-labeled 21-day-old ECs were loaded into matrigel plugs supplemented with VEGF-A and FGF-2, and injected into immunocompromised NOD-SCIDIL2Rg$^{-/-}$ (NSG) mice for 2 weeks. Following intravital labeling of the vasculature by Isolectin, matrigel plugs were then removed for analysis. Although naïve AFCs failed to form any capillaries, 21-day-old ECs formed numerous functional vessels that anastomosed with host vasculature. Taken together, these data verify the notion that ER71 and Fli1, acting in concert with TGF-b inhibition, can reprogram mid-gestational AFCs into functionally viable VEGF-A-dependent proliferative ECs that are capable of developing bona fide functional blood vessels.

Multiple cDNAs: ER71, Erg-2, and Fli1: Clone into the pCCL-PGK lentivirus vector.

- A triple Flag-tag: Subclone into the ER71 and Erg-2 constructs at the amino terminus, via Quick-Change Site Directed Mutagenesis Kit (Stratagene), then re-subclone Flag-tag ER71 into the pLVX-Tight-Puro vector (Clontech) and co-transduce with pLVX-Tet-Off vector
- Lentiviruses: Generate by cotransfecting 15 mg of our gene of interest lentiviral ECtor, 3 mg of pENV/VSV-G, 5 mg of pRRE, and 2.5 mg of pRSV-REV in 293 T cells (passage 8–10; subconfluent, 100 mm dish) by the calcium precipitation method
- Harvesting Supernatants: Collect supernatants within 40 to 64 h after transfection [174], then concentrate by Lenti-X concentrator (Clontech) and obtain viral titers determined by using the Lenti-X p24 Rapid Titer kit (Clontech).
- Recommended use to transduce AFCs: MOI = 1

5.3 Matrigel™ Plug and In Vitro Assays

Mix AFCs infected with ETS factors transduced with GFP-lentivirus with Matrigel (BD), 100 ng/mL of VEGF-A, and 50 ng/mL of FGF-2, and subcutaneously implant at the flanks of NOD-SCID gamma (NSG) mice (Jackson Laboratories, Bar Harbor, ME). After 2 weeks, inject isolectin conjugated with Alexa 568 (Invitrogen) (2 mg/kg) and sacrifice to harvest the tissue. Fix the tissue in 4 % paraformaldehyde, followed by 48 h saturation in 30 % sucrose. Prepare 20 μm cryosections and counterstain with Hoechst 33342. Enumerate the number of isolectin-positive functional vessels.

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Amniotic Fluid Stem Cells for the Treatment of Articular Cartilage Defects

8

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Abstract

Articular cartilage defects are among the most common disabling conditions of humans in the western world. The most prominent risk factors are age, weight, and trauma. Osteoarthritis is the typical outcome of chronic articular cartilage defects and up to date cartilage regeneration remains elusive. The discovery of amniotic fluid-derived stem cells has opened a multitude of new therapeutic options, one of them being their use for novel stem cell-based tissue regeneration techniques in order to treat cartilage defects. Here, we summarize developmental stages and growth factors as well as extracellular molecules involved in articular cartilage formation as well as degeneration. Furthermore, we discuss recent advances in the use of amniotic fluid stem cells as novel cell sources for the generation of cartilage tissue and how the endogenous cartilage formation process could be recapitulated during artificial tissue engineering.

Abbreviations

ACI	Autologous chondrocyte implantation
ADAM	A disintegrin and metalloproteinases
AFS cells	Amniotic fluid stem cells
BMP	Bone morphogenic protein
DZ	Deep zone
HA	Hyaluronic acid
HABP	Hyaluronic acid-binding protein
MMP	Matrix-metalloprotease
MZ	Middle zone
OA	Osteoarthritis
PCM	Pericellular matrix
PGA	Polyglycolic acid
sGAG	Glycosaminoglycan
SYM1	Symphalangism
SYNS1	Synostosis syndrome
SZ	Superficial zone

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1 Introduction

Articular cartilage starts to form during fetal joint development and subsequently covers the surface at the bony ends of articular joints, where it is maintained throughout life. Its main function is to reduce friction and therefore to facilitate movement of the joint. Defects within articular cartilage are the reason for osteoarthritis (OA), which is also described as a primarily noninflammatory, degenerative joint disease. The main histologic features of OA include disruption of articular cartilage, degradation of extracellular matrix, and reduced cellularity within cartilage [1]. The incidence of osteoarthritis is still increasing and it is the single most common cause of disability in the elderly population [2]. Cartilage is an avascular and aneural tissue and hence regeneration especially after partial injury, which does not penetrate to the subchondral bone, is limited due to poor recruitment of

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extrinsic cells. Today, marrow stimulation, autograft, and allograft-based techniques are applied to treat severe cartilage defects [3–5], but all of these procedures suffer from several drawbacks. Bone marrow stimulation like microfracture-based treatment results in the production of fibrocartilage with inferior mechanical stability compared to native cartilage. For autograft, transplantation-based treatment the patient has to undergo multiple surgeries, once to isolate chondrocytes from less weight-bearing areas of the joint and another time for re-transplantation of in vitro expanded cells. In addition, in vitro expansion of chondrocytes is associated with dedifferentiation processes, which reduce the ability of cells to produce extracellular matrix [6, 7]. Stem cells isolated from various adult tissues have been tested for their use as cell sources to regenerate chondrogenic tissue [8]. Transplantation of differentiated mesenchymal stem cells remains a promising approach for regenerative therapies, but often the tissue generated in vivo shows lower matrix deposition and, consequently, less mechanical stability as compared to chondrocyte-based transplantations [9, 10]. Amniotic fluid stem (AFS) cells possess properties that make them ideal candidates for cell-based therapy and in particular for the treatment of cartilage defects. AFS cells are genetically stable, immune-privileged, non-tumorigenic and they can be bio-banked [11, 12]. Here we describe the current knowledge on the differentiation of AFS cells towards a chondrogenic phenotype. We focus on AFS cell marker expression, use of chondrogenic differentiation factors, and briefly discuss scaffolds used for tissue generation. In vitro differentiation of AFS cells is supposed to work best if developmental processes that give rise to permanent native cartilage are recapitulated. Therefore we also review developmental processes known to be involved in articular cartilage formation, but also in degeneration.

2 Development of Articular Cartilage

Synovial joint formation is initiated with the establishment of the interzone at the prospective joint site consisting of densely packed, flattened mesenchymal cells interconnected by gap junctions [13]. The interzone plays a dual role by providing progenitor cells for all tissues constituting joints including articular cartilage, ligaments, and the synovial membrane as well as the signaling center by preventing chondrocyte differentiation at the prospective joint site and by controlling maturation of chondrocytes within adjacent future skeletal elements [14–16]. How interzone cells or subsets of them contribute to different joint tissues is not clear yet since the interzone is species-specifically organized which impedes extracting a common mechanism concerning the developmental fate of interzone cells. For example, the interzone of chicks, a model organism for developmental studies, consists of three layers [17] whereas that of humans represents a non-structured thin flattened layer [13, 18, 19]. Moreover, the developmental origin of these cells is still a matter of debate and is associated with a crucial and early event in skeletogenesis by endochondral ossification, the mesenchymal condensation, which subsequently gives rise to the cartilaginous *anlagen* [20]. Two concepts concerning the time of patterning of the future skeletal elements and hence the appearance of the interzone have been proposed by different authors (reviewed in [20] and citations therein). Those imply that interzone cells might either originate from the cartilaginous *anlagen* requiring localized cellular redifferentiation and matrix removal or from non-condensed mesenchymal cells in the area of the adjacent ends of preformed bone templates. The latter is supported by Pacifici et al. who showed that peri-articular cells actively migrate into the incipient joint and suggested that they contribute to or entirely constitute the interzone [21].

Joint formation can be divided into interzone formation, cavitation, and establishment of permanent articular cartilage (see also Table 8.1). Interzone development is controlled by BMP, TGF-β, and WNT signaling pathways [22]. GDF5 is a member of the TGF-β superfamily and is an early marker expressed by cells of the interzone and in the immediate proximity of the prospective joint [16, 23]. GDF5 is pivotal for joint maintenance rather than for induction of joint formation since a dominant-negative mutation in GDF5 indeed impairs but its ectopic expression is not sufficient to induce joint formation [24, 25]. Sox11 might contribute to the regulation of GDF5 expression in joint development although its ectopic expression cannot induce GDF5 [26]. GDF5 binds to and oligomerizes type I and type II serine-threonine kinase receptors and signals through phosphorylation of transcription factors of the SMAD family [27]. Two type I receptors are expressed in the interzone and perichondrium, BMPRIA and BMPRIB, but GDF5 specifically binds to and signals through BMPRIB [28, 29]. The importance of

Table 8.1 Articular Cartilage Development

Developmental stage	Genes	Extracellular matrix proteins	References
Interzone formation	GDF5 (TGFß-superfamily), Sox9, WNT9a, TGFß, BMP2, BMP4, BMP7	Versican, Collagen I, fibronectin	[23, 37, 111–114]
Cavitation	Sox5, Sox6, Sox9, CD44, BMP2, BMP4, BMP14	Hyaluronan	[39, 115–118]
Permanent cartilage	Sox9	Aggrecan, Collagen II, Collagen IX, cartilage link protein, cartilage oligomeric matrix protein (COMP)	[34, 111, 119–124]

GDF5-BMPRIB-mediated signaling is underlined by the fact that mutations in either one or the other lead to similar skeletal malformations (e.g., brachydactyly, symphalangism) and that many mutations of GDF5 are in its receptor-binding loop [29]. Notably, BMPRIA expression seems to be important for cartilage homeostasis and repair since conditional knockout leads to postnatal erosion of articular cartilage of normally developed joints in mice [30]. In addition, GDF6 and GDF7 are expressed at joint sites distinct from that of GDF5 and inactivation of GDF6 causes defects in the formation of joints, ligaments, and cartilage distinct from that observed due to GDF5 mutation indicating a joint-specific role of the different members of the BMP family [31]. Noggin is a BMP antagonist and binds to and thereby limits the activity of GDF5 [32]. This interaction is important for joint development since certain mutations in NOG and GDF5 are associated with symphalangism (SYM1) and multiple synostosis syndrome (SYNS1) [32, 33].

WNT9a (former WNT14) is pivotal for joint formation. In developing chick limbs, it is expressed at the prospective joint site appearing as a transverse stripe at stage 27, which separates as joint development progresses to form two stripes possibly setting the interzone boundaries. Eventually, WNT9a is detected in the fibrous part and the synovial lining of the joint capsule [34]. However, the notion that WNT9a is sufficient to induce joint formation as proposed by some authors has recently been challenged by Später et al. [35, 36]. The latter showed that Wnt9a acts cooperatively with WNT4 to suppress chondrogenic differentiation of interzone cells and that the fusion of the three carpal elements observed in *Wnt9a*:*Wnt4* double mutant mice was not due to the failing of joint induction [37].

A critical step in joint formation is cavitation, the formation of the joint space between opposing skeletal elements. It precedes the morphogenesis of synovial joint structures. The importance of mechanical stimuli for cavitation by movement of the developing joint has early been appreciated. In ovo application of neuromuscular blocking agents leading to either flaccid or rigid paralysis revealed the differential impact of dynamic and static stimuli for initiation of cavitation and maintenance of the joint cavity [38]. The molecular link to mechanostimulation is the expression of hyaluronic acid (HA). Indeed, the lack of static and dynamic stimuli prevents cavity formation and leads to reduced cartilage volume occurring concomitantly with reduced amounts of HA in the interzone and the lack of menisci and ligaments [38]. HA emerges at the interzone concomitant with cavitation [39]. Moreover, the activity of uridine diphosphoglucose dehydrogenase (UDPGD) which synthesizes precursors of HA precedes cavitation and is maintained within articular surfaces and synovium during cavitation [40], and CD44, an HA receptor, is expressed by interzone cells. The sequestering of HA-binding proteins (HABPs) to high-molecular HA is suggested to be crucial for cavitation since HA oligosaccharides applied in ovo exert an effect similar to the lack of mechanical stimulation by preventing cavitation and being hallmarked by decreased CD44 expression and UDPGD activity [41].

3 Native Articular Cartilage Organization

Articular cartilage is avascular and nutrient supply occurs by diffusion. It has been postulated that nutrition is mediated by synovial fluid as well as diffusion through calcified cartilage and the subchondral bone [42]. Especially synovial fluid plays a critical role in maintaining adult normal cartilage homeostasis [43].

The importance of mechanical stimulation in the regeneration has been shown through zone-specific gene expression after stimulatory effects of compression [44–46]. Mature articular cartilage consists of three zones, which constitute also different subpopulations of chondrocytes: the superficial zone (SZ), the transitional or middle zone (MZ), and the radial zone or deep zone (DZ). The superficial or tangential zone (10–20 %) harbors small and flat chondrocytes and collagen fibrils are orientated parallel to the cartilage surface. Cells within this zone produce and secret lubricin and proteoglycan 4, which are the most important joint lubricant superficial zone proteins [47, 48]. Human articular cartilage was predicted to not contain any source of stem cells because of the avascular architecture. However, recent discovered studies show phenotypic and functional properties of mesenchymal stem or progenitor cell populations in the SZ of mature articular cartilage [49–51]. These cells are characterized by expression of mesenchymal markers CD105, CD166, Notch-1, STRO-1, and VCAM-1 [52]. Superficial zone cells are positive for alpha-smooth muscle actin, which is also present in progenitor cells [53, 54].

The middle zone represents the thickest part (40–60 %) of the cartilage where collagen fibers are less organized and the pericellular matrix (PCM) is located. The PCM is a thin layer of extracellular matrix that immediately surrounds the chondrocyte. It plays a major role in the communication between chondrocytes and extracellular matrix [55]. The PCM is best studied in the form of chondrons which are chondrocytes clustered in one unit and form a functional and metabolic microenvironment. The PCM is different from the rest of the extracellular matrix in cartilage concerning vicinity, matrix density, and matrix composition. Collagen type VI proteins built up a fibrillar network in PCM [56]. Further identified PCM proteins are triosephosphate isomerase, transforming growth factor-ß induced protein, peroxiredoxin-4, ADAM (A disintegrin and metalloproteinases) 28, and latent-transforming growth factor ß-binding protein-2 [57].

The deep zone is characterized by ellipsoid cells and fibers, orientated perpendicular to the surface and oxygen tension is below 1 % [58]. Underneath the DZ is the tidemark to calcified cartilage and subchondral bone.

4 Articular Cartilage Degeneration

Age, trauma, and disease are the factors that facilitate joint cartilage damage, which subsequently can lead to osteoarthritis (OA) formation. The so-called primary OA develops without any known cause, but genetic, biomechanical, or biochemical factors often play a role [59, 60]. OA is a big burden of society since the majority of individuals over the age of 65 show evidence for OA on hands, knees, hips, and spine which cause symptoms like inflammation, pain, and loss of mobility [61]. All joint structures are affected by OA, but articular cartilage is affected most by degeneration. In the initial phase of the disease chondrocytes become clustered and show increased cell proliferation. When OA persits, proteinases like MMPs and aggrecanases are expressed and type II collagen and proteoglycans undergo changes in composition and are eventually degraded [62]. The excessive reorganization of hyaline cartilage finally leads to fibrillation of the surface and localized production of fibrocartilage which contributes to the observed joint stiffness during OA. Progressive OA is accompanied by hypertrophy of articular chondrocytes as evidenced by a thickening of the zone of calcified cartilage [63]. Hypertrophy is hallmarked by release of pro-angiogenic as well as osteogenic factors with subsequent apoptosis of chondrocytes [64].

Hypertrophy in articular chondrocytes is thought to recapitulate hypertrophy during endochondral ossification. During bone formation chondrocytes undergo several developmental stages which end with post-mitotic hypertrophy [65]. Upon hypertrophy, growth plate chondrocytes downregulate SOX9 and type II collagen expression, whereas RUNX2, type X collagen, alkaline phosphatase, and VEGF-A are up-regulated [66–68]. Research on articular cartilage regeneration has profited from studies on endochondral ossification because the mechanisms of hypertrophy induction are now better understood and it has become clear that for permanent cartilage formation hypertrophy induction has to be avoided.

5 Novel Approaches for the Treatment of Degenerative Joint Disorders

Articular cartilage defects can be divided into full thickness and partial thickness defects. In the first group not only cartilaginous tissue is lost but also part of the subchondral bone mass. This kind of defect will trigger an intrinsic repair response, activating mesenchymal stem cells residing in the bone marrow and leading to the formation of fibrocartilaginous repair tissue [69]. Fibrocartilage is structurally and biomechanically inferior to articular cartilage, still this endogenous repair program is the basis for so-called marrow-stimulating technique [70]. These include arthroscopic procedures where the subchondral bone is partially abrased, penetrated by drilling or microfractured. On the contrary, partial thickness defects that do not lead to stimulation of cells residing within the subchondral bone do not heal spontaneously. One reason for the persistence of partial cartilage loss is the inert nature of adult cartilage, which basically only consists of chondrocytes. In a healthy state it neither contains blood vessels, lymph vessels, nor does it contain nerve cells. Therefore a promising approach to treat partial, but also full thickness defects are cell-based transplantation approaches. The most widely applied method is autologous chondrocyte implantation (ACI), where chondrocytes are isolated from less weight-bearing areas of the joint, expanded in vitro and retransplanted into the defective area. Since this method relies on two separate surgical procedures it poses a burden for patients. Additionally, ACI leads to increased donor site morbidity, loss of chondrocytic phenotype in the course of in vitro expansion, and also fibrocartilage formation at the site of the defect [6, 7]. To reduce the risk as well as the discomfort associated with two surgical procedures potential future treatment options will rely on the application of stem cells for the treatment of degenerative joint disorders. Embryonic stem cells as well as induced pluripotent stem cells could be used, but on the one hand they potentially give rise to tumors [71–73] and on the other hand for high yield chondrogenic differentiation embryonic stem cells will first need to be differentiated towards mesenchymal precursor cells and then further on to chondrocytes, which makes the whole differentiation process more complex [74]. Mesenchymal stem cells are nowadays directly available from patient-derived tissues like bone marrow, muscle, and adipose tissue [8, 75, 76]. These cells are an attractive cell source for cartilage tissue engineering since they show strong proliferation and are easy to differentiate into chondrocytes, but their proliferation capacity can be exhausted and, when isolated from older patients, a mutation free status cannot be guaranteed. For the isolation of fetal cells the amniotic fluid is the best accessible compartment and it has been shown that cells within the amniotic fluid can be used to generate 3D fetal constructs able to be used for fetal surgery [77, 78]. With the discovery of AFS cells novel approaches in regenerative medicine have been enabled [79]. AFS cells are termed broadly pluripotent and are hallmarked by the expression of the stem cell-associated transcription factor OCT-4 [80–82]. AFS cells can be grown in large quantities and show a higher differentiation potential compared to adult stem cells [83]. AFS cells are devoid of a risk for tumor development and do not raise the ethical issues of embryonic stem cells. Furthermore AFS cells do not need exogenic treatment to induce pluripotency, are chromosomally stable and do not harbor the epigenetic memory and accumulated somatic mutations frequently found in adult source cells. Accordingly, AFS cells are now a much appreciated cell source for regenerative therapy approaches [84–87].

6 Use of Undifferentiated Amniotic Fluid Stem Cells for the Treatment of Degenerated Articular Cartilage

It is not always necessary to differentiate stem cells before they are used for cell-based regenerative approaches. For AFS cells for example it has been shown that they can contribute to renal tissue when co-cultured with embryonic kidney cells [85], or they can contribute to heart muscle after induction of heart failure [88]. The downside of this approach is that the number of differentiated cells is expected to be drastically lowered compared to the use of already predifferentiated cells (see next paragraph), but transplantation of stem cells is known to elicit a plethora of beneficial effects not directly related to novel tissue formation. These effects include specific homing to the site of injury, attraction of cells necessary to stimulate the healing process and modulation of the host's immune response. Interestingly, these effects can be of a transient nature since most transplanted stem cells do not integrate into the host tissue and will therefore be abandoned. For example, recently it was shown that amniotic fluid-derived cells with mesenchymal stem cell characteristics have the potential to accelerate the wound-healing process by stimulating proliferation and migration of dermal fibroblasts [89]. This phenomenon was attributed to the secretion of growth factors and cytokines including IL-8, IL-6, TGF-beta, TNFRI, VEGF, and EGF by the stem cells, which are important in normal wound healing. Up to date there a numerous clinical trials, which study the beneficial effects of mesenchymal stem cells directly transplanted to the region of articular cartilage degeneration, but to our knowledge AFS cells have not been used for this purpose so far.

7 Differentiation of Stem Cells from the Amniotic Fluid as a Novel Cell Source for Regenerative Therapy

Research on fetal tissue engineering lead to the discovery that amniotic fluid-derived cells harbor chondrogenic potential [90]. Ovine amniotic fluid cells were isolated and grown in culture medium without serum, but containing dexamethasone, ascorbic acid, sodium pyruvate, insulin, transferrin, and selenium. Additionally cells were treated with TGF-β2 and kept in culture for a total of 12 weeks. Compared to fetal hyaline cartilage the engineered constructs displayed less amounts of type II collagen, but similar amounts of glycosaminoglycans. Basically the culture conditions used in this first study reassembled the differentiation protocol described for mesenchymal stem cells derived from adult bone marrow aspirates [75]. First reports on human AFS cells used c-kit magnetically sorted cell populations and demonstrated increased production of type II collagen and sGAG after treatment with TGF-β1 and IGF-I [91]. The use of c-kit selected cells started a new field of research based upon the finding that c-kit selected single cell clones are broadly pluripotent [84]. Comparing c-kit positive and negative populations for their chondrogenic potential revealed that the c-kit positive stem cell population, after differentiation, displayed significant higher alcian blue staining and therefore stronger chondrogenic differentiation [92]. Therefore for chondrogenic differentiation either c-kit selected amniotic fluid cells or stem cell marker positive cells as depicted in Table 8.2 are used. The culture medium used is basically identical and Table 8.2 also shows that chondrogenesis can be triggered by differ-

Table 8.2 Chondrogenic differentiation of amniotic fluid stem cells

c-kit Selection	Marker expression	Species	Factors used for differentiation	Scaffolds used	References
Yes	CD117	Human	TGFβ1 TGFβ3 IGF-1 BMP-2	Pellet culture Alignate gel	[91]
Yes	CD117	Human	TGFβ1	Pellet culture Agarose hydrogel	[92, 125]
No	CD44, CD54, CD31, CD106, TrA-1-60, SSEA3, SSEA4	Human	TGFβ1	Two-dimensional culture	[80]
No	CD44, CD90	Murine	TGFβ3 BMP6	Pellet culture	[126]
No	CD29, CD44, CD90, CD105	Ovine	TGFβ1 TGFβ2 IGF-1	Polyglycolic acid (PGA) matrix	[104]
No	CD13, CD29, and CD44	Human	TGFβ1	Pellet culture	[89]
No	CD44	Human	TGFβ3	Pelllet culture	[127]
No	CD29, CD44, CD166	Human	TGFβ3	Fibrin gel	[103]

ent TGF-beta family members and by IGF-1, which has been shown in humans and also in other species. What can be concluded from chondrogenic differentiation of AFS cell up to now is that supplementation of chondrogenesis promoting factors is necessary for successful reprogramming of AFS cells and that the isolation of c-kit positive cells also enhances the differentiation process.

8 Spatial and Temporal Regulations of Chondrocytic Differentiation of Amniotic Fluid Stem Cells

The aim for the future is to improve in vitro protocols for the generation of artificial cartilage that can be transplanted to previously degenerated joints and will furthermore permit a permanent lubrication and full function of the joint (see Fig. 8.1). As already discussed human AFS cells represent a valuable cell source for ex vivo tissue regeneration, and ongoing work now focuses on the correct choice of differentiation factors, scaffolds and mechanical force. Optimal differentiation conditions are achieved when developmental processes regulating articular joint formation are recapitulated during in vitro culturing. This requires control of spatial and temporal changes, which on the one hand lead to efficient induction of chondrogenesis and on the other hand prevents the so-called hypertrophic state of chondrocytes. Adult chondrocytes reside in a hypoxic environment with very little nutrient supply. They are hallmarked by the expression of the chondrogenic transcription factors Sox5, Sox6, Sox9, and by formation of extracellular matrix components like Collagen type II, Collagen type IX, Collagen type XI and proteoglycans. As also shown in Table 8.2 generally TGF-β family members are sufficient for chondrogenesis in amnion fluid-derived cells, but recent data has also shown that in vitro expanded autologous chondrocytes, when treated with TGF-β1, show onset of hypertrophy [93]. Chondrocyte hypertrophy is evidenced, among others, by morphologic changes, expression of transcription factor Runx2 and production of Collagen type X and endothelial growth factor VEGFA. Hypertrophy generally leads to calcification of the tissue and subsequently to the loss of functional chondrocytes, an undesirable process within the joint region. Here we propose that developmental programs able to suppress onset of hypertrophy can also be used during cartilage engineering. For example Noggin, an antagonist of BMP signaling, has been identified to play an essential role in joint formation, since its deletion results in lack of GDF5 expression, a prominent member of the TGF-β family, and affected mice do not form joints [94]. Also WNT-4, WNT-14, WNT-16, and the WNT signaling mediator β-catenin show anti-chondrogenic activity during joint formation [35, 95]. The concept of exchanging factors during differentiation has already been used in a recent study were mesenchymal stem cells have been treated with TGF-β3 combined with β-catenin activation, which resulted in the prevention of hypertrophy and gave rise to a more stable chondrogenic phenotype than the induction with TGF-β3 alone [96]. Another way of achieving full differentiation without signs of hypertrophy is by forcing continuous expression of the master regulator SOX9. Studies in growth plate chondrocytes have shown that permanent induction of SOX9 inhibits terminal differentiation and hypertrophy by repression of the transcription factor RUNX2 [97, 98]. RUNX2 and RUNX3 are the pivotal inducers of type X and type I collagen as well as osteocalcin and MMP13 [99–101]. Therefore, it is tempting to speculate that treatment with other anti-chondrogenic stimuli during chondrogenic differentiation will result in the generation of fully functional and non-hypertrophic chondrocytes.

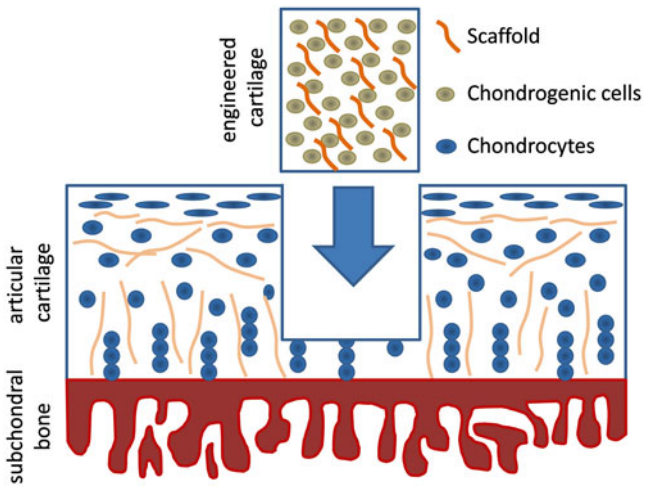


Fig. 8.1 AFS cells as a cell source for articular cartilage regeneration. Cartilage is solely composed of chondrocytes and the extracellular matrix they produce. Articular cartilage can be divided into different zones, starting from the top with the superficial zone, where collagen fibers are arranged horizontally to the surface. Underneath resides the transitional zone with spherical chondrocytes. The thickest part of cartilage consists of the radial zone with collagen fibers and chondrocytes orientated vertically to the subchondral bone. The figure shows a partial cartilage defect which does not elicit a bone marrow response. This type of defect is best healed by transplantation with artificial generated tissue. As shown AFS cells can be used to generate artificial tissue finally consisting of cells and extracellular matrix similar to native cartilage. This can be achieved by the use of growth factors, scaffolds and environmental conditions reflecting developmental processes during cartilage formation

As shown in Fig. 8.1, partial cartilage defects need to be filled with regenerated tissue consisting of appropriate extra cellular matrix. AFS cells have mostly been induced for chondrogenic differentiation in so-called pellet culture

systems which is a scaffoldless approach were approximately 250,000 cells are used to self assemble into a 3D structure. These culture conditions are reminiscent of in vivo joint formation where mesenchymal cell condensation precedes the induction of chondrogenesis and extracellular matrix molecules like type I collagen and type II collagen are produced. Since pellets are difficult to attach at sites of injury within cartilage, for transplantation approaches cells are frequently grown and differentiated on various scaffolds, which are supposed to exhibit biomechanical stability and aid in the construction of artificial tissue. Commonly used biomaterials are either biodegradable like agarose, alginate, hyaluronic acid, and collagen, or of synthetic origin like polyethylene glycol or poly-lactic-co-glycolic acid. Human amniotic fluid cells have so far been differentiated in three-dimensional pellets [92, 102] and in hyaluronan-based hydrogels [103]. Ovine amniotic fluid cells have been differentiated in biodegradable polyglycolic acid scaffolds [90] and in synthetic polyglycolic acid [104].

Articular cartilage is devoid of blood vessels, hence oxygen levels from the superficial zone to the calcified zone gradually decrease. Low oxygen tension termed hypoxia enhances the differentiation process of human mesenchymal stem cells and of human articular cartilage cells [105, 106], whereas in AFS cells, as far as we know, the impact of hypoxia on the chondrogenic potential has not been evaluated. Interestingly, chondrocyte hypertrophy, which is also a hallmark of osteoarthritis, is marked by extensive VEGF-A production. Secretion of this growth factor leads to increased angiogenesis in the affected organ area, leading to a resupply with oxygen and a subsequent mineralization process. This generation of osteophytes during progression of OA has been shown to occur in the knee joint of rabbits induced to form OA by anterior cruciate ligament transection [107]. On the contrary, hypoxia has the potential to inhibit upregulation of RUNX2 and thereby leads to a block of osteoblastic differentiation as demonstrated in mesenchymal stem cells [108].

Another physiologic process, which can be observed in native cartilage, is autophagy, since it protects chondrocytes from age-related cell death and preserves homeostasis within the tissue [109]. This process can protect cells under starvation conditions by turnover of intracellular organelles and molecules. Especially during aging-related cellular changes as well as biochemical stimuli and mechanical stress, all this can lead to osteoarthritis and during this process the competence to undergo autophagy is lost [110]. Therefore autophagy protects from aging-related cell death and probably it is also an important process during chondrogenic differentiation, even though its regulation during the differentiation process of amniotic fluid-derived stem cells has not been thoroughly studied.

9 Future Perspectives

Stem cells derived from the amniotic fluid represent a novel and promising source for cell-based therapy approaches in regenerative medicine. Cells can either be applied directly to the site of injury in order to stimulate repair processes or cells are used for chondrogenic differentiation in order to form artificial cartilage constructs. Differentiation of AFS cells has already been shown many times, most often via induction with TGF-β family members either embedded within matrix or in a scaffoldless approach. Still the molecular differentiation process is incompletely understood and up to date the regeneration of cartilage defects is elusive because novel generated tissue does not show the required toughness and hardness required for long term stability. Therefore differentiation protocols need to be improved and this is best achieved via recapitulation of the spacio-temporal aspects during chondrocyte differentiation. The hope is that future tissue regeneration will be based on stem cells stimulated to undergo self organized cartilage formation with almost native extracellular matrix composition and without signs of chondrocyte hypertrophy which will finally lead to durable repair tissue. Ways to achieve this includes the treatment with growth factors only for specific time periods and in combinations of pro and anti-chondrogenic treatment regiments in a defined ratio. This will yield the beneficial effects of chondrogenic stimuli, while preventing the end stage maturation effects of hypertrophy. Scaffolds are important three-dimensional environments, which regulate cell–cell interaction as well as oxygen supply, but usually scaffolds show too little mechanic stability and a negative impact on cell survival upon degradation. Recent efforts promote a scaffoldless approach where differentiated cells provide their own extra cellular matrix, like it is achieved when AFS cells are differentiated in a pellet culture approach. In conclusion, the demand for novel strategies to heal articular cartilage defects is high in our society and AFS cells have proven to harbor therapeutic potential. Especially the modification of existing chondrogenic differentiation approaches holds the promise to yield durable articular cartilage.

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Amniotic Fluid-Derived Cells: An Autologous Cell Source for Cardiovascular Tissue Engineering

9

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Abstract

Amniotic fluid-derived cells represent a promising autologous cell source for treatment of cardiovascular diseases, in particular congenital valvular heart defects detected prior to birth. Prenatally accessible via low-risk procedures, amniotic fluid has shown to be a source of multipotent, immature and non-tumorigenic mesenchymal stem cells. In order to overcome current therapeutic limitations, the combination of the appropriate cell source with tissue engineering approaches would allow the replacement of diseased tissue structures with prenatal autologous in vitro engineered constructs. In this regard, amniotic fluid-derived stem cells have shown to generate living autologous heart valve leaflets in vitro with a functional endothelial layer and mechanical properties comparable to the native heart leaflets. Successful in vivo translation of amniotic fluid cell-based engineered heart valves into the ovine fetal model has also been achieved. Therefore, amniotic fluid represents a promising fetal cell source for potential future therapeutic applications; however, careful in vivo assessment is mandatory prior to first clinical applications.

Abbreviations

AFCs	Amniotic fluid cells
AFSCs	Amniotic fluid-derived stem cells
αSMA	Alpha-smooth muscle actin
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
MSC	Mesenchymal stem cells
vWF	von Willebrand factor

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1 Human Amniotic Fluid: *A Heterogeneous Cocktail of Fetal Cells for Therapeutic Use*

Amniotic fluid is accessible prenatally via a low-risk prenatal harvesting procedure [58] and contains a heterogeneous population of fetal cells deriving from all three germ layers [14]. The majority of these cells are of epithelial origin and derive from different fetal organ systems including the skin, the urogenital, respiratory, or digestive tract. In addition, also cells are found from extra-embryonic structures, such as the placenta or the amniotic membranes [29]. Besides the presence of multipotent (mesenchymal) stem cells (MSCs) with common human MSC stem cell marker expression [5, 36, 57], several groups demonstrated the isolation of broadly multipotent c-kit (CD117)-positive stem cells. These cells have been demonstrated to be capable of differentiating in vitro into cell types of all three germ layers [8, 21, 22] including adipogenic, myogenic, osteogenic, hepatic, neuronal, and endothelial lineages [8, 32]. Importantly, this high degree of plasticity of amniotic fluid cells (AFCs) has been associated with a retainment of an immature differentiation state result-

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ing in higher degrees of plasticity, proliferation rate, and differentiation potential when compared to adult stem cells. In addition, subpopulations of AFCs also show expression of common stem cell factors, such as Oct-4, Nanog, and the stage-specific embryonic antigen-4 (SSEA-4) [34, 36]. This high plasticity and expression profile of stem cell factors, partly resembling the nature of embryonic stem cells (ESCs), make AFCs a promising and versatile autologous fetal cell source for therapeutic application. However, in the contrary to blastocyst-derived ESCs, AFCs are not associated with ethical controversies and tumorigenic concerns involved in the use of ECSs [2, 46].

2 Amniotic Fluid Cells: *A Promising Cell Source for Cardiovascular Regeneration?*

Cardiovascular disease remains to be the primary cause of death worldwide. Although the mortality decreased in recent years the burden of disease is still high and the amount of cardiovascular surgical interventions increased by 28 % from 2000 to 2010 in the USA [13]. Besides ischemic heart disease, which is caused by an impaired perfusion of the cardiac muscular tissue, structural heart diseases represent a major cardiovascular pathology [53].

The use of (stem) cell-based therapies is currently investigated as a potential treatment concept in ischemic heart disease. In order to promote revascularization of the cardiac tissue and limit ischemic damage a wide variety of different adult stem and progenitor cells have been evaluated as to their possible regenerative potential after intracoronary or intramyocardial injection [45, 56]. Bone marrow-derived cells are the mostly used cell source in clinical studies nevertheless only modest improvements in cardiac function have been reported so far [1]. Optimization may be achieved in the future by improving our knowledge on the underlying cellular mechanisms of differentiation and integration as well as possible paracrine effects either from the ischemic heart or the injected cells. After injection into a rat model also human amniotic fluid-derived stem cells (AFSCs) improved cardiac function in a model of myocardial infarction following their systemic or intramyocardial injection [3, 27, 64].

Besides ischemic heart disease structural heart disease is a major cardiovascular disorder. Here, the use of tissue engineering technologies for replacement of the diseased structures has been investigated as a potential future therapeutic concept. With regards to the sophisticated physiological in vivo functionality, a clear differentiation between tissue engineered vascular grafts versus tissue engineered heart valves seems crucial. Both concepts aim at the repair and replacement of diseased tissue structures by introducing an artificial autologous in vitro engineered construct with the ability to restore structures in situ, also resulting in biological functionality in vivo.

3 Amniotic Fluid Cells for Heart Valve Tissue Engineering

3.1 Heart Valve Tissue Engineering: *A Versatile Approach for the Structural Regeneration of Heart Valves*

Valvular heart disease—such as congenital malformations or age-dependent progressive calcific degeneration—are responsible for a high disease load worldwide with increasing prevalence, mortality, and morbidity each year [13]. Therefore, also heart valve replacement procedures are expected to triple globally from currently approximately 290,000 interventions per year to 850,000 in 2050 [63]. In industrialized countries the major factor contributing to this increasing epidemiology is found in the demographic development within the population leading to a higher prevalence of degenerative valve disease.

The common therapy of heart valve disease currently focuses on the surgical repair or replacement of the insufficient heart valves by either mechanical or bioprosthetic prostheses [55]. These valve prostheses are associated with several limitations including surface thrombogenicity and progressive calcific degeneration [6, 9]. In addition, repeated surgeries as to the nonadaptive nonviable replacement materials are of particular concern in still growing children and young adults [37]. In this regard, the fabrication of autologous tissue engineered heart valves represents a promising alternative with potential to overcome these limitations. Autologous cell-based valve replacements could allow for lifelong somatic growth, remodeling, and regeneration. In addition, immune rejection, reoperations, and thromboembolic complications could be prevented.

3.2 Principal Strategies of Heart Valve Tissue Engineering: *From In Vitro to In Situ Tissue Formation*

Tissue engineered heart valves can be fabricated using two different strategies. For the traditional in vitro fabrication of tissue engineered heart valves ("in vitro heart valve tissue engineering") autologous cells isolated from the affected patient are seeded onto biodegradable scaffolds, designed in the shape of a heart valve. The seeded constructs are then placed into a bioreactor system in order to promote tissue formation and maturation in vitro in an environment resembling mechanical and biological properties of the heart itself. Additionally, the orthotopic implantation into the patient results in physiological valvular function and promotes

further in vivo remodeling of the in vitro fabricated constructs. In particular several promising autologous cell sources have already been used in the attempt to generate in vitro engineered heart valve constructs such as adipose tissue-derived cells [7], amniotic fluid-derived cells [38, 39, 60], bone marrow-derived cells [19, 54], chorionic villi-derived cells [40], and umbilical cord-derived cells [42, 52]. Most seeded cells showed characteristics of either endothelial progenitor cells (EPCs) or myofibroblasts. Especially isolated MSCs have attracted major interest in the last few years due to their capacity to differentiate into various cell lineages [26], their privileged immunological characteristics [25], their extensive in vitro proliferation capacity as well as their paracrine mechanism inducing host-guided regeneration and recruitment of EPCs via inflammation-mediated pathways [17, 35].

For the in situ tissue engineering approach, a cell-seeded scaffold is directly implanted without significant in vitro conditioning in order to achieve autologous host cell repopulation, in-growth and remodeling in vivo. This second approach takes advantage of the intrinsic regenerative potential of the body, potentially also representing a simplified "off-the-shelf" strategy. One way of supporting the in situ remodeling and host cell repopulation is the pre-seeding of synthetic scaffold matrices with autologous cell in vitro. The most prominent approach in this regard is the bone marrow mononuclear cell approach, which has extensively been investigated preclinically for tissue engineered heart valves [11, 12, 62] and clinically for tissue engineered vascular grafts [30, 47]. Interestingly, cytokine release from unseeded scaffolds has shown to achieve similar host response as those seeded with bone marrow mononuclear cells [35]. These results hold great promise due to the simplified fabrication protocols and reduction of costs when considering the absence of any in vitro preseeding procedures. Mechanisms responsible for neotissue formation might also be triggered by a defined release of specific cytokines or therapeutics integrated into drug delivery systems of biodegradable scaffolds [31]. However, in spite of these promising results and technologies, the in situ approach is significantly limited by the fact that it depends mainly on the regenerative capacities of the host, which might be problematic in patients with severe disease and defects of the immune system. In addition, metabolic diseases—such as diabetes mellitus—may have an impact on the in situ remodeling capacities.

3.3 From Animal to Human: *Experiences on the In Vivo Functionality of Bioengineered Heart Valves*

In 1995, Skinoka et al. demonstrated for the first time a successful implantation of an in vitro tissue engineered autologous single pulmonary valve leaflet in a lamb model using mixed cell populations of endothelial cells and fibroblasts for the seeding procedure [49, 65]. In vitro engineering of complete trileaflet heart valves seeded with ovine myofibroblasts and endothelial cells have also shown to be principally feasible [20, 51]. Implantation of these constructs resulted in neotissue formation with appropriate functionality on a follow-up of 120 days [50]. Tissue engineered vascular grafts implanted in a lamb model have been demonstrated to have the ability of in vivo growth and adaptation as demonstrated by two independent studies [4, 18]. Therefore, in an attempt to define the underlying cellular and molecular mechanisms of neotissue formation and remodeling of tissue engineered constructs, the introduction of small animal models, especially mice, has provided first implications on how the in situ remodeling processes are orchestrated [15, 28].

In 1999, human cells harvested from the peripheral vein were seeded onto biodegradable scaffolds leading to the first autologous tissue engineered graft transplanted into a 4-year-old girl suffering from single ventricle and pulmonary atresia [48]. In the following years, tissue engineered patches that utilized bone marrow mononuclear cells have been successfully used in human clinical trials [30, 47]. Late term results revealed no graft-related mortality and complications within these patients [16]. The option of using tissue engineered grafts as potential therapy is promising, nevertheless some further analysis related to the described graft stenosis, as a main cause of graft failure, has been defined as an indispensable prerequisite for large scale clinical translation [17]. Therefore the characterization of the ongoing cellular processes is of major importance. In 2011, clinical trials for tissue engineered vascular grafts have for the first time been started in the USA, representing an important step for the entire field of cardiovascular tissue engineering [10, 59]. This blood vessel replacement consists of a synthetic scaffold which is seeded with autologous bone marrow cells. Data on their follow-up will provide a better understanding on the feasibility of tissue engineered constructs as a replacement therapy in cardiovascular diseases.

3.4 Fetal Adnexa-Derived Cells for Heart Valve Tissue Engineering: *Autologous Fetal Cells for Pre- or Postnatal Therapy*

Congenital heart valve malformations are routinely detected prior to birth by ultrasound examinations [24]. Making use of this prenatal screening, pediatric tissue engineering aim AT the in vitro fabrication of autologous heart valve constructs ready to be used at or shortly after birth. Besides amniotic fluid-derived cells, several fetal cell sources such as umbilical cord-derived cells and chorionic villi-derived cells can be obtained during or shortly after pregnancy with low-risk procedures and have shown to be attractive for the concept of pediatric heart valve tissue engineering.

Human umbilical cord-derived cells—such as Wharton's jelly-derived myofibroblasts and umbilical cord-derived progenitor cells (EPCs)—seeded on biodegradable vascular scaffolds have been successfully used for the generation of native blood vessels [41, 44] (see also Chap. 29 Section 3; K). In addition in vitro generation of living autologous cardiovascular replacements (patches) with umbilical cord-derived fibroblasts and EPCs showed significant tissue formation and confluent endothelial surface coverage with properties comparable to native tissues [43]. In 2006, engineering of biologically active heart valve leaflets cultured in a biomimetic system under biochemical and/or mechanical stimulation has firstly been reported using umbilical cord-derived cells [44]. In vitro generation of viable and functional human heart valves have furthermore been demonstrated using cryopreserved human umbilical cord cells [52]. An additional attractive cell source for pediatric tissue engineering applications is fetal MSCs and progenitor cells provided from the human placenta, particularly its chorionic villi [33]. First attempts for the engineering of living heart valve leaflets have successfully been conducted using prenatal fetal progenitors [42].

In summary, prenatal or perinatal harvesting of stem and progenitor cells represents a promising technology in cardiovascular tissue engineering. The opportunity to produce an autologous tissue engineered construct suitable for each particular patient would overcome restrictions in current therapeutic approaches and may reduce the chance of relapse including repeated operations. Besides the improvement of the involved bioengineering technologies, the establishment of human cell banks may represent another crucial prerequisite for future tissue engineering therapies in pediatric and adult patients. Nevertheless it remains still unclear which fetal cell source holds the greatest potential for clinical use and might therefore represent the most suitable therapy considering harvest procedures and tissue formation. In the future systematic comparison studies are indispensable in order to identify the most promising cell sources with regard to remodeling and tissue formation.

3.5 Amniotic Fluid Cells for Heart Valve Tissue Engineering: *A Prenatal Autologous Cell Source for Prenatal Therapy*

MSCs isolated from amniotic fluid have been determined as a reliable and clinically feasible source for tissue engineering [22, 23]. Also with regards to cardiovascular tissue engineering, human AFSCs have been shown to generate living autologous heart valve leaflets in vitro (Fig. 9.1) [38, 39]. Cells isolated from the amniotic fluid by CD133− magnetic beads demonstrated characteristics of MSCs in comparison to the endothelial like CD133+ cells [38]. Expression of characteristic mesenchymal surface markers like CD44 and CD105 were confirmed in CD133− cells. In addition expression of CD141 and eNOS could not be detected in contrast to CD133+ cells (Fig. 9.2). Functional heart valve leaflets were generated by firstly seeding CD133− cells onto biodegradable polymers, before coating the construct with CD133+ cells. Engineered heart valve leaflets exhibit a functional endothelial layer with production of common extracellular matrix elements also found in native valve leaflets. Analyses of the produced neotissue revealed functionality in the low-pressure environment as well as sufficient opening and closing behavior of the tissue engineered heart valve in an in vitro valve tester. To define the potential of these cells for adult applications, cryopreserved amniotic fluid-derived cells were furthermore compared to their non-cryopreserved counterparts in terms of stem cell phenotype, differentiation potential, and tissue formation [39]. In vitro fabrication of heart valves leaflets with similar mechanical properties could be demonstrated, indicating amniotic fluid-derived cells as potential life-long available autologous cell source for heart valve tissue engineering. In addition, in a proof of concept study the in vivo functionality of the amniotic fluid cell-based in situ (prenatally) engineered heart valves was investigated in the ovine fetal model [60]. In this study, tissue engineered heart valves were implanted into the fetal sheep pulmonary position using an in utero closed-heart hybrid model and tissue engineered construct were analyzed in terms of tissue formation, remodeling, and cell invasion.



Fig. 9.1 Amniotic fluid cell-based engineered heart valve after 28 days in vitro conditioning in a diastolic pulse duplicator system (from [38])

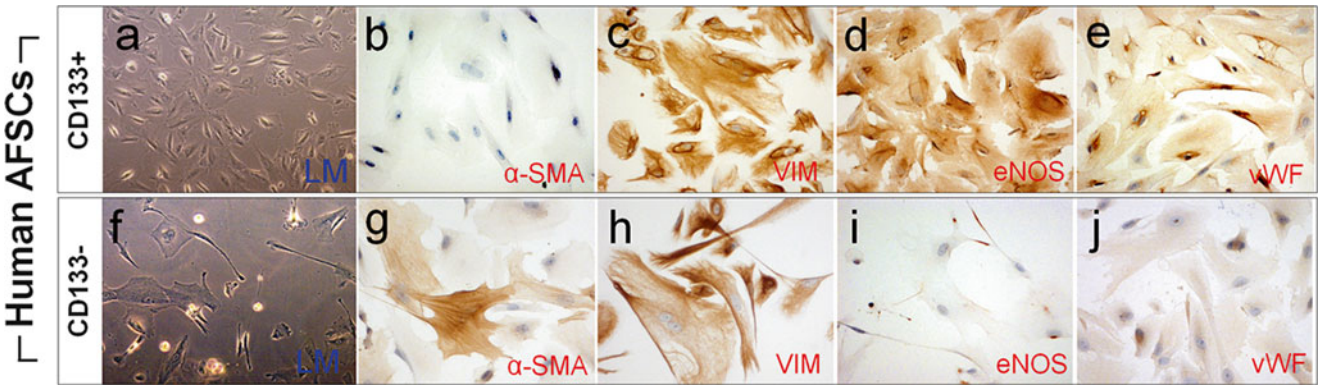


Fig. 9.2 Human amniotic fluid cells (AFCs) sorted with CD133 magnetic beads resulted in endothelial-like CD133+ cells (**a**–**e**) and mesenchymal-like CD133− cells (**f**–**j**). While CD133+ cells showed positive staining for Vimentin, eNOS and vWF, CD133− cells stained positive for αSMA and Vimentin (from [61])

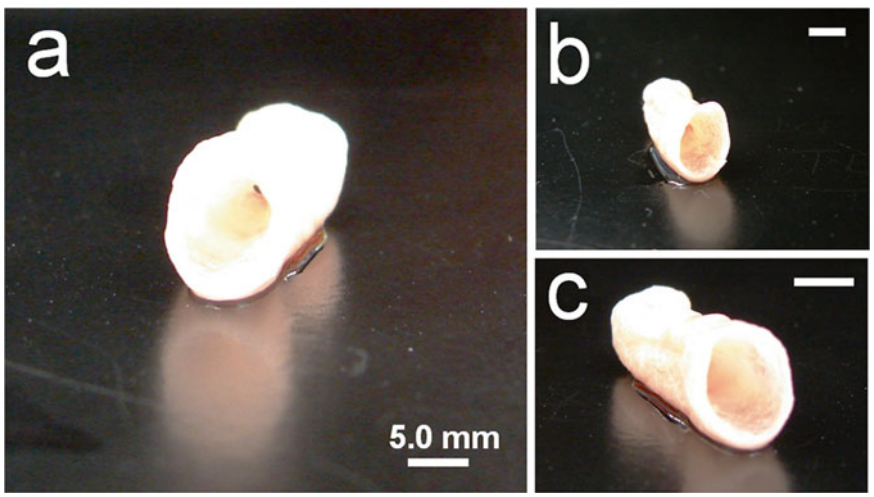


Fig. 9.3 (**a**–**c**) In vitro fabricated tissue engineered vascular grafts (small and large diameter) with the use of ovine amniotic fluid-derived cells (from [61])

Interestingly, constructs seeded with AFSCs showed high cellular infiltration and some initial tissue formation in vivo. However, the valves also showed structural changes in the leaflets—including radial leaflet shortening—representing a major limitation of this concept.

In the light of a careful preclinical evaluation of AFCs for cardiovascular tissue engineering applications, the characterization of amniotic fluid harvested from an ovine preclinical model—representing the standard model in cardiovascular medicine—has recently been reported [61]. Several biochemical parameters, like total protein amount or electrolytes, have been suggested as possible indicators for a specific isolation of amniotic fluid in sheep. Moreover, isolated cells showed a stable karyotype with expression of common mesenchymal stem cell surface markers as well as of some stem cell factors like NANOG and STAT-3. In contrast to the successful sorting of human amniotic fluid-derived cells based on AC133.1 [38], isolation of sheep endothelial cells was not possible using this technique. Nevertheless functional in vitro fabrication of tissue engineered vascular grafts and cardiovascular patches could be shown using amniotic fluid-derived cells (Fig. 9.3). However, more in vivo experiments in preclinical models are indispensable to evaluate this cell source for a possible human clinical translation. The exact in vivo fate of the cells needs to be defined in order to guarantee safety and exclude adverse effects for a potential prenatal therapy. In conclusion, amniotic fluid-derived cells represent an effective cell source for the treatment of congenital defects. In particular their early availability via low-risk procedures as well as their wide differentiation capacities hold great potential for autologous tissue engineering applications.

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Amniotic Fluid-Derived Stem Cells for Bone Tissue Engineering

10

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Abstract

Implantation of bone substitute materials and autologous bone grafting have been used for the treatment of extensive bone defects. Recent advances in tissue engineering have led to integration of viable, biological bone grafts composed of osteogenic cells proliferating within three-dimensional (3D) scaffolds. Not only could these novel grafts be used for implantation, but also they could serve in basic and translational studies of bone development, disease, and drug discovery. The ability to isolate human cells, expand them to a large density, and differentiate them into bone-forming cells remains critical to the success of human bone graft engineering. This chapter will focus on the characteristics and limitations of human amniotic stem cells and their application in bone tissue engineering.

Stem cells have proved to be an ideal source of cells for bone tissue engineering due to their ability to differentiate into osteogenic lineages. Recently, amniotic fluid-derived stem (AFS) cells that are isolated from amniotic fluid removed by amniocentesis have been identified as a novel stem cell source. The proliferative potential of these cells, along with their anti-immunogenic and non-tumorigenic properties, make them an ideal therapeutic candidate for bone tissue regeneration without ethical issues. In this chapter, the current knowledge of AFS cell isolation, characterization, and osteogenic differentiation, as well as tissue engineering approaches for the use of these cells in bone regeneration, is discussed.

1 Introduction

Bone diseases affect many millions of people who suffer from a number of pathological conditions including osteoporosis, osteoarthritis, osteosarcoma, and bone defects such as bone fractures. These conditions require bone grafts that are used to enhance bone healing and reconstruct bone defects in accordance with two distinct mechanisms: osteoconduction and osteoinduction. Osteoconductive grafts induce formation of new bone by attracting pre-existing neighboring cells while osteoinductive grafts function as a source to stimulate and differentiate progenitor cells to induce bone formation.

Autografts using tissue obtained from the patient's own body, usually the iliac crest and fibula, have been the gold standard for bone repair and regeneration is autografts [1]. As autografts are osteoconductive and ostoeinductive, conventionally, these are considered ideal for bone grafts. Although there are several advantages to autografts, including high success rate, reduction of the risk of rejection and disease transfer, the disadvantages, which include donor site morbidity, excruciating pain, limited graft supply, and the risk of complications such as resorption and fracture, present challenges that must be overcome [2]. Allografts, usually prepared from demineralized bone from cadaver, serve as

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an alternative to autografts by eliminating donor site morbidity and a secondary surgery site. Unfortunately, high potential for immune rejection and transmission of disease still exists [2].

Bone tissue engineering provides an alternative treatment modality for traditional bone grafts. The tissue engineering strategy involves the combination of a scaffold and stem cells, which are capable of osteogenic differentiation, to create constructs that mimic natural bone tissue and promote bone formation. Recently, many studies have demonstrated that human amniotic fluid-derived stem (AFS) cells present a promising alternative cell source for bone repair and regeneration. In this chapter, we provide descriptions of these cells in terms of procurement, isolation, expansion, and differentiation, as well as in vitro and in vivo studies that demonstrate the therapeutic potential of these cells. To begin, we will briefly describe the biology of bone formation and remodeling.

2 Bone Formation

2.1 Function of Bone

Human bone is a highly dynamic tissue comprised of a variety of cell types, blood vessels, and nerves embedded in a mineralized extracellular matrix, which provides structural support and protection for the vital internal organs, as well as facilitating movement and locomotion. In addition, the cytokines and growth factors present in bone maintain bone strength and mineral homeostasis of the body.

2.2 Bone Formation During Development and Wound Healing

During development, there are two main processes facilitating bone formation and growth: intramembranous and endochondral ossification. Intramembranous ossification involves a dense aggregation of mesenchymal cells that differentiate into osteoblasts and directly generate bone tissue. During endochondral ossification, mesenchymal stem cells are formed into hypertrophic cartilage that mineralizes and forms extracellular matrix, resulting in the formation of a template for long bone formation [3, 4]. Environmental factors will influence wound healing in bone, which may proceed by either process, and include juxtaposition of the ends of the bone and the degree of movement relative to each other, with motion favoring the endochondral process.

2.3 Bone Dynamics: Remodeling

Remodeling of bone increases during adolescence, with newer, healthier bone replacing older bone. This remodeling is finely controlled to prevent accumulation of microdamage while maintaining mechanical stress, as well as regulate calcium and phosphate homeostasis. Osteoblasts and osteoclasts within the basic multicellular unit replenish old bone with new bone triggered by micro-crack formation or osteocyte apoptosis [5]. Approximately 25 % of cancellous bone and 3 % of cortical bone are renewed each year [6, 7], and this remodeling is essential for ensuring functional competency of bone. When osteoclastic resorption exceeds osteoblastic formation, bone loss occurs and results in osteoporosis [8], which results in fragile bone and increased risk of fractures.

3 The Cells of Bone

Based on locations, morphology, and functions, bone contains two main types of bone-specific cells: osteoclasts and osteoblasts. Both types of cells have an important role in creating and maintaining skeletal architecture.

3.1 Osteoclasts

Osteoclasts are the only cells that are capable of resorbing bone. Osteoclasts are derived from mononuclear precursor cells of hematopoietic lineage [9]. While monocyte-macrophage precursor cells have been identified in various tissues, bone marrow-derived monocyte-macrophage precursor cells are known to be the source of most osteoclasts [5]. Osteoclasts trigger bone resorption through binding to bone matrix via integrin receptors and dissolving the mineral and proteinaceous components of bone matrix through a combination of localized acidification and protease secretion, respectively [10].

3.2 Osteoblasts

Osteoblasts are mononuclear cells that are responsible for bone formation in a dynamic process of remodeling. Osteoblasts are the direct descendants of osteoprogenitor cells that reside in the periosteum and the bone marrow. These cells are specialized to synthesize and secrete a unique collagen-rich extracellular matrix in bone and produce alkaline phosphatase, which is a key enzyme in bone mineralization.

4 The Ideal Source of Cells for Bone Formation

The current gold standard to reconstruct bone defects is autologous bone grafts (from the patient) [1]. Autografts, usually harvested from the patient's iliac crest, fibula, or ribs, are osteoconductive and osteoinductive; furthermore, they possess osteogenic potential because they are the patient's own cells [11]. It has also been reported that they might recruit and differentiate mesenchymal cells into such progenitor cells [2]. Most importantly, autografts reduce the risk of immune rejection and disease transfer. Although the success rate in the clinical practice is high, a number of disadvantages remain. Major drawbacks for autologous grafts include donor site morbidity and the limited amount of bone available for autografts [12], as well as complications from infection, resorption, and even fracture [13]. More importantly, the surgical processes of harvesting the patient's own bone can be extremely painful. One alternative source for reconstructing skeletal defects is the allogenic graft. Allografts consist of natural human bone derived from demineralized cadaveric bone. Although allografts eliminate the need for an additional surgery on the recipient, they carry high potential for host immune rejection and disease transmission [14]. Synthetic grafts, on the other hand, consist of artificial bone grafts that are made of ceramics and polymers. They have the advantage of possessing osteoconductive properties and unlimited availability; however, they are not osteoinductive and carry high risk of structural failure, which limits their application to repair large bone defects [15, 16]. Thus, more effective approaches to treat these bone defects are needed.

5 Cell-Based Tissue Engineering for Bone Formation

Recently, bone tissue engineering has emerged as one of the most promising approaches towards the development of biological bone substitutes that restore, maintain, and improve function of damaged or lost bone tissues. Another emerging class of bone tissue engineering is cell-based tissue engineering, which has proven to be a powerful tool for regenerating bone. In this setting, living cells are combined with biocompatible scaffolds to provide biological substitutes that mimic the structural integrity and function of native bone.

Lineage-committed osteoblastic cells seem an obvious choice, but results have been unsatisfying mainly due to the limited potential for proliferation. More promising results have been obtained using self-renewing stem cells. The following sections provide a brief summary of stem cells that have been used for regeneration of bone.

6 Amniotic Fluid-Derived Stem Cell

6.1 Isolation and Cultivation

The isolation of human AFS cells is appealing due to the relative ease and safety of procurement. AFS cells can be isolated from 5 mL of second aspiration of amniotic fluid by amniocentesis during 14–20 weeks of pregnancy. First, the recovered cells from amniotic fluid collected by amniocentesis are cultured in feeder-free medium containing 15 % of fetal bovine serum and Chang supplement. After expansion to confluence, the cells are harvested by trypsinization and subjected to immunological selection via CD117 (c-Kit) staining. Purified AFS cells can be expanded without spontaneous differentiation in vitro [17, 18].

6.2 Characteristics

The isolation of human and mouse AFS cells from a heterogeneous population of cell types in the amniotic fluid that are capable of extensive self-renewal and give rise to adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hypotogenic lineages was first reported by Atala's group [19]. AFS cells represent approximately 1 % of the heterogeneous population of cell types found in the amniotic fluid. These cells stained positively for c-Kit, a surface marker expressed by stem cells of mesenchymal origin [20]. Almost all clonal AFS cell lines express embryonic markers such as OCT 4 and SSEA4, which indicates their pluripotential capability [17, 21]. AFS cells also express a number of surface markers that are characteristic of mesenchymal and/or neural stem cells such as CD29, CD44, CD73, and CD105. More importantly, AFS cells descended from a single cell are capable of differentiating into all three embryonic gem layers.

Undifferentiated AFS cells display a high proliferation rate in culture (doubling time = 36 h) without the need of feeder cells, and retain long telomere length (20 kbp) and normal karyotype even after expansion to 250 population doublings [17]. Importantly, AFS cells do not induce tumor formation in severe combined immunodeficient (SCID) mice [17].

7 AFS Cells for Bone Tissue Engineering

7.1 Osteogenic Differentiation Media

In culture, AFS cells can be differentiated into bone cells based on the culture media supplemented with osteogenic supplements. Typical osteogenic differentiation media contains dexamethasone, ascorbic acid, and β-glycerophosphate.

Dexamethasone, a synthetic corticosteroid, induces osteogenic differentiation of AFS cells, resulting in the expression of osteogenic genes and mineralization [22, 23]; however, it is also reported that dexamethasone may not be sufficient for mineral formation [24]. The effect of the glucocorticoid depends on cell type, the gene regulatory proteins enclosed within the cell, and the regulatory region of the gene [25]. The intracellular signaling mechanism responsible for the stimulatory effect of dexamethasone on osteogenic differentiation is unknown.

Ascorbic acid is required for the survival of human osteoblasts in vitro [26], up-regulates osteoblast differentiation and proliferation, and assists maintenance of the osteoblastic phenotype by enhancing alkaline phosphatase activity and the total protein [27]. It has been reported that the presence of ascorbic acid is not a requirement for alkaline phosphatase expression in hMSCs. As with dexamethasone, exact mechanism of ascorbic acid on osteogenic differentiation is not known.

β-Glycerophosphate is known to promote osteogenesis by inducing mineralization in cell cultures [28]. The exact mechanism through which β-glycerophosphate can induce mineralization is not clear; however, inorganic phosphate released due to hydrolysis of organic phosphate by alkaline phosphatase is believed to promote mineral deposition onto the substrate on which the cells are plated.

8 Osteogenic Potential of AFS Cells and In Vitro Production of Tissue-Engineered Bone

Bone tissue engineering using AFS cells is still in its infancy. Few in vitro and in vivo studies have been performed; however, a variety of research results provide strong evidence that AFS cells could serve as a powerful tool in regenerative medicine. Here, those studies will be reviewed from a perspective of osteogenic potential and the production of tissue-engineered bone. A summary of bone tissue engineering using AFS cells is provided in Table 10.1.

The majority of studies have both proven that AFS cells are capable of differentiating into osteoblasts in 3D and confirmed that, following induction, these stem cells have potential therapeutic utility. Kim et al. [22] demonstrated that differentiation of human AFS cells were induced in the 2D osteogenic medium, containing dexamethasone, ascorbic acid, and β-glycerophosphate. This was evidenced by both qualitative and quantitative analysis of Alizarin Red staining that indicates calcium deposition as shown in Fig. 10.1. Subsequently, a composite scaffold using a collagen matrix derived from porcine bladder submucosa matrix (BSM) and poly(lactide-*co*-glycolide) (PLGA) was developed and studies were performed to determine whether human AFS cells seeded in this composite scaffold retained their ability to induce osteogenic differentiation in the osteogenic medium. The results from these studies showed that human AFS cells adhered to the composite scaffolds with uniform distribution and proliferated over time. Osteogenic differentiation on the composite scaffold was also assessed using real-time PCR, and expression levels of three major transcription factors associated with osteogenic differentiation, RUNX2, OPN, and OCN, were consistently higher in the scaffold groups treated with osteogenic medium than untreated scaffolds. Mineralization of the differentiated samples was also confirmed by calcium content measurement.

AFS cells can also contribute to bone formation in vivo. Dupont et al. [29] investigated the effect of human AFS cells on bone formation using a nude rat critical-sized femoral segmental defect model. In this study, poly(ε-caprolactone) (PCL) seeded with human AFS cells showed higher bone ingrowth compared to those receiving acellular scaffolds. Various types of stem cells have been investigated for bone formation; however, there have been only few comparisons. Using this model, human AFS cells were compared to human MSCs for their effect on bone repair. 2D X-ray images and micro-CT quantification of bone volume showed that there were no significant differences between these cell sources (Fig. 10.2).

In Peister's study [23], however, direct comparisons were made between AFS cells and MSCs in terms of mineralization potential in 3D environments where cells were cultured on scaffolds constructed of PCL for 15 weeks. MSCs differentiated more quickly than AFS cells on 3D scaffolds, but mineralized matrix production slowed after 5 weeks. In contrast, AFS cells showed an extended duration of mineralization that continued to increase to 15 weeks. The total mineralized content in AFS constructs were five times more than MSC constructs (Fig. 10.3). Based on these findings, it was suggested that MSCs would be a good choice for the clinical application where immediate matrix production is needed while the AFS cells would be more effective when robust mineralization is needed for a longer period of time. This study provided a basis of cell sourcing strategies for efficient treatment depending on clinical needs.

De Coppi et al. [17] also demonstrated in vitro the differentiation of AFS cells in culture medium with osteogenic supplements, and these cells yielded functional osteoblasts evidenced by the production of mineralized calcium and expression of alkaline phosphatase. For the in vivo assessment, AFS cells in an alginate/collagen scaffold exposed to osteogenic medium for 1 week showed bone formation after subcutaneous implantation into immunodeficient mice, which was not observed in the unseeded scaffolds.

Table 10.1 Bone tissue engineering applications of amniotic fluid-derived stem cell

	Testing condition		Result		
Indication	In vitro	In vivo	In vitro	In vivo	Reference
To verify the capability of hAFSCs of osteogenic differentiation forming tissue-engineered bone	Printed hAFSC-alginate/collagen scaffold are differentiated to osteogenic lineage in osteogenic medium	Subcutaneous implantation of ASFCs/scaffold into immunodeficient mice	Osteogenic differentiation: mineralization, ALP activity and gene expression RUNX2, OCN, ALP	– Ectopic bone formation (hard mineralized tissue generation after 18 weeks)	De Coppi et al. [17]
To verify the capability of hAFSCs to osteogenic differentiation	Differentiated in osteogenic medium		Differentiation of osteoblast with calcium mineralization (von Kossa stain)		Kim et al. [31]
To see the potential of AFSCs to produce 3D mineralized ECM within porous mPCL scaffold	– hAFSCs initially differentiated in 2D culture in osteogenic medium – 2D-cultured hAFSC seeded in 3D mPCL scaffold	Subcutaneous implantation of pre-differentiated AFSCs/mPCL scaffold into athymic rats	– 80 % of colonies are mineralized – AFSC are viable after 15 weeks in 3D culture	– Pre-differentiated AFSCs produce seven times more mineralized matrix – AFSCs produce mineralized bioengineered constructs in vitro and in vivo	Peister et al. [32]
To repair bone defect (to determine osseous grafts engineered from AFSCs)	Rabbit AFSCs seeded in PLLA electrospun nanofiber in bioreactor	Implantation of pre-differentiated electrospun nanofibrous scaffold to the defective intercostal space		Ectopic bone formation: full thickness repair of sternal defects, graft density increased	Steigman et al. [33]
Osteogenic differentiation of hAFSCs in nanofibrous scaffold induced by rhBMP-7	hASFCs on the NF scaffolds induced by 50 ng/mL rhBMP-7	Subcutaneous implantation into athymic nude mice	– Osteogenic differentiation by mineralization (calcium) and expression of gene (RUNX2, OSX) – Synthetic nanofibrous scaffolds enhance osteoblastic differentiation of hAFSCs – rhBMP-7 induce mineralize hAFSCs more than MSCs	Ectopic bone formation	Sun et al. [30]
Compare osteogenic capacity of hMSCs and hAFSCs, on electrospun nanofiber meshes	hMSCs and hAFSCs are seeded in 3D nanofiber mesh in osteogenic medium		hAFSC show delayed ALP peak, but elevated mineral deposition, compared to hMSCs		Kolambkar et al. [18]
To explore the feasibility of fabrication a macroscopic bone tissue at centimeter scale	Two stage culture – hAFSC-laden CultiSpher S microcarrier (from porcine gelatin) to prepare modular tissue – Assembly of microcarrier in cylindrical perfusion culture chamber		Osteogenic differentiation: mineralization (calcium), ALP, expression of gene (collagen type I, OCN) Good viability and homogenous distribution of AFSCs and bone characteristic ECM		Chen et al. [34]
To compare osteogenic differentiation capacity between MSCs and AFSCs	A1hAFSC and H1hAFSC seeded within mPCL scaffold – 2D culture in osteogenic medium and hAFSC seeded in 3D mPCL/collagen gel scaffold		AFSCs have delayed, robust osteogenic differentiation compared to MSC		Peister et al. [23]
To repair critical size cranial bone defects (to evaluate fibroin scaffolds with hAFSCs and hDPSCs)	Fibroin scaffold seeded with hAFSC or hDPSCs in osteogenic medium	Cell/scaffold implant into critical-sized cranial defect in immunocompromised rat	Osteogenic differentiation by radiology, confocal microscopy, histologic method	Bone formation and defect correction with higher bone formation by hAFSC-seeded scaffold	Riccio et al. [35]
To investigate BSM with 3D PLGA scaffold to facilitate osteogenic differentiation	hAFSC seeded in 3D composite scaffold system using collagen matrix from porcine BSM and PLGA in osteogenic media		Osteogenic differentiation: mineralization, ALP activity and gene expression RUNX2, OPN, OCN		Kim et al. [22]

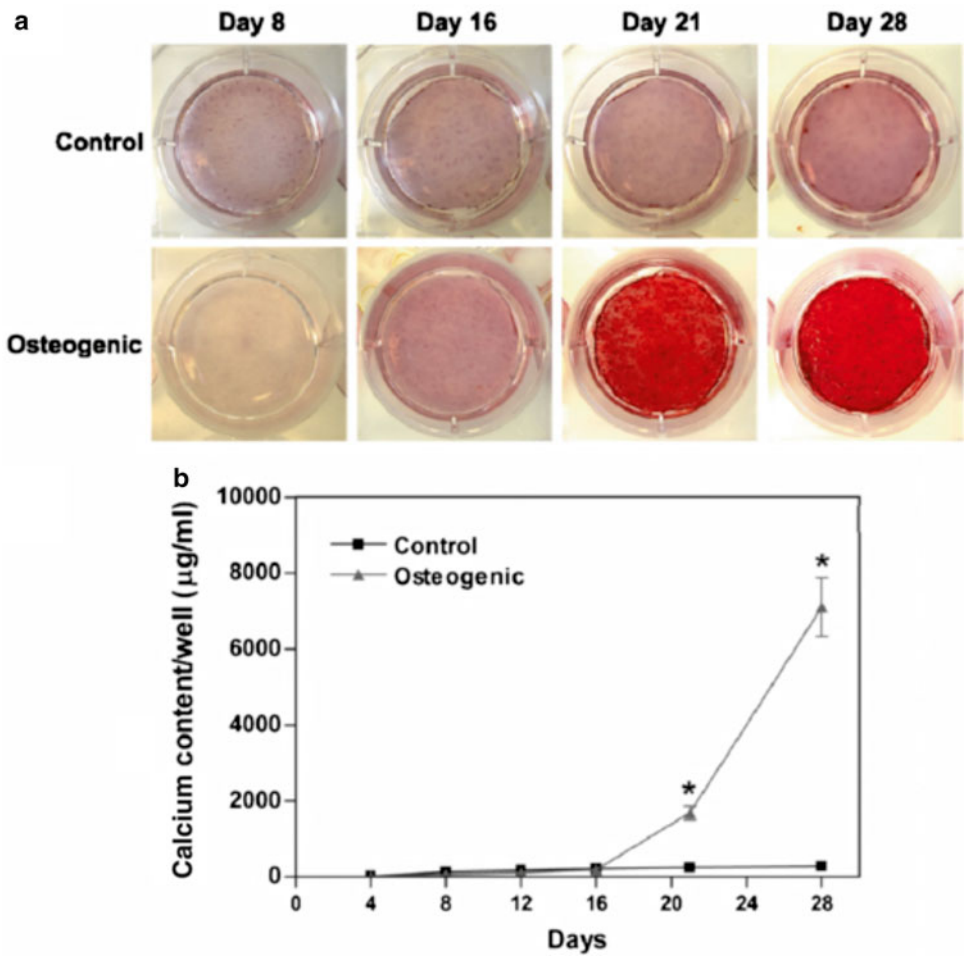


Fig. 10.1 Effect of osteogenic medium on the mineralization was qualitatively analyzed by Alizarin Red staining after 8, 16, 21, and 28 days: (**a**) gross examination (**b**) quantitative data (*$P<0.01$, $n=6$). From Kim et al., *Biomedical Materials*, 8(1):014107, Copyright © 2013. Adapted by permission of IOP Publishing

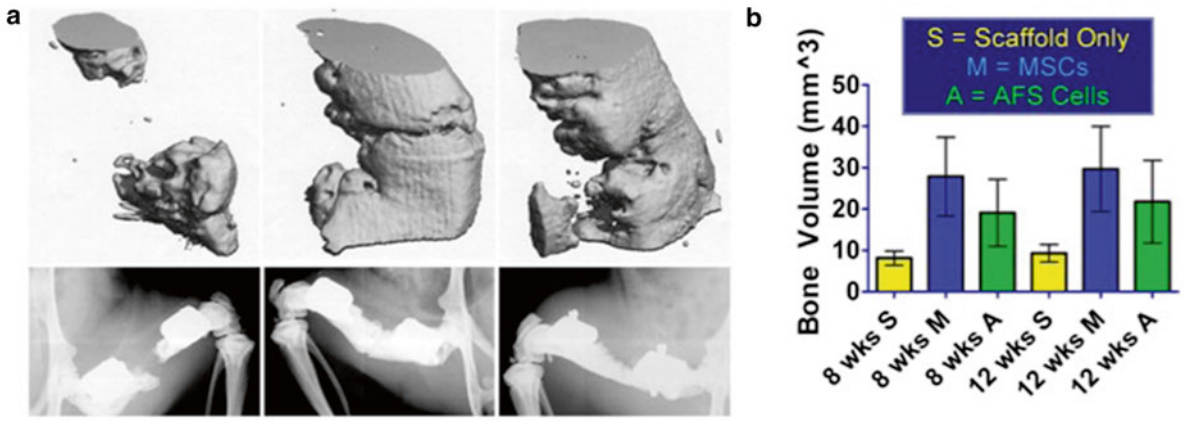


Fig. 10.2 Structure results from in vivo delivery of stem cell-loaded scaffolds. (**a**) Micro-CT (*upper*) and X-ray (*lower*) images of the best bone formation per group in defects receiving acellular scaffold (*left*), hMSC-seeded scaffold (*center*), or hAFS cell-seeded scaffold (*right*). (**b**) In vivo bone volume comparison showing no significant differences between groups. From Dupont et al., *PNAS*, 107(8):3305-10, Copyright © 2013. Adapted by permission of PNAS

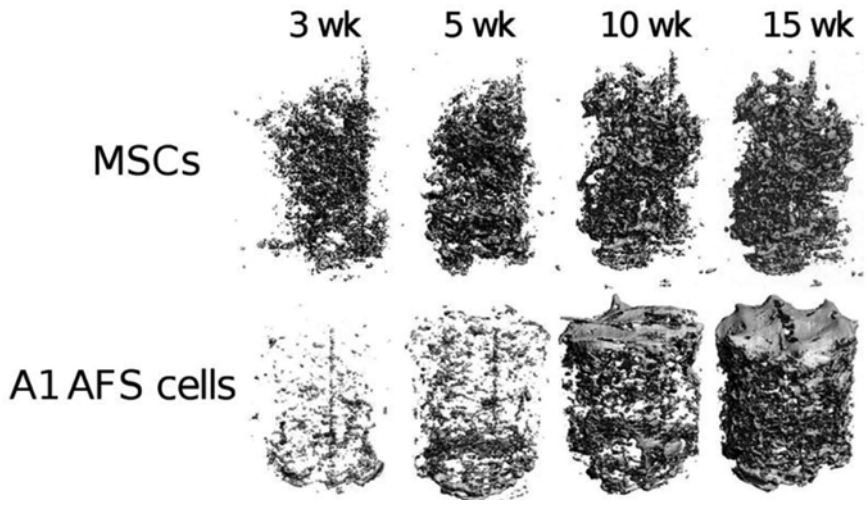


Fig. 10.3 MSCs and AFS cells induced to produce mineralized matrix within the PCL scaffold. The scaffolds were aseptically scanned by micro-CT at weeks 3, 5, 10, and 15. The mineral volume of the same scaffold is shown for the A1 AFS cells and MSCs to illustrate the change in mineralized matrix over time. The mineralization of the PCL scaffold by the AFS cells is much more extensive, with the mineral distributed throughout the scaffold. The mineral produced by the MSCs is primarily found on the interior of the scaffold. $N = 12$. From Peister et al., *Stem Cell Research*, 7:17-27, Copyright © 2013. Adapted by permission of Elsevier

While traditional osteogenic supplements have been widely used in many studies to induce osteoblast differentiation, Sun et al. [30] showed that rhBMP-7 induced differentiation of human AFS cells to the osteogenic lineage, revealing more active synthesis of a matrix stimulating OCN and BSP gene expression. In addition, a stronger mineralization was observed in human AFS cells than in human MSCs at the same concentration. The underlying mechanism has not been suggested.

9 Conclusion

While various types of stem cell populations have been studied to date, little is known about the optimal cell source for bone tissue engineering, and the best candidate has yet to be established. Unique features of AFS cells present opportunities that are not shared by other cell sources. The recent discovery of AFS cells with easily accessible ex vivo culture from the amniotic fluid, high proliferative capacity, little or no ethical concerns, and differentiating capacity into osteogenic lineages illustrates its usefulness for the development of novel bone tissue engineering strategies. In addition, AFS cells represent an immediate stage between embryonic stem cells and adult stem cells and do not form teratomas in vivo. Their behavior and characteristics have not yet been investigated in as much detailed as other types of cells, and, so far, no therapeutic approach has begun as AFS cells have only recently been identified. Additional studies are needed to elucidate the regenerative properties of AFS cells in vivo and their therapeutic potential. A variety of new research results provide strong evidence that AFS cells could indeed serve as a promising alternative source of cells for use in bone tissue engineering and cell-based therapies.

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Amniotic Fluid Stem Cells for the Repair of Prenatal and Perinatal Defects

11

Weerapong Prasongchean and Patrizia Ferretti

Abstract

Congenital abnormalities are relatively frequent and often require multiple surgical interventions with child growth to ameliorate structure and function of the affected organ. Cell-based therapies and tissue engineering may help to reduce the need for multiple surgeries and improve outcome. Amniotic fluid-derived stem (AFS) cells offer a very suitable cell source for the establishment of such therapeutic approaches for the treatment of birth defects in uterus and perinatally. Together with their high plasticity, AFS cells have the advantage, compared for example to bone marrow stem cells, that they are harvested during standard amniocentesis. This avoids the need for any additional invasive procedure to the fetus, while accessing an autologous cell source. The focus of this review is on current experimental and clinical evidence of the potential of AFS cells for the treatment of birth defects either in uterus or early postnatally. Although clinical translation in this field is still in its infancy, AFS cells offer much promise both because of their paracrine effects and of their ability to differentiate into a variety of tissues with the required functional properties and shape when seeded onto appropriate scaffolds.

1 Background

Congenital defects refer to abnormalities in appearance, structure or function present, though not always already apparent, at birth. Congenital abnormalities can be caused by teratogenic agents, genetic abnormalities, alterations in the intrauterine environment or mechanical damage to the developing fetus [1]. Many morphological abnormalities can now be identified in uterus in the course of routine antenatal screening tests. The incidence of birth defects is high (approximately 3 % of live births), with some body structures, such as nervous system, heart, limbs, and palate, being more frequently affected than others. They can cause long-term disability and be a significant burden to the patients themselves, their carers and society. It is estimated that nearly eight million children worldwide are affected by congenital birth defects [2].

The management of severe morphological abnormalities usually requires surgical intervention to attempt to restore structure and function of the affected organ. Reconstructive surgery, however, is often complex, and not always able to achieve satisfactory results. Furthermore, multiple interventions are often required as a child grows. Hence, over the last few years additional strategies for the repair of tissue and organ defects have been sought, fuelled by the technological advances in biomaterials, stem cells, and tissue engineering [1]. Approaches to induce tissue neo-formation or repair include: (1) delivery of tissue-inducing compounds; (2) grafts of cultured progenitor or stem cells; (3) delivery of cell-containing microcapsules, if paracrine factors are the therapeutic agents; and (4) transplantation of 3D tissue constructs [3].

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The issue of cell source choice in cell grafting strategies is of great importance particularly in children, where the use of autologous stem cells, or at least cells with very low immunogenicity, is highly desirable in order to circumvent the need for life-long immunosuppression [4]. It is also important to consider which cell source will minimize distress to the young patient and how they can be "applied".

In this chapter we will focus on amniotic fluid-derived stem (AFS) cells and discuss their possible use for the treatment, either in uterus of postnatally, of birth defects identified during gestation on the basis of current experimental and clinical evidence.

2 Amniotic Fluid Stem (AFS) Cells

Somatic stem cells of fetal origin are found in the umbilical cord, the placenta, the amniotic membranes and the amniotic fluid [5]. Amniotic fluid cells have been collected from amniocentesis since the 1950s and routinely used for prenatal genetic screening and diagnosis [6]. Amniocentesis is normally performed around 16 weeks of gestation and is a relatively safe procedure [7]. Second-trimester amniocentesis might be safer than early amniocentesis and allows collection of a higher volume of amniotic fluid [6, 8].

The amniotic fluid is a complex and dynamic mixture of fluid and cells of both fetal and maternal origin [9–11]. Its composition changes with the time of gestation [12]. Proteomic analysis has suggested that the cells present in the amniotic fluid are heterogeneous and originate from different embryonic and extra-embryonic tissues [13]. Indeed, cells from the three germ layers, ectoderm, mesoderm, and endoderm, have been detected in amniotic fluid. Furthermore, expression of some proteins present in embryonic stem cells has been detected in cells isolated from the amniotic fluid [13, 14]. A more recent genome-wide study of AFS cells supports their heterogeneity and great variations in gene expression profile depending on gestational age, and supports the view that AFS cells display an intermediate phenotype between pluripotent stem cells and somatic stem cells, as they express both some pluripotency and lineage-specific markers [15].

The amniotic fluid was proposed as a source of cells for fetal tissue engineering [16] before extensive information on the differentiation potential of human AFS cells was available [17]. The presence of cells expressing Oct4, a pluripotent stem cell marker, in the human amniotic fluid was first reported in 2003 [18]. Subsequent studies showed that both the human and rodent cell population isolated from amniotic fluid on the basis of c-Kit expression, that is the membrane receptor for SCF (stem cell factor), expressed markers of embryonic and adult stem cells. This c-Kit-positive population could be maintained in culture without a feeder layer for at least 250 population doublings without any apparent karyotypic alteration [17]. Furthermore these cells were shown to differentiate into derivatives of all three germ layers. It was proposed that under appropriate conditions, human AFS cells could differentiate into adipocytes, osteocytes, chondrocytes, and even into neuronal cells, though this has been a matter of debate, and that they were less differentiated than bone marrow-derived mesenchymal stem cells [14]. When injected into murine embryonic kidneys growing in vitro, the human AFS cells were found to differentiate and integrate into the host tissues to form primordial kidney structures [19]. Human AFS cells can also be more efficiently reprogrammed to induced pluripotent stem (iPS) cells than adult somatic cells. Furthermore, given their younger age, they will have the advantage of carrying fewer environmentally induced mutations [20–22]. Hence, they could be very valuable for establishing birth defect-specific iPS cells to study the mechanisms underlying the disease. Feasibility of reprogramming AFS cells from a diseased fetus has been recently reported in a study where iPS cells originating from AFS cells from a fetus with beta-thalassemia were obtained [21].

3 Rationale for the Use of AFS Cell-Based Therapy Prenatally and Perinatally

Interest in stem cells isolated from the amniotic fluid as a potential source for cell-based therapy to aid the management of birth defects has been rapidly increasing [23]. These cells can be obtained before birth through a routinely used, minimally invasive, and ethically acceptable procedure (Fig. 11.1). As a small aliquot of the amniotic fluid obtained by amniocentesis is required as the starting material, AFS cell collection for therapeutic development/use poses no additional risk to either the mother or the fetus.

Crucially, AFS cells display high proliferative activity and plasticity typical of fetal cells and can therefore be rapidly expanded to obtain sufficient number of cells for therapeutic purposes. As we will discuss later, they appear to maintain their differentiation potential in a variety of scaffolds that are needed to provide the required tissue shape and size according to the anatomical defect to be managed. Furthermore, AFS cells appear to lack tumorigenicity and have low immunogenicity following transplantation into adult animals [24]. Hence they could be a valuable stem cell source both for autologous and allogeneic transplantation. It has been reported that cryopreservation of AFC cells does not affect their stemness and biological activity [25, 26]. Although the clinical and economical value of long-term stem cell banking is still a matter of debate, and much attention should be paid to stringent quality control for banking, the first private amniotic stem cell bank was opened

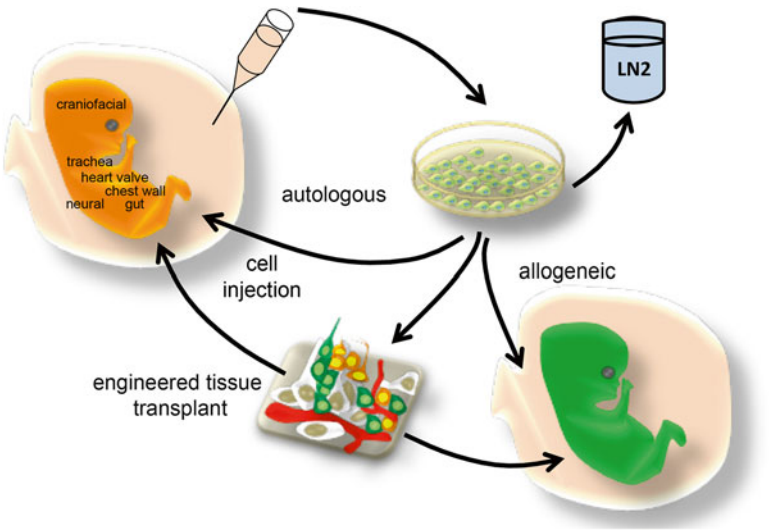


Fig. 11.1 AFS for prenatal and perinatal intervention. AFS collected from the amniotic fluid are expanded and either directly administered, or incorporated into scaffolds for tissue engineering, and subsequently transplanted, or stored for future use (LN2: liquid nitrogen). They could be used for treating birth defects in the fetus (*orange*) or neonate (not shown) from which they were derived (autologous transplantation), or for treating a different fetus (*green*) or neonate (not shown, allogeneic transplantation). Some of the tissues/structures that may be repaired in the fetus or neonate using AFS cells are indicated

by Biocell Center in October 2009 in the USA (http://www.biocellcenter.com/).

In summary AFS cells can offer several advantages for inducing tissue repair and tissue engineering prenatally and perinatally in comparison to other stem cell sources that include:

- No major ethical issues attached
- Ease of harvesting cells from the amniotic fluid
- Rapid expansion time
- Multilineage potential
- Low immunogenicity

Recently, the long-term safety and efficacy of tissues engineered using AFS cells has been validated, paving the way for clinical trials [27]. Altogether, AFS cells seem on course to become one of the cell sources of choice for cell-based therapies in the fetus and perinatally.

4 AFS Cell Behavior in a Prenatal Environment

As AFS cells can be expanded relatively rapidly, autologous AFS cells can in principle be easily available for the treatment of a range of defects identified during routine antenatal screening. Furthermore, given the immunotolerance of the developing fetus and AFS cell low immunogenicity, allogeneic AFS cells might also be used. The pros and cons of the two approaches have yet to be fully evaluated, and they may differ depending on the pathology to be tackled and the stage of development at which medical intervention is deemed appropriate.

We have recently investigated the effect of AFS cell transplantation in an easily accessible embryonic injury model, the chick (Fig. 11.2a) [28]. In this severe thoracic crush injury model that results in extensive damage of the early developing spinal cord and surrounding mesenchymal tissues, most embryos die within 24 h. Grafting AFS cells into the spinal cord at the time of injury significantly reduced tissue damage and increased cell survival assessed over 5 days. The effect of grafting different cell types or using a pharmacological treatment at the time of injury was also investigated. No significant difference in survival was observed in injured embryos grafted with either 3T3-NIH fibroblasts or C17.2 neural stem cells, or treated with the anti-haemorrhage drug, desmopressin. Therefore, the rescue response observed appeared to be specific to AFS cells.

The extensive reduction in tissue damage observed in live embryos grafted with AFS cells at 24 h after surgery could be due to integration and differentiation of grafted AFS cells at the injury site, cell–cell interaction between AFS and host cells, that would allow to fill the gap created by the injury, or by trophic effects. These mechanisms are not mutually exclusive. While there was clearly tissue sparing after injury in embryos injected with AFS cells, no evidence of neural differentiation was observed at the injury site [28]. Significantly, however, there was a clear relationship between embryo survival over time and detection of grafted AFS cells. Evidence that tissue sparing and embryo survival was due to secreted factors was demonstrated in vitro. These experiments showed great reduction in cell death and a parallel increase in the presence of healthy neurons in organotypic spinal cords co-cultured, but not in direct contact, with AFS cells. This is consistent with a paracrine mechanism (Fig. 11.2b). A key paracrine role for AFS cells has been proposed also for the closure and re-epithelialization of skin wounds in mice [29].

In addition to increasing survival within the injured tissues, AFS cells may boost endogenous repair mechanisms

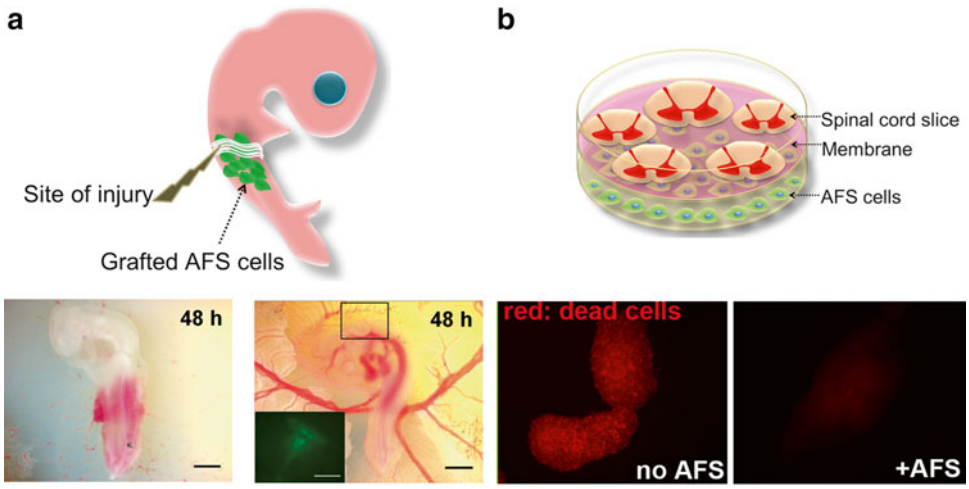


Fig. 11.2 Tissue damage following injury is reduced by AFS via a paracrine effect. (**a**) Upper panel: schematic representation of embryonic chick injury model. GFP-positive AFS cells (*green*) are injected at the injury site. Lower panel: 48 h after injury the embryo without AFS cells has died (*left*), whereas the one injected with AFS cells (*right*), is still alive and development has progressed; the insert from the region indicated by the box shows a fluorescent image of the GFP-labelled AFS grafted cells. (b) Upper panel: schematic representation of the co-culture system. E15 chick spinal cord organotypic slices in the upper chamber are cultured with or without AFS cells in the lower chamber. Lower panel: analysis of cell death using propidium iodide shows extensive cell death in a slice cultured without AFS cells (*left*), but not in the one maintained in their presence (*right*) [28]

that are known to occur spontaneously via the recruitment of endogenous stem cells in the injured spinal cord of lower vertebrates [30]. Indeed, scaffolds containing human AFS cells, but not empty ones, implanted subcutaneously in nude mice have been reported to recruit host cells to the scaffold [31].

Damage to the fetal nervous system caused by a variety of insults, such as oxygen deprivation, can be greatly increased by concomitant infection and consequent inflammatory responses [32]. Given the early stages of development used, it is unlikely that the AFS cells-dependent rescue observed in the chick spinal cord injury model is mainly due to an anti-inflammatory response. However, in later models of neural injury, AFS cells may provide additional significant beneficial effects because of their anti-inflammatory properties. For example, they may also help to reduce injury to the fetal brain associated with pathological exposure to maternal antigens [33, 34]. An anti-inflammatory role for AFS cells has been recently supported in perinatal and adult models of disease including inflammatory bowel diseases and vasculopathies that occur as a consequence of organ transplantation [35, 36].

The ability of AFS cells to reduce neural damage and stimulate epithelialization could also be of great value in developing therapies for spina bifida. This defect can be detected at early stages of gestation, when neural damage is believed to be still limited. Early covering of the exposed neural tissue using AFS cell-based constructs could have the dual advantage of being neuroprotective and stimulate epithelialization over the exposed spinal cords, as suggested in wound healing models [29, 37]. In a recent study AFS cells were injected in the amniotic fluid of mouse fetuses that had been treated with retinoic acid to induce neural tube defects mimicking spina bifida. AFS cell-derived neural cells were detected on the surface of the exposed neural tissues in most of the embryos examined [38]. Therefore, for some fetal abnormalities intra-amniotic delivery of AFS cells could represent a convenient administration route worth additional investigation.

Molecules secreted by AFS cells have been proposed to be responsible for tissue repair also in non-neural tissues, such as following heart tissue ischemia [39], lung moderate hyperoxia [40], and lung hypoplasia [41]. A paracrine effect may also underlie increased proliferation observed when collagen plugs with platelets and AFS cells are used, as compared to plugs without AFS cells, to seal an iatrogenic membrane defect in a fetal rabbit model [42]. However, which of the factors secreted by AFS cells, that are likely to include cytokines and neurotrophic factors, are "protective/repair inducing" in different disease models has yet to be established.

Whatever the specific molecular mechanisms underlying the efficacy of AFS cells may be, in order for them to be useful therapeutic tools, they need to be able to home to the desired organ, ideally following simple administration protocols. As discussed above, in some cases intra-amniotic delivery might provide a suitable administration route [43]. Recent studies have investigated the distribution of autologous AFS cells injected intraperitoneally in the fetal sheep. The injected GFP-labelled AFS cells were detected in several organs, including liver, heart, placenta, membrane, umbilical cord, adrenal gland, and muscle [44]. Furthermore, injection of AFS cells in mouse heart ventricles within 24 h after birth, resulted in engrafting of the injected cells within the heart, airways, and lungs [45].

Table 11.1 Examples of studies where AFS cells (AFSC) were transplanted prenatally

Host	AFS source	Pathology	Intervention	Outcome	Reference
Chick	Rat	Injury to spinal cord and surrounding tissues	Injection at the injury site	Decreased tissue damage and increased embryo survival	[28]
Rat	Rat	Neural tube defect	Intra-amniotic injection	Undifferentiated AFSC homed to the neural placode	[43]
Sheep	Sheep	None	Intraperitoneal injection	Widespread distribution of grafted cells	[44]
Sheep	Sheep	Heart valve defect	Construct implantation (synthetic material)	Intact functional valves/absence of thrombus formation	[46]
Sheep	Sheep	Circumferential tracheal defect	Construct implantation (synthetic material)	Remodeling to fibrous cartilage pattern	[47]
Sheep	Sheep	Circumferential tracheal defect	Construct implantation (decellularized rabbit trachea)	Enhanced remodeling, epithelialization and growth	[48]
Rabbit	Rabbit	Damaged fetal membrane	Construct implantation (synthetic material)	Increased local cell proliferation	[42]

Table 11.2 Examples of studies where AFS cells (AFSC) were transplanted perinatally

Host	AFS source	Pathology	Intervention	Outcome	References
SCID mouse	Human	None	Injection in heart ventricle	Engraftment in heart, lung, and airways	[45]
Rat	Allogeneic rat	None	Intraperitoneal injection	Migration, homing and integration into various organs	[49]
Rat	Allogeneic rat	Necrotizing enterocholitis	Intraperitoneal injection	Reduced inflammation, improved function	[35]
Sheep	Autologous sheep	Diaphragmatic hernia	Construct implantation (collagen hydrogel)	Improved tensile strength/structure decreased diaphragmatic hernia recurrence	[27, 50, 51]
Rabbit juvenile	Allogeneic rabbits	Diploic nasal bone defect	Construct implantation (synthetic material)	Enhanced mineralization	[52]

Studies in diseased fetuses and newborns to establish whether there is effective homing to the damaged organ of interest and a therapeutic effect following injection of AFS cells have yet to be carried out. Nonetheless, there is a least proof of principle that AFS cells injected in utero in the sheep model can reach a variety of tissues, and that intra-amniotic delivery and intraventricular and intraperitoneal injection perinatally also result in some AFS cell engraftment as indicated in Tables 11.1 and 11.2 [35, 38, 44, 45, 49].

As there have been only a few studies on prenatal therapy of AFS cells in different animal models (Table 11.1), they will be discussed in the next section together with examples of postnatal intervention using tissue-engineering approaches (Table 11.2).

5 AFS Cell-Based Tissue Engineering for the Management of Birth Defects

AFS cells are amenable to be used for tissue engineering Kaviani et al., [53]. Postnatal transplantation of construct containing AFS cells for the management of various birth defects has been suggested to be of potential clinical relevance in the few studies currently available in animal models (Table 11.2). Examples of birth defects that could benefit from the use of AFS cell-based implants to repair or reconstruct the abnormal tissue for which some experimental evidence is available include enteric, cardiovascular, diaphragmatic, and skeletal defects.

5.1 Necrotising Enterocolitis

Necrotising enterocolitis is the most common gastrointestinal surgical emergency occurring in neonates, with high mortality rates ranging from 15 % to 30 %. However, there is no effective therapy for NEC, and surgery remains the treatment of choice for necrotic bowels [35]. The most widely accepted hypothesis is that enteral feeding concomitant with intestinal hypoxia-ischemia-reperfusion and pathogen colonization stimulates an inappropriate inflammatory response by the immature intestinal epithelial cells [54]. There is a growing body of evidence that stem cells can play a therapeutic role in inflammatory bowel diseases [55]. Significantly, AFS cells showed therapeutic effects in a well-established neonatal rat model of necrotising enterocolitis. When grafted in the bowel wall of a diseased newborn rat, they could integrate, decrease apoptosis and bowel inflammation, increase enterocyte proliferation, and improve intestinal function and survival [35]. Also in this model, paracrine

effects on endogenous cells, rather than significant repopulation of the diseased gut, appear to underlie the beneficial effects of the grafted AFS cells.

5.2 Craniofacial Defects

Craniofacial defects include defects of skull bones (e.g., premature suture fusion), facial cartilages (e.g., incomplete, microtia, or absent, anotia, ears), palate, and soft tissues (e.g., cleft lip and palate). AFS cells have been shown to stably differentiate into all the major cell types present in craniofacial tissues, such as bone, cartilage, muscle, and blood vessels [17, 56, 57]. Notwithstanding the relative frequency of craniofacial birth defects and the skeletogenic potential of AFS cells, information on the potential use for repair of these tissues perinatally is still lacking. Allogeneic AFS cells mixed with electrospun biodegradable poly-L-lactic acid nanofibers were tested for their ability to repair full thickness diploic nasal defects in young adult rabbits, as grafting a bioengineered construct in neonates seemed to be difficult in this species [52]. Histological examination of AFS-cellularized constructs showed evidence of bone formation with levels of extracellular calcium significantly higher in these constructs than in the acellular implants. Although some areas of AFS-constructs displayed fairly normal bone architecture, this was neither homogeneous nor consistently observed in all cellularized constructs. Nonetheless, the overall extent of bone formation was significantly higher in these constructs than in the acellular implants. This suggests that further studies on the use of AFS cells for perinatal nasal defect repair are worth pursuing, possibly selecting different scaffolds and using different species for the validation of this therapeutic approach.

5.3 Tracheal Defects

Abnormalities of the tracheal cartilage can cause narrowing of the airway, a birth defect known as congenital tracheal stenosis. This results in breathing problems that can be very severe and difficult to resolve, particularly when several segments of tracheal cartilage are defective. Successful replacement of defective airway segments with decellularized donor cartilage seeded with stem cells was first achieved in an adult female patient [58], and more recently in a paediatric patient [59]. Comparison of AFS cells and other mesenchymal stem cells for cartilage tissue engineering in vitro showed an overall higher content of extracellular matrix proteins in cartilage derived from AFS cells [60].

Approaches to fetal cartilage engineering have involved the use of AFS cells combined either with synthetic scaffolds or decellularized donor scaffolds [47, 48]. In both cases, constructs containing AFS cells were shown to be superior to acellular scaffolds for airway reconstruction. The constructs consisting of decellularized rabbit trachea seeded with AFS induced to differentiate into cartilage and implanted in fetal sheep were of particular interest [48]. Although some degrees of stenosis were reported in both the AFS-containing and acellular tracheal scaffolds, the extent of epithelialization was much greater in grafts with AFS cells. Significantly, these grafts were found to have grown in size post-implantation. This is a crucial feature for the treatment of a birth defect in a growing organism. In addition, AFS cells were shown to effectively enhance normal fetal wound healing, another highly desirable feature both when performing in utero and perinatal surgery [61]. Altogether, there is some evidence that transplantation of cartilage tissue-engineered using AFS cells could be used to treat congenital tracheal abnormalities.

The tracheal transplantation field has been fast moving, and given the clinical experience rapidly accumulating into the use of cellularized scaffolds together with the encouraging results on the use of AFS cells emerging in this context from animal models, it is conceivable that their clinical use in paediatric patients with tracheal defects might not be too far down the line.

5.4 Congenital Defects of the Chest Wall

Congenital defects of the chest wall are the consequence of abnormal formation of the thoracic cavity or of skeletal development. Congenital diaphragmatic hernia, which has an incidence of about 1/2,500–5,000 live births, is the consequence of a hole in the diaphragm. This results in movement of abdominal organs into the chest and restriction of lung development. The value of using stem cells and tissue-engineering approaches for patch repair in severe congenital diaphragmatic hernia has been recently discussed [62]. Over the last few years, a series of studies from the Fauza's group has investigated the potential of AFS cells for diaphragm tissue engineering when combined with collagen hydrogel. These constructs were implanted after creating a large diaphragmatic defect in neonate sheep, and reherniation and overall failure of the implants compared to acellular hydrogels and standard Teflon patches [27, 50, 51]. Overall, the outcome was more favorable following implantation of constructs containing AFS cells, where better diaphragmatic repair with improved mechanical properties and function was observed.

Sternal abnormalities are very rare defects of the chest wall. In these patients surgery needs to be carried out during the first few months of life, when the rib cage is still highly flexible, to ensure appropriate heart protection and reduce the occurrence of respiratory infections. The use of AFS cells to bio-engineer bone constructs for chest wall repair has been

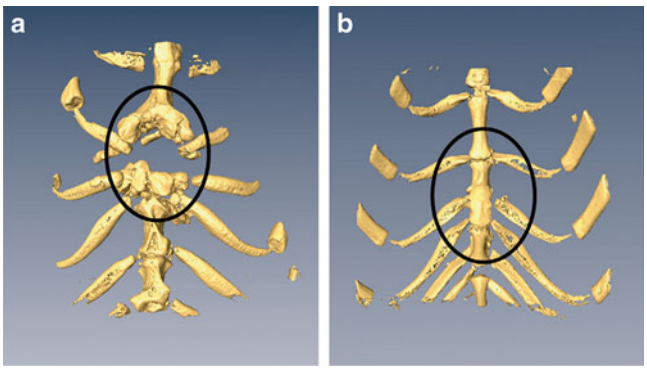


Fig. 11.3 Example of a sternal defect repair in rabbit using a poly-L-lactic acid (PLLA) nanofibrous scaffold seeded with autologous AFS cells taken from Klein et al. [63]. Three-dimensional micro-CT scans of repaired full thickness sternal defects, in front view (within the ellipses). (a) Acellular scaffold implant showing some evidence of local neobone formation, but with nonunion of the sternum. (b) Engineered osseous graft showing sustained complete closure of the defect, albeit also without a fully normal appearance

explored at the time of weaning in a rabbit model of full thickness partial sternal resection [63, 64]. Bone grafts were produced by incorporating rabbit mesenchymal ASF cells in electrospun poly-L-lactic acid scaffolds. Micro-computed tomography images showed complete union of sternal defects in these animals, whereas nonunion was observed in animals implanted with acellular grafts (Fig. 11.3). While alkaline phosphatase activity was not higher in cellular grafts than in acellular ones, increased calcium deposition was found in the former. These initial studies support the view that grafts containing AFS cells could be further developed for closing sternal defects early after birth.

5.5 Cardiovascular Defects

Congenital cardiovascular defects are very common types of birth defect, with an incidence of approximately 1/10 live births. Significant abnormalities in valve development and consequent stenosis can be very serious and result in major breathing and feeding problems. Early intervention is often crucial in order to minimize damage to the heart.

The first tissue-engineered vascular construct consisting of autologous bone marrow cells (BMC) enriched for the mononuclear fraction (BM-MNCs) in a poly(chitosan-g-lactic acid)/PGA matrix was implanted in a 4-year-old child just over a decade ago [65, 66]. Positive outcome has been reported for a cohort of young patients with single ventricle physiology that were treated using vascular grafts generated from autologous bone marrow seeded onto a biodegradable polyglycolic acid and epsilon-caprolactone/L-lactide scaffold over 12 years [66, 67]. However, when prompt intervention is required, as for example in newly born babies as well as in uterus surgery, BMC harvest is undesirable as it involves an additional invasive procedure. The possibility of using AFS cells for the repair of cardiovascular defects therefore provides an attractive alternative.

The use of AFS cells for engineering heart valve tissue has been explored by Schmidt et al. [68], who studied the $CD133^-$ and $CD133^+$ AFS cell populations. They showed that $CD133^-$-AFS cells displayed characteristics of mesenchymal progenitors, as they expressed CD44 and CD105. The $CD133^-$-AFS cell population was found to differentiate into osteogenic cells and adipogenic cells. In contrast, $CD133^+$-AFS cells differentiated into endothelial cells that expressed eNOS and CD141. Therefore, for the construction of cellularized heart valves, a two pronged approach was used. First, $CD133^-$-AFS cells were seeded onto heart valve leaflet scaffolds fabricated using rapidly biodegradable polymers. Second, these constructs were seeded with $CD133^+$-AFS cells. The heart valve tissue obtained had a smooth surface and mechanical testing showed adequate ability of these valves to open and close under low pressure conditions. Histologically, the valve tissue appeared fairly well organized, with endothelial-like cells present in the upper layer and abundant extracellular matrix.

The same group investigated the effect of cryopreservation on the performance of AFS cells when used for heart valve tissue engineering and showed that after cryopreservation, they both retained their stem cell-like properties [69]. Cryopreserved $CD133^-$-AFS cell production of extracellular matrix was comparable to that of their non-cryopreserved counterpart, and so were the mechanical properties of valves fabricated from non-cryopreserved cells. On the basis of these results it has been proposed that banking of fetal AFS cells might offer a life-long available autologous cell source for heart valve tissue engineering [69]. Nonetheless, as already discussed, the effect of long-term storage of AFS cells on their stability and the cost-benefit balance has yet to be established.

Recently, autologous AFS cells from pregnant sheep were isolated and fabricated with biodegradable PGA-P4HB composite matrices to create tissue-engineered heart valves. Using an in utero closed-heart hybrid approach for implantation, in vivo function of the engineered heart valves was successfully shown [46].

6 Conclusions

AFS cells can differentiate into several cell types, and have been proposed as valuable therapeutic cells for regenerative medicine. AFS cells can reduce tissue damage, increase survival in injured embryos, and reduce inflammation via paracrine mechanisms. Transplantation of AFS cell-based tissue constructs for the repair of anatomical defects in both prenatal and postnatal periods has started to be explored in

many disease models. Interestingly, some constructs appear to support tissue remodeling, a crucial feature for paediatric applications. Although the properties displayed by AFS cells point at them as potentially valuable therapeutic agents for prenatal and perinatal repair of defects diagnosed during gestation, thorough consolidation of current proof of concept findings will be necessary to underpin progression towards robust and informative clinical studies.

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Stimulation of Therapeutic Angiogenesis Using Amniotic Fluid Stem Cells

12

Teodelinda Mirabella

Abstract

Amniotic fluid is an appealing source of multipotent, fetal, extra-embryonic stem cells (AFSCs), which could be potentially utilized for intrauterine therapies or banked for future uses. In their undifferentiated state AFSCs can serve as an incredible pro-angiogenic burst and drive endogenous repair. Besides the feasible in vitro manipulation to direct AFSCs toward a vascular phenotype, there is also the opportunity to use their secreted pro-angiogenic soluble factors to promote neoangiogenesis in ischemic tissues. AFSCs are the optimal cell candidate for both tissue engineering and regenerative medicine approaches, exogenous and endogenous repair. The present chapter discusses the current approaches to differentiate AFSCs to endothelial cells and the emerging evidence of AFSC paracrine effect on the vascularization of ischemic sites. In particular, the discovery that AFSC secretome can be used for therapeutic angiogenesis has opened to the possibility that a cell-free treatment could be employed as a safe alternative to cell and tissue transplantation.

1 Therapeutic Angiogenesis for Ischemic Diseases: An Overview

Angiogenesis is defined as the formation of new blood vessels from preexisting vessels. In postnatal life, angiogenesis can occur either as a physiological or a pathological process, such as during wound healing or tumor growth, respectively. Pro-angiogenic stimuli activate endothelial cells (ECs) releasing matrix metalloproteinases, which degrade the interstitial matrix and are essential for vascular guidance tunnel formation. Finally, the deposition of a basement membrane and the association with mural cells are key steps for the neo-vessel stabilization. All these steps are needed in order for a neo-vessel to sprout from the parent vessel, reach and perfuse tissues.

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Tissue repair is naturally accompanied by vascular remodeling which includes angiogenesis. The poor vascularization of ischemic tissues is the target of a recently established experimental area of treatment called therapeutic angiogenesis. The concept of therapeutic angiogenesis was born in 1971 from the observation of Judah Folkman that "hot bloody" tumors produced angiogenic factors that sustain the vascularization and growth of the tumor itself [1]. After the identification of VEGF as a secreted angiogenic growth factor [2], cardiovascular investigators could finally test the hypothesis that the administration of certain molecules could stimulate the vascularization and the rescue of ischemic tissues [3]. A modern definition of therapeutic angiogenesis could be "any therapeutic strategy making use of cells, proteins, genes, etc., capable of converting an ischemic tissue into a viable and functional tissue through maintenance and remodeling of existing vasculature and stimulation of the formation of new vasculature."

Cardiovascular diseases (CVD) are the number one cause of death worldwide. Only in the United States one third of the annual deaths are due to coronary artery, cerebrovascular, aortic and peripheral arterial diseases [4]. If we consider that the quality of life and the ability to work of people who have

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experienced CVD is seriously affected, CVD represent a global medical and economic burden. Especially for those patients who are ineligible for traditional surgical approaches of re-vascularization, such as angioplasty or bypass grafting, therapies stimulating the growing of preexisting blood vessels are in high demand.

Among the proteins that have been used for therapeutic angiogenesis, VEGF and FGF-2 have reached human clinical trials. The administration of those growth factors by intracoronary and intravenous route in patients with severe myocardial ischemia was proven to be safe and well tolerated with improvements in symptoms and quality of life [5–7]. However, when the two recombinant proteins were used in randomized double blinded, placebo controlled trials (VIVA and FIRST trials), no differences in myocardial perfusion and exercise time between the control and the treated group were reported [8, 9]. The clinical response to growth factors is limited by their poor stability and short serum and tissue half-lives in vivo which would result in frequent and high dose administration with the risk of uncontrolled vascular formation in undesired sites.

Despite the numerous gene targets that may be envisioned for therapeutic angiogenic purposes, the development of gene therapies has been slow with few clinical studies performed on small numbers of patients [10]. Moreover, the efficacy of gene angiogenic therapies remains elusive and the long-term safety concerns include angiogenesis-triggered malignancies, inflammatory response to viral vectors and in vivo recombination.

Many cell types known to produce angiogenic factors have been used for the purpose of vascular regeneration in ischemic tissues. Putative endothelial progenitor cells (EPCs) are immature cells mobilized from the bone marrow to ischemic sites through the systemic circulation [11]. EPCs express the trilogy of CD34, CD133, and VEGFR2 markers, but their identity remains unclear because of significant overlap in antigen expression with other cell types such as monocytes/macrophages. While EPCs can directly participate in the re-endothelialization of injured vessels [12], mesenchymal stem cells (MSCs) present in adult tissues, including bone marrow and adipose, have been recently proposed to mediate a therapeutic effect through the paracrine release of angiogenic factors rather than through their transdifferentiation into endothelial cells [13, 14]. Nevertheless, MSCs are optimal cell candidates for therapeutic angiogenesis with advantages like lacking of MHC class II and co-stimulatory molecules making them suitable for allogeneic transplantation, a good rate of expansion *ex-vivo* and migratory capabilities in vivo.

So far, controversial results have emerged from clinical randomized trials, such as REPAIR-AMI [15] and ASTAMI [16] where autologous mononuclear bone marrow cells (BMCs) were delivered via the intracoronary to patients with acute myocardial infarction. The absence of clear benefits to LV function and contractility with respect to the placebo group could be likely due to either the relatively low abundance of EPCs in the BMC total population, to the insufficient engraftment of BMCs in the ischemic tissue or to the heterogeneity of cell populations recovered from the mononuclear fraction of bone marrow aspirates.

Although bone marrow-derived progenitors are already used in clinical settings and the most studied source of cells for therapeutic angiogenesis protocols, many drawbacks can limit their use and have pushed researchers to look for new cell sources. Some of those drawbacks are common to all the stem cells derived from adult tissues. These limitations include the need for surgical procedures to harvest the tissue of interest, the limited amount of isolated cells as well as the number of passages in culture that the cells can undergo without acquiring chromosomal aberrations [17].

Recently, amniotic fluid (AF) has been demonstrated to be an accessible reservoir of multipotent fetal cells [18]. Progenitor cells can be isolated from as little as 2 ml of extracted AF from second trimester pregnancies and expanded to >180 million cells within 4 weeks in three passages in In't Anker culture setting [19]. The use of human amniotic fluid stem cells (AFSCs) would avoid ethical concerns that plague embryonic stem cells (ESCs) as AFSCs are isolated from tissues normally discarded at birth. Besides, AFSCs have not showed the safety concerns that ESCs or iPS cells have in that they cannot be transplanted in an undifferentiated state because of their potential to form tumors and/or teratomas.

In this chapter, the present and the potential applications of AFSCs for therapeutic angiogenesis purposes will be discussed.

2 Current Approaches to Differentiate AFSC Toward the Endothelial Lineage

From a tissue engineering point of view, therapeutic angiogenesis might be pursued by designing, generating, and then supplying implantable blood vessels to the patient. With the aim to search for cell populations that can be induced to differentiate toward an endothelial phenotype, it would be reasonable to utilize a new and high-yielding source of multipotent progenitors such as the amniotic fluid.

De Coppi et al. and Perin et al. [18, 20] used the following culture conditions to differentiate human AFSCs, isolated from c-kit positive fraction of amniocentesis specimens, into ECs: a cell density of 3,000 cells/cm^2, gelatin precoated dishes, endothelial cell medium-2 (EGM-2), supplemented with 2 ng/ml recombinant human FGF-2 added at intervals of 2 days. After 8 days in culture, detectable expression levels of CD31 and VCAM-1 mRNA were observed.

Schmidt et al. [21] exposed CD133 positive selected AFSCs to endothelial cell medium for 28 days. Although CD133+ cells were positively stained with eNOS (endothelial nitric oxide synthase), cytofluorimetric analysis revealed that both CD133+ and CD133- cells lacked CD31 and von Willebrand factor (vWF) protein.

Zhang et al. [22] isolated AFSCs by centrifugation and washes of unfractionated amniotic fluid and then performed endothelial differentiation in EGM-2 medium with VEGF, FGF-2, EGF, IGF-1, heparin, ascorbic acid at the manufacturer's suggested concentrations. After 2 weeks, cells were able to uptake ac-LDL (acetylated low-density lipoprotein), stained positive for lectin and formed tube-like structures in Matrigel. Additionally, transcript levels of CD31 and vWF, measured by real time PCR, increased during the culture in EGM-2, with further up-regulation when AFSCs were stimulated by shear force (12 dyn/cm^2) for 48 h. In this study a proper time course for the protein level of CD31 and vWF was not shown, but for the first time some in vitro endothelial properties, such as ac-LDL uptake and tube formation in a 3D extracellular matrix were investigated.

Later on, other authors demonstrated that the non-adherent fraction of the amniocyte cultures used for prenatal cytogenetic diagnosis can be replated and differentiated into ECs in 1 week as demonstrated by the positive immunofluorescent staining for CD31 and vWF [23, 24]. The differentiation conditions were similar to the previous studies (EGM-2 medium supplemented with VEGF 10 ng/ml) except for the fact that 3,000 hAFSCs were plated onto fibronectin coated dishes.

Recently, Benavides et al. showed that c-kit positive selected human AFSCs can undergo endothelial differentiation when cultured onto gelatin coated dishes in endothelial growth media with concentrations of $VEGF_{165}$ up to 50 ng/ml [25]. After 2 weeks, AFSCs were stained positive for vWF, eNOS, CD31, VE-cadherin, VEGF receptor-2, while a decrease of the stem cell markers c-kit and SSEA4 occurred. In functional assays, differentiated AFSC formed tubular networks in Matrigel and metabolized ac-LDL. For the first time these authors documented the functionality of endothelial cells differentiated from human AFSCs compared to a primary endothelial cell source such as human umbilical vein endothelial cells (HUVEC).

Although in vitro differentiation protocols are important steps for an initial assessment of AFSC potential to be directed toward specific lineages, the in vivo traceability of the implanted cells within a vascular niche is the goal to achieve in order to provide functional vessels to patients. Whether human AFSCs are able to differentiate into an endothelial phenotype in vivo or whether pre-differentiated AFSCs are able to serve as a functional endothelium in vivo, requires further investigations.

Employing an immune-suppressed rat model with experimental myocardial infarction, Yeh et al. demonstrated that undifferentiated human AFSCs injected directly into the peri-infarct areas attenuated the development of myocardial fibrosis and enlargement of left ventricle cavity. Also, AFSCs increased the numbers of capillaries and arterioles in both the border and the infarcted zone compared to the saline injection group [24]. Human nuclei, meaning AFSCs, were found within some microvessel walls. In this study, because of the poor resolution of the images, the presence of cells double positive for the human nuclear antigen (HNA) and vWF does not discriminate between the endothelial differentiation of injected AFSCs, rather than the integration of AFSCs into the host endothelium.

Liu et al. differentiated human AFSCs in EGM-2 medium supplemented with bFGF and assessed the in vivo efficacy of intramuscularly injected endo-differentiated AFSCs (AFSC-ECs) in an athymic nude mouse model of hindlimb ischemia. Using this methods they obtained a better limb salvage (85 %) compared to undifferentiated AFSCs (56 %) and HUVEC (25 %) [26]. The authors stated that transplanted AFSC-ECs were incorporated into vessels in the ischemic region as confirmed by immunofluorescent staining for human smooth muscle 22α or vWF. The mechanism of endothelial differentiation of AFSCs remains unexplained as well as the higher in vivo survival and vascular incorporation rates of endo-differentiated AFSCs compared to both undifferentiated AFSCs and HUVECs.

VEGF-A, which is known to induce endothelial differentiation in ESCs and MSCs [27–29] has been the growth factor mainly used by researchers to convert AFSCs into ECs, but it has not been elucidated which cellular processes and biochemical responses are induced in human AFSCs following VEGF stimulation.

To date, the most efficient reprogramming of amniotic fluid-derived cells into endothelial cells was achieved by Rafii's group [30]. The authors use an interesting approach that is based on the regulation of vasculogenesis and angiogenesis during embryonic development and adult life by transcriptional factors (TFs) of the E-twenty six (ETS) family [31–35]. Devoiding second trimester amniotic fluid-derived cells (ACs) of c-kit$^+$ subpopulation to exclude the possibility that ECs originate exclusively from multipotent or pluripotent progenitors, the authors transduced ACs with a combination of lentiviral ETS-TFs (ETV2, FLI1, and ERG1) and demonstrated a robust induction of multiple EC markers within 7 days lasting for over 1 month. On the contrary, adult fibroblast and mesenchymal cells were minimally induced by ETS-TFs, while ETS-reprogrammed human ESCs were poorly proliferative and often drifted into non-endothelial lineages. Following 3 weeks of reprogramming with ETS-TFs transduction and TGF beta inhibition (to prevent the endothelial-mesenchymal transition), endothelial cells derived from ACs (VE-Cadherin$^+$) showed a permanently functionalized $VEGFR_2$ signaling pathway and maintained

long-term vascular identity and stability. The transcriptome profile of reprogrammed ACs was similar to HUVEC and liver sinusoidal endothelial cells (LSECs), but not to bone marrow stromal cells (BMSCs) and $CD34^+$ hematopoietic cells. Importantly, non-EC genes such as smooth muscle actin, musclin, calponin-1, as well as hematopoietic markers, were not expressed by reprogrammed ACs. After 21 days of reprogramming with ETS-TFs and TGF beta inhibitors, AC-derived ECs formed tubes in vitro and uptook Ac-LDL similarly to HUVECs. In vivo implantation of AC-derived ECs (GFP labeled) into Matrigel plugs, revealed that reprogrammed ACs were able to form patent vessels of varying caliber that anastomosed to the host vasculature. Finally, intrasplenic transplantation of AC-derived ECs resulted in the engraftment of these cells into the 5–10 % of the regenerated liver sinusoidal vessels of mice that had undergone 70 % partial hepatectomy. Thus, the protocol by Ginsberg et al. ensures a durable reprogramming of ACs into expandable, mature and stable endothelial cells capable of forming functional perfused vessels in vivo. This study opens the concrete possibility of banking AC-derived ECs for the treatment of different pathologic vascular conditions.

3 AFSCs and Endogenous Repair: Pulling the Trigger of Angiogenesis?

Vessel engineering requires costly and time consuming protocols involving expansion and differentiation of cells; selection of biocompatible materials for adherence and possibly instruction of cells; and bioreactors where the tissue can grow while retaining its viability and functionality. Finally, providing the engineered vessel to the patient often implies invasive surgeries and postoperative immunosuppressant drug administration.

Thus, therapeutic approaches capable of stimulating endogenous mechanisms of angiogenesis and repair are highly desirable. In this perspective, the systemic or local supply of biologically active products, spanning from proteins to cells, would allow exploitation of the inner regenerative capacity of the patient.

Apart from specific studies made on models of ischemia, the literature offers some examples of endogenous tissue repair and remodeling achieved, at least in part or indirectly, by the angiogenic response evoked by transplanted AFSCs. Following is a list of pathological experimental conditions where AFSCs have been proved to be: (1) not retained in vivo, (2) beneficial, (3) stimulating vascularization.

For instance, bronchopulmonary dysplasia in preterm infants and adult-onset emphysema, characterized by arrested alveolar development or loss of alveoli, respectively, are urgent global health problems and lack effective therapeutic treatments. The development of a functional vasculature in the lung is essential to promote alveolar growth during development and maintain alveolar structures throughout postnatal life [36–38]. Bronchopulmonary dysplasia (BPD) in preterm infants is a serious condition, often requiring intensive care. It may persist beyond childhood leading to chronic respiratory diseases in adults [39]. Rat pups exposed to hyperoxia during the first 14 days of life develop abnormal lung tissue characterized by parenchymal thickening similar to BPD with scarring and inflammatory infiltrates that finally destroy the alveolar structure [40]. Grisafi et al. delivered a single dose of human AFSC intratracheally in rat pups, previously exposed to a normobaric oxygen concentration set at $FiO_2 = 0.6$ [41]. Despite a low level of pulmonary AFSC retention, the lung histopathology of AFSC-treated animals was ameliorated, showing a recovery of alveolar growth, no evidence of inflammatory cells, fibrosis, and thick septa. More important, for the purpose of this part of the chapter, in AFSC-treated animals capillaries of the alveolar walls were more clearly appreciable and homogeneously distributed. In this report, the reparative potential of AFSCs appears to depend on their direct and indirect involvement in alveolarization and angiogenesis.

Another serious condition where therapeutic angiogenesis would be useful is post-ischemic acute tubular necrosis (ATN). Renal artery stenosis or embolism can cause a long-term hypoperfusion of the kidneys [42, 43] leading to the destruction of renal tubular cells and renal failure. It has been demonstrated that VEGF therapy can prevent glomeruli loss and tubular damage in an experimental set of renal thrombotic microangiopathy [44]. Perin et al. injected human AFSCs directly into nu/nu mouse kidneys with glycerol-induced ATN [45]. A bioluminescent signal from luciferase-transfected AFSC was detected in the kidney after 21 days. However, the number of injected AFSC found in the kidney decreased over time as shown both by the bioluminescence images and by the immunohistochemistry data. Thus, no clonal expansion of integrated AFSCs might be postulated; on the contrary, the beneficial effect of AFSCs in preventing the progression of ATN (increased viability of tubular epithelial cells, decreased cast formation), might be likely due to cytokine-mediated paracrine mechanisms. The same group had recently demonstrated that a single intracardiac injection of murine AFSCs into mice with Alport syndrome ($Col4a5^{-/-}$ mouse model) during the very early phase of disease can prolong animal survival and delay the progression of chronic kidney disease (CKD) exhibited by interstitial fibrosis and glomerular sclerosis [46]. Five days after injection, Qdot-labeled AFSCs could be detected by histological analysis within the interstitial space and the glomeruli. Cytofluorimetric analysis revealed that AFSC were almost untraceable after 1 month. As in the previous report, integration of the injected cells and their differentiation into tubular epithelial cells was negligible. Authors postulated that the

mechanism of renal protection driven by AFSCs was probably due to the paracrine/endocrine modulation of both cytokine expression and recruitment of macrophages M2 promoting tissue remodeling. Of notice, AFSC injection prevented the scarring and the collapse of small and fragile glomerular blood vessels.

Healing of skin wounds, occurring after traumatic injuries, surgeries or secondary to pathologic conditions, is another therapeutic area requiring the attention of scientists and experts in therapeutic angiogenesis. Particular conditions, then, such as diabetes, infections, or venous ulcer may cause non-healing, chronic wounds [47]. Wound healing is a complex biological process involving initial hemostasis and inflammation, subsequent formation of granulation tissue, re-epithelialization, contraction, and remodeling of the scar tissue [48]. Angiogenesis is imperative not only during the different stages of wound repair, but also for restoring morphology and functionality of the damaged tissue, namely, wound regeneration. Skardal et al. recently tested AFSC-mediated wound healing in a nude mouse model of skin repair using bioprinting technology to treat full-thickness skin injury [49]. AFSCs drove wound closure and re-epithelialization. They also increased microvessel density and capillary diameters compared to wounds treated only with fibrin-collagen gels. Tracking analysis of fluorescently labeled AFSCs revealed that the cells remained transiently in the injury site and did not permanently integrate in the skin, suggesting that the wound healing and the angiogenesis may be due to delivery of secreted trophic factors.

Bone autograft and allograft surgeries are increasingly requested to replace segments of bone after trauma or malignant tumor invasion [50]. The lack of vascularization can cause necrosis of the graft. Despite the lack of a definitive and convincing proof of the capability of AFSCs to differentiate toward an osteo-lineage and engraft in vivo into a bone environment, it has been shown that AFSC-seeded polymer scaffolds, orthotopically implanted, can repair rat critically sized femoral defects with significantly higher bone ingrowth compared to acellular scaffolds [51]. The outcome of the in vivo healing of large bone defects is even more significant if AFSCs are pre-committed in vitro [52]. Recently, Cancedda's group used an ectopic bone model system to investigate AFSC contribution to the formation of the ectopic ossicle, the response of the host to implanted AFSCs, and the crosstalk between human osteoblasts and human AFSC [53]. Using a bioluminescence tracking system and histological analysis, they found that: (1) AFSCs do not osteo-differentiate, nor osteo-integrate; (2) AFSCs drive a pro-inflammatory and pro-angiogenic host response; (3) AFSCs do not disturb the osteoblast-mediated deposition of bone, but do model the ectopic ossicle by increasing the vessel density and the total perfusion area. Although AFSC do not last longer than 2 weeks in vivo, AFSC-mediated vascularization could facilitate the attraction of circulating progenitors and trophic factors as well as the survival of bone grafts or the endogenous bone formation.

To conclude, in none of these studies is a formal and scrupulous demonstration that the tissue repair achieved by transplanting AFSCs is due to therapeutic angiogenetic mechanisms. Specific experimental designs are needed to address this question. However, many of the observations collected so far show that the beneficial effect of AFSCs could be attributed more to paracrine factors triggering neovascularization and cytoprotective effects on injured tissues, rather than to AFSC differentiation toward a specific cell lineage and direct replacement of the damaged organ. The vascularizing properties of AFSCs, as well as the consequent recruitment of circulating progenitors and vessel-resident progenitors, could be one, if not the most important, mechanism of tissue repair mediated by these cells.

4 AFSC-Mediated Recruitment of Host Pro-angiogenic Progenitors

The recruitment or activation of progenitors toward or within the injured site is an important step in tissue repair. A bidirectional flux, termed "homing" in the case of progenitors entering the blood stream to reach a specific tissue niche, and "mobilization" in the case of progenitors leaving the tissue to enter circulation cannot occur without the presence of a patent vasculature. Saving the bone marrow-derived hematopoietic progenitors, the existence of other circulating tissue-specific progenitors remains elusive, although some authors have postulated the existence of early tissue-committed stem cells (TCSCs) that can be isolated from the peripheral blood mononuclear cells [54]. The presence of a small subpopulation of resident stem/progenitor cells in most tissues, mediating the homeostatic maintenance of the adult tissue itself, has affirmed the notion that it is possible to stimulate their in vivo proliferation and differentiation [55].

AFSC-mediated paracrine and juxtacrine signaling may account for the recruitment of circulating progenitors and for the proliferation and differentiation of local resident progenitors (Fig. 12.1). Both processes can be considered part of the more complex "tissue activation" occurring after an injury or a regenerative treatment. To date, the mechanistic basis of the interaction between donor AFSCs and host cells has not been disclosed and very few studies have investigated how the activation of host progenitors guides the tissue repair. Especially, in those experimental settings where AFSCs have a therapeutic effect without any proof of AFSC direct involvement in the repair process, it becomes mandatory to investigate whether AFSCs trigger "tissue activation."

We showed for the first time that human AFSCs recruit host progenitors when implanted in the subcutis of nude

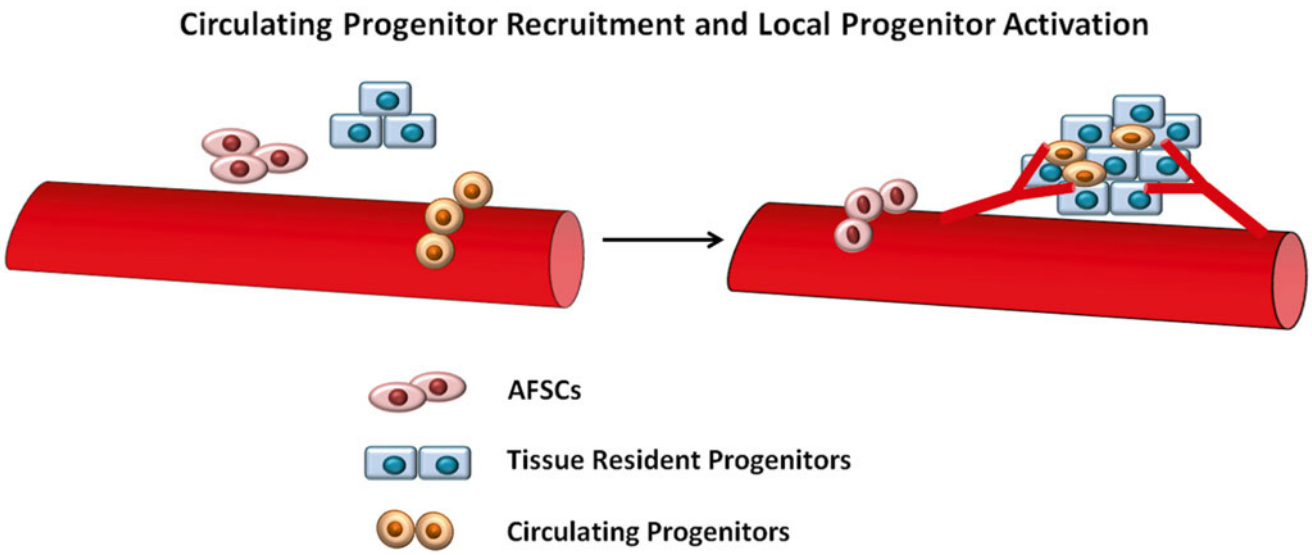


Fig. 12.1 Proposed model for AFSC-mediated tissue repair. Implanted undifferentiated AFSCs evoke a strong pro-inflammatory and pro-angiogenic response, which accounts for the rapid vascularization of the implantation site. Circulating progenitors are recruited through the neo-formed vessels. At the same time, tissue-resident progenitors can be "activated," while AFSCs are progressively cleared out. The regeneration is endogenous in nature, meaning that AFSCs only function as an initial burst and host progenitors fulfil the repair

mice [56]. In that study, more than two million AFSCs were incorporated in porous ceramic scaffolds with fibrin glue and subcutaneously implanted in mice. Animals were sacrificed 1, 2, 4, and 8 weeks after implantation and the grafts removed and processed for histological analysis and flow cytometric quantification of specific cell populations present in the explanted scaffold. During the first couple of weeks, three basically important events happened: (1) inflammatory cells colonized the scaffold, (2) vessels infiltrated the scaffold, (3) donor AFSCs were not retrieved in the scaffold and part of them reached local and distal sites through systemic circulation. Between 1 and 2 months, different types of tissues of mesoderm origin (adipose, muscle, loose and dense connective tissues) were observed inside the scaffolding structure. Importantly, none of these tissues were of human origin meaning that AFSCs did not directly participate in tissue formation.

Finally, cytofluorimetric analysis of cells harvested from the scaffold offered an interesting insight into the host populations recruited at different time intervals. The first 1–2 weeks are particularly significant in order to study the initial phases of the host response. Murine cells positive for CD14 were detected at 1 week post-implantation and were absent at 2 weeks. Since cells extracted from late implants (2 and 4 weeks) were CD14- and CD45+, the authors speculated that CD14+ mature monocytes of the first inflammatory wave of recruited cells might represent the host immune response (residual in nude mice) versus the xeno-transplanted human cells. On the other hand, CD45+ leukoblasts (minimally differentiated) might be, instead, continually recruited from the systemic circulation, and exert still unclear immunoregulatory and remodeling functions on the tissue homeostasis. Cells positive for CD31 (PECAM-1), which is expressed in monocytes and platelets other than in endothelial cells, were retrieved in high amount during the first week and were progressively decreasing from 2 to 4 weeks post-implantation suggesting that an instant blood-mediated inflammatory reaction may occur or that angiogenic and vasculogenic progenitors were acutely mobilized from the bone marrow [57]. The activation of the host endothelium, proved by the detection of CD106 (VCAM-1) positive murine cells at 2 weeks, is important in order to orchestrate localization and diapedesis of circulating blood cells as well [58]. The authors also observed an increasing amount of host CD146 + cells. Being CD146 a component of endothelial junctions [59] and also a marker of pericyte-like cells that surround the endothelial layers and of MSCs [60–62], the identity of these CD146+ cells remains unclear as it is debatable if they originate from the activation of the pericytes associated to the recruited vessels or from the activation of local (subcutaneous in the proposed model) resident stromal cells or from both. Nevertheless, there are many reports indicating that signals from transplanted stem cells, with bone marrow-derived MSCs being the most well characterized, can induce the mobilization of host reparative cells toward the site of a lesion [63–66].

We have also investigated the capability of AFSC-secreted factors to recruit host progenitors in a rat model of ischemic skin [67]. In this study, the subcutaneous tissue from the perfused part of the skin flap outlining a necrotic region was used to quantify the number of murine CD31, CD34, and $VEGFR_2$ positive cells by cytofluorimetry. Only when the skin flap was treated with the AFSC secretome (see the next part of this chapter), were cells positive for CD34 observed in the subcutis. Again, since CD34 cannot be considered solely a marker of EPCs—on the contrary, CD34 positive cells can be isolated also from the adipose stromal-vascular-fraction (SVF) [68]—it is not understood if these cells come from the local subcutaneous niche or from circulation. Nevertheless, a consistent and well-defined population of cells double positive for CD31 and $VEGFR_2$ was recruited by AFSCs secretome. The cytofluorimetric analysis does not prove the endothelial origin of the CD31+/$VEGFR_2$+ cells and, also, does not exclude the hematopoietic origin of the same cells. However, the isolation in culture of cells growing in colonies, with cobblestone morphology, and positive for the vWF, proved that AFSC secretome had recruited and/or activated endothelial progenitors in the subcutaneous niche.

As previously mentioned, Asahara and colleagues [11] were the first to describe EPCs, originally reported as circulating angioblasts for their property to home to ischemic tissues and form *de novo* vessels, obtained from CD34+ peripheral blood mononuclear cells (PBMCs) by using culture conditions favoring endothelial differentiation. Further studies revealed that the majority of EPCs derive from circulating monocytes which can express endothelial markers, form tubes in Matrigel, be integrated into injured endothelium and accelerate re-endothelialization [69–72]. Monocytes were also found to be progenitors for fibroblasts promoting physiologic scar formation and wound repair or pathologic fibrosis [73]. Originally named fibrocytes by Bucala and colleagues [74] and later described as monocyte-derived mesenchymal progenitors (MOMP) [75], these cells are characterized by a fibroblast-like morphology; expression of CD14, CD45, CD34, and type I collagen; potential to differentiate into osteoblasts, chondrocytes, adipocytes, skeletal myoblasts. Zhao et al. reported that a subset of peripheral blood spindle-shaped CD34+ monocytes even have multipotency features, as for their capability of differentiating along several distinct lineages: mature macrophages, T lymphocytes, endothelial cells, neurons, hepatocytes [76]. Thus, blood circulating monocytes are not only precursors for macrophages and dendritic cells; on the contrary, their plasticity degree, as well as their ontogeny, has not been completely explored.

From the information we have to date, it is plausible that macrophages, and circulating, as well as vascular resident, endothelial progenitors are recruited in the site of AFSC implantation. Because of both the intrinsic difficulty of these recruitment studies and the lack of sufficient data, at present, we are not able to unequivocally identify the cell populations (EPCs, monocyte/macrophages, mesenchymal progenitors, or a single progenitor for all of them) which are mobilized by AFSCs, but, it would be useful to know the mechanism underneath these incredible AFSC- proper regenerative properties.

Also, most studies have focused on the capability of AFSCs to migrate in response to tissue injury. Systemically and locally injected AFSCs were found to distribute to the epineuria and neuromuscular junction of a crushed sciatic nerve [77], stably integrate into skeletal muscles in a model of muscular dystrophy [78], or incorporate into the inflamed bowel wall of a model of necrotizing enterocolitis [79]. Despite the copious availability of data about AFSC response to tissue damage, very little is known about the tissue response to exogenous AFSCs. Therefore, AFSC-mediated recruitment and activation of host progenitors should be further investigated, with the perspective of using these cells as regenerative therapeutic agents, rather than simple substitutes of dysfunctional organs.

5 AFSC Secretome as a Cell-Free Treatment for Ischemic Conditions

The term secretome was originally coined by Tjalsma et al. [80]. The secretome is the ensemble of all the products secreted by a cell, tissue, or organism. An increasing amount of studies, largely conducted on acute myocardial infarction, have revealed that only a very small amount of MSCs engraft and are functionally integrated in the tissue during the post-transplantation period [81, 82]. This has prompted cardiovascular scientists to propose a paracrine effect of MSCs (both trophic and immunomodulatory) as the main mechanism of cardiovascular repair. Many studies have investigated the factors that are secreted by human MSCs from different sources, such as bone marrow, adipose tissue, and ESCs [83–85]. The studies by Mirabella et al. were the first reporting pro-angiogenic properties of the AFSC secretome and the creation of a therapeutic cell-free product named *ACM*—since it is made from *A*FSC *C*onditioned *M*edia, capable of recovering and preventing ischemic damage [67, 86]. ACM is a collection of all the soluble factors secreted by AFSCs and represents the total secretome of these cells at least in certain in vitro culture conditions.

First, in a hind limb ischemia murine model [86], Mirabella and colleagues ligated the right femoral artery immediately distal to the origin of the deep femoral branch. Intramuscular treatment with ACM, or PBS for the control group, was started 10 days after the surgery. It was shown by the authors that ACM prevented atrophy and gangrene of the ischemic limb. ACM-treated mice showed a normal hind limb functionality already 1 week after the first injection and

their walking time was comparable to the non-ischemic animals. ACM treatment also stimulated arteriogenesis as evaluated from counting the number of arterioles in the semimembranosus (SM) muscle. The density of endomysial arterioles in ACM-treated mice was significantly higher than in PBS-treated and non-ischemic mice. In the PBS group, the arteriole density was comparable to the non-ischemic group, but the arterioles were enlarged, had thin mural walls and low deposition of collagen fibers in *adventitia*. On the contrary, in the ACM group, the arteriole showed a regular lumen, a thick *tunica media* and a remodeled *tunica adventitia* with hyper-deposition of collagen fibers and fibrocyte infiltration/proliferation. Distal angiogenesis and muscular degeneration, generally occurring in the muscles below the knee, were evaluated on the gastrocnemius (GC) muscle. In the PBS group, distal capillaries and muscle fibers were degenerated. No significant differences in the capillary density and in the distribution of muscle fiber diameter were observed between the ACM group and the non-ischemic group indicating that ACM prevents ischemia-mediated distal tissues damage.

In a second rat model of ischemic fasciocutaneous flap, Mirabella and colleagues [67] elevated a 7×7 cm^2, abdominally located, full-thick skin flap, and ligated one inferior epigastric neurovascular bundle with the contralateral bundle remaining pervious to perfuse half of the flap (vital part). At the time of the surgery, right before the flap was sutured back, a gelatin membrane, previously embedded with ACM or saline (CTRL), was locally positioned beneath the skin flap. Blood perfusion through the flap was evaluated by laser doppler imaging (LDI) through the week following the surgery. ACM treatment was shown to mediate vessel growth and sprouting from the vital part to the ischemic region of the flap. In the ischemic zone of the ACM-group, the perfusion level at day 7 was 50 % higher than the initial baseline (perfusion before ligation), while the initial perfusion was never recovered in the CTRL group. Since the survival of the flap is linked to the blood supply, the ensuing necrosis development was progressively increasing in the CTRL group, while it was delayed and significantly less pronounced in the ACM group. The histological analysis showed a very thick, dead cutaneous layer in the CTRL group while the ACM group showed normal arrangement of epidermal and dermal structures (glands, follicles, collagen fibers) and a high density of vessels in subcutaneous tissues.

The human AFSC conditioned media was then used by Bollini et al. in an experimental setting of myocardial ischemia (30 min)/reperfusion (2 h) damage [87]. The systemic injection of AFSCs, as well as their conditioned medium, was shown to be cardioprotective. The infarct size and the myocardial cell survival were improved compared to the PBS group and no significant difference between the cellular (AFSC)-group and the relative secretome (ACM)-group was reported.

Other groups have used conditioned media as a surrogate of the secretome from MSCs to achieve repair in vivo.

For instance, human embryonic MSC-derived conditioned medium (CM) was found to reduce progression of chronic kidney disease (CKD) in a rat model: twice daily intravenous injections of CM for four consecutive days after the experimental induction of the CKD increased the glomerular filtration rate and the renal plasma flow in 6 weeks [88].

Concentrated, conditioned medium from cultured (both in normoxia and hypoxia conditions) human bone marrow-derived MSCs was intravenously administered in rats subjected to traumatic brain injury (TBI). Media treated rats performed significantly better than the controls in both motor and cognitive functional test, and the increased neurogenesis accounted for less brain damage [89].

The conditioned medium generated from human amniotic mesenchymal tissue cells (AMTC) was administered to mice with bleomycin-induced lung fibrosis. Two weeks later AMTC-CM-treated mice showed significantly lower fibrosis, as indicated from fibroblast proliferation, collagen deposition, and alveolar obliteration scores [90].

Taken together, both the observations regarding the limited presence/integration of transplanted stem cells in host tissues, and the studies regarding the beneficial effect of the administered conditioned media, strongly suggest that the mechanisms by which stem cells promote the tissue repair are paracrine in their nature.

Is ACM's capability to attract vessels an ACM prerogative? Of course, the probability that any cell-conditioned medium would provoke an anti-angiogenic reaction, when compared to PBS or saline, is very unlikely. Anyway, it is essential to understand if AFSC pro-angiogenic properties are specific, or at least superior to other cell types.

Mirabella et al. compared the AFSC conditioned medium (ACM) with the medium conditioned by an equal number of Bone Marrow Stromal Cells (BMCM) in a Matrigel plug assay and found that ACM is almost 50 % more angiogenic than BMCM [86]. The same authors compared AFSC- and bone marrow stromal cells (BMSC)-mediated recruitment of host cells after 3 days from implantation and found a significant increment of murine CD31+, CD14+, and VEGFR2+ cells in AFSC constructs compared to BMSC constructs suggesting that AFSCs trigger a strong pro-angiogenic and pro-inflammatory response in the host [53].

In a study by Roubelakis and colleagues, conditioned media (CM) from human BMSCs, dermal fibroblasts (DFs) and AFSCs (isolated by plastic adhesion criteria from fibroblastoid colonies), were analyzed with proteome profiler arrays for angiogenesis growth factors [91]. Although AFSCs and DFs shared about 95 % of the secreted proteins analyzed by the authors, some factors secreted by AFSCs were not detected in CM from DFs, such as EGF and PDGF-AB/BB. More interestingly, the BMSC-CM lacked many of the

inflammatory response molecules found in AFSC-CM and DF-CM (IL-8, MCP-1, MIP-1α, GM-CSF, IL-1β), molecules involved in vessel stabilization (angiostatin, endostatin, thrombospondin 2, TGFβ-1, PDGF-AB/BB), enzymes involved in tissue remodeling (MMP-8, MMP-9), growth factors important for tissue repair (EGF, HGF, FGF4), and peculiar pro-angiogenic factors (EGVEGF, PD-ECGF). Although the technique adopted by the authors is semi-quantitative and not very sensitive for detecting low abundant proteins, it offers an important panoramic view on the secretome of different cell types.

Previously, Mirabella et al. used Luminex's xMAP Technology to detect the amount (pg/ml) of some AFSC-secreted factors, normalized by the DNA content of the secreting cells [86]. The authors found that 1 μg of DNA (meaning about 170,000 AFSCs) secreted about 1 ng of VEGF-A, IL-8 and SDF-1α, about 0.5 ng of IL-6 and MCP-1, about 0.2 ng of TGFβ-1. The same authors also demonstrated an up-regulation of the cited molecules after TNF-α stimulation of AFSCs [53].

Between the soluble factors detected at high level in the ACM [86], VEGF-A, one of the main factors that prime endothelium development, would be considered a possible candidate causative of the in vivo pro-angiogenic reaction. But CM from BMSCs releases almost double the amount of VEGF-A compared to ACM [91]. Thus, the factors released by AFSCs must act in combination to achieve the strong pro-angiogenic response so far reported. It is known that SDF-1α is chemoattractive for EPCs [92]. It could be conceivable that VEGF-A and SDF-1α act in synergy to promote angiogenesis, as this has been proven in tumors [93]. Another powerful chemokine secreted by AFSCs at higher levels than BMSCs is IL-8 [86, 91], which directly enhances endothelial cell survival and matrix metalloproteinases production which is important for capillary sprouting [94, 95].

In general, angiogenesis is a complex biological process:

- It is driven by many factors and each of them can be necessary but not sufficient to the process.
- The removal of one factor can result in no phenotype changes because of biological redundancy.
- The over-supply of a non-necessary factor can result in pronounced phenotypic changes.

Indeed, angiogenic growth factors (GFs) have been used for therapeutic angiogenesis. Few studies have compared and combined different GFs to identify which could be critical, since in most of the cases a single factor is not sufficient to promote angiogenesis and two or more GFs can act in synergy [3, 96–99].

AFSC-derived secretome, thus ACM, has been proposed as a cell-free cocktail of different growth factors and chemokines capable of stimulating the migration and proliferation of vessel progenitors as well as maturation of neo-formed vessels. In vitro studies have demonstrated that when HUVEC were cultured for 16 h with ACM, the expression level of CD105 (endoglin) was doubled and the level of CD146 significantly upregulated as well [86]. The glycoprotein CD105, a part of the TGFβ-receptor complex, is required for the development of the cardiovascular system and the neo-formation of blood vessels [100]. It also antagonizes the inhibitory effects of TGFβ-1 on endothelial cell growth [101]. The glycoprotein CD146 is an adhesion marker of endothelial cells, which has also been identified on other cell types, such as pericytes and MSCs, and it is involved in the control of cell–cell cohesion and vessel integrity [59]. Taken together, these results suggest that ACM can promote both vessel growth and stabilization. Unfortunately, all the studies published to date are descriptive and mechanisms by which AFSCs stimulate angiogenesis have not been elucidated. However, it is clear that AFSC secretome does contain the paracrine signals needed to stimulate therapeutic angiogenesis, and a successful use of ACM in clinical protocols for therapeutic angiogenesis would shift the current tissue engineering (TE) paradigm of implanting exogenous bioengineered vessels to a newer regenerative medicine (RM) breakthrough of inducing endogenous vessel growth (Fig. 12.2).

6 Remarks and Future Challenges

There is stronger and stronger evidence that the stem cell-based therapy could serve as a "chemotactic" recruitment of host progenitor cells. To engineer a tissue in vitro is a very difficult operation both theoretically and practically. Since we do not possess the complete knowledge of developmental biology for each cell type, it is at least compelling to recreate the same identical conditions of a developing organism through the large availability of grow factors, scaffolds, and bioreactors. The in vivo cell therapy approach may consist of systemic infusions or topic implantations of cells. In both cases, after the eventual migration toward the injured site, cells should be retained in the compromised tissue, at least for a certain time, in order to establish a bidirectional, communicating network with the host microenvironment which consists in:

- Receiving stimuli from healthy and damaged resident cells and from inflammatory infiltrating cells.
- Giving a feedback by juxtacrine (contact-dependent) interactions with cells and by the release of cytokines and grow factors that exert a short distance (paracrine) activation of the tissue niche and/or a long distance ("endocrine") recruitment of circulating progenitors or progenitors settled in anatomically separated sites.

The focus of this chapter was to discuss the emerging evidence that:

1. AFSCs *need to be rationally reprogrammed* toward the endothelial lineage in order to be functional vessels

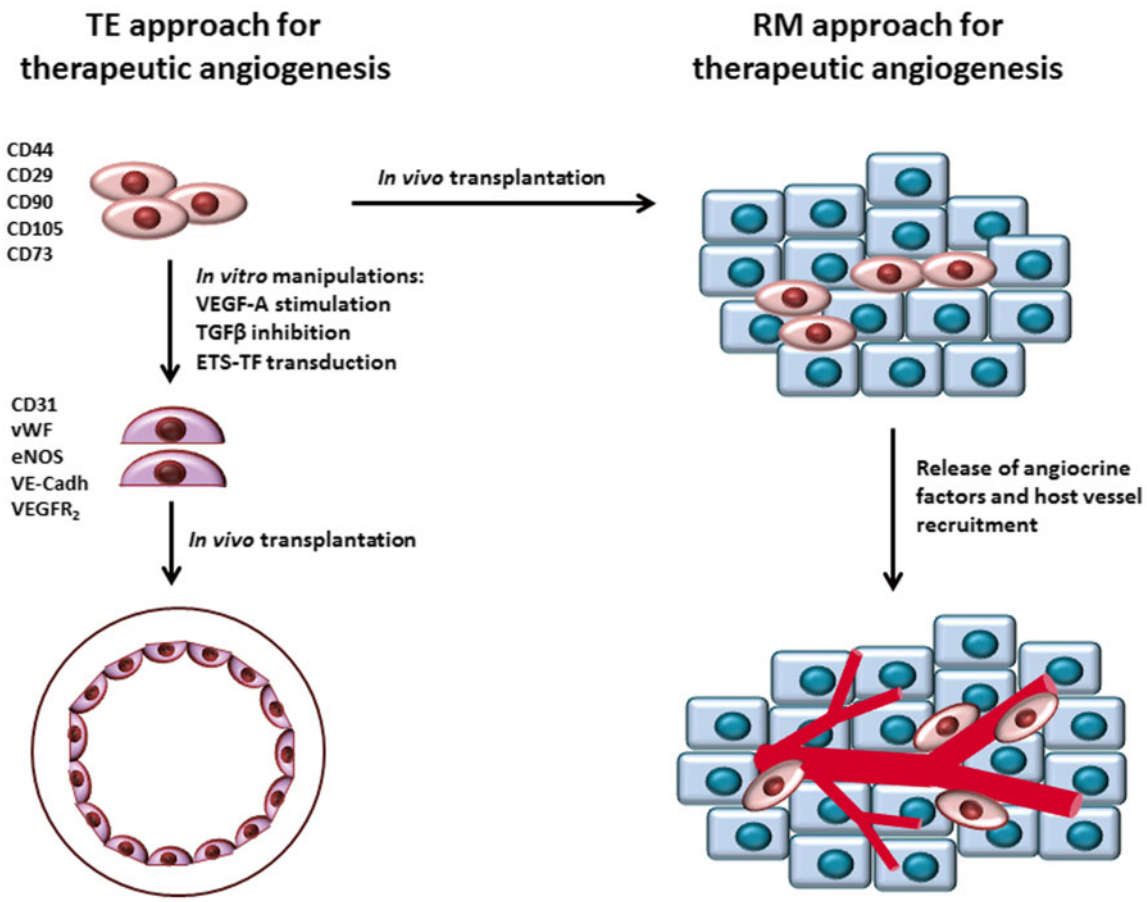


Fig. 12.2 Proposed model to switch from a tissue engineering (TE)—to a regenerative medicine (RM)-based therapeutic angiogenesis. In the TE approach, AFSCs, as other stem cells expressing mesenchymal markers (CD44, CD29, CD90, CD105, CD73, etc.) in culture, have to be differentiated or reprogrammed to express endothelial cell markers (CD31, vWF, eNOS, VE-Cadh, $VEGFR_2$); subsequently, the engineered cells or vessels will be transplanted in the patient. In the RM approach, the release of angiogenic factors from AFSCs, as well as the simple use of ACM, is sufficient to drive the vascularization of ischemic tissues in the patient

in vivo. We need to learn more about the *biology of blood vessel development* to optimize differentiation protocols.

2. The crosstalk between AFSCs and specific tissue niches has to be elucidated each time a new application of these cells results in a beneficial effect. *Mechanisms of repair* can be mediated by the recruitment of *host reparative progenitors*, rather than by a direct integration of AFSCs in the tissue.
3. The administration of *soluble factors secreted by cultured AFSCs* may achieve regenerative effects equivalent to cell therapy. The research and development of *secretome-based therapy* would be free from the limitations of immune rejection and possible tumorigenesis observed in cell transplantation.

The future challenges that the field will encounter, can be summed up as follows:

– Which amniotic fluid-derived cell fraction has to be used? Also, is the maintenance of the stem potential essential in order to get a therapeutic effect? Amniotic fluid contains heterogeneous cell types. To date, c-kit-positively selected amniotic fluid cells are considered multipotent stem cells [18]. On the other hand, c-kit negative amniotic cells are considered lineage-committed, phenotypically marked as CD324, CD326, and CD24 positive cells [30]. Finally, Arnhold et al. showed that magnetic c-kit selection does not influence the growth in culture but differentiation capacity through different lineages is affected [102].
– What is AFSC fate in vivo? As discussed above, many studies have reported that AFSCs are poorly or not retrieved at the site of implantation. It is known that AFSCs acutely recruit a wave of inflammatory cells, and that they have migratory properties through the circulatory system [51, 53, 56]. Both these processes can account for in vivo AFSC clearance.
– Which strategies must be envisioned to force AFSCs to be retained in vivo? Protocols of differentiation and reprogramming, or selection of tissue-committed subpopulations could improve both the engraftment and the functionality of transplanted AFSCs.

- AFSC secretome and its most immediately translational surrogate, the ACM, have to be better characterized. Liquid Chromatography with Tandem Mass Spectrometry Detection (LC-MS/MS) can be useful for characterizing the secretome profile, although not very sensitive as to detect low abundant proteins. Antibody-based methods (e.g., ELISA and antibody arrays) are limited by the availability of antibodies to detect secreted proteins. A systematic integrated approach would include LC-MS/MS detection, antibody arrays, microarrays, and bioinformatics [85, 103].
- In order to overcome short protein half-lives and rapid diffusion, the delivery of AFSC secretome could be achieved as a sustained and controlled release of ACM factors in a temporal and spatial-controlled manner, or in an environment-responsive manner.
- In the future, it will be necessary to investigate pathways involved in the secretion of growth factors, such as PI3K/Akt, ERK1/2, p38 MAPK. These signaling cascades have been already studied in other MSCs [104–109].

When I search PubMed for "amniotic fluid stem cells," I get less than 600 hits! We are on a good track, but we need to combine criticism and enthusiasm for a more in depth examination of AFSCs. We have in our hands a cell source with a great potential for many still unexplored future applications.

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Part II

Placental and Placental Membrane Stem Cells

Placental Stem/Progenitor Cells: Isolation and Characterization

13

Ornella Parolini, Debashree De, Melissa Rodrigues, and Maddalena Caruso

Abstract

Mounting evidence suggests that the human term placenta could be a source of stem/progenitor cells with therapeutic potential and wide applicability for regenerative/reparative medicine approaches.

Over recent years, we have learned that different cell types can be isolated from various regions of the human placenta.

This chapter aims to discuss recent significant developments regarding the isolation of these cells and to present what is to date known in terms of their phenotype, differentiation potential, and immunological properties. We will begin with a brief description of the structure of the placenta in order to provide readers with a clear picture of the target regions for stem/progenitor cell isolation, and we will then discuss trends which are evident in the properties of these cells by drawing on different characterization studies which have been performed. Furthermore, we have highlighted that although significant progress has been made, further improvements are required both for the establishment, and in particular the standardization, of isolation protocols for placental cells, and also for characterization methods which would lead to a better understanding of the phenotype of these cells and of their potential in terms of cell differentiation, immunogenicity, and other properties which would be relevant to their clinical application.

Abbreviations

ABCG2	ATP-binding cassette transporter G2
ANP	Atrial natriuretic peptide
AT	Antitrypsin
CD	Cluster of differentiation/designation
CK	Cytokeratin
CYP	Cytochrome
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
EVT	Extravillous cytotrophoblast
FGF	Fibroblast growth factor
FISH	Fluorescence in situ hybridization
FP	Fetoprotein
FZD9	Frizzled family receptor 9
GATA	Gata binding protein
GFAP	Glial fibrillary acidic protein
Glut	Glucose transporter
hAEC	Human amniotic epithelial cells
hAMSC	Human amniotic mesenchymal stromal cells
HBR	Hyaluronan mixed ester of butyric and retinoic acid
hCG	Human chorionic gonadotropin
hCMSC	Human chorionic mesenchymal stromal cells
hCTC	Human chorionic trophoblastic cells

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HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
IL	Interleukin
MAP2	Microtubule-associated protein 2
MLC	Myosin light chain
MMP	Matrix metalloproteinase
MSC	Mesenchymal stromal/stem cells
NKx 2.5	Nk2 homeobox5
OCT	Octamer-binding protein
PAX	Paired box gene
PC	Proprotein convertase
PDX-1	Pancreatic and duodenal homeobox 1
REX-1	Reduced expression protein-1
α-SMA	Alpha smooth muscle actin
SOX-2	SRY (sex determining region Y)-box 2
SP-1	Pregnancy specific β1 glycoprotein
SP-B	Surfactant protein B
SSEA	State specific embryonic antigen
STRO	Stromal cell surface marker
TGF-β	Transforming Growth factor-beta
TRA	Tumor rejection antigen
vCTB	villous Cytotrophoblast
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	von Willebrand factor

1 Placenta Structure

Term human placenta is oval or round in shape with varying diameter (15–20 cm) and thickness (2–3 cm). The fetal side of the placenta is bordered by chorionic plate while the maternal side by decidual plate. The decidua is derived from the maternal endometrium which is comprised of three regions defined by their location relative to the implantation site: the decidua basalis which is the region beneath the implantation site; the decidua capsularis which is the portion covering the implantation site and adjacent to the chorion leave; and the decidua parietalis which covers the remainder of the endometrium (Fig. 13.1a) [1].

The fetal part of the placenta is composed of the placental disc and the amniotic and chorionic membranes. The placental disc is comprised of the chorionic plate and the basal plate, which form the base and cover respectively, to enclose the intervillous space. The chorionic plate is multilayered and consists of a spongy layer, followed by chorionic mesodermal layer, and a Langhan's fibrinoid layer interspersed with highly variable proliferating extravillous cytotrophoblast cells. The amnion covers the face of the chorionic plate, which is closest to the amniotic cavity, while chorionic villi project from the other side of the chorionic plate and either

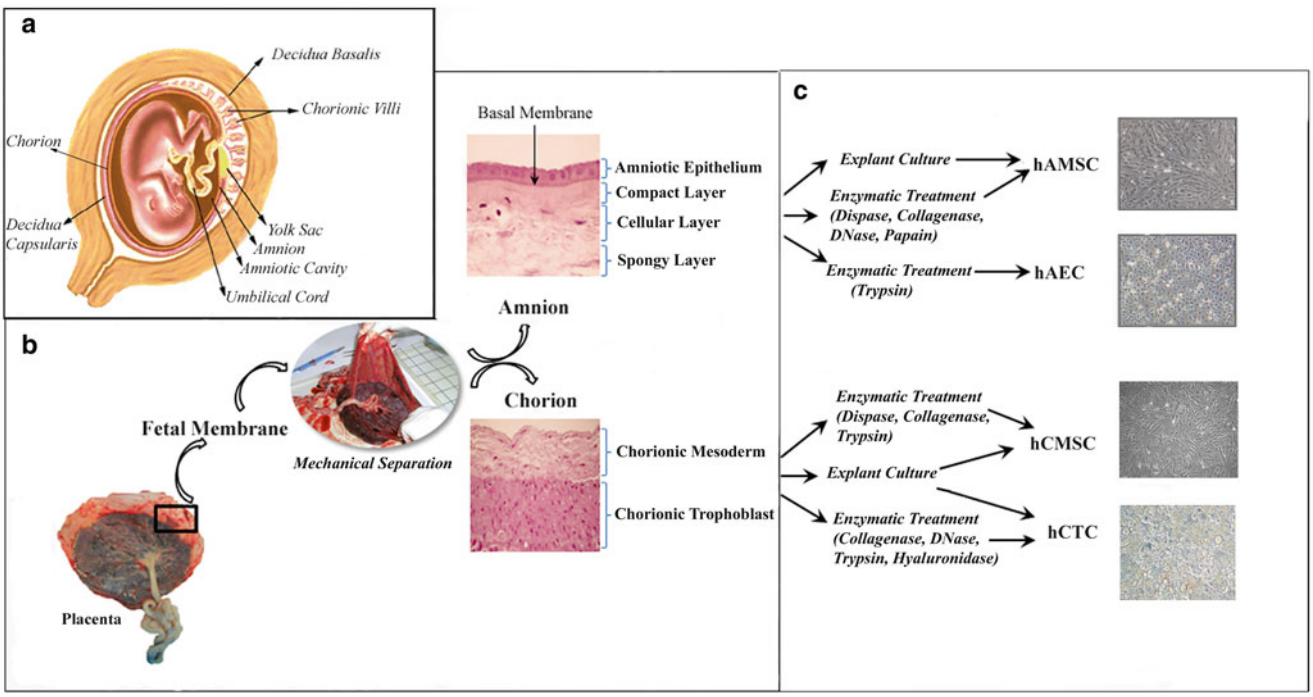


Fig. 13.1 Placenta structure and placenta-derived cells. (**a**) Illustration of the structure of placenta. (**b**) Mechanical separation of the fetal membrane and depiction of the different layers of amnion and chorion. (**c**) Schematic representation of the different cells derived from the fetal membranes

terminate freely in the intervillous space where maternal blood flows, or anchor the placenta through the trophoblast of the basal plate to the endometrium (Fig. 13.1a) [2].

The basic structure of villi comprises of an inner stromal core and a trophoblast layer separated by a basement membrane. The stromal core consists of fetal vessels and connective tissue, in which mesenchymal cells, fibroblasts, myofibroblasts, and fetal tissue macrophages, also known as Hofbauer cells, are dispersed. The trophoblast comprises of two layers—an outer uninterrupted multinucleated layer of cells called synsytiotrophoblast and an inner single or aggregated cytotrophoblast cells [2].

The basal plate is the most important contact point between the maternal and fetal tissues; composed of a superficial stria of Rhor's fibrinoid facing the intervillous space, an extravillous cytotrophoblast and connective tissue layer, followed by Nitabuch's fibrinoid layer. At the regions of placenta that are in contact with the decidua capsularis during gestation, the intervillous space is obliterated where the chorionic plate and the basal plate fuse with each other to form the chorion leave, which consists of chorionic mesoderm and trophoblast respectively. The chorionic membrane consists of a network of collagen bundles intermingled with finer fibrils of fibroblasts and macrophages. A basal lamina separates the chorionic mesoderm from the highly variable extravillous trophoblast layer.

The amniotic membrane is in contact with the amniotic fluid on its inner surface, while on the other side it is in contact with chorion laeve, chorionic plate and the umbilical cord. The amnion is contiguous over the umbilical cord with the fetal skin. The amniotic membrane is a thin avascular sheet which can be differentiated into amniotic epithelium and amniotic mesoderm [3]. The epithelium is composed of a single layer of flat, cuboidal to columnar epithelial cells, and is in turn connected to the mesoderm through a distinct basal lamina. The amniotic mesoderm is acellular compact layer of interstitial collagens I, III, and fibronectin where a deeper network of widely dispersed fibroblast-like cells and rare macrophages are also distinguishable.

2 Placenta-Derived Stem/Progenitor Cells

Cells with features of stem/progenitor cells have been isolated from different placental regions, of both fetal (Fig. 13.1b, c) and maternal origin. According to the first international workshop on placenta-derived stem cells, which was held in Brescia, Italy in 2007 [4], four major regions of fetal placenta, thought to harbor potential stem/progenitor cells, can be discriminated: amniotic epithelial, amniotic mesenchymal, chorionic mesenchymal, and chorionic trophoblastic tissues. Consequently, a consensus was reached according to which at least four different cell populations with stem or progenitor properties have been distinguished: human amniotic epithelial cells (hAEC), human amniotic mesenchymal stromal cells (hAMSC), human chorionic mesenchymal stromal cells (hCMSC), and human chorionic trophoblastic cells (hCTC) [4]. Cells with properties of mesenchymal stromal/stem cells (MSC) have also been isolated from other portion of fetal placental regions (e.g., chorionic villi [5–8]), from decidua [9], and from at least five compartments of the umbilical cord: the umbilical cord blood, the umbilical vein subendothelium, and three regions of the Wharton's jelly, that is, the perivascular zone, the intervascular zone, and the subamnion [10]. In this chapter we have focused on placenta-derived stem/progenitor cells isolated from the fetal membranes (Fig. 13.1b), villi and the maternal decidua and for the cells isolated from umbilical cord, we refer readers to other chapters of this book specifically dedicated to these cells.

2.1 Amniotic Epithelial Cells

The hAEC are isolated from the amniotic epithelium which develops from the epiblast, 8 days after fertilization and before gastrulation [11], bearing the possibility that they might maintain the plasticity of pregastrulation embryonic cells. Several studies have demonstrated that these cells can be easily recovered from the amnion and demonstrate the ability to differentiate in vitro towards cells of all the three lineages, namely ectoderm, endoderm and mesoderm [4].

2.1.1 Isolation

Different protocols have been established for the isolation of hAEC. Current research is directed towards developing isolation protocols to introduce hAEC in clinics for therapeutic purpose [12–14].

The isolation of hAEC begins with peeling off the amniotic membrane from the underlying chorionic membrane, followed by extensive washing in an appropriate buffer [12, 15–17] and subsequent cutting of the membrane into small pieces. The washing step is crucial as residual blood clots tend to reduce the efficiency of the trypsin digestions. The basis for isolation includes a series of trypsin digestions to release the hAEC, filtration using filters of varying pore sizes (70–100 μm), centrifugation and thereafter suspending in an appropriate medium best suited to culture the adherent epithelial cells. The most commonly followed protocol constitutes treating the amnion pieces with trypsin (0.05 %) three times, with incubation intervals lasting 10–40 min, with a reported yield of $80–300 \times 10^6$ hAEC from a single term placenta [18].

Various factors such as the trypsin concentration, duration of incubation, as well as the freshness of placenta influence the yield and viability of hAEC post isolation [17, 19]. Single

30 min incubation in trypsin has also been reported, highlighting that a reduction in the incubation time decreases the probability of causing cellular damage due to enzymatic exposure, preserving a greater cellular viability [17]. Therefore, attempts to improve the isolation protocol and to achieve better yield led to modification of trypsin concentrations (ranging 0.05–2.5 %) depending on which incubation periods have also been standardized [16, 17, 19–22]. Alternate to these isolation procedures, hAEC isolation post hAMSC removal from the amnion has also been reported [23, 24]. This is achieved by either manually scraping out the amniotic mesoderm without affecting the epithelial layer [17], or enzymatic removal of hAMSC following which the remaining tissue is subjected to trypsin digestion [23]. Furthermore, use of density gradient (Percoll) centrifugation for enrichment of state specific embryonic antigen (SSEA)-4-positive cell populations from the isolated hAEC prior to culture has been described [18].

hAEC are plastic adherent and on culturing, readily adhere to the culture dish without any feeder layer or any specific pretreatment of the culture substrate [25]. It was observed that hAEC do not proliferate well at low densities but replicate faster for longer duration when cultured in media with low calcium [25]. In addition, when cells are cultured in DMEM supplemented with 10 % serum and epidermal growth factor (EGF), the cells grow for 2–6 passages. Removal of the EGF results in a rapid decrease in proliferation and what appears to be terminal differentiation [26]. Proliferating hAEC display a normal karyotype [11], and have an average doubling time of 38.4 h [27].

Interestingly, it has been reported that after 5 days in culture, two fractions of cells become evident; first a population of adherent cells and second loosely attached cells over the adherent ones [11]. On collecting these two fractions by differential trypsinizations, it was observed that the clusters remaining in culture over the basal layer of adherent cells contained more cells with stem cell characteristics than in the adherent fraction [11].

2.1.2 Phenotypic Characterization

In terms of morphological appearance, hAEC in culture form a confluent monolayer of cobblestone-shaped epithelial cells [21, 22]. In terms of marker expression, nearly 100 % of hAEC are positive for pan-Cytokeratin (CK) [28], markers generally used to differentiate epithelial from mesenchymal cell lineages. Interestingly, there are reports of hAEC expressing some of the mesenchymal markers, namely vimentin [28] and alpha smooth muscle actin (α-SMA) [29]. Molecular evidence of a bona fide epithelial to mesenchymal transition (EMT) undergone by cultured hAEC (i.e., increased expression of several genes associated with EMT such as snail homologue 1 (Drosophila) protein, matrix metalloproteinase (MMP)-9, plasminogen activator inhibitor 1 and α-SMA) was also provided, possibly due to hAEC autocrine production of transforming growth factor-β (TGF-β) [29]. In regard to the expression of other markers, freshly isolated/naive hAEC are not homogenously positive for all the markers analyzed. Reportedly, hAEC present with markers typically expressed by human embryonic stem cells, namely, SSEA-3, SSEA-4, tumor rejection antigen (TRA)1-60 and TRA1-81, but lack the expression of SSEA-1 [4]. Other markers expressed include crypto, FRL-1, criptic family 1, developmental pluripotency-associated protein 3, prominin 1, paired box gene (PAX)-6 [21] and GCTM2 [30], while expression of forkhead box D3, growth differentiation factor-3, and telomerase reverse transcriptase are not observed [22, 31]. Along with these markers, molecular markers of pluripotent stem cells like octamer-binding protein (OCT)-4, SRY (sex determining region Y)-box 2 (SOX-2), Nanog, Lefty-A, fibroblast growth factor (FGF)-4, reduced expression protein-1 (REX-1) and teratocarcinoma-derived growth factor-1 [32] are also observed. Among these molecular markers, OCT-4 which is one of the transcription factors that plays a critical role in maintaining pluripotency and self-renewal in undifferentiated cells, is found to be expressed in majority of hAEC [28].

Expression of mesenchymal and hematopoietic markers such as human leukocyte antigen (HLA)-A, HLA-B, and HLA-C, cluster of differentiation/designation (CD)10, CD13, CD29, CD44, CD49e, CD73, CD90 (Thy-1), CD105, CD117 (c-kit), CD166 and stromal cell surface marker (STRO)-1 are reported; however HLA-DQ, HLA-DR, CD14, CD34, CD45, CD49d are found to be negative [4, 22, 33]. Low expression of HLA-A, HLA-B, and HLA-C was reported immediately after isolation, which surprisingly increased to significant levels in culture [34]. Similarly, the expression of CD90 is reported to be low initially, but after 6 days of culture, approximately 50 % of the cells express CD90 [28].

In addition, hAEC show expression of CD9, E-cadherin (CD324), integrin α_6, integrin β_1, CD24, c-met and ATP-binding cassette transporter G2 (ABCG2/BCRP) [35]. The expression of ABCG2 in hAEC indicates that they may have some properties similar to the so-called side population of hematopoietic stem cells found in bone marrow [28].

Along with these markers, undifferentiated hAEC also show expression of neural (glutamic acid decarboxylase, myelin basic protein, neurofilament medium chain, neuron-specific enolase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase, proteolipid protein/DM-20, microtubule-associated protein 2 (MAP2), MAP2 kinase, glial fibrillary acidic protein (GFAP), neurofilament proteins), lung (NK2 homeobox1, mucin, occludin, aquaporin-5, caveolin-1), hepatic (albumin, α-fetoprotein (FP), α-1 antitrypsin (AT), CK18, glutamine synthetase, carbamoyl phosphate synthetase-1, phosphoenolpyruvate carboxykinase, cytochrome (CYP)2D6,

CYP3A4, CYP2C9, transthyretin, tyrosine aminotransferase, hepatic nuclear factor 3-α, CCAAT/enhancer binding protein-α), cardiomyogenic (Gata binding protein (GATA)-4, Nk2 homeobox5 (NKx 2.5), myosin light chain (MLC)-2A, MLC-2V, myosin regulatory light chain-7, atrial natriuretic peptide (ANP), calcium channel, voltage-dependent, L type, alpha 1C subunit, potassium voltage-gated channel, Shal-related family, member 3), and pancreatic (pancreatic and duodenal homeobox 1 or PDX-1) lineage associated markers [22]. Some differences in the percentage of cells expressing the reported markers have been observed, possibly depending on different gestational age [5], and variable culture conditions used by different groups and expansion in vitro [13, 36, 37]. Culturing of hAEC in a serum free medium led to the expression of hematopoietic and monocytic markers, high telomerase activity and elongated telomeres whereas hAEC normally cultured in media supplemented with fetal calf serum lacked these markers and also showed no telomerase activity [22].

It is evident that a standardization of isolation as well as culture protocols is necessary for obtaining uniformity in the cell populations, to guarantee accurate data interpretation of the experiments using these cell populations and enabling reliable comparison between results obtained from different research groups.

2.1.3 Differentiation

Since the discovery of hAEC, several groups have been keenly interested in exploring their differentiation potential with the hope of using them in clinical/translational medicine. Pioneering studies by Sakuragawa and colleagues showed that hAEC express neuronal and glial markers such as neurofilament and MAP2, or GFAP [38], oligodendrocyte markers [39], synthesize catecholamines from L-tyrosine [40] and have the ability to convert 3,4-dihydroxyphenylalanine (L-DOPA) into dopamine [41], suggesting therefore their potential use for the treatment of neurological disorders. Thereafter, the potential of hAEC to differentiate towards the neural (ectodermal) lineage has been investigated, both in vitro and in vivo.

hAEC cultured in a medium containing supplements, including *-trans* retinoic acid and FGF-4, differentiated in vitro towards glial- and neuronal-like cells [11, 21]. Niknejad and colleagues reported that the capability of hAEC to express neural cell markers upon induction of neural differentiation in vitro is affected by some factors including serum, noggin, basic-FGF and retinoic acid [19]. The neural and neurotrophic potential of hAEC under in vivo conditions have shown that they can facilitate neuroregeneration/repair in disorders like Parkinson's disease, stroke, and spinal cord injury. For instance, hAEC grafted into the dopamine-denervated striatum of a rat immunosuppressed model of Parkinson's disease survived and improved the neurobehavioral deficit [42]. Liu et al. transfected hAEC, previously infected with a recombinant lentivirus to express the glial cell line-derived neurotrophic factor, into the brains of rats with a transient middle cerebral artery occlusion, and observed that hAEC survive and migrate to the ischemic area of rats, significantly ameliorating behavioral dysfunction and reducing infarct volume [43]. In animal models with spinal cord injury the administration of hAEC promoted hind limb motor function recovery; the atrophy was ameliorated and the size of injured neurons partially restored [44]. Recent preliminary findings indicate the feasibility to potentially use hAEC to treat multiple sclerosis. Indeed, transplantation of hAEC in an experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis ameliorated EAE and reduced infiltration of T lymphocytes and monocyte/macrophages, and demyelination within the central nervous system [45]. Similar findings, as well as decreased central nervous system inflammation, demyelination, and axonal degeneration in the mouse spinal cord and brain were reported on intraperitoneal injection of hAEC in a mouse model of multiple sclerosis [46]. Reportedly, the conditioned medium derived from cultured hAEC exhibits neurotrophic effects on rat cortical cells [47].

The differentiation potential of hAEC towards cells of the endodermal lineage has also been investigated. In terms of hepatic differentiation, after Sakuragawa and colleagues [48] first demonstrated that albumin and α-FP-producing hAEC are promising transgene carriers for allogeneic transplantation into liver, other works have encouraged the use of placental cells for restoring functionality of hepatic tissues. Takashima and colleagues reported that cultured hAEC, even in the absence of hepatic differentiating stimuli, express several hepatocyte-related genes and demonstrated albumin production, glycogen storage, and albumin secretion [49]. Further, reports of hAEC differentiation in vitro towards hepatocyte-like cells have been put forward by different groups using variable inducing factors [11, 21, 50, 51].

Even though there are no clear reports on in vivo differentiation, many in vivo studies provide evidences supporting the potential application of hAEC in the treatment of hepatic diseases. The anti-fibrotic effect of hAEC was demonstrated by transplanting them into CCl_4-induced cirrhosis animal model which resulted in cell engraftment with reduction of hepatocyte apoptosis, inflammation, and fibrosis. The observed therapeutic effect of transplanted hAEC as reported was more likely due to hAEC-mediated reduction of pro-inflammatory and pro-fibrotic cytokines and induction of a collagen-degrading phenotype [52]. The same group, using mice chronically injured with long-term CCl_4 treatment, also reported that hAEC engrafted in injured livers lead to significant changes in hepatic macrophage numbers and phenotype, associated with a significant reduction in the extent of established fibrosis [53]. In another study, hAEC were transplanted into the liver of retrorsine-treated immunodefi-

cient mice and post transplantation were found in the liver expressing mature liver genes, including cytochromes, plasma proteins, transporters, and other hepatic enzymes [50].

The differentiation potential of hAEC towards pancreatic cell type has also been investigated. Pancreatic differentiation was induced on culturing the hAEC in media containing nicotinamide [11]. The differentiated hAEC showed the expression of the downstream transcription factors PAX-6, Nk2 homeobox2, and the mature hormones insulin and glucagon [11] along with formation of masses of cells with a cyst like appearance, expressing the pancreatic exocrine cell marker amylase 2B [21]. Interestingly, transplantation of hAEC reported a reduction in the hyperglycemia levels in streptozotocin-induced diabetic mice, thereby indicating potent future therapeutic application for the treatment of type I diabetes mellitus [54, 55].

The capability of hAEC to differentiate towards other cell types of endodermal lineage has also been reported. Moodley et al. demonstrated that naive hAEC differentiate into cells with features of type II pneumocyte 2 weeks post parenteral injection into a severe combined immunodeficiency (SCID) mouse model of bleomycin-induced lung injury. The transplanted hAEC reduced inflammation and reduced fibrosis post-lung injury [30]. Meanwhile, our group found that transplantation of a mixture of amniotic and chorionic membrane-derived cells, including hAEC, into bleomycin-challenged immunocompetent mice, by different delivery routes, significantly reduces the severity of lung fibrosis; likely owing to paracrine actions exerted by the soluble molecules they secrete [56], rather than differentiation of the transplanted cells, as supported by the fibrosis-reducing action of conditioned medium generated from amniotic membrane-derived cells when delivered in the same lung fibrosis animal model [57].

Additionally, there are in vitro reports indicating that hAEC possess a mesodermal differentiation potential as well. For example, it is reported that naive hAEC can differentiate into cells with characteristics of mesodermal-derived adipocytes, osteocytes [5, 11, 21, 33, 58], and chondrocytes [22]. hAEC were also reported to differentiate towards the myogenic lineage comparable to bone marrow-MSC [5]. Differentiation towards cardiomyocyte-like cells was induced on using ascorbic acid supplemented media and after induction, the expression of cardiac-specific genes atrial and ventricular MLC 2 and the transcription factors GATA-4 and Nkx 2.5 was observed [11, 21]. The differentiated cells expressed Troponin T and contained T tubules, numerous myofilaments and myofibrils, and H bands characteristic of relatively mature cardiomyocytes [21].

Taken together the above evidences indicate the potential of hAEC to differentiate towards cells of all the three germ layers in vitro, while there are no strong evidences of their in vivo differentiation capacity. However, several preliminary studies mainly in the field of hepatic and neural diseases support their possible role as an effective therapeutic agent.

2.2 Mesenchymal Stromal/Stem Cells

MSC isolated from human placenta has gained increased importance in the recent years on account of their demonstrated differentiation potential, together with their immunomodulatory properties, features that enable them as an attractive source in regenerative/reparative medicine [59–62]. As already mentioned above, cells with characteristics of MSC have been isolated from different placental regions. Though these isolated cells exhibit most of the features of bone marrow-MSC, their characterization is getting complicated as volumes of work are being unraveled and hence the identification of a set of reliable markers still remains elusive. In the passages to follow, reports on the current criteria for MSC phenotypical characterization has been described.

2.2.1 Isolation of MSC from Maternal Side of Human Term Placenta-Decidua

Various groups described the isolation of MSC from the decidua basalis as well as the parietalis [9, 63–67]. Isolation of decidua-MSC is usually initiated by mechanical mincing of the tissue followed by enzymatic digestion employing collagenase [68], or trypsin (with or without EDTA) [66, 67, 69], or collagenase in combination with trypsin [65, 70, 71]. Cocktails of enzymes such as collagenase and DNase with dispase or hyaluronidase and pronase [72] have also been employed. The enzymatically digested tissue is filtered through meshes of varying pore size (25–100 μm), centrifuged [63, 70] and the pellet is then suspended and cultured in an appropriate medium. Use of density gradient centrifugation for cell enrichment post enzymatic digestion has also been reported [65, 71].

Non-enzymatic method of isolation of MSC has also been reported [9]. The procedure briefly entails mechanical mincing of the desired tissue, following which cell suspension is prepared by extensively flushing the minced tissue with an appropriate washing media through a filter. The cell suspension is then cultured in an appropriate media and the adherent cells are selectively grown to confluence [9]. The non-enzymatically prepared decidual MSC usually results in a certain degree of contamination of fetal alleles [9]. However, other researchers clearly indicated that the isolated cells in culture at varying passages are largely of maternal origin, as demonstrated by fluorescence in situ hybridization (FISH), short tandem repeat analysis, and karyotypic analysis [66, 67].

The decidua-derived MSC exhibit high proliferative ability and can be continuously cultured until passage 20 after which they undergo senescence [63, 67, 73].

The telomere progressively shortens with each cell division and as telomere length is an indicator of replicative senescence, decidual MSC would not proliferate indefinitely thereby indicating the safety issues concerning telomerase-associated teratoma formation [67].

2.2.2 Isolation of MSC from the Fetal Portion of Human Term Placenta

The regions of the fetal part of term placenta that have been of particular interest in terms of isolation of MSC include chorionic villi, chorionic membrane, and amniotic membrane. Irrespective of the placental region of interest, the basis for isolation of MSC broadly entails mechanical separation of the desired tissue, mincing and enzymatic digestion followed by filtration, centrifugation, and suspension in an appropriate medium to culture adherent stromal cells.

A few initial reports on isolation of MSC employed the whole unfractionated placenta, wherein all the different regions of the fetal part are used as a starting material [74, 75]. The tissue is minced, enzymatically digested using trypsin-EDTA [74–76] and finally processed and cultured. Contaminating erythrocytes are removed either by flushing the working medium through the arterial-vein circuit prior to any enzyme treatment or by lysing them using ammonium chloride solution after enzymatic treatment [75, 77].

Isolation of MSC from the chorionic villi popularly involves the explant culture method, in which the decidual layer is usually removed and a central cotyledon of the villous vascular bed of the fetal tissue is minced. The minced tissue, either treated with trypsin and DNase or untreated, is washed and the tissue pieces are allowed to adhere on a suitable surface enabling the cells to migrate out from the cut end of the tissue pieces which are then harvested [78]. Other enzymes reported to have been employed on tissue fragments for culture include collagenase and dispase [5]. Alternative to the explant procedure, the isolation of MSC from the villi may also employ enzyme treatment (trypsin or collagenase-DNase) and the tissue suspensions obtained are further processed and cultured [8, 79]. The duration of trypsin treatment may vary, that is, from as less as 10 or 20 min to as much as 24 h [8, 78, 80].

In terms of MSC isolated from the fetal membranes (the chorionic and amniotic membranes), the hCMSC are relatively less characterized compared to hAMSC and hence their descriptions are relatively limited [81, 82]. The hCMSC are isolated from the deflected part of the chorionic membrane through an initial mechanical and enzymatic removal of the trophoblastic layer, followed by a combination of enzymes to release the MSC [4]. The amniotic membrane on the other hand comprises of epithelial cells on one side of the membrane adjacent to the amniotic fluid with the mesodermal layer facing the other side. Thus the isolation of hAMSC entails either prior removal of contaminating epithelial cells or isolating without affecting the epithelial layer [23, 83, 84].

Various enzymes employed in the isolation of MSC from the amnion and the chorion have been reported; such as dispase and collagenase [82, 85] in combination with DNase [23, 86]. Some workers have reported on the use of trypsin solely [87] or in combination with collagenase [88–90]. The use of papain with dispase and DNase has also been described [91]. Trypsin employed in the isolation procedure helps in removal of the contaminating epithelial cells [25, 84] and the relatively purified tissue is then subjected to appropriate enzymatic digestion to release the corresponding hAMSC [83, 90]. Reports on scraping out of the amniotic mesoderm without affecting the epithelial layer have been described. The isolated mesoderm is then subjected to collagenase digestion to liberate the stromal cells [17, 92]. Other popular methods on selective isolation of hAMSC mostly entail enzymatic process [23, 35, 89, 91]. The digested tissues are generally filtered, centrifuged and the pellet is then suspended in an appropriate medium. Use of gradient ultracentrifugation of digested tissues for enrichment of the MSC prior to culture has also been reported [83]. The isolated cells are often accompanied with contaminating blood cells and removal of these contaminants requires lysis with ammonium chloride, $KHCO_3$, and EDTA [34], or employs anti-CD45 and glycophorin A immunomagnetic beads [86].

The MSC from amnion and chorion are usually of fetal origin as indicated by FISH and polymerase chain reaction analysis [23, 82, 87]. Nonetheless, presence of maternal cells in fresh as well as cultured preparations of hCMSC cannot be ruled out [23, 82].

It has been reported that the isolated hAMSC and hCMSC adhere and proliferate in culture and can be kept until passages 5–10 [4, 61]. However, recent report indicated that hCMSC could be propagated until 55 passages (more than 100 population doublings) and maintains telomere length and lack telomerase activity [82].

2.2.3 Phenotypic Characterization

According to the above mentioned consensus reached by the first international workshop on placenta-derived stem cells [4], the minimum criteria for identifying hAMSC and hCMSC include adherence to plastic; formation of fibroblast colony-forming units; expression of mesenchymal markers CD90, CD105, and CD73 by greater than 90 %; absence or less than 2 % expression of hematopoietic markers such as CD45, CD34, CD14, MHC class II; fetal origin; and potential to differentiate towards at least one or more lineages such as osteogenic, adipogenic, chondrogenic, and vascular or endothelial. hAMSC and hCMSC isolated from bulk populations retained the mentioned phenotype for at least 15 passages despite the acquisition of morphological changes [23]. In addition expression of CD166 [93], CD29 (β1-integrin receptors) [4], CD106 or vascular cell adhesion molecule-1 [8, 87], CD54 [7, 23, 94], α-SMA, vimentin [73], CK7 [23]

at varying levels have been reported. Conflicting reports on STRO-1 expression have also surfaced [63, 66, 67]. In addition expression of pluripotency markers such as OCT-3, OCT-4, SOX-2, Nanog, SSEA-3, and SSEA-4 have been inconsistently reported [93]. Moreover, OCT-4A, isoform of OCT-4, associated with pluripotency, may be a case of false positive expression and therefore warrants critical refinement in characterization scheme [95].

Detailed characterizations by researchers are revealing that marker expression varies at different time points of the culture and therefore necessitates passage specific marker profiling. Freshly isolated hAMSC expressed CD44, CD73, CD90, and CD105 at low to moderate levels as compared to up to 90 % of its expression after 4–5 passages [90, 96–98]. Hematopoietic and vascular cell related markers such as CD14 [90], CD45, and MHC class II [96–98] have been reported in relatively low but distinguishable amounts in freshly isolated hAMSC [96] which were lost or absent after subsequent passages [97, 98].

A plethora of markers in addition to these are being increasingly reported and hence the characterization of MSC isolated from different parts of the placenta is getting far more complex than previously imagined. A few freshly isolated hAMSC showed positive staining for epithelial makers such as CK5 and CK18, epithelial cell adhesion molecule and CD49f [84, 90] and low affinity nerve growth factor receptor (CD271). Increased surface expression of CD349 or frizzled family receptor 9 (FZD9) has been reported suggesting that it could be a suitable marker for MSC isolation and important for stem cell renewal [79]. CD51/61 and β3 integrins [87], CD146 (also known as the melanoma cell adhesion molecule or cell surface glycoprotein MUC18) [8], and REX-1 [67, 85] have also been noted. Other reported markers include organogenesis regulator GATA-4 [67, 99] and hepatic nuclear factor-α [22].

2.2.4 Differentiation

MSC isolated from the different parts of the placenta have successfully shown to differentiate towards cells of the classic mesodermal lineages, namely adipogenic, osteogenic, and chondrogenic [4, 9, 67], with reportedly different magnitude of commitment towards a specific lineage among cell types depending on the placental tissue source and gestational age, passages in culture and protocols to induce/evaluate differentiation [5, 23, 24, 63, 68, 72–74, 85, 87, 88, 90, 92, 94, 100]. Interestingly, MSC from placenta have also demonstrated increased osteogenic differentiation on microcarriers [101], nanofiber scaffolds [102] and slowly degradable polyurethane foams [103] indicating potent application for future in vivo bone regeneration and tissue engineering.

Successful reports of in vivo cartilage repair in rat and rabbit animal models have also been demonstrated [90, 104]. For example, hAMSC transplanted into rat with collagen-scaffold into defective cartilage underwent characteristic morphological changes concurrently with deposition of collagen type II, suggesting their differentiation into chondrocytes in vivo thereby alleviating ostochondral defect [104]. Likewise, hAMSC seeded onto poly lactic-co-glycolic acid blocks and transplanted into rabbit knee joints were detectable up to 8 weeks, without evident inflammatory response or tumorigenic proliferation, suggesting that they represent potent allo-transplantable cell resource for cartilage repair [90].

Induction of myogenic differentiation in vitro of MSC from amnion [5, 94] and from other placental regions [5] results in the formation of myotube precursors in the form of long multinucleated cells [5] coupled with the expression of transcription factor MyoD1 [5, 94], myogenin [67, 94], and desmin [94]. hCMSC and first trimester villous MSC exhibited more pronounced myogenic differentiation with respect to first trimester and term hAMSC [5].

Cardiomyocyte differentiation of decidual MSC led to the expression of ANP, cardiac-specific transcription factors homeobox Nkx [67] and GATA-4, genes such as atrial MLC-2a, ventricular MLC-2v, and the cardiac troponins cTn1 and cTnT. Further, integration of hAMSC into cardiac tissue and their differentiation into cardiomyocyte-like cells have been observed after transplantation into rat hearts following myocardial infarction [105]. It has been revealed that mixed ester of hyaluronan and butyric and retinoic acids (HBR) promoted cardiogenic/vasculogenic differentiation of fetal membrane-derived MSC and enhanced the expression of genes essential for cardiomyogenesis and cardiac markers such as the sarcomeric myosin heavy chain and the alpha sarcomeric actin [106]. HBR treated cells reflected expression of von Willebrand factor (vWF) and enhanced cardiac repair in infarcted rat hearts [106]. Moreover, hAMSC seeded onto an elastic combined scaffold, composed by a fibrin layer and by a microporous synthetic layer, have shown increased disposition to cardiac and vascular marker expression [107] indicating avenues for potent future therapeutic applications.

In addition to the traditional mesodermal lineages, placenta-derived MSC have shown to exhibit ectodermal as well as endodermal differentiation. Ectodermal (neural) changes result in formation of long bipolar, complex multipolar or branching processes [71, 91, 92]. Nestin, the widely accepted neuroectodermal maker is expressed in almost all placental MSC in both undifferentiated and differentiated states [71, 74, 80, 82, 86, 91, 108] at variable levels (20–87 %) [5, 80, 91, 109]. Neuronal markers post differentiation such as β-tubulin-III [80, 87], mushashi [91], NSE [68, 71, 74, 75], neurofilament-200 [67, 82], Tuj 1 [91, 92], MAP2 [86, 87, 92], Neu-N [92, 108] and NMDA receptor NR1 [87], GFAP [68, 71, 75, 91, 92] and CD133 [5] have been reported. Transcription factors related to midbrain dopaminergic neurons including expression of LIM homeobox transcription factor 1-beta, PAX-2 and nuclear receptor

related-1 protein have also been documented [92]. Expression of dopamine secreting neuron marker tyrosine hydroxylase [71, 92, 108] and markers such as FZD9 further support neurodifferentiation potential [71]. Neural differentiation of chorionic villi MSC was observed when the same was co-cultured with astrocytes from newborn rats without the addition of exogenous differentiation factors [80]. The authors have demonstrated that the presence of astrocytes (i.e., neural environment) influenced the neural differentiation of villi MSC, indicating the importance of microenvironmental stimuli in regulating differentiation cues [80]. Reports by Park et al. on in vivo differentiation potential of placenta-derived MSC in a neonatal rat model of stroke as well as Parkinson's disease rat model was reflected when it led to subsequent recovery of locomotor activity upon formation of dopaminergic cells in the host brain [109, 110]. Facilitation of neuronal differentiation with placenta-derived MSC seeded on to gold coated collagen nanofibers have also been demonstrated, indicating possible application in neuroregeneration for future therapeutic purposes [111].

Endodermal (hepatic, alveolar, and pancreatic) differentiation potential has also been reported [4, 99, 112]. In terms of hepatic differentiation, it has been observed that undifferentiated decidual MSC already express albumin and c-met [69] while undifferentiated hAMSC express α-FP, CK18, and α-1AT in addition to albumin [113] even in the absence of differentiating stimuli. Following differentiation, by means of various protocols, increased expression of α-1AT and albumin [113] and expression of CK19 and CK7 [84] were observed. However, contradictory results with α-FP expression were reported; from induction of expression post differentiation [113], to reduced expression within 48–72 h [69], to absolute undetected levels [84]. Urea synthesis, low density lipoprotein uptake [114], CYP450 mixed function oxidase activity [84, 114] as well as generation and storage of glycogen have also been shown [113]. Moreover, placental MSC transplanted into injured pig models exhibited differentiation resulting in hepatic regeneration and better survival rate [114].

In terms of alveolar differentiation, decidual MSC expressed prosurfactant protein C and surfactant protein B (SP-B) immunostaining and expression of SP-B and GATA-6 genes post differentiation [67].

Pancreatic differentiation has been exhibited by in vitro differentiation into pancreatic beta cells [70, 115]. Formation of epithelial-like cells and many islet-like clusters usually accompany the changes [115]. Pancreatic cell specific genes such as PDX-1, Islet-1, PAX-4, PAX-6, neurogenin 3, neurogenic differentiation-1, glucose transporter (Glut)-1, Glut-2, proprotein convertase (PC)1/3, PC2, glucokinase, pancreatic polypeptide, somatostatin, and guanylate cyclase are significantly expressed post differentiation [115, 116]. Further they are positive for insulin, glucagon, somatostatin, and c-peptide [115, 116]. Transplantation of placental MSC into kidneys of diabetic mouse model reflected improved recovery with normalized blood glucose owing to co-expression of human as well as mouse insulin and c-peptide [116]. Human cell specific gene hAlu and other pancreatic specific genes were also detected in the graft [116]. These findings indicate potent future application in diabetes.

The angiogenic differentiation of placental MSC has been controversial. Increased expression of vascular endothelial growth factor receptor (VEGFR)-1 (FLT-1) [82, 94, 117] and VEGFR-2 (KDR) [94, 117] along with the expression of intercellular adhesion molecule-1 [94], CD34 [94, 117], CD31 [68, 82, 117] and mature endothelial marker vWF post differentiation have been reported in hAMSC, hCMSC as well as decidual MSC. In addition, post differentiated expression of angiogenic factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) [68, 118], angiopoietin [68, 118, 119], EGF [118, 119], interleukin (IL)-8, and insulin-like growth factor-1 [118] have also been demonstrated. The angiogenic potential is further reflected by the ability to form capillary tube-like structures in vitro [82, 94, 119]. In vivo differentiation with the ability to augment angiogenesis in chick chorioallantoic membranes has also been reported [117]. The authors suggest the involvement of α5β1 and fibronectin to mediate the key angiogenic steps through VEGF-A induced pathway [117]. In contrast to these findings, however, a report on the ability of hAMSC to exhibit angiogenic properties but resistance to mature endothelial cell differentiation has been demonstrated [119]. The authors reported that hAMSC exhibited significant downregulation of pro-angiogenic genes and proteins such as tenascin C, angiopoietin receptor Tie-2, VEGF-A, CD146, FGF-2, IL-8, MMP-1, urokinase type plasminogen activator receptor with concomitant up-regulation of anti-angiogenic factors endostatin, serpinF1, the FGF-2 signaling antagonist sprouty1 and angioarrestin. Interestingly, despite this observation, hAMSC exhibited angiogenic properties but resisted expression of vWF and vascular endothelial-cadherin [119]. It is speculated that the niche or the microenvironment of MSC may influence the observed differences in angiogenic differentiation. Castrechini et al. for example reported that MSC isolated from human chorionic villi reside in a vascular niche and are able to contribute to angiogenic features such as vessel maturation and stabilization. However, the stromal cells isolated from this region were negative for vWF [8].

The above findings indicate that placenta-derived MSC demonstrate considerable plasticity. However, numerous studies have indicated considerable variations and therefore further investigations in terms of assessment of the functionality of the differentiated cells both in vitro and in vivo, with robust and persistent assessment of the engrafts of transplanted cells is warranted.

2.3 Chorionic Trophoblastic Cells

Within the chorionic trophoblastic derived cells (hCTC), four major types of trophoblast cell population are distinguishable; namely, relatively undifferentiated villous cytotrophoblast (vCTB), intermediate cytotrophoblast, terminally differentiated villous syncytiotrophoblast and extravillous cytotrophoblast (EVT). The syncytiotrophoblast is the outer most layer of terminally differentiated trophoblast cells and is derived from the inner mononuclear cytotrophoblast layer by a process of proliferation, differentiation, and fusion with the adjacent syncytium [120]. A fine balance between the cytotrophoblast and syncytiotrophoblast cellular layer is regulated by apoptosis and cellular shedding of syncytial knots into the maternal circulation [121]. The vCTB is thought to harbor putative stem cell population; however reliable methods to successfully identify the markers expressed by them have not yet been proposed [122].

2.3.1 Isolation

Research on trophoblast has been mainly focused for many years on understanding the basis of implantation and factors that regulate the formation of villous and extravillous trophoblast cells. Therefore, isolation of vCTB by distinct groups employing various protocols is relatively well standardized. One of the traditional procedures for isolation of vCTB employs explant culture method and it has been suggested that hypoxic conditions favor selective proliferation of cytotrophoblast cells [123]. The explant culture however results in potential contamination by non-trophoblastic cells [123]. James et al. have reported further processing of the explants using trypsin and DNase, following which the isolated cells were cultured in an appropriate media [124].

The most widely employed procedure for isolation entails enzymatic processing of the villous tissue as an initiation step. Some prominent enzyme combinations used are trypsin-EDTA [125, 126] or trypsin in combination with DNase [127–129]. Use of collagenase prior to trypsin and DNase or simply collagenase-DNase combinations in the isolation steps have also been reported [122, 130]. Another enzyme used in combination with collagenase and trypsin is hyaluronidase [131]. As the syncytial layer is sensitive to enzymatic treatment, this procedure mostly entails release of cytotrophoblast cells along with leukocytes and mesenchymal cells among other contaminants [123]. Prolonged enzymatic digestion results in more contamination of non-trophoblastic cells which prolong in the cultures and hence various methods have been further employed to eliminate these contaminants. The most popular method employs discontinuous [127] or continuous [132] density gradient centrifugation often followed by immunomagnetic enrichment of the vCTC [133]. A negative selection employing immunomagnetic procedure usually exploits the fact that non-trophoblastic cells express MHC class I and II molecules and elimination of labelled cells is facilitated with immunomagnetic microspheres [123]. Use of CD9 for elimination of fibroblasts has also been reported [128]. Enrichment of vCTC is employed by trophoblast-specific monoclonal antibodies and their subsequent absorption by panning in plates coated with anti-mouse IgG [134] and fluorescence activated cell sorting using trophoblast-specific antibodies [135]. Contaminating leucocytes are usually eliminated using anti-CD45 immunomagnetic beads [131, 136] and erythrocytes by centrifugation on lymphoprep [125]. The isolated cells are then cultured in appropriate extracellular matrix-coated dishes for high yield and better viability.

2.3.2 Phenotypic Characterization

The isolated vCTC have been characterized according to morphology, cytoskeletal proteins, enzyme histochemistry, hormone production, and other antigens [123]. Ultrastructure analysis employing transmission electron microscope revealed that these cells exhibit fine structural appearances, typical of trophoblast-derived cells. These cells contain numerous microvilli, lipid droplets and some glycogen. Branched segments of rough endoplasmic reticulum, many coated pits, well developed golgi and scattered multivesicular bodies have also been identified [137].

In terms of marker expression, however, no universally acceptable marker for identifying this population of cells has been defined. The most widely acceptable marker expression nonetheless includes expression of cytokeratin and absence of vimentin [123]. Up to 99 % positivity for cytokeratin has been reported [129, 132]. In addition, positivity for E-cadherin [129, 138] and pregnancy specific β1 glycoprotein (SP-1) have also been reported [132]. vCTC were reported to express human chorionic gonadotropin (hCG) [132, 138] and progesterone [138], usually detected 2-3 days after culture [132, 138]. Enzymes such as interstitial collagenase and 92 kDa type II collagenase (during early gestation) have also been reported [131]. Further, vCTC lack expression of MHC class I and II molecules [132].

The surface antigen expression may often be damaged during isolation employing proteolytic enzymes thereby yielding a false-negative reaction with respective antibodies [123]. In addition, proteins loosely attached to the cell surface may also be lost and get attached to non-trophoblastic cells resulting in a false-negative reaction. Thus, antibodies that recognize intracellular antigens and cytokeratins and steroidogenic enzymes are considered to be more reliable. Distinguishing between macrophages and vCTC is usually achieved by presence of cytokeratin, staining for hormones and absence of α-antichymotrypsin, a marker of Hofbauer cells [123].

Recently, it has also been shown that vCTC express stem cell specific markers such as SSEA-4 and OCT-4 [130]. In addition, high levels of alkaline phosphatase (~92 %) and CD117 have been reported [130]. These authors also demonstrated that SSEA subpopulation of vCTC cells exhibited Hochest efflux capability, which is considered to be another stem cell characteristic. Further, they have also reported the expression of Nanog and SOX2 [130].

2.3.3 Differentiation

The isolated vCTC traditionally exhibit limited replicative activity with spontaneous morphological and functional differentiation in vitro [129]. It is therefore speculated that majority of these isolated cells are well along in the progression to terminal differentiation. The vCTC have shown to differentiate into functional syncytiotrophoblast and systematically express placental proteins SP-1 and hCG followed by human placental lactogen [137]. The formation of syncytia is both time and cell density dependent with rapid differentiation in densely seeded cultures. Morphologically differentiated cells show diminished staining for cytotrophoblast antibodies but express increased immunostaining for alkaline phosphatase, expressed by syncytiotrophoblast [123]. It has further been reported that vCTC are not bipotential, that is, exhibit the ability to differentiate into both syncytiotrophoblast and EVT. Instead distinct populations of vCTC exist with the potential to differentiate into EVT or syncytiotrophoblast [124]. The subpopulation of vCTC that differentiate into EVT expresses HLA-G (~20 %) [124].

Recently, a group of workers have identified a subpopulation of vCTC SSEA4-positive cells with multipotent differentiation capacity both in vitro and in vivo. In vitro differentiation into ectoderm, endoderm, and mesoderm has been exhibited [130]. Neural (ectodermal) differentiation was reflected by the ability to yield elongated cells with spindle-like extensions, and expression of β-tubulin and nestin along with the expression of neuronal transcripts such as neuromodulin (GAP43). Myogenic or mesodermal differentiation resulted in approximately 60 % of elongated mononuclear and multinuclear cells with increased expression of actin, desmin, and α-sarcomeric actinin. Endodermal (pancreatic) differentiation yielded cells with an enlarged cytoplasm with the expression of peptide C (~30 %) and PDX-1 (20 %) [130]. A fraction of the peptide C+ cells exhibited granular insulin staining. These SSEA-4-positive cells have shown in vivo ability to engraft damaged hematopoietic tissue and when injected into a mouse blastocyst, they were incorporated into inner cell mass and could be traced in hematopoietic and other tissues of the adult chimeric mice. Further, they have shown that SSEA-4-positive cells isolated from fetuses affected by spinal muscular atrophy can be genetically corrected employing small fragment homologous recombination, a gene targeting approach [130]. These cells may therefore serve as a safe and convenient source for cell-based as well as gene therapy.

3 Immunological Properties of Placenta-Derived Cells

The immune paradox of the pregnancy, first put forth by Sir Peter Medawar towards the beginning of 1950s, is the paradox of how the maternal immune system tolerates the genetically distinct fetus, while maintaining immune reactivity against infections/foreigners. Considering the essential role of placenta in maintaining this fetomaternal tolerance, it seemed conceivable that placental cells may have immunomodulatory properties, which could take part in this process. These features make these cells also potentially useful in allo-transplantation settings and for the treatment of pathological conditions where immunomodulatory actions may have a therapeutic effect [4, 112, 139]. Over recent years, mounting evidence has accumulated in support of both of these hypotheses. In this chapter we will focus on the information currently available from in vitro studies about the immunomodulatory properties of different placental stem/progenitor cells; and at the same time invite readers to refer to other reviews for the discussion of the link between placental cells and the fetomaternal tolerance [140], as well as to the in vivo studies in support of an immunosuppressive/immunomodulatory effects of placental cells in animal models of different disorders, ranging from organ fibrosis to acute graft-versus-host disease [22, 112, 139, 141].

3.1 Low/Absent Immunogenicity of Placental Cells

An aspect of the immune-related properties of placental cells is represented by their low/absent immunogenicity. Indeed, cells from different placental regions are reported to be poor antigen-presenting cells, due to the expression of low to moderate levels of the highly polymorphic HLA class I antigens (HLA-A, HLA-B and HLA-C) and the presence of low or even absent levels of MHC class II (HLA-DP, HLA-DQ and HLA-DR) and costimulatory molecules (e.g., CD80 and CD86) on their surfaces [4, 13, 67]. These characteristics may potentially reduce the risk of immune-rejection after transplantation, as suggested by several in vivo studies, for example, prolonged survival of fetal membrane-derived cells after xenogeneic transplantation into immunocompetent animals [22, 86, 112, 142–144]. Additionally, long-term engraftment was observed after intravenous injection of human amniotic and chorionic cells into newborn swine or rats, with human microchimerism detected in several organs [86].

3.2 Immunomodulating Properties of Placental Cells In Vitro

Increasing number of studies demonstrate that placental stem/progenitor cells, like MSC isolated from different sources [145], exert immunomodulatory actions on the major cells of the immune system.

T cells are the primary cells in immune response. Through in vitro studies, it was shown that both hAMSC and hAEC inhibit T cell proliferation in response to alloantigens [86, 146, 147], mitogens [146, 147], as well as activation by CD3 and CD28 antibodies [96]. The proliferation of T cells activated by different stimulators is significantly inhibited also by MSC isolated from placental tissues other than amniotic membrane [100, 148–152]. Notably, the immunosuppressive properties of placental cells on T cells may be influenced by the conditions under which they are cultured and expanded [13].

Interestingly, differential expression of one or more surface markers on subpopulation of cells isolated from the same placental regions may modify the corresponding immunomodulatory actions. For example, two subpopulations isolated from the mesenchymal region of the amniotic membrane and discordant for the expression of CD14, CD45, and HLA-DR, surprisingly had suppressive versus stimulatory ability, with the HLA-DR-positive cells being able to cause strong proliferation of anti-CD3-primed allogeneic T cells in vitro [96]. Meanwhile, chorionic villi MSC expressing CD106 were more effective in modulating T helper subsets, and expressed more cytokines likely involved in the immunoregulatory activities with respect to the CD106-negative cellular counterpart [153].

Although the mechanisms underlying the T cell immunosuppressive effects of placental cells have not been fully clarified, both cell-to-cell contact and the secretion of bioactive soluble molecules seem somehow involved [96, 146, 147, 149, 150, 154, 155]. In regard to the secreted molecules, placental cells have been shown to produce a number of cytokines and chemokines, which together with the expression of different chemokine receptors, and a variety of growth factors, could have an important effect on their immunomodulatory potential [139]. Though the specific role of all of these molecules in the placental cell immunomodulatory actions remains to be fully elucidated, recently we provided insights into the mechanism by demonstrating that the conditioned medium generated from the culture of amniotic membrane-MSC possesses anti-proliferative effect on lymphocytes in vitro, with prostaglandins as one of the key effector molecules in this immunosuppressive activity, along with a minimal involvement of IL-6 and IL-10, but not of indoleamine 2,3-dioxygenase, nitric oxide, TGF-β, or HGF; which are instead proposed to be involved in anti-proliferative action of MSC from other sources [98].

We further demonstrated that placental cells also target dendritic cells (DCs). DCs are "professional" antigen-presenting cells crucial to initiate and modulate the immune responses [156, 157]. Strategies that could control DC maturation and/or the cytokine microenvironment might provide valuable tool to counteract many clinical situations that involve T cells, including for example graft rejection after transplantation. Cells derived from the amniotic membrane mesenchymal tissues have shown to strongly inhibit the generation and maturation of monocyte-derived DCs in vitro, preventing the expression of the DC marker CD1a and reducing the expression of the costimulatory molecule CD80, activation molecule CD83, and HLA-DR. Moreover, the monocyte maturation block resulted in impaired allostimulatory ability of these cells on allogeneic T cells. The presence of amniotic cells in differentiating DC cultures arrested the stimulated monocytes into the G0 phase of the cell cycle and abolished the production of inflammatory cytokines such as tumor necrosis factor-α, CXCL10, CXCL9, and chemokine (C-C motif) ligand 5 [158].

In addition placental cells appear to target neutrophils and macrophages. Li and colleagues [154] reported that the supernatant obtained from the culture of hAEC is able to reduce the number of murine neutrophils, and inhibits migration of murine neutrophils and macrophages in vitro, likely through the action of migration inhibitor factor. This group also reported that the hAEC supernatant significantly reduced the proliferation of B cells after mitogenic stimulation, and induced apoptosis of murine B lymphocytes. In addition, Tan et al. [159] have recently suggested that hAEC-conditioned media could alter macrophage polarization, migration, and phagocytosis, without affecting macrophage survival or proliferation in vitro. Meanwhile, modulation of macrophages in vivo has also been invoked to explain the mechanisms underlying the beneficial effects of hAEC transplantation in animal models of hepatic [53] and lung fibrosis [159].

4 Conclusions

In this chapter some of the recent developments in the isolation and characterization of placental stem cells have been elucidated. From the volumes of works that have been unraveled, it is evident that although a marked progress in this regard has been made, further studies in assessing the credentials of the isolated cells to ensure efficient applicability in regenerative/reparative medicine are warranted. In particular, a better understanding of their precise location within the placental tissues from which each different cell types are derived along with an extensive knowledge of their phenotypic characteristics and stem cell potential, is required in order to gain a deeper understanding of these cells. Most of these isolated cells display a spectrum of differentiation

capacity, even across germinal boundaries outside their specific lineage. Although these findings open avenues in regenerative medicine with wider applicability, exhaustive studies to demonstrate the functional state of the differentiated cells are necessitated.

Furthermore, it is also pertinent to address that most of the reparative processes observed are owing to paracrine factors released by many of these isolated cells and not differentiation per se. Coupled with these features, the placental stem/progenitor cells finds importance owing to their ability to demonstrate immunomodulatory properties. Though, further studies in understanding the precise pathways of immunomodulation are required, the placental stem cells definitely hold a promising future in the field of regenerative/reparative medicine.

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14 Treatment of Liver Disease Using Placental Stem Cells: Feasibility of Placental Stem Cells in Liver Diseases: Potential Implication of New Cell Therapy-Based Strategies for Hepatic Diseases

Gi Jin Kim

Abstract

Translational studies have explored the therapeutic effects of stem cells, raising hopes for the treatment of numerous diseases. Liver diseases are the most common medical diagnoses worldwide and millions of people suffer from difficult-to-treat liver diseases. Currently, orthotopic liver transplantation is the only effective treatment of end-stage hepatic disease, but this procedure is associated with many problems, including the donor scarcity, operative damage, high cost, risk of immune rejection, and the lifelong immunosuppressive treatments. Therefore, the development of therapies is required and currently under investigation all over the world. Use of cell therapy is increasing for the treatment of damaged tissue or organ regeneration. Mesenchymal stem cells (MSCs), an adult stem cell population, have characteristics of self-renewal, multipotency, and immunomodulation without ethical problem and risk for teratoma formation. Placenta-derived stem cells (PDSCs), which have received much research attention, display multi-lineage differentiation potential, and they are free of ethical concerns, easily accessible, abundant, and strongly immunosuppressive. There are several types of PDSCs. In this chapter, we will explain the characterization of several kinds of PDSCs and discuss recent investigations that study the therapeutic potential of PDSCs in repair of injured liver with a view to its utility in regenerative medicine. Although there remain many problems we should solve, many results suggest that human stem cells therapy including PDSCs is one of the new promising technologies for intractable human liver damage/diseases.

Abbreviations

AD-MSCs	Adipose-derived mesenchymal stem cells
AECs	Amniotic epithelial cells
AM-MSCs	Amniotic mesenchymal stem cells
BM-MSCs	Bone marrow-derived mesenchymal stem cells
CCl4	Carbon tetrachloride
CP-MSCs	Chorionic plate-derived mesenchymal stem cells
CV-MSCs	Chorionic villi-derived mesenchymal stem cells
DTP	Direct transplantation into the liver
EPCs	Endothelial progenitor cells
ESCs	Embryonic stem cells
HLA	Human leukocyte antigen
IDO	2,3-Dioxygenase
MHC	Major histocompatibility complex
NTP	Non-transplantation
PD-MSCs	Placenta-derived mesenchymal stem cells
PDSCs	Placenta-derived stem cells
STP	Intrasplenic transplantation
TTP	Intravenous transplantation via the tail vein
WJ-MSCs	Wharton's jelly-derived mesenchymal stem cells of the umbilical cord

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1 Introduction

Transplantation of stem cells has previously been used to treat various degenerative diseases because stem cells could be stimulated to develop into specialized cells that represent renewable sources of cells, tissue, or even organs by transplantation [1]. Also, stem cell therapy might be used to treat injuries as well as a great variety of degenerative disorders by cell replacement and/or stimulation in transplanted environment. Especially, mesenchymal stem cells (MSCs) are defined by adherence to tissue culture plastic and capacity to differentiate into multiple lineages with profiles of certain cell surface markers without ethical problem and risk for teratoma formation [2]. These MSCs were originally isolated from bone marrow (BM) with subsequent isolation from other organs including adipose tissue, placenta, and umbilical cord blood [3–6]. MSCs isolated from several sources including bone marrow, adipose, cord blood, and so on represent an attractive therapeutic candidate for treating degenerative diseases, however, their use is limited by low cell yields, dependence on donor age, limitations to autologous use, and difficulty in recruiting donors. Furthermore, the characteristics of differentiation in tissue-specific manner endow a great promise to the use of these multipotent stem cells in the fields of regenerative medicine [7, 8].

Recently, placenta-derived stem cells (PDSCs), which have received much research attention, exhibit characteristics similar to those of BM-MSCs, but they enjoy several advantages [9, 10]. PDSCs display multi-lineage differentiation potential, and they are free of ethical concerns, easily accessible, abundant, and strongly immunosuppressive [4, 9, 11]. Parolini et al. described the minimal criteria for defining PDSCs: (1) fetal origin; (2) generation of fibroblast colony-forming units; (3) specific patterns of surface antigen expression; and (4) potential to differentiate into one or more lineages [10]. Moreover, PDSCs have the potential to differentiate in vitro into hepatocyte-like cells and insulin-positive cells, as well as mesodermal lineages [12, 13]

Liver is responsible for several vital functions such as glycometabolism, toxicant metabolism, biotransformation, excretion, and hormone production. Liver disease by hepatic dysfunction caused by infection of hepatitis C and B, toxins, alcoholism, nonalcoholic fatty liver disease, autoimmune, and drug induced hepatic disorders is one of the most common medical diagnoses in the over the world. Many of these conditions lead to series of precisely regulated physiologic events and prevented or treated because liver has evolved to have a unique ability to regenerate itself to heal massive injuries [14]. But if not healed by endogenous mechanisms they lead to progressive liver injury, liver fibrosis and ultimately cirrhosis, liver failure, and, in some cases, cancer [15]. End-stage liver diseases such as liver cirrhosis, represent a worldwide health problem. Due to this reason, liver transplantation is the only effective treatment, but it is associated with many problems, including a shortage of donors, operative damage, risk of immune rejection and high costs. Furthermore, liver transplantation comes with considerable long-term side effects, such as chronic renal failure, post-transplantation lymphoproliferative disorder and cardiovascular complications [16, 17]. The emerging field of stem cell therapy has raised great hope for improving the treatment of liver disease, because it has the potential to promote liver repair and regeneration with fewer complications. Therefore, stem cells transplantation may offer the possibility of a simple and effective way of repairing the damaged liver [18, 19].

The natural repair of liver is mainly dependent on endogenous stem/progenitor cell pools, including hepatocytes, hepatic progenitor cells, and oval cells [20]. Currently exogenous stem/progenitor cells are under investigation in the context of repair of liver injury [19]. Although many studies have been undertaken in animals to analyze the efficacy and safety of this new approach with various stem cells including embryonic stem cells (ESCs), endothelial progenitor cells (EPCs), bone marrow- or adipose-derived mesenchymal stem cells (BM-MSCs or AD-MSCs), and perinatal stem cells, such as umbilical cord blood-derived MSCs (UCB-MSCs), placenta-derived mesenchymal stem cells (PDSCs), however, the clinical feasibility of this approach remains controversial because of the differences between humans and model animals [21–24]. Therefore, the development of new therapies using stem cells is required and currently under investigation all over the world.

In the chapter, we will explain the characterization of several kinds of PDSCs and discuss recent investigations that study the therapeutic potential of PDSCs in repair of injured liver with a view to its utility in regenerative medicine.

1.1 Part I. Characterization of PDSCs

Stem cells are believed to have great potential for both research and eventually clinical applications. ESCs derived from the inner cell mass of blastocysts have unlimited self-renewal activity and participate in the development of all organs and tissues in the adult animal, confirming their intrinsic potency [25]. Although ESCs have a great potential, however, problems about ethical, political, safety still remain for future regenerative medicine applications. Recent years have witnessed an explosion in the number of adult stem cell populations isolated and characterized. Every tissue or organs, apparently contains a stem cell population, therefore, easily harvested and low immunogenic stem cells might be used clinically to treat disorders of more vulnerable and less accessible internal organs. Until now, bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose-derived mesenchymal stem cells (AD-MSCs) have been studied for the purpose of cell therapy [26]. However, potentials of these

adult stem cells are decreased significantly with donor age-dependent as well as invasive procedures. In contrast, perinatal stem cells derived from amniotic fluid, fetal membranes, placenta, umbilical cord, and Wharton's jelly have advantage of in the view of their intermediate state between adult and ESCs [10, 27]. Therefore, fetal-originated stem cells are strongly attractive source of stem cells in the future study for cell therapy. These perinatal stem cells have been isolated from multiple extra-embryonic tissues are interesting due to their potential clinical utility because they were capable of long-term self-renewal and differentiation to multiple lineages when they compared to those of other adult MSCs as well as reminiscent of gradual broadening of stem cell sources seen in the adult [28].

Among the several perinatal stem cells, cord blood-derived stem cells were successfully employed in a bone marrow transplant in 1989 clinically [29]. In some countries, banking of the cord blood of their newborns against the advent of childhood hematological diseases is performed routinely. Amniotic fluid, Wharton's jelly, amnion, and placenta have all generated putative stem cells and the relative potency of these stem cell populations needs to be fully determined, and further investigation is ongoing. Especially, PDSCs originated from extra-embryonic tissues, which collected afterbirth are routinely discarded, have minimal ethical problems for the harvest of the stem cell populations. Furthermore, they facilitate isolation, without patient risk that attends adult stem cell isolation and the comparatively large volume of extra-embryonic tissues and ease of physical manipulation theoretically increases the number of stem cells that can be isolated [30, 31].

The fetal-derived placenta has neonatal and maternal components and plays critical roles including nutrient transporting, gas exchanges, hormone production, and immunomodulation as a feto-maternal organ to support fetal development during pregnancy [9]. Since it has been reported that human placenta-derived cells isolated term placenta have mesenchymal stem/progenitor cell potential, many scientists explored the characterization and therapeutic potentials of PDSCs [12]. As result, PDSCs which have received much research attention, exhibit characteristics similar to characteristics of BM-MSCs, but they enjoy several advantages [4, 32–34]. Especially, PDSCs display multi-lineage differentiation potential, and they are free of ethical concerns, easily accessible, abundant, and strongly immunosuppressive [4, 9, 10, 35]. Under defined in vitro conditions, PDSCs differentiated to putative osteoblasts and adipocytes as well as ectodermal neuron-like derivatives and even endodermal hepatocyte-like cells [11, 36].

However, there are many discrepancies for characterization of PDSCs because their anatomical complexity and different location or mixed lineages of stem cells which exist in placental tissues. To order the confusion for PDSCs, Parolini and their colleagues described that the minimal criteria for defining PDSCs: (1) fetal origin; (2) generation of fibroblast colony-forming units; (3) specific patterns of surface antigen expression; and (4) potential to differentiate into one or more lineages [10]. The placenta is composed of the placental disc, chorioamniotic membranes, and umbilical cord. According to their characteristics by localization and isolation methods of stem cells in human term placenta, chorioamniotic membranes contain amniotic epithelial cells (AECs) and mesenchymal cells including amniotic mesenchymal stem cells (AMSCs) and chorionic mesenchymal stem cells (CMSCs) in the collagenous stroma that underlies the amnion epithelium. Also, mesenchymal stem cells derived from the chorionic villi (CVMSCs) and the Wharton's jelly of the umbilical cord (WJ-MSCs) were isolated from placenta [27, 37–39]. After these fundamental categories based on the placenta-derived stem cells had been reported, researches no longer considered the placenta as a discarded organ, but as a source of valuable cells for field of regenerative medicine [40, 41]. In addition, PDSCs are regarded as ideal candidates for reprogramming to the pluripotent status in the view of their intermediate state between adult and ESCs [42].

In previous reports, we also analyzed and compared the characterization of several kinds of PDSCs according to derivation from placental anatomy [27] (Fig. 14.1). First, the human amniotic membrane (AM) contains two distinct cell populations including AECs and AMSCs. AECs are cuboidal to columnar cells that form a monolayer lining the membrane as shown on Fig. 14.1. The origin of hAEC arise from the embryonic epiblast, and AMSC are derived from the extra-embryonic mesoderm [43]. Interestingly, their morphology of subcultured AECs changed from epithelial to mesenchymal like cell, which has been suggested possibility of epithelial-mesenchymal transition by previous report [27, 44]. Second, MSCs isolated from chorionic plate of placental membrane, through mechanical and enzymatic dispase, termed as CMSCs or chorionic plate-derived mesenchymal stem cells (CP-MSCs) [10, 12, 22, 45]. These cells express the germinal layer makers as well as the pluripotent stem cell markers, Oct4, Nanog, and Sox2, as reported by others [46, 47]. Their advantages are the ability to acquire a homogenous MSCs isolated from mesenchymal layers of placental membranes. Third, mesenchymal stem cells derived from the chorionic villi (CVMSCs) isolated from placental villi contaminated with maternal cells because the chorionic villous stroma contains different cell types (stromal fibroblasts, capillary endothelial cells and macrophages) and the isolation of CVMSCs may be very complex and ineffective compared with that of other placental MSCs [45] as shown in Fig. 14.1. Furthermore, CVMSCs and CMSCs are easily contaminated with maternal cells [48]. Finally, the abundant extracellular matrix of Wharton's jelly resides a recently described stem cell population called umbilical cord Wharton's jelly stem cells (WJSCs) or umbilical cord matrix-derived stem cells (UCMSCs). The cells are present in relatively high numbers,

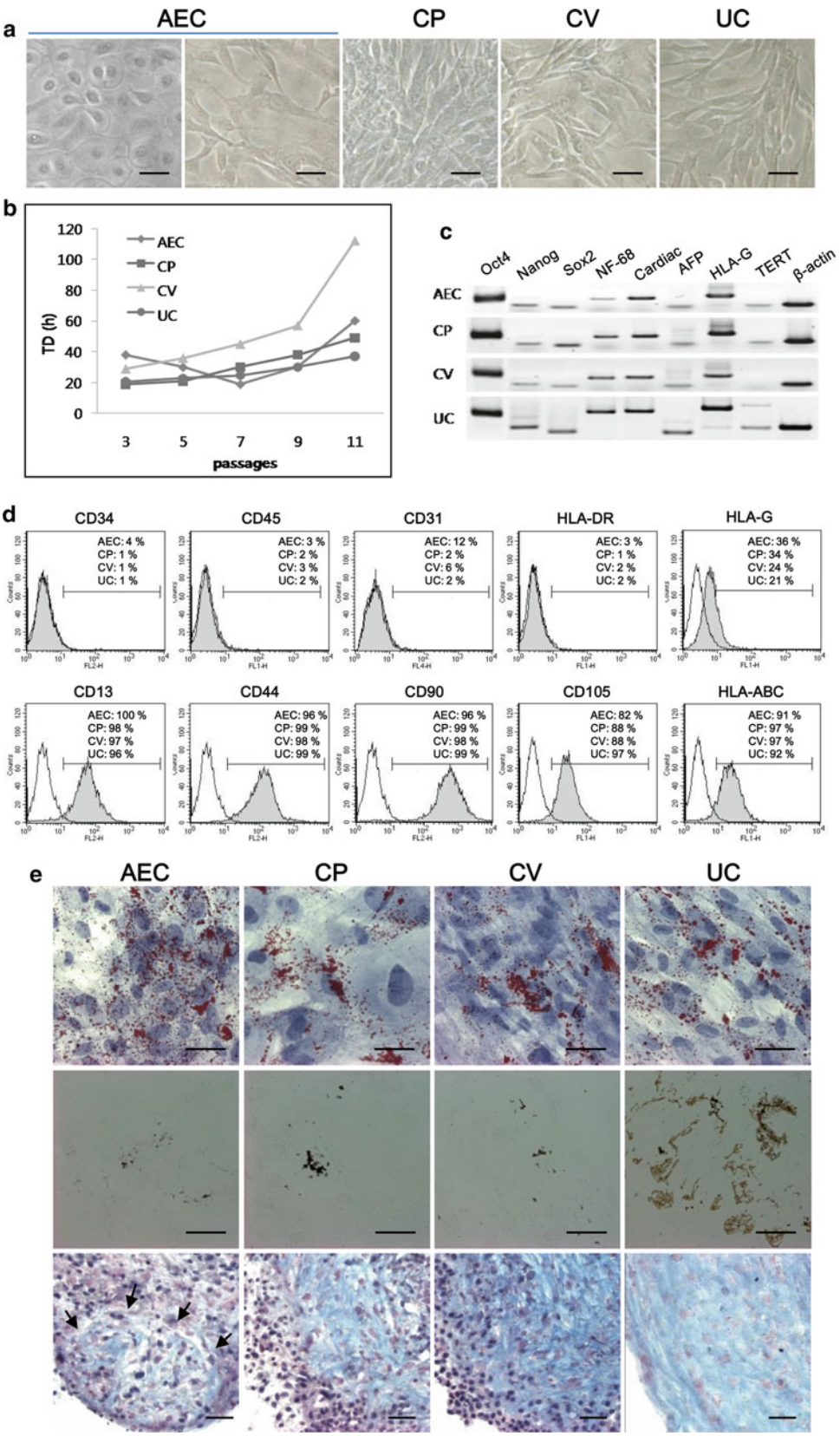


Fig. 14.1 Characterization of several placenta-derived stem cells. (**a**) Morphology of amnion epithelial cells (AEC), chorionic plate-derived mesenchymal stem cells (CP), villi-derived mesenchymal stem cells (CV), and umbilical cord-derived stem cells (UC). Scale bars = 50 μm. (**b**) Doubling time analysis of third to eleventh passage placenta-derived stem cells. (**c**) RT-PCR for self-renewal markers (Oct-4, Nanog, Sox-2 and TERT), three germinal layer markers (NF-68, Cadiac, AFP) and immunomodulatory marker (HLA-G). β-actin was used as internal control. (**d**) Immunophenotype of placenta-derived stem cells. Third passage CP-MSCs and UCMSCs were positive for CD90, CD44, CD13, and CD105 and negative for CD45, CD34, and CD31 by analysis of flow cytometry. It shows the data of HLA-ABC, HLA-DR, and HLA-G. Positive cells were evaluated compared with signal of isotype control and the percentages were indicated along with the fluorescence intensities. (**e**) Differentiation potentials of placenta-derived stem cells into mesodermal lineages including adipogenic (Oil-red O staining, *upper*), osteogenic (von Kossa staining, *middle*) and chondrogenic (Alcian Blue staining, *lower*) lineages. The cells were counterstained with hematoxylin. Scale bars = 50 μm. (Adapted from [27])

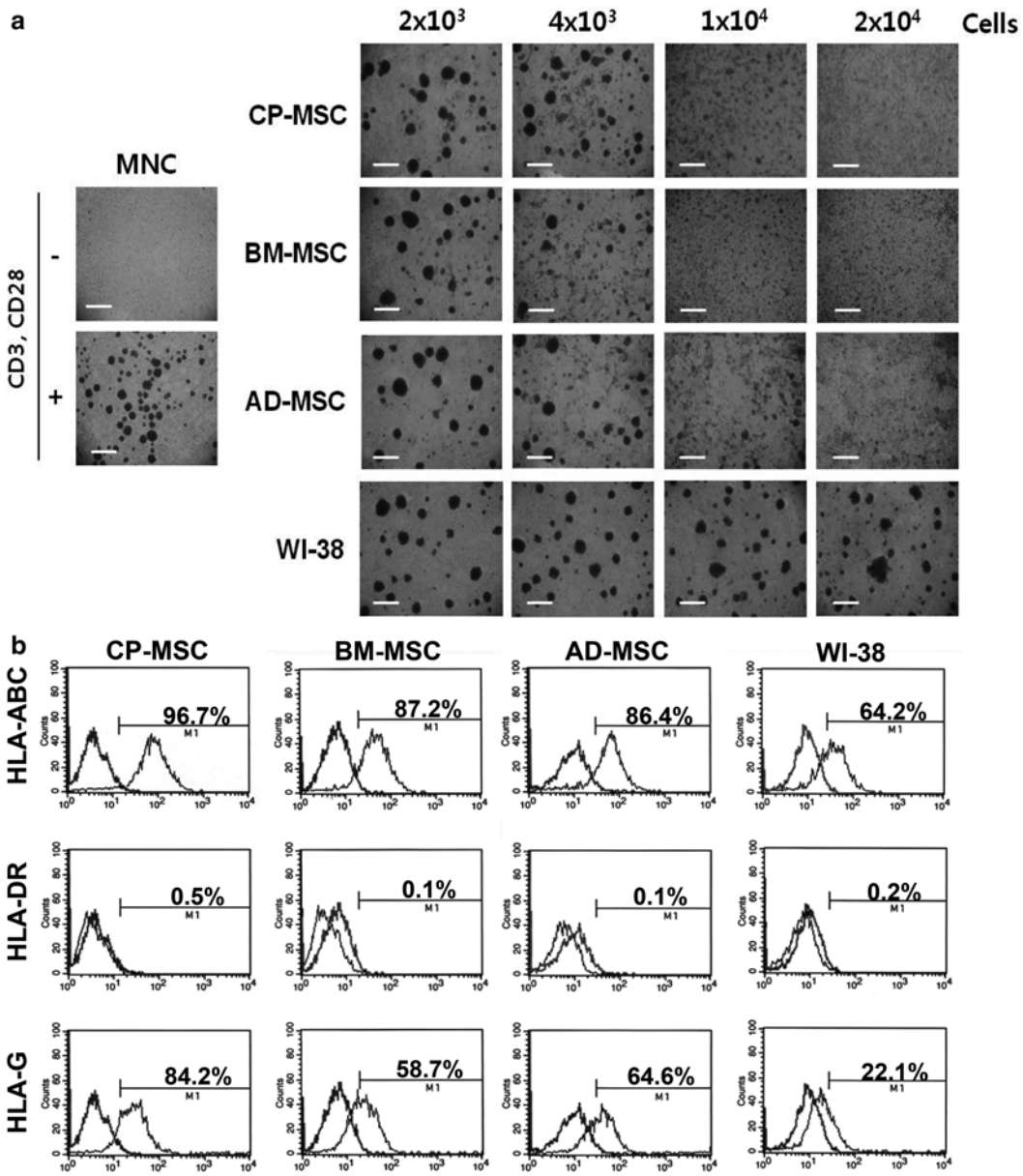


Fig. 14.2 Immunomodulatory effects of MSCs derived from placenta, bone marrow, and adipose on activated T-cells depend on co-cultured MSCs in dose-dependent manner. (**a**) MNCs isolated from umbilical cord blood were cultured with or without 1 μg/mL anti-CD3 and anti-CD28 for 72 h, which are T-cell activating mAbs, and clustering of activated T-cells decreases with increasing number of co-cultured MSCs. Scale bar = 50 μm. (**b**) Expression of surface markers for immunomodulation in MSCs derived from placenta, bone marrow, and adipose using flow cytometry analysis. Notably, the expression of HLA-G, which plays a role in immune tolerance, was highly positive in CP-MSCs. The percentages are indicated along with the fluorescence intensities. *MSCs* mesenchymal stem cells, *HLA* human leukocyte antigen, *CP-MSCs* chorionic plate-derived MSCs, *BM-MSCs* bone marrow-derived MSCs, *AD-MSCs* adipose-derived MSCs, *WI-38* normal fibroblast cell line (Adapted from [35])

with an average of 400,000 cells isolated per umbilical cord [49]. This is significantly greater than the number of MSCs that can be routinely isolated from adult bone marrow, emphasizing a primary advantage of stem cell harvest from extra-embryonic sources. Furthermore, UCMSCs have immunomodulatory properties without tumor formation up to 12 weeks post-transplantation [50, 51]. According to our data, the characteristics of CP-MSCs and WJ-MSCs were similar to those of traditional MSCs [27].

Besides the regenerative function, another important potential of placenta-derived MSCs is immune-related property [9, 52, 53]. In our data, placenta-derived MSCs were positive for HLA-ABC and negative for HLA-DR, which are similarly expressed in BM-MSCs and AD-MSCs, however, HLA-G was strongly positive only in CP-MSCs in contrast to BM-MSCs and AD-MSCs, consistent with previous report [35] (Fig. 14.2). In a number of in vitro studies, immunosuppressive effects of MSCs and their mechanism have been

well described in which suppression of T-cell proliferation and inhibition of dendritic cell differentiation have been suggested as the key events. As well as, placenta-derived MSCs suppress allogeneic T-cell proliferation through 2,3-dioxygenase (IDO) induction secreted by IFN-r [9, 54]. Immunosuppressive effects have been further confirmed in in vivo studies and are being evaluated in clinical trials in diseases such as refractory graft-versus host-disease and Crohn's disease. In addition, immune privilege and hypoimmunogenicity of placenta-derived MSCs are other aspects of immune-related property. Although controversy exists, there are evidences that MSC might be immune privileged to freshly isolated NK cells preventing them from lysis and that low expression of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I with no expression of co-stimulatory molecules may play an important role. These properties are anticipated to give advantage to survival and engraftment of MSCs in transplanted setting [55, 56].

1.2 Part II. Therapeutic Potentials of PDSCs in Hepatic Diseases

Transplantation of hepatocytes has been suggested as a useful therapeutic approach for liver disease [57]. However, there are several stumbling blocks to the use of hepatocytes in cell-based clinical applications, including difficulty of access and low yield [58, 59]. Due to the reason, many scientists have been reported and developed that the potentials on hepatogenic differentiation of MSCs derived from bone marrow, umbilical cord and adipose, and their efficacies when they transplanted into animal model with hepatic diseases such as liver fibrosis and cirrhosis, even hepatocellular carcinoma [22, 60–62].

Placenta-derived MSCs originated from mesodermal lineages also have the potential to differentiate in vitro into hepatocyte-like cells and insulin-positive cells, which are endodermal lineages [12, 13, 22]. In previous reports, isolated various types of stem cells from placental tissues including amniotic fluid, AECs, chorionic plates, villi, and umbilical cord have been characterized the stem cell capacities and hepatogenic differentiation potentials. Especially, human amniotic membrane-derived mesenchymal stem cells (hAMCs) and chorionic plate-derived mesenchymal stem cells (CP-MSCs) have been reported that their capacity to differentiate into hepatocyte-like cells, as well as their ability to reduce chronic fibrogenesis [22, 63, 64]. Also, Jung et al. reported that human umbilical cord blood (UCB)-derived MSCs could improve liver fibrosis in rat with carbon tetrachloride (CCl_4)-induced cirrhosis [65]. However, these results are controversial, as they vary somewhat among different animal models, CCl_4 treatment conditions, and cell conditions [62, 66]. So, it is necessary to establish animal model with hepatic diseases to validate the therapeutic effect of PDSCs on hepatic failures. Generally, CCl_4 is widely used experimentally to elicit liver damage; however, it decomposes in the lipids of damaged cells and leads to rapid breakdown of the endoplasmic reticulum and loss of its function. CCl_4-induced liver injury is both severe and extremely rapid in its onset, and it ultimately manifests histologically as hepatic steatosis, fibrosis, hepatocellular death, and carcinogenicity, Nevertheless, damaged hepatocytes regenerate easily in the absence of further exposure to CCl_4 [67]. Animal injuries caused by low-dose and short-term injection of CCl_4 show rapid hepatocyte regeneration on a time scale dependent on the species used, making it necessary to confirm liver damage comparable with cirrhosis in any CCl_4-injured liver model [68, 69]. Therefore, many scientists utilize a rat model of severe liver injury such as early stage of cirrhosis through high-dose, long-term treatment with CCl_4, or late stage of hepatic failure model by bile duct ligation (BDL) model [22, 70, 71].

Until now, several hypotheses for therapeutic mechanism of PDSCs on hepatic diseases have been introduced [22, 64, 72–74], however, it is still unclear. The therapeutic mechanism could be categorized as a cell-to-cell interaction and paracrine effect of PDSCs transplantation [75, 76]. Growing studies have explained two important aspects of MSC for cell therapy: (1) MSCs can modulate immunological responses, and (2) systemically administered MSCs home to sites of injury. Fetal-origin cells have a nature of migration ability cross the placental and blood–brain barriers. Due to their characterization, PDSCs also observed to home to the damaged liver after intravenous, portal vein and intrasplenic transplantation [77, 78]. The effect of engrafted PDSCs has been shown to increase depending on their transplantation [79]. So, it is important factor that transplantation technology of the cells via an appropriate route is the key to maximizing the therapeutic effects of PDSCs based therapies in liver injury. Because of the effect on the homing efficiency and biological activity of stem cells, route of transplantation is one of the most important factors determining therapeutic efficacy. In our previous report, we further investigated whether different transplantation routes, namely direct transplantation into the liver (DTP), intrasplenic transplantation (STP) and intravenous transplantation via the tail vein (TTP), exhibited different therapeutic effects and which is the optimal transplantation technique for the carbon tetrachloride (CCl_4)-injured liver rat model [80]. Homing to liver tissues damaged was confirmed by tracing cells labeled with PKH26. The differences for these three different transplantation routes can be explained as follows: (1) DTP may cause loss of the fewest transplanted cells, with many cells being expected to settle in damaged liver tissues; however, surgical procedures are unavoidable and direct injury to the liver cannot be ruled out, (2) STP is known to be an effective method for cell transplantation, with efficient homing of cells to the liver [81], and does not cause direct surgical damage to the liver, and (3) TTP may result in the loss of large numbers of

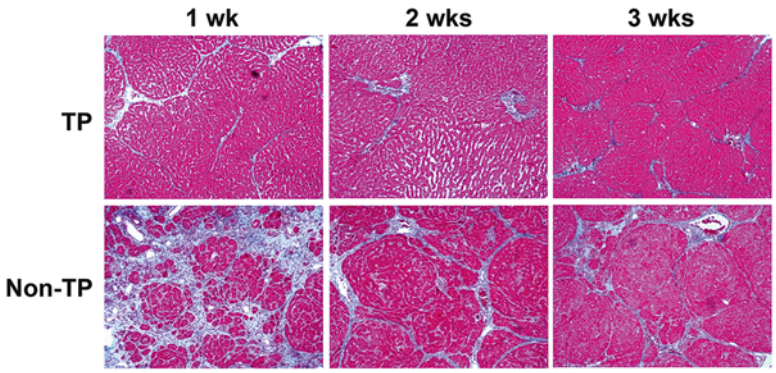


Fig. 14.3 Therapeutic effects of the transplantation of placenta-derived stem cells into the CCl_4-injured rat liver. Liver tissues were obtained from TP group (*Upper*) and Non-TP group (*Lower*) at 1, 2 and 3 weeks after generation of the CCl_4-injured rat liver disease model for 9 weeks. A: After CP-MSCs transplantation into liver tissues, the degree of liver fibrosis was analyzed from 1 week to 3 weeks using MT staining. (×100). (Adapted from [22])

transplanted cells during homing to damaged liver tissues, but is relatively easy and does not involve surgical procedures. Although it was revealed that DTP and STP were superior to TTP on anti-fibrotic effects, direct surgical application of stem cells to an already injured organ may exacerbate the damage. Therefore, STP would be desirable to transplant the cells by a similarly effective method that is potentially less damaging to the target organ. It was found that liver regeneration was more greatly enhanced in the STP group than in the DTP group although the CCl_4 administration cause injury of spleen called megalosplenia as well as liver. The therapeutic effects of engrafted PDSCs were systematically investigated by comparing DTP, STP, and TTP. Based on these findings, STP may represent an appropriate method for administering cell-based therapies in the treatment of liver disease.

The major therapeutic effect of PDSCs on a rat model with fibrosis or cirrhosis is an anti-fibrotic effect through increased MMP-2 and MMP-9 activities [22, 72, 74, 75, 78] (Fig. 14.3). Engrafted PDSCs inhibit collagen synthesis, trigger the expression of MMPs, and modulate MMP activities in TGF-β-exposed T-HSC/Cl-6 cells in an in vitro co-culture system [22]. These mechanisms are similar to those of previous reports, which have shown that transplanted bone marrow-derived mesenchymal stem cells (BM-MSCs) increase MMP expression in liver failure models [82, 83]. Manuelpillai et al. reported that human amnion epithelial cells (hAECs) engrafted onto immunocompetent CCl4-injured mice show lower expression of TNF-a and IL-6, and higher IL-10 comparing to mice model without transplantation of hAECs. Also, engrafted hAEC induced to decrease the expression of hepatic TGF-b levels, which is capable of controlling the expression of the collagen through regulation of degrading enzyme (e.g., MMP-2) [78]. These histopathological improvements in animal model transplanted with PDSCs may be linked to the observed improvement in function of the damaged liver. Furthermore, Cargnoni et al. reported that transplantation of allogenic and xenogenic PDSCs reduced both neutrophil infiltration and fibrosis in mice with bleomycin-induced lung fibrosis within 2 weeks [84]. These reports suggest the possibility that PDSCs may be useful therapeutic reagents in the treatment of degenerative diseases characterized by abnormal collagen deposition.

Also, PDSCs have immunomodulatory properties. The utility of placenta-derived stem cells is being explored towards wider range of areas and is incorporated in clinical trials as well based on the reports of their superior immunomodulatory effects. Indeed, considering the fundamental role of placenta as a bridge between mother and fetus, the immunomodulatory function might be inherited by PDSCs [35, 85]. Jung et al. demonstrated the transplantation of PDSCs into the injured rat liver resulted in a dramatic reduction of leukocytic infiltration. Additionally, IL-10 expression was significantly increased compared to the NTP group at 1 week and 3 weeks post transplantation (Fig. 14.4). These findings suggest that transplantation of PDSCs improves injured liver tissue conditions, at least in part through anti-inflammation processes [79]. The results are similar to previous reports [9, 51, 78, 86]. The anti-inflammatory effects of PDSCs suppress the chronic inflammation condition of hepatic diseases resulting in an improved environmental condition to recover liver damage.

The balance between cell death and survival is critical for several cellular processes, including embryogenesis, organogenesis, repair systems and carcinogenesis and tissue and organ repair consists of complex, multicellular processes involving the coordinated efforts of inflammation, survival and/or cell death regulation [87, 88]. Fujiyoshi et al. reported that, in liver regeneration, cell proliferation and cell growth are achieved through interleukin (IL)-6/STAT3 and PI3K/PDK1/Akt pathways via downstream molecules, such as cyclin D1/p21 and mammalian target of rapamycin (mTOR) expression, and protect against cell death by upregulating anti-apoptotic factors (e.g., FLIP, Bcl-2, Bcl-xL, Ref1 and

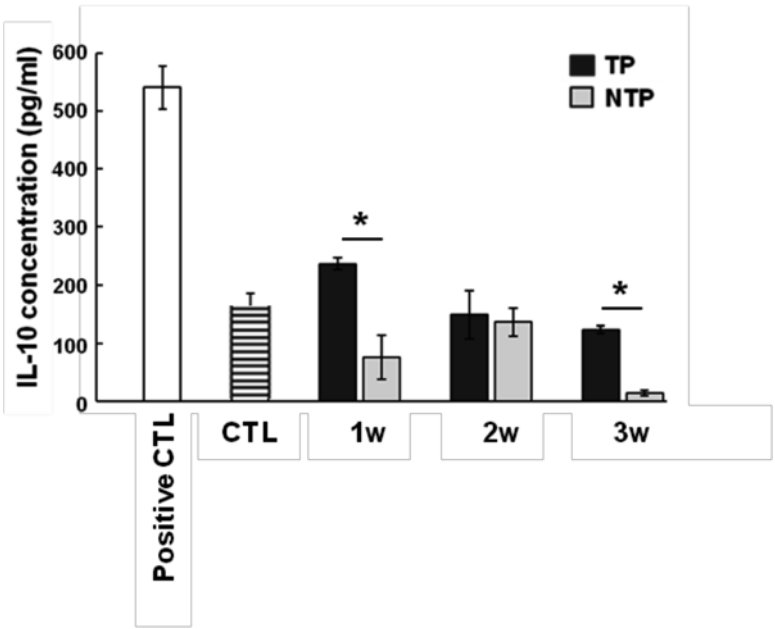


Fig. 14.4 Anti-inflammatory effect on rat injured liver according to placenta-derived stem cells transplantation. Quantitative ELISA analysis of the anti-inflammatory cytokine IL-10. Equal amounts of protein from individual animals were pooled from control rats ($n=5$), TP 1 week ($n=6$), TP 2 weeks ($n=6$), TP 3 weeks ($n=7$), NTP 1 week ($n=6$), NTP 2 weeks ($n=6$), NTP 3 weeks ($n=7$) and analyzed by ELISA. All reactions were performed in triplicate. Data are expressed as the mean ± SD of triplicate experiments. Significant differences were observed at 1 week and 3 weeks post transplantation (*$p<0.05$). *CTL* control rat, *TP* CP-MSCs-transplanted group, *NTP* non-transplanted group, *wk.* week(s) (Adapted from [79])

MnSOD) [89]. Recently, it was reported that proliferation or apoptosis mechanisms are regulated by autophagy, which is considered a key-regulating factor of disease-repair processes [90, 91]. Jung et al. demonstrated that placenta-derived mesenchymal stem cells promote hepatic regeneration in CCl4-injured rat liver via through upregulation of autophagy-inducing factors (e.g., PI3K class III, Beclin1, ATG7, ATG5-12 and LC3 II) as well as downregulation of negative regulator (e.g., p-mTOR) of autophagy [79] (Fig. 14.5). This finding suggests a new therapeutic mechanism that PDSCs transplantation induces hepatic cell regeneration in injured livers via upregulation of autophagy-related signaling molecules.

2 Conclusions

Stem cell-based therapy represents a novel approach for regenerative medicine and treatment of a wide spectrum of disorders affecting diverse organ systems. Being derived from the placenta, PDSCs (e.g., amnion, chorion, umbilical cord, and Wharton's jelly) have immunomodulatory properties, suggesting that the transplantation of PDSCs may reduce inflammatory responses in injured organs [44, 54]. Recently, Ichim and their colleagues reported that a clinical trial with placenta-derived allogenic mesenchymal stem cell treatment in patients with symptoms of either acute or chronic graft-versus host-disease was well tolerated and no adverse side effects were observed either acutely or 11 months posttreatment period [92]. These properties of PDSCs are considered very strong advantages for cell therapy through allogenic transplantation for stable engraftment via escape from host immune rejection. Furthermore, PDSCs have been added to the growing list of putative stem cell populations with providing some advantages such as easily accessibility from the tissues generally discarded after birth, harvest a lot of early stage of several distinguishable adult stem cells as well as overcomes ethical concerns. Also, PDSCs grow well in culture and appear capable of differentiation to multiple cell types including hepatocyte-like cells. The therapeutic potential of PDSCs have been demonstrated several degenerative diseases (e.g., neuronal-, skeletal disease, diabetes, as well as liver disease) by differentiation into the functional cells.

Many scientists have been demonstrated that PDSCs have the therapeutic potential in hepatic diseases. Based on the above studies, we propose that the repair mechanism triggered by PDSCs transplantation in injured liver acts through multiple events as follows: (1) environmental rehabilitation through anti-fibrosis and anti-inflammation, (2) damaged cells clearance through apoptosis and autophagy, (3) cellular protection and recycling of cellular products through autophagy, and (4) functional cells repopulation through activation of cell proliferation in injured hepatic cells (Fig. 14.6). PDSCs are an attractive cell source for degenerative diseases including hepatic diseases.

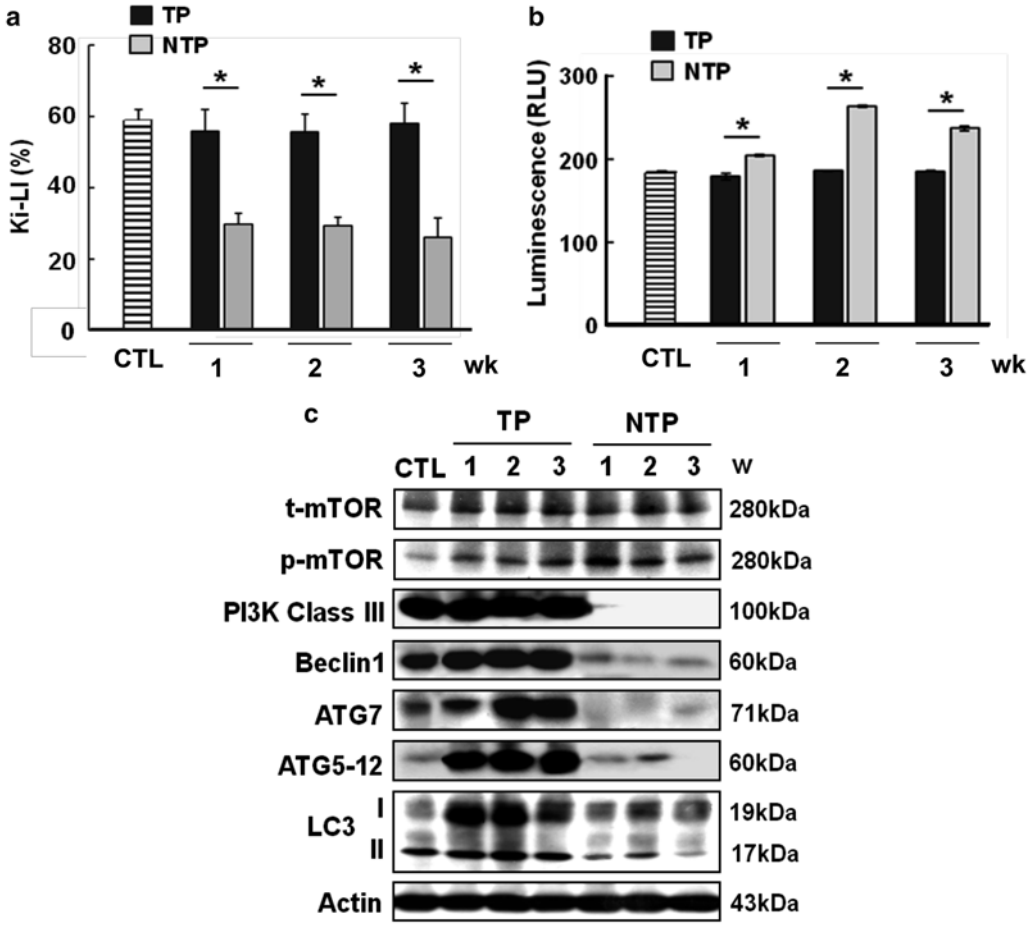


Fig. 14.5 Expression levels of cell cycle-, liver regeneration- and tissue regeneration-related factors in liver tissues from the TP and NTP groups. (**a**) The proliferative activity of liver tissues from rats in the control, TP, and NTP groups assessed through immunohistochemical analysis of Ki67 and the Ki67 labeling index (Ki-LI) and presented as the percentage of Ki67-positive nuclei in the total number of hepatocytes. Data expressed as mean ± SD. *Depicts significant differences between the TP and NTP groups ($p < 0.001$). (**b**) Caspase 3/7 activities from liver tissues of the TP and NTP groups. The activities of caspase 3/7 by ELISA in the liver tissues of the TP and NTP groups. Equal amounts of protein from individual animals were pooled from control rats (CTL; $n = 5$), TP 1 week ($n = 6$), TP 2 weeks ($n = 6$), TP 3 weeks ($n = 7$), NTP 1 week ($n = 6$), NTP 2 weeks ($n = 6$), NTP 3 weeks ($n = 7$) and loaded each well of ELISA kit. All reactions were performed in triplicate. Data are expressed as the mean ± SD of triplicate experiments. *Depicts significant differences between the TP and NTP groups ($p < 0.05$). (**c**) The expression of total- (t-), phosphorylated- (p-) mTOR, PI3K III, Beclin1, ATG7, ATG5-12, LC3 I, and LC3 II in the liver tissues of the TP and NTP groups. Actin was used as a loading control. (Adapted from [79])

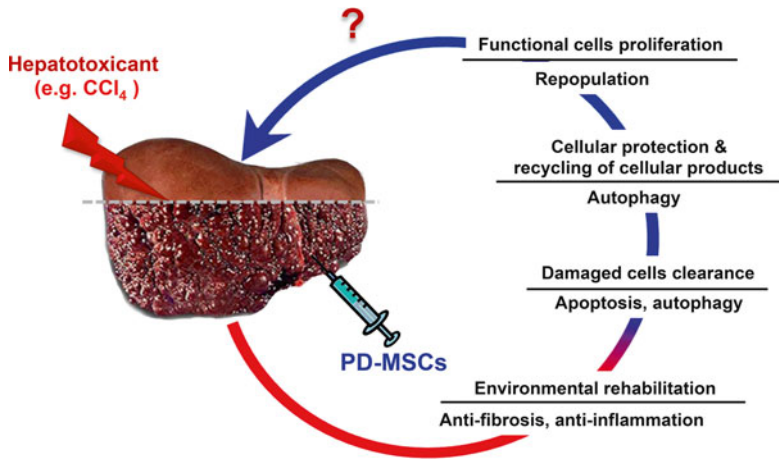


Fig. 14.6 Therapeutic mechanism of placenta-derived stem cells in hepatic diseases. The repair mechanism triggered by placenta-derived stem cell transplantation in injured liver acts through multiple events as follows: (1) environmental rehabilitation through anti-fibrosis and anti-inflammation, (2) damaged cells clearance through apoptosis and autophagy, (3) cellular protection and recycling of cellular products through autophagy, (4) functional cells repopulation through activation of cell proliferation in injured hepatic cells

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The Human Term Placenta as a Source of Transplantable Hematopoietic Stem Cells

15

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Abstract

In this chapter we summarize current knowledge on the hematopoietic potential of the human placenta throughout gestation and speculate about the possible use of this tissue at birth for the harvest of hematopoietic stem cells (HSCs) and progenitors. Placental $CD34^+$ cells could be used to augment those harvested from umbilical cord blood (UCB), which are routinely banked and used for stem cell transplantation. The placenta is an organ that develops from embryonic tissue, even before formation of the embryo, and it performs multiple critical transport functions throughout gestation. The discovery, a decade ago, that the placenta harbors HSCs and is an active and important hematopoietic organ in mice and humans dramatically changed our knowledge of the ontogeny of the hematopoietic system, adding a new extra-embryonic site to our models of hematopoietic development. We think that unveiling the hematopoietic potential of the placenta did more than shift our understanding of developmental hematopoiesis; the finding that the full-term placenta also contains sizable numbers of HSCs, which could readily be harvested at birth and combined with those present in the UCB, might also change the way we collect, bank, and transplant neonatal HSCs. Moreover, this finding might also further expand the use of UCB-HSCs for adult allogeneic transplantation by significantly increasing the harvested number of HSCs and thus avoiding the use of multiple units of UCB in single transplants, ex vivo expansion of UCB-HSCs or any other manipulation of this precious, but limited, source of HSCs prior transplantation.

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1 Introduction

Transplantation of allogeneic hematopoietic stem cells (HSCs), mostly obtained from bone marrow (BM), has been a successful treatment for a number of hematological malignancies, syndromes resulting in BM failure, hereditary immunodeficiencies, and metabolic disorders [1]. The realization, 35 years ago, that human neonatal umbilical cord blood (UCB) contains a relatively high frequency of HSCs ranks among the most important discoveries in the field of HSCs transplantation [2] and opened up a new source of HSCs that had a relatively low risk of infection and could be banked. Ende and Ende were the first to report that UCB could be used for the treatment of a child with leukemia [3]. Intensive research on the hematopoietic potential of UCB and the subsequent improvement in

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isolation techniques of UCB-HSCs led to the first human leukocyte antigen (HLA) matched UCB transplant into a 5-year-old boy with Falconi anemia one decade later [4]. During the last 25 years, the field of UCB banking and transplantation has expanded considerably, with over 600,000 UCB units cryopreserved worldwide and more than 30,000 UCB transplants having been performed. Additional advantages of UCB-HSCs transplants are that unrelated UCB donors (2–3 HLA antigens mismatch) an yield for successful long-term engraftment and survival [5, 6] and that the risk of acute and chronic graft-versus-host disease (GVHD), frequently associated with allogeneic HSCs transplantation was considerably lower in comparison with transplants of BM-HSCs [7].

Despite these impressive achievements, only 30 % of patients in immediate need of a HSC transplant have the chance to receive an HLA-matched transplant and a high number of patients with mixed racial backgrounds and belonging to ethnic minorities do not find a match in time [8]. The use of UCB as a widely employed source of HSCs for transplantation faces a crucial challenge: the number of HSCs in a single UCB unit is small in comparison with those isolated from adult bone marrow or HSC-mobilized peripheral blood. The limited cell dose in UCB transplants results in slow engraftment; low numbers of neutrophils, lymphocytes, and platelets during the first 4–5 months post-transplant increase their chance of graft failure, infection, and mortality [9]. Clinical trials have evaluated the co-infusion of 2 UCB units. Encouraging results have been obtained suggesting that administration of a higher HSC doses can result in improved engraftment and survival in adults when compared with results obtained with single donor UCB transplantation [10]. One major disadvantage of using multiple UCB units per patient is that it greatly diminishes the availability of HLA-matched UCB and good HLA-matching is essential, regardless of cell dose, to achieve good levels of engraftment and overall survival [11].

The approach we have taken to improve UCB transplantation has been to evaluate the feasibility of isolating HSCs from the placenta, an organ that is routinely discarded at birth. The HSCs obtained from the placenta could be banked to supplement the UCB-HSCs, thus increasing the cell dose harvested from a single donor. In the following chapter we review our knowledge on the hematopoietic potential of the placenta and discuss the role that this organ plays during development. In addition, we discuss the potential advantages of harvesting HSCs from placenta at birth, simultaneously to UCB-HSCs, to boost the yield of total neonatal HSCs that can be banked at birth and transplanted into patients whose long-term survival depends on the engraftment of as many HSCs as possible.

2 The Placenta Is an Active Hematopoietic Site During In Utero Development

Although the observation that the mouse placenta contains transplantable hematopoietic cells was first reported several decades ago [12–14], the issue was not thoroughly investigated until 10 years ago, when first in mouse [15–17] and later in human [18–20], it was recognized that this organ was an important contributor to the overall hematopoietic system during gestation.

To understand the role of the placenta as a hematopoietic organ in development, it is necessary to take into consideration the dynamic nature of embryonic and fetal hematopoiesis, which occurs in orchestrated stages as a number of anatomical sites become transiently active centers of hematopoiesis. This sequential activation involves both extra-embryonic and intra-embryonic tissues and organs. Figure 15.1 shows a model summarizing the major players (tissues and organs) in the early ontogeny of the human hematopoietic system. The first phase of embryonic angio-hematopoiesis occurs around 16 days of development in the extra-embryonic niches provided by yolk sac blood islands [21]. The hematopoietic output of the yolk sac is gradually replaced by sequential intra-embryonic sites, such as the aorta-gonad-mesonephros (AGM) region, also defined as the region generated from the para-aortic splanchnopleura. Subsequently, the embryonic liver—once thought to be the first site of intra-embryonic hematopoiesis—becomes hematopoietic [22]. Cells resembling primitive hematopoietic progenitors are first detected in the human AGM at day 27 [23, 24] and the population quickly expands until their disappearance by day 40. Recent studies demonstrated that definitive HSCs emerge first in the AGM region, specifically in the dorsal aorta, and only later appear in the yolk sac, liver, and placenta [25], suggesting that the first definitive HSCs are indeed of intra-embryonic origin and spreading towards other extra- or intra-embryonic sites. The embryonic liver is the next hematopoietic tissue to become active around 5–6 weeks of gestation [26], peaking around the end of the second trimester/early third trimester and gradually diminishing throughout the remainder of fetal development [27]. Finally, in the first weeks of the second trimester of gestation, the fetal bone marrow (BM) begins to function [28]. The bone marrow remains the major site of hematopoiesis throughout life, but the contribution of different anatomical sites changes over time. Bone marrow first becomes hematopoietic in the long bones found in the limbs, which are the largest bones in the body and are the first bones in the fetus to attain a size capable of forming a marrow cavity.

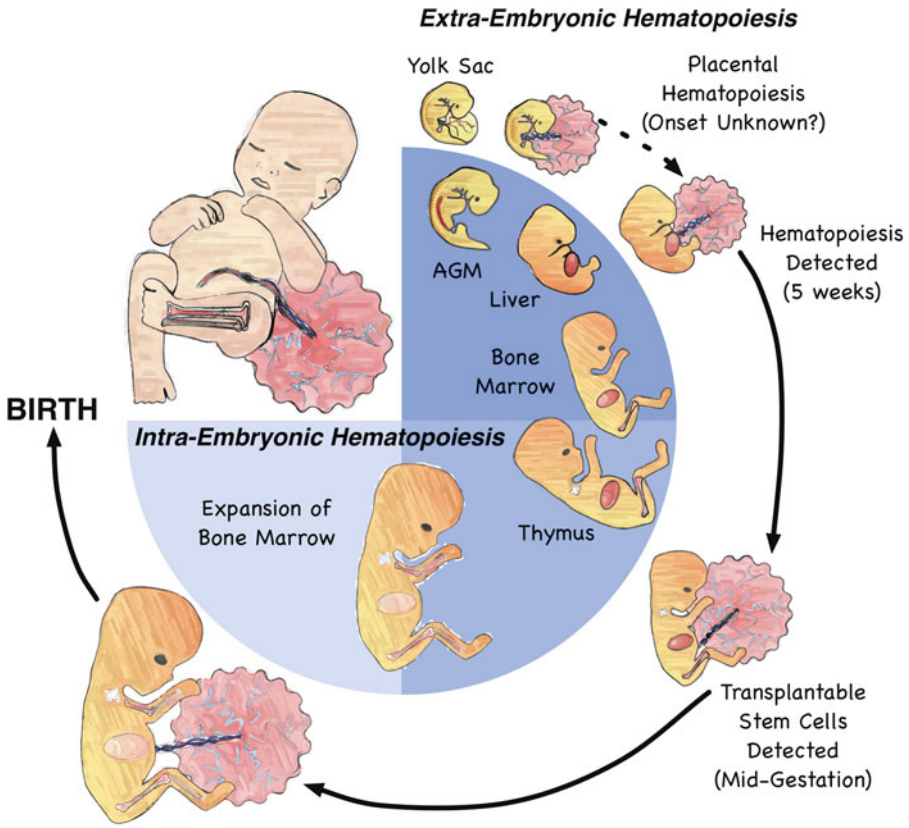


Fig. 15.1 Role of the placenta in the ontogeny of the human hematopoietic system. The sequential role of various intra-embryonic and extra-embryonic tissues in hematopoiesis is represented

We have updated this model of hematopoietic development (Fig. 15.1) to now include the placenta as an active site of hematopoiesis during gestation. Whether the human placenta produces HSCs early in development or, as in the mouse, peaks in its activity during mid-gestation has not yet been discerned. We are currently analyzing data to specifically answer this question and will report our findings in the near future (authors, manuscript in preparation). Nonetheless, one of the key factors governing the itinerant nature of prenatal hematopoiesis is the availability of physical space for hematopoietic activity. Early in embryogenesis, the yolk sac offers more space than exists in the embryo. The liver—the largest internal organ—serves as a hematopoietic organ until the bone marrow develops. In small mammals such as mice, the spleen acts as another major site of hematopoiesis during the perinatal period until the bones develop sufficient size to support hematopoiesis in their marrow cavity. In contrast, splenic hematopoiesis is negligible during human fetal development, presumably because of the prenatal development of the bone marrow allows for an earlier shift in hematopoiesis from the liver. The placenta, however, offers a large potential reservoir for hematopoietic activity throughout gestation. Beyond the considerable blood volume found in the placenta, the placenta also contains significant numbers of hematopoietic elements within its tissues, cells that may have a direct placental origin rather than migrating from the blood.

Many questions remain unanswered, particularity in the human system, about the ultimate reason for the placenta to possess hematopoietic potential. For instance, we do not presently know whether HSCs found in the placenta are produced in situ or they are generated at other locations and migrate to this organ at some point in gestation. Reports in the mouse point to an endogenous production of placental HSCs, since these cells were detected in placentas prior to the onset of fetal circulation and the heart beat begin [29, 30]. However, to unequivocally determine their presence, their functional status and their origin in humans is extremely difficult, particularly early in development, as the embryonic circulation begins at the fifth gestational week [31]. The connection between developing placental vessels and the embryonic circulatory system is established by the connective stalk (the predecessor of the umbilical cord) around 6.5 gestational weeks [32, 33]. Equally enigmatic is the function of the placental HSCs, whether they are differentiating in

the placenta to efficiently serve its own needs of hematopoietic cells (there are abundant macrophage-like cells, named Hoffbauer cells, present very early in gestation) while its vascular connection to the embryo is being established or if they are stored in this organ to serve as a pool of HSCs that can migrate out to intra-embryonic sites later in gestation.

What we have observed is that cells resembling primitive hematopoietic progenitors, defined by their cell surface phenotype of $CD34^{++}CD45^{low}CD38^{-}$ are present in the earliest samples of placenta we have been able to obtain, 5 weeks of gestation [18]. These cells are detected at a very low frequency (0.03–1.2 % of light density placental cells, $n=59$) and shared many features with HSCs/progenitors isolated from intra-embryonic hematopoietic organs, such as fetal liver and bone marrow. For instance, they express fetal stem cells markers CD133, CD90, HLA-DR, CD117, and CD31 [18]. We also demonstrated that these cells were of fetal origin capable to produce abundant myeloid colonies and some mixed myeloid-erythroid colonies in in vitro clonogenic colony-forming assays [18]. Moreover, placental cells are capable of full long-term and multilineage engraftment in immunodeficient NOD-SCID mice, thus proving their HSC properties in vivo [34].

The fact that the placenta is an active hematopoietic organ was established by the presence of not only HSCs but also of more differentiated $CD34^{+}CD45^{+}$ progenitors as well as mature progeny. These lineage-committed progenitors displayed a limited hematopoietic potential in in vitro clonogenic colony-forming [18] assays, they were not able to reconstitute immunodeficient NOD-SCID mice [34] and they contained myeloid-committed ($CD34^{+}CD13^{+}CD33^{+}$) and erythroid-committed ($CD34^{+}CD71^{+}EpoR^{+}$) precursors [18]. The finding of a spectrum of hematopoietic progenitors in the placenta is indicative of active hematopoiesis and differs from the profile of hematopoietic precursors found in blood, which is enriched in stem cells and contains relatively fewer committed progenitors.

One interesting observation derived from our studies is that the density of $CD34^{++}CD45^{low}CD38^{-}$ cells changes during gestation, possibly reflecting functional changes of the cells and the hematopoietic output at different stages. The number of HSCs was determined in 59 placental cell suspensions from 5 to 40 weeks of gestation by flow cytometry [18]. Although the total number of $CD34^{++}CD45^{low}CD38^{-}$ cells increased over gestation as the organ increased in size, the largest numbers of cells per gram of tissue were observed during the embryonic period (up to 8 weeks). Their density in the placental tissue dramatically decreased (sevenfold) from the ninth week onward, remaining quite constant for the rest of the pregnancy (Fig. 15.2).

We presently do not know the physiological reasons behind the substantial change in the density of $CD34^{++}CD45^{low}CD38^{-}$ cells during the transition from the embryonic to the fetal period. We can speculate that the enhanced hematopoietic function early in gestation may be due to the critical requirement of mature hematopoietic cells in the placenta. Placental hematopoiesis could provide, in situ, critical numbers of erythrocytes to provide O_2 to the embryo and Hofbauer cells that could aid in the immunological protection of the embryo/fetus from pathogens while the fetal-placental circulation and the immune system of the embryo are being established. During the period of 5–8 weeks the embryonic liver gradually becomes hematopoietic and thus, more HSC and mature blood cells will be exported into the blood stream. These fetal liver derived hematopoietic cells will presumably be able to reach the placenta, in which case the endogenous production of HSCs might not be as crucial as earlier in development. Another possible explanation is that the placenta contributes to the early embryonic pool of HSCs and once that the intra-embryonic hematopoietic sites, such as the fetal liver, are fully functional, declines its potential accordingly. Finally we should consider the possibility that the embryonic $CD34^{++}CD45^{low}CD38^{-}$ population contains not only hematopoietic progenitors but also could possess endothelial progenitor potential. The placenta is a highly vascularized organ with a large need of endothelial cells early in gestation. In addition, there are strong evidences suggesting the existence of a common hematopoietic and endothelial precursor, or hemangioblast, in the hemogenic endothelium during embryogenesis in mice and humans [35, 36]. Although direct evidence demonstrating the existence of hemogenic endothelium in placenta is lacking, there are indirect suggestions that it may exist. In Ncx1−/− circulation deficient mice, the emergence of HSC has been shown in the placenta in association with placenta vessels [37]. Our own observations of placental $CD34^{+}CD45^{+}$ cells in direct contact with endothelial cells also suggest the possibility of hemogenic endothelium in the early gestation placenta [34]. It would be very interesting to assess the endothelial potential of early $CD34^{++}CD45^{low}CD38^{-}$ placental cells, as it is the aim of the current research in our laboratory.

Another relevant aspect of our studies was to address the question whether placental HSCs/progenitors are functionally similar to intra-embryonic stem cell populations. Although placental HSCs/progenitors are capable to produce multilineage progeny in cytokine-supplemented liquid cultures in vitro and in vivo after being transplanted into mice [18, 34], our in vitro studies point to unique features of placental hematopoietic precursors. Sorted placental $CD34^{++}CD45^{low}$ cells and the more mature population constituted by $CD34^{+}CD45^{low}$ cells were cultured in in vitro clonogenic assays and hematopoietic colonies of different types were counted as well as individually analyzed by flow cytometry [18]. Most of the colonies, at any gestational age,

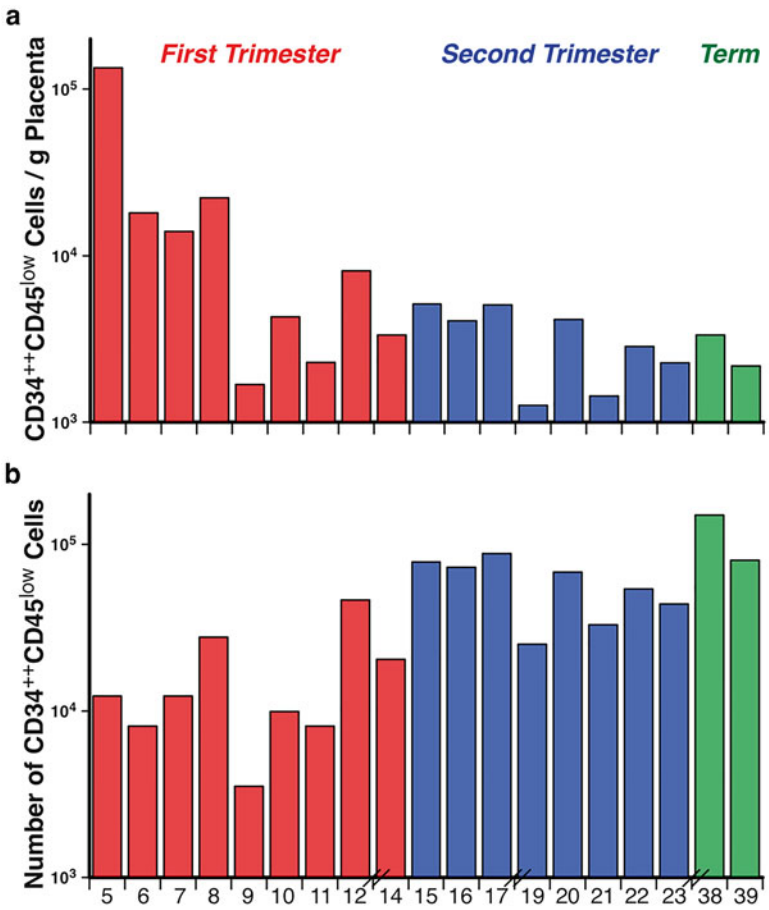


Fig. 15.2 *Changes in the hematopoietic compartment of the placenta during development.* (**a**) Bar chart of the density of $CD34^{++}CD45^{low}$ cells in the placenta expressed as cells per gram of tissue grouped by gestational age. (**b**) Bar chart of the total number of $CD34^{++}CD45^{low}$ cells contained in the placenta by gestational age. *Bar charts* represent the median value of measurements made for each gestational age ($n = 1$–5 observations for each week, $n = 59$ in total). These data were previously published in a different format [18]

produced only myeloid cells (i.e., colony-forming unit-granulocyte monocyte or CFU-GM) and a small number of colonies showed a mixture of lineages containing both myeloid and erythroid cells (CFU-Mix). The finding of myeloid precursors resonates with the high number of macrophage-like cells, Hofbauer cells, present in the cores of placental chorionic villi; therefore, one could interpret that the hematopoietic potential of placental HSC might be skewed to keep up the number of Hofbauer cells during this organ's expansion and growth along gestation. One surprising observation is that neither $CD34^{++}CD45^{low}$ cells nor $CD34^{+}CD45^{low}$ cells produce pure burst-forming unit-erythroid (BFU-E), while their intra-embryonic counterparts, in particular the fetal liver, generate abundant BFU-E [38]. This observation is in contrast with a recent report showing that early first trimester placenta is a site for terminal maturation of primitive erythroid cells [39]. The lack of BFU-E activity by placental precursors might be due to the absence of critical signals from the environment that are missing in vitro. Nonetheless, the in vitro behavior of placental erythroid precursors appears to differ from those of the fetal liver or fetal BM.

The second functional difference we have detected is the strict dependence of placental HSCs/progenitors on high fetal bovine serum content in the methylcellulose semi-solid medium used in colony-forming assays (unpublished observations). While fetal liver and fetal BM progenitors grow abundantly in the absence of serum (in fact, the presence of fetal bovine serum—FBS—inhibits the number of CFUs that can be obtained [27]), placental progenitors generate 60 % less CFU-GM and CFU-Mix in serum free media. The ability of producing CFUs of any type is restored by adding back increasing amounts of FBS, with plateau activity observed around 30 % of FBS.

All together these data suggest different growth requirements and/or different hematopoietic potential of placental HSCs in comparison with intra-embryonic HSC and require further investigation. This aspect is particularly pressing when considering the framework for the potential clinical application of term placental HSCs to expand the number of UCB-HSC for banking and transplantation. Additionally we might take into consideration the influence of the microenvironment where these cells arise. One possible cause of the functional differences between extra-

embryonic and intra-embryonic HSC might be the molecular and cellular signals that the hematopoietic niche delivers to the stem cells. Our experiments aimed to characterize the placental hematopoietic niche have led to the conclusion that there are two separate environments where placental HSCs reside: a vascular niche, in which $CD34^{+}CD45^{+}$ hematopoietic progenitors/HSCs are in direct association with $CD34^{+}CD45^{-}$ endothelial cells and a mesenchymal cell niche, composed by cells expressing stromal markers on the cell surface and vimentin intracellularly (A. Bárcena, M.O. Muench, M. Kapidzic, M. Gormley, S.J. Fisher, manuscript in preparation). Therefore, the placental hematopoietic niches seem, in appearance, similar to the bone marrow niches, although in-depth studies aimed to identify the individual cellular and molecular elements, in particular regarding the mesenchymal cell niche, have not been performed. We think that the information obtained from these studies could help explaining the functional in vitro divergence of placental HSC/progenitors in comparison with other fetal and perinatal stem cell populations and we are currently working on a manuscript that reports some of these data.

3 Term Placenta Contains Hematopoietic Stem Cells/Progenitors

Our research on embryonic/fetal placenta was not only designed to address developmental questions about the emergence of the hematopoietic potential of this transient organ, but also to determine whether HSCs are contained in the term placenta. The prospective of harvesting HSCs from this new hematopoietic source is very exciting, as it could lead to important clinical applications in the field of UCB banking and transplantation. In Table 15.1 we summarize our findings from six placental cell suspensions obtained at birth of full-term neonates. The samples were stained with specific monoclonal antibodies against CD34 and CD45 antigens and subjected to flow cytometric studies. The density of $CD34^{++}CD45^{low}$ cells was fairly similar between samples, ranging from 1 to 11×10^{3} cells/g, while the total number of cells recovered ranged from 1 to 12.2×10^{6} from the light density (mononuclear cells) fraction. In one of the specimens (sample D) we obtained the corresponding UCB and the light density fraction was also analyzed by flow cytometry. We observed that 75 mL of UCB contained a total of 1.5×10^{6} $CD34^{++}CD45^{low}$ cells, while the placenta, weighting 590 g contained 12.2×10^{6} $CD34^{++}CD45^{low}$ cells. Although it can be argued that this placenta sample was singularly enriched in $CD34^{++}CD45^{low}$ cells, the yields of $CD34^{++}CD45^{low}$ cells that we regularly observe in UCB and in placenta are very much comparable to those reported in the literature. In a study of 300 UCB samples that were similarly processed and analyzed by FACS, the mean number of total $CD34^{+}CD45^{+}$ cells was 3.1×10^{6} cells per UCB unit [40] and different groups have published similar results, $0.5–2.6\times10^{6}$ $CD34^{+}CD45^{+}$ cells/UCB unit [41, 42].

An example of flow cytometric analysis of light density cells freshly isolated from 50 g of term placental tissue is shown in Fig. 15.3. Although the frequency of $CD34^{++}CD45^{low}$ cells is very low (less than 1 % of the light density fraction), the placenta is quite a large organ and typically weighs 500 g on average. Therefore it is, in principle, possible to purify several millions of $CD34^{++}CD45^{low}$ cells from a single placenta. We investigated their functional status in vivo, by transplanting them intravenously into sub-lethally irradiated immunodeficient NSG mice. Figure 15.4 shows the analysis of a representative mouse 4 months after transplantation of term placental cell suspension (38 weeks of gestational age) enriched on HSCs/progenitors by lineage magnetic depletion. The detection of human mature hematopoietic cells in the mouse bone marrow belonging to multiple lineages (erythroid, myeloid, and lymphoid) proves the long-term engraftment (after 10 weeks post-transplantation) capability of the transplanted cells, which is currently considered the golden standard to demonstrate functional human HSCs. Other researchers in the field have reported similar results, which is highly relevant since they used different methods to dissociate the placental tissue than the one we employ [19] or, by perfusion of the placenta and in the absence of tissue dissociation [20]. We believe that this is an important consideration: besides the method by which the cells are harvested from term placenta, different groups of investigators have been able to observe their hematopoietic potential. This finding suggests that a side-by-side comparison of different methods for harvesting placental HSCs, with an emphasis on those techniques that could be applicable in the clinical setting, and that allow for an improved yield of cells, is an important step towards the utilization of this organ as a source of transplantable HSCs.

Table 15.1 Abundant $CD34^{++}CD45^{low}$ cells are present in placenta at birth[a]

Tissue	Gestational age (wks)	C-section (C) labor (L)	# $CD34^{++}$ $CD45^{low}$ (cells/g)	Total # of $CD34^{++}$ $CD45^{low}$ cells
Placenta A	36	C	1.1×10^{4}	7.2×10^{6}
Placenta B	37	C	1.4×10^{3}	9.8×10^{5}
Placenta C	39	C	3.0×10^{3}	1.6×10^{6}
Placenta D	39	C	2.1×10^{4}	12.2×10^{6}
Placenta E	41	L	1.5×10^{3}	1.0×10^{6}
Placenta F	38	C	3.2×10^{4}	8.5×10^{6}

[a]Some of the data shown in this table was previously published [34]

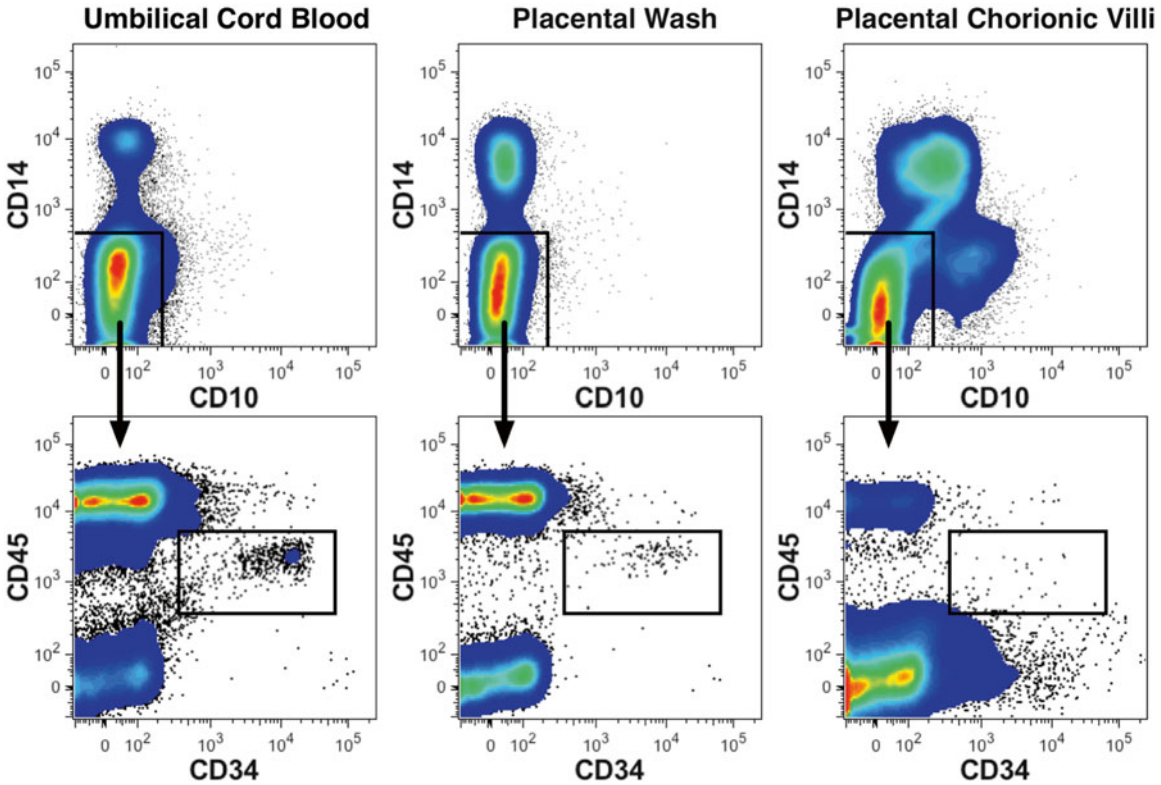


Fig. 15.3 *Term placenta contains CD34^{++}CD45^{low} cells*. Flow cytometry analyses of freshly isolated light density fractions from a placenta (39 weeks and 2 days of gestational age), the blood that drips from the placenta without perfusion (placenta wash) and the corresponding UCB. In the upper panels we show our gating strategy, where a gate was set to exclude the abundant Hofbauer cells (CD14^{+}) and stromal cells (CD10^{+}) present in the placenta. The *box* indicates the presence of CD34^{++}CD45^{low} cells in the different tissues

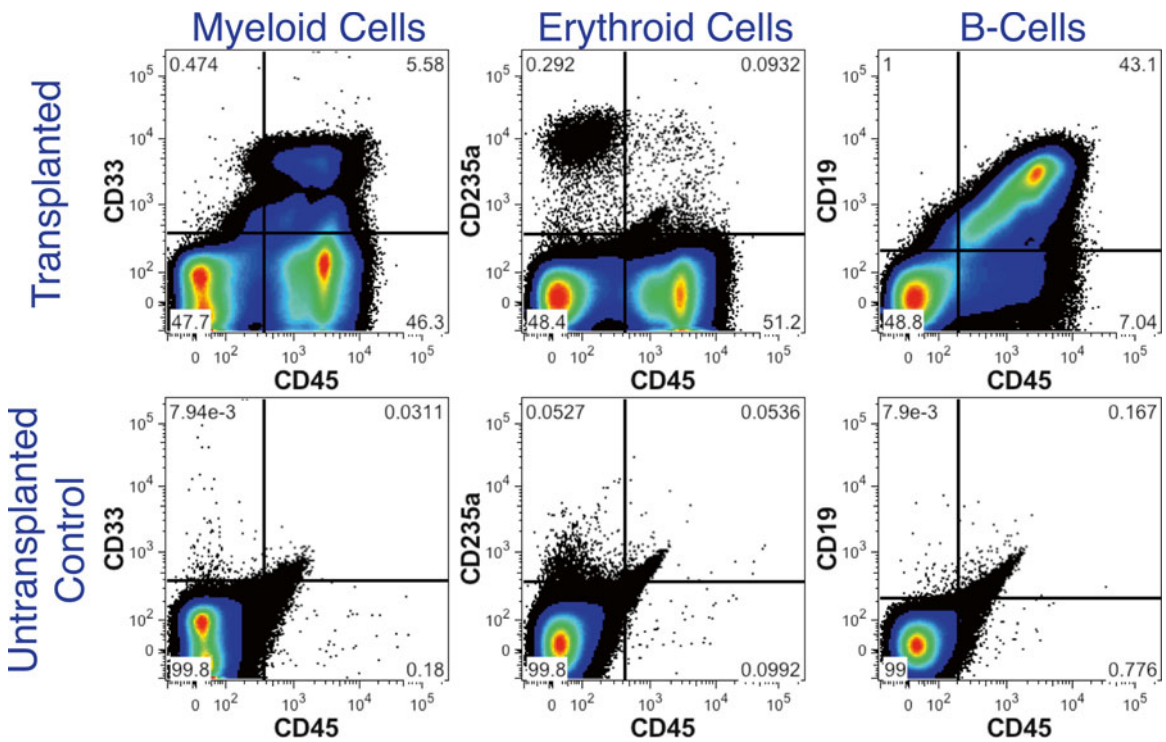


Fig. 15.4 *Hematopoietic reconstitution by placental chorionic villi cells*. Chorionic villi cells, harvested at term—38 weeks' gestation, were transplanted into an immunodeficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mouse and analyzed 4 months later. Bone marrow engraftment by human cells is shown in the top row and staining of the bone marrow of an untransplanted mouse is shown in the bottom row. Total bone marrow cells were analyzed using gates that define live, single cells based on lack of propidium iodide staining and the height and width properties of the forward light-scatter signal, respectively (not shown). The expression of human myeloid, erythroid, and B-lymphoid markers is shown together with the human common leukocyte marker, CD45. Numbers associated with each quadrant represent the percentages of live single cells found in the respective quadrant

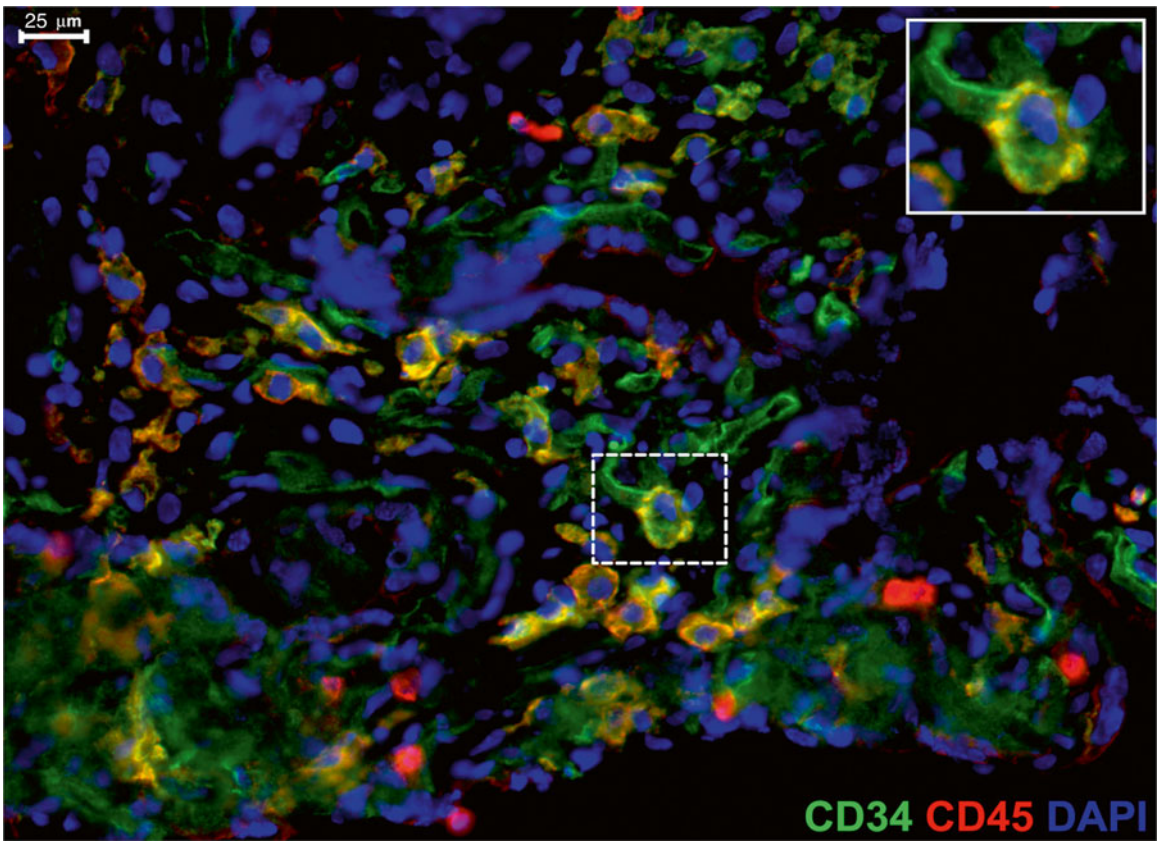


Fig. 15.5 *Immunolocalization of CD34⁺CD45⁺ cells in term placenta.* Tissue sections of a 38 weeks full-term placental villus (delivered by C-section) were subjected to double indirect immunolocalization to evaluate expression of CD34 (FITC) and CD45 (Rhodamine). Nuclei were stained with DAPI (blue). Double positive cells appear yellow. The photographs were taken at 40× magnification. *Inset* on the top right corner shows a magnified CD34⁺CD45⁺ cell anchored to a CD34⁺CD45⁻ endothelial cell. This figure was originally published in Barcena et al. [34]

We also investigated the anatomical localization or CD34⁺CD45⁺ cells in term placenta by immunolocalization studies. The results of these experiments could aid in designing future methods to improve their isolation. In Fig. 15.5 we show a representative experiment of immunostaining and fluorescent microscopy, where several CD34⁺CD45⁺ cells can be readily identified in fixed tissue sections of term placenta. The sensitivity of this technique does not allow us to distinguish between cells expressing variable levels of antigens, such as CD34⁺⁺ and CD34⁺ cells, as can be done by flow cytometry. Hence we cannot make a strict correlation between the data obtained by FACS versus the immunolocalization results. Nevertheless, the paucity of CD34⁺CD45⁺ cells detected by immunostaining is very much in line with the low frequency data obtained by FACS. Hematopoietic progenitors and HSCs are a sparse populations that in term placenta are mostly associated with endothelial cells (CD34⁺CD45⁻ cells) [34] lining the abundant vessels that vascularize the chorionic villi. Their contact with endothelium suggests that purification methods based on placental perfusion might be ideal to mobilize the majority of these cells, as discussed in the next section.

4 The Potential Contribution of the Placenta Towards UCB-HSC Transplantation

The idea of harvesting placental HSCs/progenitors to enhance the efficacy of UCB transplantation should be considered and integrated in the context of many other recent and successful efforts to boost the yield of UCB-HSCs and expand their use in treating patients. The main strategies that have been experimentally and clinically explored to improve the therapeutic potential of UCB-HSCs have focused on enhanced collection, ex vivo progenitor expansion and the potential of co-transplanting "accessory" mesenchymal stem cells (MSCs). We discuss the possible contribution of harvesting placental HSCs in light of these other efforts.

4.1 Enhanced Collection of UCB

Since the small numbers of HSCs contained in a single unit of UCB are one of the most critical factors that determine the

success of a transplant, we propose the harvest of placental blood as a relatively simple method to efficiently augment the number of $CD34^+CD45^+$ cells obtained. There is evidence showing that this strategy might be worth considering in addition to routine UCB harvest and banking. Bornstein et al. demonstrated that a modified collection of UCB, in which a single injection of 50 mL of heparinized saline was used to perfuse the placenta right after birth, resulted in a 20 % increased yield of HSC, evaluated by both flow cytometry (enumeration of the frequency of $CD34^+CD45^+CD133^+$ cells) as well as by functional colony-forming unit assays [43]. This method combined the standard venipuncture of the umbilical cord with a secondary placental perfusate. Important considerations were the lack of increased risk of maternal cell contamination and the observation that the bacterial contamination rate was identical to the risk followed by traditional UCB collection. In agreement with these findings are other reports describing augmented collection of HSCs by placental perfusion per se [44, 45] and by including in the perfusate HSC's mobilization agents such as AMD 3100, an antagonist to the CXCR4 receptor [20, 46, 47]. Moreover, our data on the isolation of placental precursors shows a clear population of hematopoietic cells that can be recovered by washing the placental tissue after UCB harvest (Fig. 15.3). All together, these data strengthen the notion that the term placenta is an untapped reservoir of HSCs and that we should explore improved methods to maximize the extraction of the majority of these cells in a timely manner right after birth.

4.2 Ex Vivo Expansion of UCB-HSCs

One of the utopias for researchers focused on human HSC transplantation is to be able to safely multiply the number of HSC in vitro without altering the "stem cell" state. Studies on ex vivo expansion of BM-HSCs have been performed for several decades and only recently have we begun to observe some success. It has been reported that the exposure of UCB-HSCs to the soluble notch ligand Delta1 for up to 14 days supports the expansion of short-term repopulating cells and an accelerated myeloid engraftment [48]. This protocol is currently being tested in clinical trials and early results indicated that the culture of UCB-HSCs with Delta1 producing cells that did not contribute to long-term hematopoiesis. Other investigators have shown that the culture of $CD34^+$ UCB cells in the presence of aryl hydrocarbon receptor antagonists promotes the expansion of HSCs that fully reconstituted transplanted immunodeficient mice [49]. A very recent report showed that a short exposure of UCB to 16,16 dimethyl prostaglandin E2 promotes accelerated neutrophil recovery and long-term engraftment in a phase I clinical trial [50].

Another interesting approach has been the expansion of UCB $CD34^+$ cells in the presence of stroma prior transplantation. The idea for this method is not novel [51], indeed, it has its origins in decades of study of in vitro hematopoietic culture systems employing mixed cell types such as "Dexter" long-term bone marrow cultures. Reports have shown that co-culturing one unit UCB $CD34^+$ cells with BM-derived MSC for 1 week, followed by 1 week of culture in the presence of cytokines only, had a positive effect on double UCB unit transplants, as indicated by faster neutrophil and platelet engraftment in patients compared with unmanipulated double UCB unit transplant [52]. In this regard, it might be worth considering that the placenta is an organ plenty rich in MSC, opening the possibility of ex vivo expansion of UCB $CD34^+$ cells on autologous placental MSCs that could be harvested, expanded, and banked at the time of UCB collection.

4.3 Co-transplantation of MSCs and UCB-HSCs

This approach has been studied by several groups in transplantation protocols used to treat children and adults in which HSCs were obtained from several sources, including BM, cytokine mobilized peripheral blood and UCB [53–56]. The source of MSCs also varied, from allogeneic to haploidentical (parental). Doses of infused MSC varied as well. However, despite the differences between the reports, the results obtained coincided in several aspects: the co-infusion of MCSs and HSCs is safe; myeloid engraftment is accelerated in comparison with HSCs infusion alone; there is less incidence of acute GVDH but not all the patients show chimerism from the MSCs donor. We believe that these promising results are just the beginning of many future clinical trials that will continue evaluating the therapeutic properties of MSCs. Moreover, as mentioned, the term placenta is a rich source of MSCs that have been shown to reside near the vascular niche [57], the same area where we and others have immunolocalize HSCs. The proof of principle that placental MSCs enhance single and double UCB engraftment has been shown co-transplanting these cells into immunodeficient mice [58]. These investigators demonstrated that the co-infusion of MSCs with UCB-HCSs increased engraftment by enhanced homing to the mouse BM and that the engrafted human cells were able to maintain self-renewal potential by serial transplantation. Another interesting observation that arose from this study was the decrease incidence in the dominance of one unit of UCB in double UCB transplants. Double UCB transplants often result in long-term engraftment by only one of the two transplanted units, possibly due to a graft versus graft phenomenon similar to GVHD. The co-transplantation of MSC might be sufficiently immunosuppressive, as evidenced by their potential to decrease GVHD risk, that they increase the chances of both UCB units to engraft without rejection.

Our interest in placental MSCs derives from our previous research on the nature of the cells that support HSCs in the extra-embryonic hematopoietic niche. We have observed the presence of stromal cells (vimentin$^+$) of the niche earlier in gestation (first and second trimester) and based on these observations, we have derived several MSC primary cultures from 5 weeks to term placenta (A. Bárcena, A. Ahmed, M.O. Muench et al., manuscript in preparation). We are currently investigating the properties of these cells regarding their ability to support HSC renewal in vitro and/or enhance reconstitution in vivo. The intimate relation of the placenta, in particular perivascular stromal cells and endothelial cells, with the fetal UCB suggests that the placental MSCs might be ideal "accessory" cells that could be obtained from the same donor as the UCB. We speculate that the contribution of the placenta to improving UCB HSC transplants could be twofold: to obtain more HSCs as well as autologous MSCs.

In conclusion, no matter whether the term placenta retains the fetal potential to engender HSCs or if it just provides temporary residence for these precious cells in its vast vascular network, we believe that there are many valid arguments to seek a safe, efficient, reliable and clinically applicable technique to retrieve placental HSCs immediately after birth. Furthermore, we think that the simultaneous harvest of placental HSCs and MSCs might hold the key for significantly improving UCB transplantation. Studying these possibilities could change how UCB banks operate, as well as increase operation costs, but more importantly, it might facilitate an unprecedented expansion in the use of neonatal HSCs to perform life-saving transplants.

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Placental Stem Cells for Cartilage Tissue Engineering

16

Hsi-Yi Yeh, Betty Linju Yen, and Shan-hui Hsu

Abstract

Preparation of functional biocartilage substitutes in vitro for repairing cartilage—a tissue with poor self-healing capacity—is an attractive therapeutic approach with the recent advances in tissue engineering. Human placenta-derived mesenchymal stem cells (PMSCs) represent a promising source of stem cells, but the application of these fetal-stage stem cells for cartilage tissue engineering, however, has only been minimally explored. Within the literature, there are diverse opinions regarding the chondrogenic differentiation capacity of PMSCs. In this chapter, we review the research findings currently in the literature to clarify the potential use of PMSCs in cartilage repair. It has been reported that PMSCs displayed limited chondrogenic differentiation capacity in traditional pellet culture or when grown on regular tissue culture polystyrene dish. Conversely, when PMSCs were incorporated into 3D scaffolds, superior neocartilage formation was observed in vitro as well as in vivo, with results superior than using bone marrow MSCs. The potential of human PMSCs as a candidate cell source for cartilage tissue engineering was further supported by several recent studies, which also confirmed the essential role of 3D scaffolds for PMSCs to promote cartilage repair.

1 Cartilage Repair

Cartilage is a type of connective tissue composed of chondrocytes, extracellular matrix (ECM), and water, which can be found in many areas in the body of human and other animals. Based on the differences in molecular components of the ECM, the location, and specific function, cartilage can further fall into one of three major categories: hyaline cartilage, elastic cartilage, and fibrocartilage [1, 2]. Hyaline cartilage covers the surface of many joints with a white glassy appearance. The ECM is composed of type II collagen and proteoglycans, which have a protein core associated with glycosaminoglycan such as hyaluronic acid (HA). Elastic cartilage is found in the airway and the external ear and is distinguished by the presence of elastin in the ECM. Fibrocartilage exists in the intervertebral discs, temporomandibular joint disc, and knee meniscus. Unlike the hyaline cartilage, fibrocartilage has a higher proportion of type I collagen in the matrix. The photomicrograph of normal articular cartilage is shown in Fig. 16.1. There are no blood vessels in the tissue, and chondrocytes are the only cell type trapped within the ECM matrix. The cells are contained in spaces called lacunae and represent only 1–2 % of cartilage volume. The essential nutrients for chondrocytes are supplied by diffusion from the synovial fluid [1, 2].

Articular cartilage is referred to the hyaline cartilage on the articular surface of bones, which can bear a force up to six times the body weight and a stress approaching 10 MPa.

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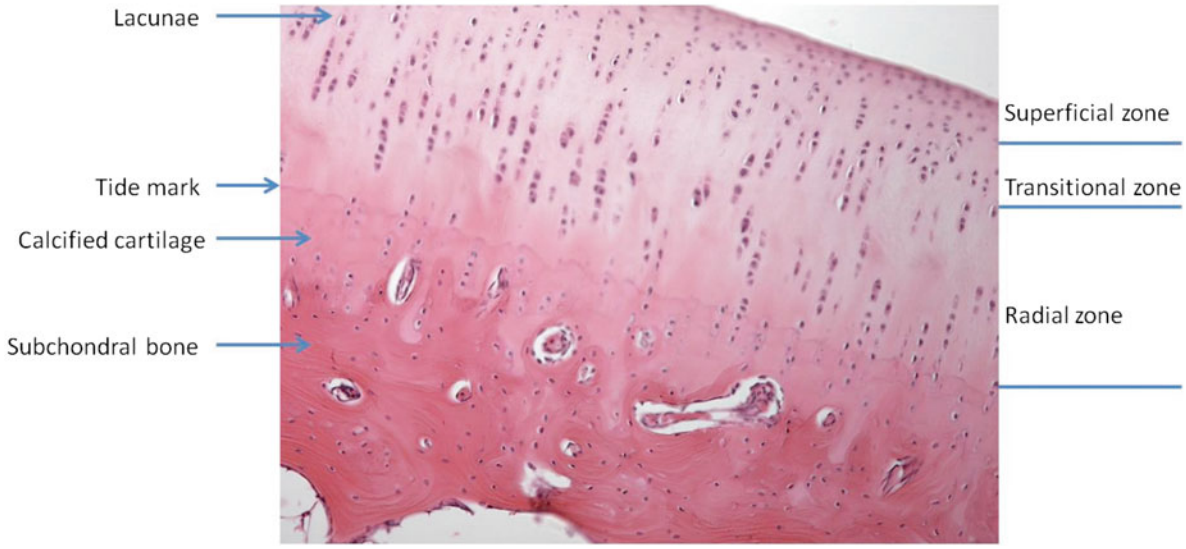


Fig. 16.1 Photomicrograph of a typical articular cartilage stained by hematoxylin and eosin staining. Articular cartilage can be subdivided into different zones from the cartilage surface to subchondral bone. In the superficial zone, collagen fibers are parallel arranged to cartilage surface and thus resistant to shear force. On the other hand, the radial zone can resist to compression, in which collagen fibers are vertical to the surface, and cells are arranged in columns. This zone contains the largest-diameter collagen fiber and the highest concentration of proteoglycans. Calcified cartilage acts as an anchor between articular cartilage and subchondral bone

Furthermore, the kinetic coefficient of friction for cartilage is less than 0.005 [2]. Even so, articular cartilage is frequently damaged by sport injury, trauma, or overuse. After damage, the cartilage has a limited ability for intrinsic repair because of its avascular and nearly acellular nature, which limits the ability of progenitor cells from the blood or bone marrow, and the resident articular chondrocytes that are entrapped within the surrounding matrix to migrate toward the injured tissue to secret the reparative matrix.

Traditional treatments for cartilage repair include allografts and autografts. There are many limitations for applications of cartilage grafts including donor's availability, donor site injury, scarring, and pain. Allografts have a high risk of infection. In addition, the difficulty for implanted materials to integrate into the host tissue and the loss of cell viability due to graft storage often reduces the lifetime of the grafts. Autografts also suffer from the problem with integration and require additional defects to be created in the patients' remaining cartilaginous tissue [1].

2 Tissue Engineering for Cartilage Repair Is an Open Challenge

Since cartilage has relatively poor self-healing capacity, preparation of functional biocartilage substitutes in vitro for cartilage repair is an attractive concept with the recent advances in tissue engineering. Tissue-engineering aims to combine three-dimensional (3D) scaffolds, cells, and bioactive factors to generate in vitro functional constructs for implantation. In the literature, regenerated cartilage can be derived from various cell types, including chondrocytes and mesenchymal stem cells (MSCs). Autologous chondrocyte implantation (ACI) is a cell-based therapy for repairing cartilage defects. Cartilage is taken from the lesser weight-bearing area of the bone interface. After removing the matrix, chondrocytes are isolated and cultured in vitro to obtain a large number of cells for transplantation. These fresh cells are harvested and injected on the damaged area in combination with a biomembrane to aid cell adhesion or preseeded in a scaffold matrix. Although autologous chondrocytes can reduce the risk of immune rejection, these are expanded cells and have a tendency to lose their phenotype in vitro [1, 3]. On the other hand, MSCs have the capacities for self-renewal, stemness maintenance, and the potential to differentiate into a variety of connective tissues including cartilage, bone, fat, tendon, and others. Therefore, MSCs have been extensively used as a cell source for cartilage regeneration [1, 4]. Recently, there are several kinds of strategies associated with the use of MSCs for cell-based therapies [5]. The first strategy is to inject the MSCs immediately into the damaged tissue where these donor cells are expected to participate in replacing the host cells. The second approach is to incorporate MSCs into 3D scaffolds to replace the damaged tissue. In the third concept, MSCs are considered as supporting cells that provide growth factors or cytokines to stimulate the reparative events in vivo [5].

A systematic review has examined the available clinical evidence to support the various MSC treatment strategies used in orthopedics for cartilage defect regeneration, based

on the literature in PubMed database from 2002 to 2012 [6]. This systematic review showed an increasing number of published studies on this topic over time and identified 72 preclinical papers and 18 clinical trials. Among these, 50 preclinical studies and 11 clinical trials are associated with the use of cultured bone marrow-derived MSCs (BMSCs) [6]. BMSCs are the first identified and most studied adult MSCs and are commonly used as the cell source in many tissue-engineering fields. Owing to their well-characterized properties and differentiation potential, BMSCs are regarded as the "gold standard," especially in cartilage and bone regeneration [7]. However, MSCs account for only 0.01–0.001 % of the nucleated cells in the adult human bone marrow [4]. Harvesting BMSCs is a painful and invasive procedure and may lead to complications. In addition, the cell number and differentiation capacities of human BMSCs are decreased with age [5, 8]. Hence, the use of autologous BMSCs for elderly patient in cartilage repair is significantly hindered.

3 Placental Stem Cells for Cartilage Tissue Engineering

More recently, MSCs have been isolated from extraembryonic tissues including amniotic fluid, umbilical cord, and placental. Because of the perinatal origin of these tissues, these MSCs are considered to be more primitive and could potentially be candidate cell sources for cartilage and bone regeneration [5]. The multilineage differentiation capacities of placenta-derived MSCs (PMSCs) have been reported, and lineages include osteogenesis, chondrogenesis, adipogenesis, neurogenesis, and endothelialization [9–13]. In addition, the immunosuppressive properties of PMSCs have been demonstrated to be significantly stronger than that of BMSCs, which further supports the consideration of PMSCs as a clinically viable source for cell therapy [14]. However, the number of studies associated with the application of human PMSCs (hPMSCs) in cartilage regeneration is rather limited when compared with those of BMSCs and umbilical cord-derived MSCs (UCMSCs).

The first study on evaluating the chondrogenic differentiation ability of hPMSCs was reported by Zhang et al. [13]. hPMSCs isolated from chorionic villi were pelleted into micromass and the chondrogenesis was induced by serum-free medium in the presence of BMP-2, TGF-β3, and dexamethasone. The pellets of cultured PMSC were similar in size and weight as compared with those of BMSC after 3 weeks of induction. The upregulation of cartilage-specific genes, including SOX9, COL2A1, aggrecan, COL10A1, BMP-2, and BMP-6, was detected in the pellets after induction, indicating that PMSCs had the potential for chondrogenic differentiation. To further examine the chondrogenic differentiation of PMSCs, cells were cultured in atelocollagen gel with the chondrogenic medium. The cell-atelocollagen gel became white, glistening, and harder than the original gel after 3 weeks of culture. Metachromatic territorial matrices were observed in the gel by the staining of toluidine blue, and cells with lacuna formation were also examined in the cartilage-like matrix. Moreover, PMSCs were seeded in the collagen sponges and implanted into the osteochondral defects of nude rats in vivo. Histological analyses showed that the reparative tissue filled the defect with cartilage-like surface and closely adhered to the residual part of the bone. Although the formation of hypertrophic repair cartilage was also observed at the top part of newly formed tissue, the bottom one had the strongest metachromasial staining and round cells, which indicated the formation of hyaline cartilage by PMSCs. The results of this study are encouraging and suggest that PMSCs can be one of the possible allogeneic cell sources for cartilage tissue engineering.

There are several studies that compared the chondrogenic potential of PMSCs with MSCs isolated from the other tissues [15–17]. In one study, MSCs from fetal and adult tissues were phenotypically characterized and examined for their differentiation capacities, based on morphological criteria and the expression of ECM components [15]. Fetal lung-derived MSCs (FLMSCs) and PMSCs showed a more elongated and thinner shape compared with the rounder and thicker shape of BMSCs as cultured on the tissue cultured polystyrene surface. The chondrogenic differentiation potential of MSCs was evaluated in pellet culture by induction with TGF-β3, BMP-6, and dexamethasone. Although MSCs from these four different sources could all form a pellet after centrifugation, the pellets of FLMSCs and PMSCs were frequently less stable and showed a more irregular shape compared with the pellets from fetal bone marrow (fBM) and adult bone marrow (aBM) MSCs that were firm and spherical. In addition, MSCs of bone marrow origin, either fetal or adult, exhibit better chondrogenesis than FLMSCs and PMSCs, judging by the appearance of typical morphological features of cartilage, the intensity of toluidine blue staining, and the expression of collagen type II, IX, and X after being cultured under chondrogenic conditions.

In another study, the morphology, immunophenotype, and differentiation patterns of MSCs isolated from the bone marrow, umbilical cord blood (UCB), and placenta were compared [16]. Three morphologically distinguishable cell types were indentified in cultures from BM ($n=9$). The most common type observed was fibroblastoid cells, corresponding to 83 ± 7 % of all cells present in culture. In cultures of UCB-MSCs ($n=11$) and PMSCs ($n=5$), the same three morphologically distinguishable cell types were observed, and fibroblastoid cells remained the most frequent (85 ± 6 % and 86 ± 5 %, respectively). Although the morphology was similar, a distinct type of cartilage formation was observed in pelleted micromasses from BMSCs and PMSCs compared

with UCB-MSCs as evidenced by alcian blue staining after chondrogenic induction for 4 weeks. It was observed that in pelleted micromasses from BMSC and PMSC samples, cells grouped into small clusters located in the same area, a feature typical of hyaline cartilage. In contrast, pelleted micromasses from UCB-MSC showed minimal matrix formation and a predominance of fibrous collagen deposition, which indicates the presence of fibrocartilage formation.

In an attempt to establish the relative potential of alternative MSCs sources, Cavallo et al. analyzed and compared the yield and differentiation potential of human MSCs from adipose tissue (AT) and BM tissues of cadaveric origins, and from fetal annexes (placenta and umbilical cord) after delivery using standardized isolation and culture protocols [17]. After MSC selection through tissue culture plastic adhesion, cells displayed a comparable proliferation capacity with distinct colony morphologies and were positive for a pool of typical MSCs markers. To successfully isolated MSCs through adherence selection, a minimum cell density of 1×10^5 cells/cm^2 was necessary for mononuclear cells (MNCs) derived from AT, amniotic, and chorionic membranes of placenta. As for the MNCs from the mucous connective tissue of UC called Wharton's Jelly, the cell density had to be doubled to achieve MSC colony formation. On the other hand, the minimum cell density required to isolate MSCs from the BM was 5×10^5 cells/cm^2, thus implying a relative lower amount of MSCs present in BM and UC compared to AT, amniotic and chorionic membranes. Moreover, MSCs from fetal annexes had the tendency to form better-defined colonies than MSCs from BM or AT. In particular, MSCs from the chorionic membrane and in part MSCs derived from the Wharton's Jelly seemed to continuously grow in confined colonies, suggesting that these cells may have a more primitive phenotype and a higher potency likely due to developmental-age differences. In the in vitro differentiation assay, cells were plated in six well plates at a density of 1×10^5 cells/cm^2 and induced by osteogenic or chondrogenic medium. Results showed a higher osteogenic differentiation capacity of AT- and BM-derived MSCs, and a higher chondrogenic differentiation capacity of BM-derived MSCs. The expression of chondrogenic marker genes (e.g., SOX9, aggrecan, and type II collagen) for MSCs isolated from amniotic or chorionic membranes of placenta was significantly lower than those of BM-derived MSCs.

4 The Chondrogenic Potential of PMSCs in 3D Scaffolds

Based on the existing literature, PMSCs have multilineage differentiation ability, but display limited chondrogenic capacity compared with that of BMSCs while pellet cultured or seeded on tissue cultured polystyrene surface (Fig. 16.2). Although the results may be disappointing, it may also indicate that PMSCs are more sensitive to the culture environment and less able to withstand the forced aggregation or the two-dimensional (2D) adherent culture system as compared with BMSCs.

Therefore more recently, a few groups tried to incorporate PMSCs into 3D scaffolds and evaluated the potential to regenerate cartilage [18–20]. Encouraging results were revealed in these recent studies (Fig. 16.3). Some of these studies have been described by Longo et al. [5]. Among the encouraging work, Liu et al. reported the high passages of PMSCs available for chondrogenesis in vitro [18]. In their study, PMSCs were isolated by trypsinization and further induced into cartilage cells in vitro. The tissue-engineered cartilage was constructed by combining PMSCs with collagen sponges, and cartilage formation was further confirmed by implantation into nude mice. Their results showed that PMSCs featured MSC phenotype and maintained proliferation in vitro for over 30 passages while remaining undifferentiated. Cells from all passages have the potential to differentiate into functional cartilage cells in vitro and in vivo when combined with collagen sponges, which provided experimental evidence for prospective clinical applications.

Another 3D culture system that promoted the chondrogenesis of hPMSCs was established by Hsu et al. [19],

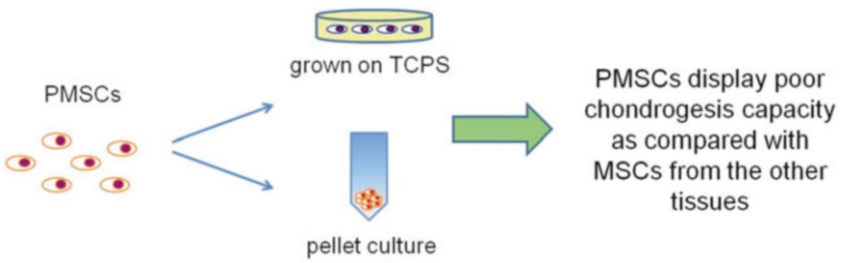


Fig. 16.2 Traditional culture of PMSCs for chondrogenic induction. PMSCs displayed poor chondrogenic differentiation potential in pellet culture or when grown on 2D tissue cultured polystyrene surface as compared with BMSCs or MSCs from the other tissues. It may imply that PMSCs are more sensitive to the culture environment and less able to withstand the forced aggregation or the two-dimensional (2D) adherent culture system

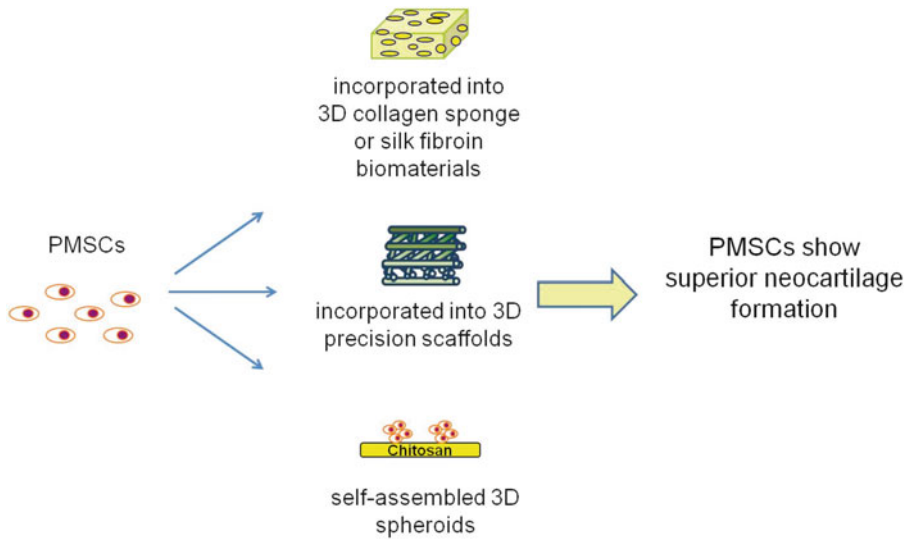


Fig. 16.3 Several encouraging studies associated with PMSCs in cartilage repair. Encouraging results were revealed in some recent studies. It was noted that when PMSCs were incorporated into 3D scaffolds, significant neocartilage formation was observed in vitro as well as in vivo, which was even more efficient than that based on BMSCs. Formation of self-assembled 3D spheroids of PMSCs on chitosan-based substrates was also shown to enhance the self-renewal properties and increase the chondrogenic differentiation capacity of PMSCs for cartilage tissue engineering. The promise of human PMSCs as a candidate cell source for cartilage tissue engineering was evidenced by these recent studies, which also pointed out the essential role of 3D scaffolds and substrate materials for PMSCs to promote cartilage repair

in which PMSCs were grown in a 3D precision scaffold for cartilage tissue formation in vitro. To select proper scaffolds for 3D culture of MSCs, AT-derived MSCs were initially employed to optimize the composition and condition of the 3D environment. Poly(D,L-lactide-*co*-glycolide) (PLGA) precision scaffolds made by solid freeform fabrication [stack angles of 4D (0°/90°/45°/135°), fiber diameter of 0.2 mm, and interval between adjacent fibers (d_h) about 0.7 mm] with a porosity approximately 50 % were employed. It was observed that cells embedded in alginate gel and seeded in precision scaffolds form cartilage-like tissue. This study also suggested that the inclusion of nano-sized calcium-deficient hydroxyapatite (nCDHA) and/or a recombinant protein containing arginine–glycine–aspartate (RGD) into the alginate gel enhanced chondrogenesis for both AT-derived MSCs and hPMSCs, and the maximal promotion occurred when both nCDHA and RGD were added in the system. The amount of ECM, such as glycosaminoglycan and type II collagen accumulated, was found to be high. Histological analyses revealed lacunae formation with ECM stained by alcian blue and type II collagen. Comparing hBMSCs and hPMSCs grown in the same scaffolds, the secretion of glycosaminoglycan from hPMSCs was twice higher than that from hBMSCs. Therefore, the gel/precision scaffold system could provide a 3D environment for the chondrogenesis of hPMSCs and offer dimensional stability to generate tissue-engineering constructs. This study also suggested that hPMSCs, when grown in a suitable scaffold, may be a better cellular source for building up the tissue-engineered cartilage.

The ability of PMSCs to grow on silk fibroin (SF) was demonstrated by Li et al. [20]. hPMSCs were maintained in vitro in an allogeneic mixed lymphocyte reaction (MLR) system to investigate the suppressive effects on T-cell proliferation. Results showed that the proliferation of T lymphocytes was inhibited to different extents by adding various ratios of hPMSCs to human or rabbit peripheral blood mononuclear cells (PBMCs), with the inhibition rate increased as the ratio of PMSC to PBMC increased. The inhibitory effect was extremely high at a 1:5 and 1:10 ratio of PMSCs to human PBMCs, and at a 1:20 ratio of PMSCs to rabbit PBMCs. These results indicated that hPMSCs could inhibit rabbit T-cell responses in the xenogeneic system, which was consistent with the earlier findings that PMSCs had superior immunosuppressive properties [14]. SF serving as a delivery vehicle for hPMSCs was then evaluated in a rabbit articular cartilage defects model. Rabbits were implanted with a PMSCs/SF complex after articular cartilage defect of the femoral condyle in the knee. The repair of the articular cartilage defect was observed after 4 weeks, 8 weeks, and 12 weeks. The grafts were well incorporated with the surrounding native cartilage. After 4 weeks, the defect was filled with semitransparent tissues. In the 8th week, the appearance of recovered tissues was as smooth as the normal cartilage tissues, and the boundary was obscured. In the 12th week, the color and luster of the recovered tissues were similar to the surrounding normal cartilage. Histological analyses revealed that the cartilage was highly cellular, with round-to-oval lacunae in binucleate and single forms. Inflammation and

foreign-body reaction were not noted. These results suggested that knee damage was repaired by the newly formed hyaline cartilage, and within 12 weeks there was neither degeneration nor infiltration with lymphocytes or leukocytes, and no residue of SF was detected. This study demonstrates that implantation of hPMSCs in SF scaffolds can regenerate the articular cartilage defects in rabbits.

Aside from the use of dispersed PMSCs, Huang et al. reported that the formation of PMSC spheroids self-assembled from PMSCs grown on chitosan-based substrates [21]. hPMSCs formed 3D spheroids on these substrates faster than AT-derived MSCs. Spheroids formation helped to maintain the expression of stemness genes (OCT4, SOX2, and NANOG) for both PMSCs and AT-derived MSCs, and more significantly for PMSCs. Blocking CD44 by neutralizing antibodies prevented the spheroid formation and decreased stemness gene expression profile moderately; while treatment by Y-27632 compound inhibited the spheroid formation and significantly decreased the stemness gene expression profile. After chondrogenic induction, the spheroids showed remarkable upregulation of SOX9, aggrecan, and collagen type II in terms of gene expression and were stained positive for glycosaminoglycan and collagen type II. Using this method, hPMSCs showed better chondrogenic differentiation potential than AT-derived MSCs upon induction. This study suggests that formation of self-assembled 3D spheroids of PMSCs may further enhance their self-renewal properties and increase the chondrogenic differentiation capacity for cartilage tissue engineering. This study also suggests that the substrate material may play a partial role in maintaining stemness and enhancing the differentiation capacity of PMSCs, by regulating the Rho/Rho-associated kinase (ROCK) signaling pathway and CD44 interaction.

Regarding genetic modification of PMSCs, Zhang et al. attempted to prolong the life span of hPMSCs by modifying hTERT and Bmi-1, and investigated whether these modified hPMSCs retained their differentiation capacity and multipotency [22]. The results indicated that the combination of hTERT and Bmi-1 was highly efficient in prolonging the life span of hPMSCs with differentiation capability to osteogenic, adipogenic, and chondrogenic cells in vitro. The immortalized cells may be a useful tool for further studies using PMSCs in tissue engineering, including cartilage repair.

5 Future Perspectives

hPMSCs represent a promising source of stem cells. Their application in cartilage tissue engineering is just beginning to be explored. Descriptions about the chondrogenic differentiation capacity of PMSCs vs. the other MSCs are highly varied in literature. It is generally noted that PMSCs have limited chondrogenic differentiation capacity in pellet culture or when grown on tissue cultured polystyrene surface. On the other hand, when PMSCs are incorporated into 3D scaffolds, they appear to be superior to the other types of MSCs in chondrogenesis. There remains some design problems for scaffolds in cartilage engineering [2, 23]. Matching the compressive mechanical properties between scaffolds and natural/endogenous cartilage may be crucial, as stiff scaffolds can shield the mechanosensitive cells from the normal loading, and soft scaffolds may fail upon implantation. Moreover, scaffolds must possess sufficient surface and tensile properties for functioning in the high shear joint environment. If lacking in these properties, the less appropriate scaffold may lead to the rubbing of the opposing or adjacent cartilage. In addition, most scaffolds may promote cell spreading, which encourages fibrous matrix production [2]. Scaffold materials may affect the morphology and phenotype of MSCs through the cell–substrate interaction. Embedding MSCs in a hydrogel before seeding may help to maintain the favorable morphology and phenotype for cells in a scaffold [19, 23].

In conclusion, hPMSCs increasingly appear to be a candidate stem cell source for cartilage tissue engineering, which has recently been demonstrated by several reports in the literature. The necessity of a 3D scaffold for PMSCs to promote well cartilage repair is also indicated. Optimization of the scaffold materials may be critical for PMSCs to be considered for therapeutic use in cartilage tissue engineering, and further investigations toward this goal is urgently warranted.

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Mesenchymal Stem Cells from Human Amniotic Membrane

17

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Abstract

The human amnion or amniotic membrane (HAM) has emerged as a novel and alternative source of stem cells. This tissue presents a reservoir of two types of stem cells from different embryological origins: human amniotic mesenchymal stem cells (hAMSCs), derived from the mesodermal germ layer, and human amniotic epithelial cells (hAECs), derived from the ectodermal germ layer. Both types of cells are different in disposition and morphology but have similar phenotypic characterization. hAMSCs are easily to isolate and can be expanded in vitro for several passages, obtaining a large amount of cells. Properties such as to be immune-privileged, antimicrobial, and antitumorogenic make themselves suitable for transplantation, cell therapy, and regenerative medicine. These cells were widely studied due to these properties and to their pluripotent capacity, being a promising clinically tool.

1 Localization of Mesenchymal Stem Cells from Human Amniotic Membrane

The amniotic sac is a resistant but thin pair of membranes that sustains the developing fetus until birth. These two membranes are: the amnion, the inner membrane which contains the amniotic fluid and the fetus, and the chorion, the outer membrane which wrap the amnion [1], separated by an intermediate layer (Fig. 17.1).

Amnion comprises three layers (Fig. 17.1): uniformly organized, a single layer of epithelium derived from ectoderm [2], the basement membrane and a mesenchymal layer. The mesenchymal layer also can be subdivided into the compact layer, the fibroblast layer, and an intermediate layer (zona spongiosa) [3].

The HAM protects against infections, traumas, and toxins. It acts as a filter and a preventive shock [4] because of their mechanical properties. It has no nerves, muscles or lymph vessels [5] and is strong, elastic, transparent, and semipermeable. It contains different types of collagens (I, III, IV, V, and VI), which provide resistance to rupture [6] and, also contains fibronectin, nidogen, laminin, proteoglycans and hyaluronan, as well as growth factors [3, 7, 8].

HAM could to be suitable for allotransplantation and regenerative medicine. There are several advantages which make it suitable: procurement of a high cell number, easily to obtain because HAM is usually discarded after parturition, and their use is within the legal and ethical framework. Also, HAM reduces pain and inflammation, inhibits scarring and shows little or no immunogenicity. It seems to be immune-privileged, and possess antimicrobial, antitumorigenic, antifibrotic and

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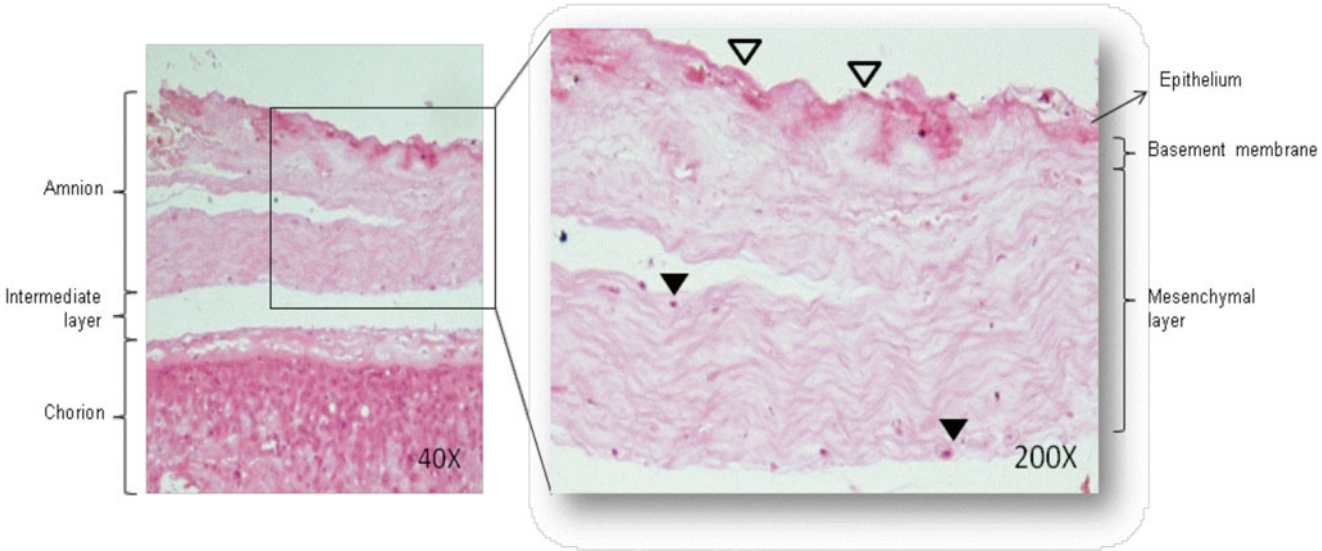


Fig. 17.1 Cross-section of the amniotic sac stained with hematoxylin–eosin. On the left hand, subdivisions of amniotic sac: amnion, intermediate layer, and chorion (magnification ×40). On the right hand, subsections of amnion: epithelium, basement membrane, and mesenchymal layer (magnification ×200)

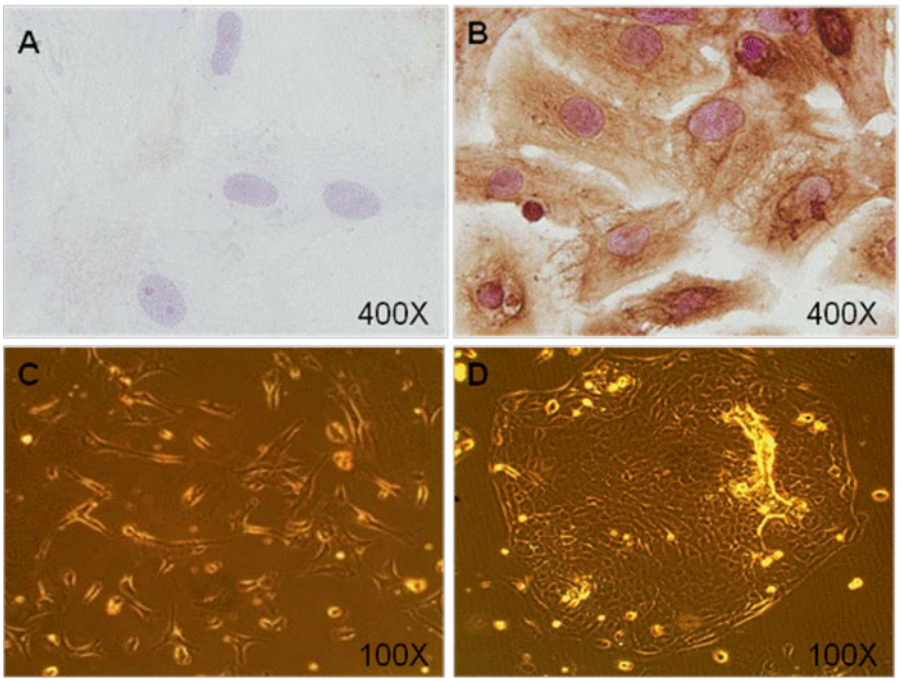


Fig. 17.2 Identity and morphology of HAM-isolated cells. (**a**) Human amniotic mesenchymal stem cells (hAMSCs) were negative for cytokeratin immunostaining. (**b**) hAECs showed positivity for cytokeratin immunostaining (magnification ×400). (**c**) Morphology of cultured hAMSCs isolated from amniotic stroma. (**d**) Morphology of cultured hAECs isolated from amniotic epithelium (magnification ×100)

antiangiogenic characteristics. It does not represent transplantation risk, it enhances wound healing and epithelialization and acts as an anatomical and functional barrier [9–14].

It has been suggested that the amniotic membrane may retain a reservoir of stem cells throughout pregnancy coming from embryonic epiblast cells, prior to gastrulation [15].

HAM contains two different cell types (Fig. 17.2): human amniotic epithelial cells (hAECs) and human amniotic mesenchymal stem cells (hAMSCs), from different embryological origins [16, 17]. Both populations show similar cell surface receptor expression pattern but many differences with regard to cell shape (Fig. 17.2c, d) and cell localization (Fig. 17.1)

[18, 19]. hAECs are located in the epithelium layer (Fig. 17.1, empty arrowhead) and hAMSCs in the mesenchymal layer (Fig. 17.1, arrowhead) [20].

Using immunohistochemical stains we can distinguish the identity of both the cell types (Fig. 17.2a, b), because cytokeratins are epithelial markers only expressed on cultured hAECs but not on mesenchymal ones [21]. hAECs forms a continuous monolayer of epithelium derived from embryonic ectoderm, in contact with the amniotic fluid (Fig. 17.1) [22]. hAMSCs, which underlies amnion epithelium [19], are cells sparsely distributed, derived from embryonic mesoderm (Fig. 17.1) [22].

2 Isolation and Culture of Mesenchymal Stem Cells from Human Amniotic Membrane

Both hAECs and hAMSCs can be isolated easily from the epithelial and mesenchymal regions of the amnion. Different methods to isolate HAM-derived cells have been published [14, 16, 20, 23–25].

First of all, the amniotic sac is obtained from scheduled cesareans, avoiding contamination through the birth canal. Then, the amniotic membrane is separated mechanically from the chorion, through the spongy layer [26] and washed to remove the red blood cells. All protocols are based only in the use of digestive enzymes to separate the two cell types [14, 16, 23–25], except the Barbati et al.'s protocol [20], which introduce a second mechanical separation (epithelium from mesenchymal layer) to avoid the cross-contamination between cells. Some of these authors [14, 16, 25] suggest a first trypsin digestion to release hAECs from the HAM and a subsequent incubation to obtain the hAMSCs, using other digestive enzymes such as dispase, trypsin and/or collagenase, alone or combined with DNAse. However, Bacenkova et al. and Soncini et al. [23, 24] isolated first the hAMSCs with those digestive enzymes and then isolated hAECs through a trypsin digestion (Fig. 17.3). These protocols avoid the reduction of hAMSCs in the quantitative cellular yield at isolation and the contamination of hAECs with hAMSCs [20].

Diaz-Prado et al. [27] compared two previously published protocols [16, 23] for the isolation of hAMSCs, with different digestive enzymes and digestion in different periods of time.

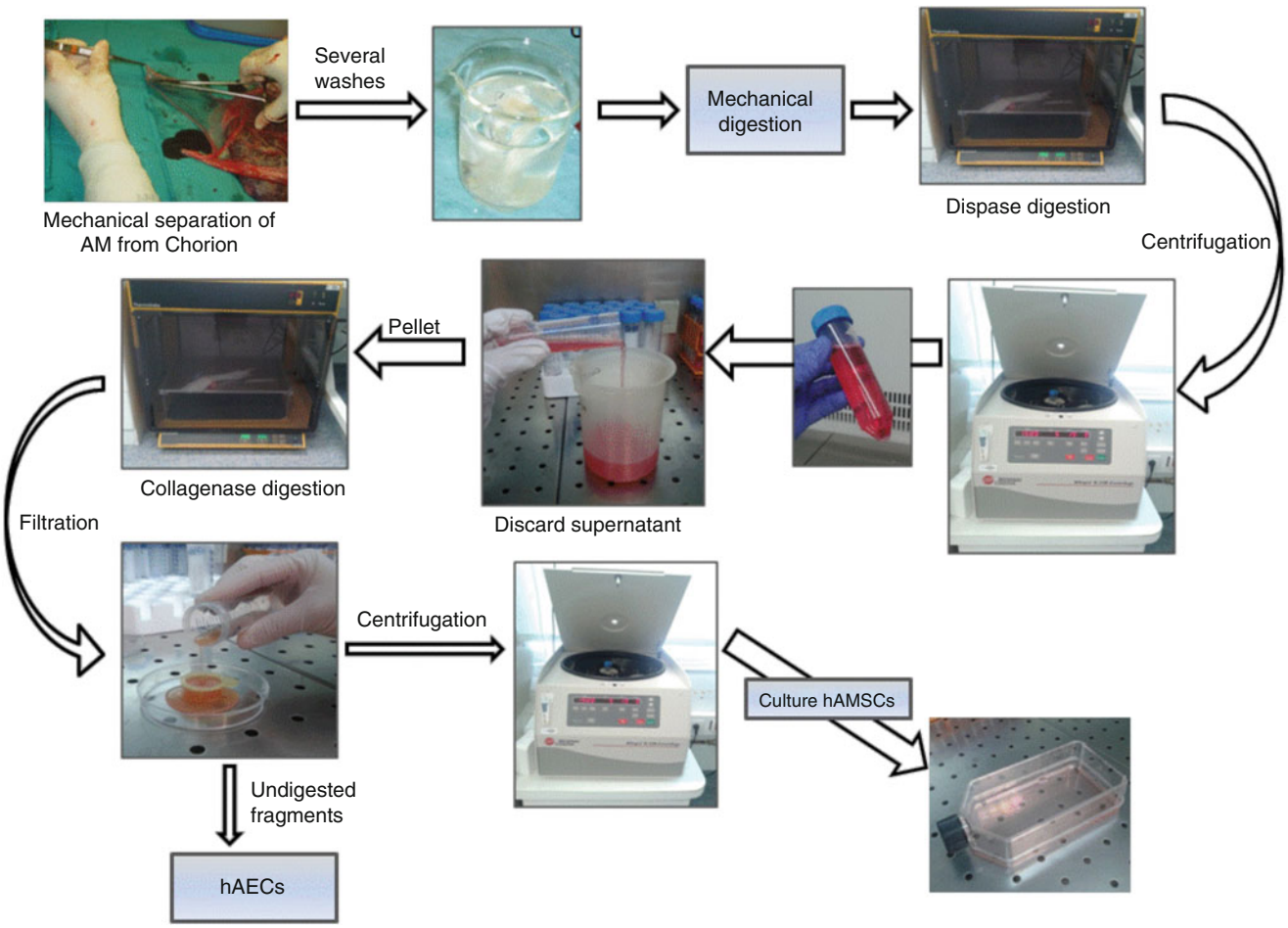


Fig. 17.3 Schematic representation of hAMSCs isolation, based on Soncini et al. [23] and Bacenkova et al. [24] protocols

This quantitative study showed that with Soncini's protocol it was obtained an increase in the hAMSCs isolation yield with regard to Alviano's protocol. Also, the former protocol allowed the isolation and expansion in a very short time of a larger number of cells. Procurement of cells in a ready and rapid availability is one requirement of a source of MSCs for it to be considered for cell transplantation.

HAM isolated cells can be grown in Dulbecco's modified Eagle's media (DMEM) or a similar culture medium (e.g., DMEM:F12), supplemented with fetal bovine serum (FBS) or fetal calf serum (FCS), glutamine and antibiotic–antimycotic, and seeded into culture flasks. Both populations should be expanded at 37 °C, in a humidified 5 % CO_2 atmosphere. Although these culture mediums are widely used in basic research, for clinical applications it is necessary to have the culture and expansion under xenobiotic-free conditions for a good manufacturing practice, which could lead to differences in expression markers, capacity to differentiate and other changes in their characteristics [28].

There is a contradiction with the passage number at which hAMSCs suffer changes. Some authors [19] founded that their proliferation stops beyond passage 5, others could expanded them in vitro at least 15 passages [16, 23]. Although phenotype of hAMSCs seems to be maintained from passage 0 to passage 9 [18], Fatimah et al. [25] showed that after serial passages, stemness gene expression decreases. However, Bailo et al. [17, 29] agreed that hAMSC's immune-inhibitory properties were not affected by serial passaging.

3 Characterization of Mesenchymal Stem Cells from Human Amniotic Membrane

The International Society for Cellular Therapy proposed three criteria to define all types of stem cells: self-renewal, multipotency, and the ability to reconstitute in vivo a tissue. Because the absence of specific markers for MSCs, there are some additional requirements for their identification, which include: adherence to culture flask plastic, fibroblast-like morphology, prolonged capacity for proliferation, the capacity to differentiate in vitro into cells of mesodermal lineage, expression of CD90, CD73, CD105 and do not express CD45, CD34, CD14 or HLA-DR [30].

The properties which make the amnion successful for transplantation and regenerative medicine are given by the inherent cell characteristics:

(a) Amnion cells seem to be immune-privileged [12, 14]. They express at a very low level HLA A-B-C, corresponding to class I of the major histocompatibility complex (MHC), and do not express the HLA-DR and HLA-DQ, corresponding to class II of the MHC [3, 15, 17, 21, 26, 31]. Furthermore, amnion cells secrete HLA-G (class I MHC) and Fas ligand [26, 32], immunosuppressive factors. They have also been shown to inhibit the lymphocyte proliferation response [17, 29].
(b) HAM-isolated cells secrete interleukin (IL)-1 receptor antagonist, activin A, tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) and IL-10, antiangiogenic and anti-inflammatory proteins which are deposited within the amniotic membrane stroma [13].
(c) The antimicrobial nature of HAM is provided by cell expression of β-defensins and secretory leukocyte protease inhibitor (SLPI) [3, 33].

hAMSCs show plastic adherence and fibroblast-like growth usually observed in MSCs from bone marrow. It is possible to obtain a population of adherent mesenchymal cells with a fibroblastic-like morphology, after 3–4 weeks of culture (Fig. 17.2). Meanwhile, hAECs are small-size cells which exhibit cuboid morphology (typical epithelial) and grow in culture into a tightly packed monolayer [34].

Studies of transmission electron microscopy showed that hAMSCs present dispersed mitochondria, glycogen lakes, and stacks of rough endoplasmic reticulum cisternae. Characteristics of higher specialization were absent, such as the presence of assembled contractile filaments, prominence of endocytotic traffic, and junctional communications [31].

Immunophenotypic characterization of hAMSCs demonstrates the presence of the common and well-defined human MSC markers (Fig. 17.4) (CD90, CD44, CD73, CD166, CD105, and CD29), described for bone marrow. It also demonstrates the absence of the hematopoietic markers (CD34 and CD45) and monocyte (CD114), macrophage (CD11) and fibroblast markers [26, 35, 36]. Other markers present in hAMSCs are CD13, CD10, CD49c, CD49d, CD49e, CD54, CD271 low, CD349, CD140, CD324, and E-cadherin [21].

hAMSCs express specific transcription factors for pluripotential stem cells such as Oct4 (octamer binding protein 4), NANOG, SOX2 (SRY-related HMG-box gene 2) and REX-1 [21, 37–39]. They may be considered as superior in their differentiation and proliferation capacity to adult MSCs due to their higher OCT4 mRNA levels [16]. These cells are also positive for embryonic stem cell markers as stage-specific embryonic antigen 3 and 4 (SSEA-3, SSEA-4), tumor rejection antigen (TRA)-1-60 and TRA-1-80 [3].

4 Multilineage Differentiation Potential of Mesenchymal Stem Cells from Human Amniotic Membrane

Koizumi et al. [40] determined that hAMSCs are capable of secreting several growth factors that support angiogenesis and tissue remodeling, and decrease inflammation. These growth factors include: epidermal growth factor (EGF),

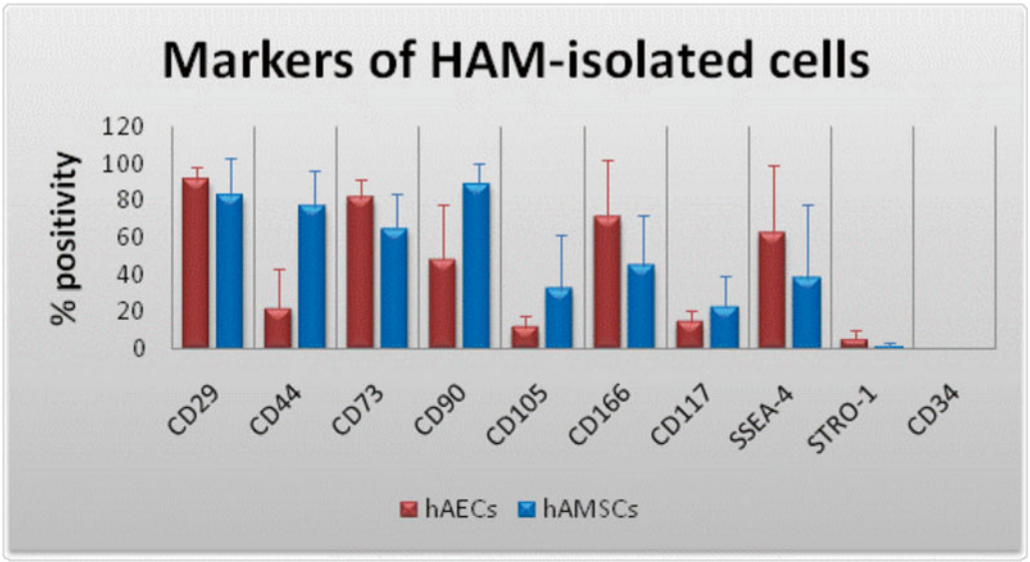


Fig. 17.4 Graphic representation of hAMSCs surface markers expression. It is represented typical (CD29, CD44, CD73, CD90, CD105, CD166, CD117) and non-typical (CD34) markers of MSCs, and pluripotent markers (STRO-1 and SSEA-4)

transforming growth factor (TGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), and basic fibroblastic growth factor (bFGF). The expression of growth factors related to different cell types gives us an idea of their pluripotency. Different authors demonstrated that hAMSCs can be induced to differentiate, in vitro, into tissues from all three germ layers: endoderm, mesoderm, and ectoderm [15, 16, 41].

hAMSCs grown in a specific-cell differentiation medium were capable of expressing specific cell markers. It was found that hAMSCs expressed typical neuronal markers (ectodermal lineage), Nestin, Musashi-1, neuron-specific enolase, neurofilament medium, microtubule-associated protein [MAP]-2 and Neu-N, and the typical glial marker GFAP (glial fibrillary acidic protein) [22, 42–44].

Alviano et al. and Fatimah et al. [16, 25] found that these cells expressed angiogenic and endothelial markers as FLT-1 (receptor of the vascular endothelial growth factor 1), KDR (receptor of the vascular endothelial growth factor 2), I-CAM-1/CD54, CD34, von Willebrand factor (vWF), PECAM-1/CD31 (platelet-endothelial adhesion molecule-1), and endothelial nitric oxide synthase (eNOS). It was found that these cells were capable of myogenic differentiation [43] due to the expression of MyoD and myogenin. Also, hAMSCs grown in a differentiation-specific medium were capable to express the cardiac-specific factor GATA4, cardiac-specific genes atrial myosin light chain (MLC)-2a, ventricular MLC-2v, and the cardiac troponins cTnl and cTnT [45]. Moreover, the expression of bone morphogenetic proteins (BMPs) and their receptors BMPR-IA and BMPR-IB indicates the capacity to induce the differentiation of mesenchymal cells into osteoblast and chondroblast lineages. The expression of oligomeric matrix protein (COMP), SRY-related HMG box family (SOX) as SOX5, SOX6, SOX9, and the presence of collagen type II and aggrecan (Fig. 17.5) in the extracellular matrix of differentiated cells indicates the capacity to differentiate into a chondrocyte-like phenotype [14]; presence of calcium deposits (Fig. 17.5) and expression of osteopontin (OP) and alkaline phosphatase (ALP) indicates the capacity to differentiate into osteoblast-like cells. hAMSCs differentiation to another mesodermal lineage, adipocytes, was also detected by the presence of lipid drops (Fig. 17.5) and the expression of adiponectin (APM1), fatty acid binding protein (FABP4) and lipoprotein lipase (LPL) [18].

Tamagawa et al. [41] found that hAMSCs could differentiate into hepatocyte-like cells. These cells expressed albumin, cytokeratin 18 (CK), α-fetoprotein (α-FP), α1-antitrypsin (α_1AT), and hepatocyte nuclear factor (HNF)-4α.

Pre-clinical and clinical studies have demonstrated possible multiple uses for hAMSCs. The most promising applications for tissue repair of hAMSCs in cell therapy are: corneal tissue [46], spinal cord [47], brain infarction [48], Parkinson's disease [49], myelodysplastic syndromes, ischemic stroke, aplastic anemia [50], Crohn's disease, diabetes mellitus [1] and urinary bladder [51], among others.

Currently, it is also arising new concepts in the use of HAM, as a possible application in oncology [52].

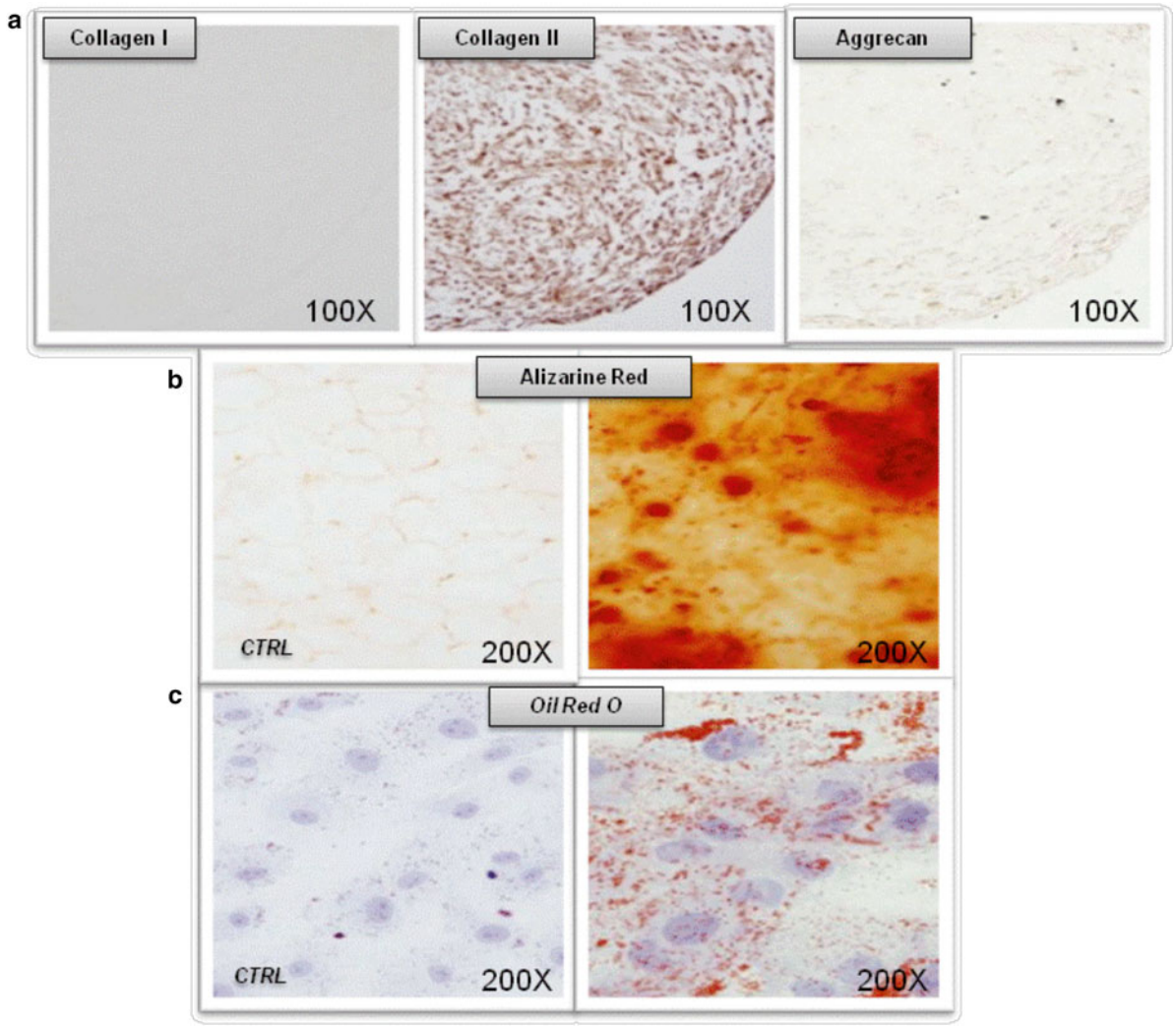


Fig. 17.5 hAMSCs differentiation. (**a**) Positivity by Col type II and little positivity by aggrecan immunostainings of hAMSCs under chondrogenic differentiation. hAMSCs showed no positivity for typical mesenchymal ECM protein, Col type I (magnification ×100). (**b**) Alizarine Red staining showed calcium deposits in osteogenic differentiation of hAMSCs but not in their controls (magnification ×200). (**c**) hAMSCs showed lipid droplets by Oil Red O staining under adipogenic differentiation and not in the control ones (magnification ×200)

5 Summary

Fetal tissues are commonly discarded postpartum. HAM is abundant, economic, and easy to obtain. hAMSCs may serve as an alternative source of mesenchymal stem cells without ethical or legal considerations associated with their collection and use. This tissue provides high efficiency in MSC procurement with no intrusive procedures. HAM represents a very useful source of progenitor cells for a variety of applications. Additional in vivo investigations into their functional potentials are necessary.

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Bladder Reconstruction Using Amniotic Mesenchymal Stem Cells

18

Tomonori Minagawa, Tetsuya Imamura, Osamu Ishizuka, and Osamu Nishizawa

Abstract

Regenerative medicine of functional urinary tracts has been vigorously investigated to provide effective treatments for severe and/or irreversibly injured tissues. We attempt to construct regenerative medicine based on tissue engineering methods. We have two strategies: the development of biomaterials and the establishment of effective cell sources. In this chapter, we indicate that human amniotic membranes (hAM) have a potential to provide great hope for reconstruction of functional urinary bladders. The hAM can be used as biomaterials to enlarge urinary bladder capacity and increase compliance. Furthermore, the hAM is composed of mesenchymal stem cells that have ability to differentiate into various kinds of cells. The human amniotic mesenchymal stem cells (hAMSCs) are one of the cell sources to form urinary bladder tissues. We show that the hAMSCs implanted into the frozen-injured bladders differentiate into smooth muscle cells. The reconstructed urinary bladders, composed of the hAMSCs-derived smooth muscles, show contractile responses to potassium and carbachol. The hAMSCs can recover functional urinary bladders. Therefore, hAMSCs will be a great cell source for reconstruction of functional urinary tracts.

Abbreviations

ES	Embryonic stem
hAM	Human amniotic membranes
hAMSCs	Human amniotic mesenchymal stem cells
HuNu	Mouse anti-human nuclei monoclonal antibody
MSCs	Mesenchymal stem cells
PBS	Phosphate buffer saline

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Bladder disease, such as bladder cancer, neurogenic bladder dysfunction, detrusor overactivity, and chronic inflammatory diseases cause serious urinary dysfunctions. These dysfunctions cause a decreased quality of life of patients. Current treatments for the urinary dysfunctions that are behavioral and drug therapy are poorly tolerated. The treatments for these dysfunctions need to overcome the small-capacity and high-intravesical storage pressure of urinary bladder. Bladder augmentation using gastrointestinal segments is one of the treatments to increase bladder capacity and compliance, and widely accepted as a standard surgical procedure [1–4]. However, gastrointestinal segments result in serious complications including metabolic and electrolyte disturbance [5], stone formation [6], perforation [7], and neoplastic progression [8]. Therefore, the development of biomaterials to enlarge urinary bladder capacity and increase compliance, and/or the establishment of reliable and effective cell sources to form urinary bladder tissues have been vigorously investigated to reconstruct functional urinary bladders.

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1 The Cell Sources for Bladder Reconstruction

Tissue engineering has a great potential to provide new functional tissues for bladder reconstruction. The final goal of bladder reconstruction needs to obtain functional bladders formed with accurately histological structures. Tissue engineering is composed of three elements; cells, scaffolds, and growth factors. In urology, regenerative medicine based on tissue engineering methods has been vigorously investigated to provide effective treatments for severe and/or irreversibly injured urinary bladders due to X-ray irradiation therapy, diabetes mellitus, or perforation. To develop regenerative medicine based on tissue engineering methods, establishment of reliable and effective cell sources is extremely important. There are many reports to investigate somatic stem and/or progenitor cells that have ability to differentiate into various kinds cells.

1.1 Embryonic Stem Cells

Embryonic stem (ES) cells are one of the pluripotent stem cell types that have great potential to reconstruct urinary bladders. Lakshmanan et al. reported that human ES cells co-cultured with the bladder mesenchymal cells were successfully differentiated into urothelium [9]. Even more, the seeding of the co-culture of ES cells and the bladder mesenchymal cells on decellularized xenogenic small intestinal submucosa in vitro led to the formation of a composite graft [10]. We also indicate that ES cells co-cultured with urothelium differentiate into smooth muscle cells [11].

1.2 Mesenchymal Stem Cells Derived from Bone Marrow

Mesenchymal stem cells (MSCs) from bone marrow also have the ability to differentiate into smooth muscle cells when treated with growth factors and corticosteroids. Becker et al. [12] report that the bone marrow-derived MSCs co-cultured with primary urothelial cells by using humoral factors significantly increase smooth muscle-specific gene expression in the treated MSCs, and that some of the cells differentiate into smooth muscle cells [12]. Chung et al. [13] performed augmentation of the rat bladder by introducing porcine small intestinal submucosa seeded with bone marrow-derived MSCs in rats. After 3 months, the histological examination shows the normal bladder structure in the implanted tissue, with fully differentiated urothelial and smooth muscle cells. Although a three-layered cellular architecture is also observed in control experiments using unseeded small intestinal submucosa, only the bone marrow-derived cell-seeded biohybrid exhibits gene expression levels similar to those of sham-operated animals [13]. Ringden et al. [14] investigated the effect of allogeneic hematopoietic stem cell transplantation in seven patients with hemorrhagic cystitis. The MSCs are taken from human donors and intravenously transplanted to the patients. In five patients, the severe hemorrhagic cystitis decreases after MSC infusion and gross hematuria disappear after a median of 1–14 days. In one advanced case, the perforation healed. In another patient, the MSCs reduced the need for further transfusions. Although the MSCs could not be detected, their DNA was found in the host bladder. These results suggest that the MSCs have the ability to home the damaged bladder tissue, and that this therapeutic approach has a great benefit in the future therapy for tissue toxicity [14]. Tian et al. [15] report that MSCs from bone marrow can be successfully differentiated into smooth muscle cells when seeded on a highly porous PLLA scaffold and treated with platelet-derived growth factor plus tumor growth factor β1 [15]. De Coppi et al. [16] also report that MSCs from bone marrow may have the role of preventing tissue damage (liquid nitrogen-cryo-injury), possibly via a paracrine mechanism [16]. These studies suggest that stem cell therapy may have not only direct reconstruction but also indirect reconstruction of urinary bladders. We demonstrated that MSCs from bone marrow are also useful for morphological and functional recovery of rat urinary bladder injured by frozen or X-ray irradiation [17, 18].

1.3 Other Stem Cells

Adipose-derived MSCs have potential to regenerate injured urinary tract and to differentiate into smooth muscle and nerve tissue of bladder as well as bone marrow-derived MSCs [19–21]. In addition, harvesting surgery of the adipose-derived MSCs is safely and minimally invasive compared to bone marrow-derived MSCs.

Sharma et al. report that endothelial progenitor cells have characteristics applicable for bladder tissue regeneration because they express similar contractile and vascular markers as bladder wall elements [22]. Drewa [23] investigate the suitability for cystoplasty augmentation by using acellular bladder matrices seeded with hair follicle stem cells in rats in vivo. The results show that bladder capacity is similar to non-cell-seeded control matrices, but the shape is regular and characteristically oval in bladders grafted with cell-seeded matrices. Muscle layers are thicker in bladders reconstructed

with cell-seeded grafts compared to controls. The urothelium are regenerated in both groups [23]. These results suggest that epithelial and dermal multipotent stem cells within hair follicles may be a cell source for tissue engineering of the urinary bladder. A subpopulation of cells isolated from urine is demonstrated to have progenitor cell characteristics and the potential to differentiate into urothelial, smooth muscle, endothelial, and interstitial cells. After a serial culture, these cells maintain normal karyotype [24]. Moreover, induced pluripotent stem (iPS) cells or multilineage-differentiating stress-enduring (Muse) cells, both which can differentiate into various kind cell types, will be a great cell source in tissue engineering of the urinary tracts.

The establishment of cell sources is essential to develop regenerative medicine based on tissue engineering. The cell sources need to overcome some limitations including securing consent of tissue donors and recipients, immunological antigenicity, and ethical concerns.

2 Human Amniotic Membrane

We believe that human amniotic membrane (hAM) is one of the great cell sources. The hAM provides two benefits. One is that the hAM provides a biomaterial to enlarge urinary bladder capacity and increase bladder compliance. Another is that the hAM is composed of numerous MSCs that have multipotency to differentiate into various kinds of cells.

The hAM is approximately 0.02–0.5 mm in thickness and composed of five layers: epithelium, basement membrane, compact layer, fibroblast layer, and spongy layer. It is an avascular tissue that forms the inner layer of the fetal membranes [25]. Since 1990s, the hAM has been considered to provide immunological advantages. The hAM is resistant to rejection, even after allo-transplantation due to a lack of expression of major histocompatibility complex class 2 [26] and overexpression of immunosuppressive factors, CD59 [27] and soluble histocompatibility antigen, class I and G [28]. Clinically, hAM is used as a wound dressing for burn patients and in other surgical procedures [29–31]. In ophthalmology, the hAM is used as an artificial cornea to treat for ocular surface reconstruction [32, 33]. To obtain and use the hAM, there are few ethical questions.

Fishman et al. report that utilization of hAM is used to augment urinary bladders in a canine model [34]. They show the regeneration of normal smooth muscle and urothelium in the area of the graft, and increases of the bladder capacity in the augmented bladder [34, 35]. Previously, we also report that hAM can be used for replacement of urinary bladders in the animal model [36]. By immunohistopathological studies, in vitro muscle-strip studies, and in vivo cystometric studies, we indicate that the hAM is superior to small intestinal submucosa. The hAM used in the bladder augmentation plays as scaffolds. Therefore, the hAM has a great potential as scaffolds for the development of regenerative medicine.

3 Human Amniotic Mesenchymal Stem Cells

Human amniotic mesenchymal stem cells (hAMSCs) isolated from hAM can differentiate into chondrocytes, adipocytes, osteocytes, and neural cells [26, 37–39]. The hAMSCs are used as cell therapies for injured nerve [40] and stroke [41]. In addition, the hAMSCs express Oct-4 (a marker of the pluripotent state) [39], as well as the phenotypes of pancreatic beta cells [39], neural cells [42], and hepatocytes [43]. The hAMSCs maintain same advantages of hAM, such as low immunologic antigenicity and few ethical concerns. Therefore, we consider that the hAMSCs will be one of the cell sources for the development of regenerative medicine [44–47].

4 Human Amniotic Mesenchymal Stem Cells Reconstruct Functional Urinary Bladders

We investigated to determine if the hAMSCs will be a useful cell source for the regeneration of damaged or diseased urinary bladders [48]. One of the our projects determined if the hAMSCs can differentiate into some cell types that compose the urinary bladder, such as smooth muscle cells, urothelium, and nerve cells. We demonstrated that the hAMSCs implanted into mice frozen-injured urinary bladders can differentiate into smooth muscle cells and promote morphological and functional recovery. In this chapter, we indicate the detail of the methods and results of the reconstruction of functional urinary bladders due to the implant of hAMSCs.

5 Isolation and Culture of hAMSCs

The hAMSCs were isolated from the amnion membrane by mechanically peeling from the placenta [39]. The obtained amnion membrane was washed with phosphate buffer saline (PBS), and cut into small pieces in PBS with 0.025 % deoxyribonuclease I and 0.03 % hyaluronidase. The minced membranes were digested with 0.2 % trypsin in Dulbecco's modified Eagle's medium (DMEM) and incubated for 30 min at 37 °C. The treated membranes were centrifuged at 130 rpm for 10 min in 37 °C, and then supernatant fluid including epithelial cells was removed. This trypsinization was repeated

several times until the number of epithelial cells is lower than 10^4 cells. After removing the epithelial cells, the cell pellets including hAMSCs were resuspended in DMEM supplemented with 10 % regular fetal bovine serum. The cells were seeded on collagen-coated 10-cm culture dishes at a cell density of 3.0×10^4 cells/cm^2. The hAMSCs were cultured at 37 °C in humid air with 5 % CO_2 for 7 days.

6 Frozen-Injured Bladder Models and Implantation of hAMSCs

We used BALB/c nu/nu female nude mice at postnatal week 5 as recipient animals. Three days prior to cell implantation, the mice bladders were exposed through abdominal midline incisions under anesthesia. Urine within the bladders was evacuated through the anterior side using a microsyringe. Following, 0.1 mL of saline was injected in the same manner to create a uniform bladder volume. The iron bar chilled by dry ice was applied to the posterior walls of bladders for 30 s. The frozen region was thawed with body and room temperature within approximately 10 s. After thawing, the injured bladder was returned into the pelvic cavity, and then the abdomen was closed.

At 3 days after the frozen-injury operation, the mice were randomly divided into the cell-implantation and the control-injection group. After anesthetizing and making a midline abdominal incision as above, the cultured hAMSCs were implanted into the freeze-injured regions by using a microsyringe. In the control-injection group, cell-free solution was injected in the injured region.

At 1, 2, 4, and 6 weeks after the cell implantation, animals of each group were killed with a pentobarbital sodium solution, and their bladders were removed. The bladders were subdivided for immunohistochemistry and muscle strip investigations. In order to evaluate the direct and indirect damage induced by freeze injury, the bladders were equally cut into anterior and posterior side for muscle strip investigation.

7 Immunohistochemistry

In order to detect the implanted hAMSCs within the recipient tissues, the specimens of the bladder were immunohistochemically stained by mouse anti-human nuclei monoclonal antibody (HuNu). Subsequently, the treated sections were immunohistochemically stained by alpha-smooth muscle actin, a marker of smooth muscle cell differentiation.

8 Muscle Strip Investigation

To investigate the role of implanted hAMSCs on functional recovery of injured bladder, muscle strip study was performed in each anterior and posterior side of bladder. The strips were attached at one end to a tissue holder and at the other end to a force displacement transducer. Each strip was stretched until a stable tension of 1 g. After the equilibration period, the bladder strips were exposed to a 124 mM potassium-Krebs solution for 1 min. After washing out the K^+-Krebs solution, contractions were evoked using the muscarinic cholinergic agonist carbachol.

9 Morphological Recovery of Frozen-Injured Urinary Bladders

At 1 week after implantation, the presence of hematoma, edema, and inflammation in the injured bladders was slightly decreased (Fig. 18.1a). The cell-implanted urinary bladders had thin and fragile smooth muscle layers composed of some SMA-positive cells (Fig. 18.2a). At 2 and 4 weeks after implantation, the injured regions had little hematoma, edema, and inflammation (Fig. 18.1b, c). In addition, SMA-positive cells formed layered smooth muscle structures similar to normal bladders (Fig. 18.2b, c). At 6 weeks after implantation, the layered smooth muscle structures of the cell-implanted urinary bladders (Figs. 18.1d and 18.2d) were similar to normal ones. In contrast, 1 week after the control injection, the hematoma, edema, and inflammation remain present without any notable decrease (Fig. 18.1e). These bladders had few distinct layered structures compared to cell-implanted ones (Fig. 18.2e). At 2 and 4 weeks after, the control regions partially showed spontaneous recovery (Figs. 18.1f, g and 18.2f, g). At 6 weeks after, the control-injected bladders had layered smooth muscle structures composed of SMA-positive smooth muscle cells similar to normal ones (Figs. 18.1h and 18.2h).

10 Differentiation of hAMSCs into Smooth Muscle Cells

In the cell-implantation group, the implanted hAMSCs that are positive for HuNu were present at the injured site in the walls (Fig. 18.2a–d). In the control-injected bladders, there were no HuNu-positive cells with the bladder walls (Fig. 18.2e–h). At 1 and 2 weeks, in the regions receiving the hAMSCs, some SMA-positive cells were simultaneously

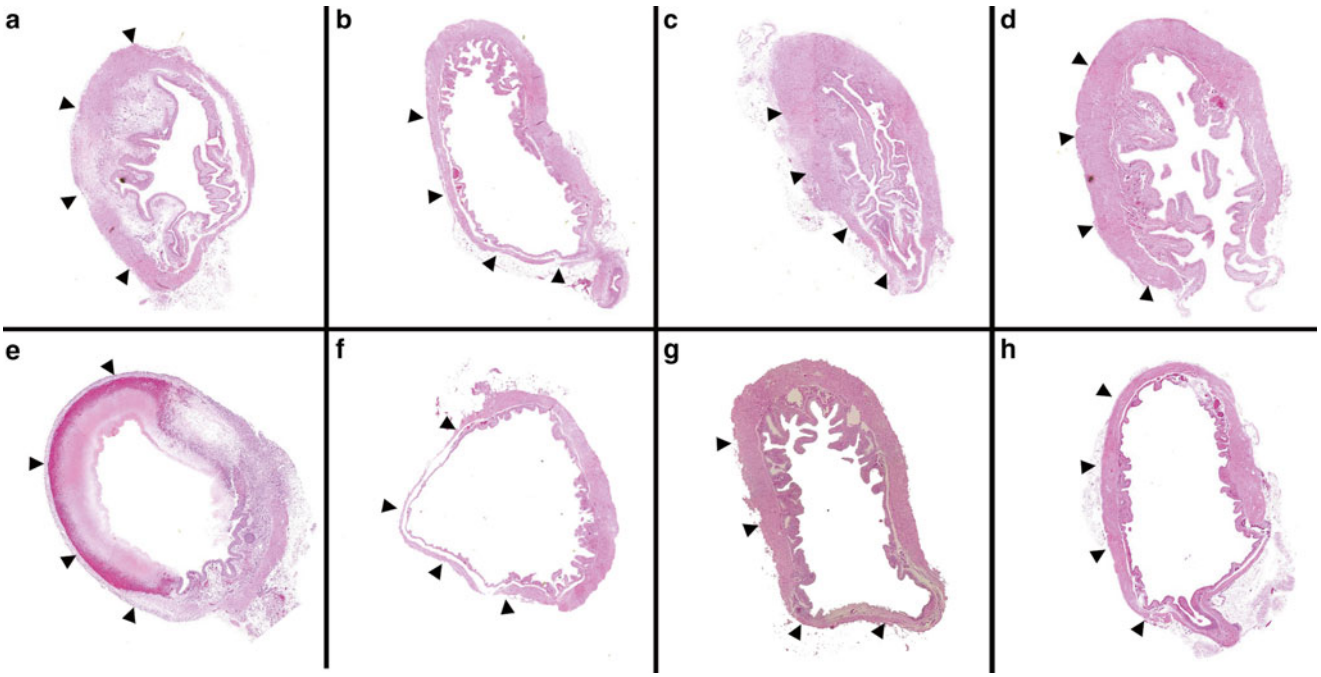


Fig. 18.1 (**a**–**h**) The histopathological course of recovery for freeze-injured bladders. Longitudinal sections of the bladders at 1, 2, 4, and 6 weeks after the cell-implantation (*upper*) or the control-injection (*bottom*) showed the injury site on the posterior sides (*arrowheads*). ×50 [48]

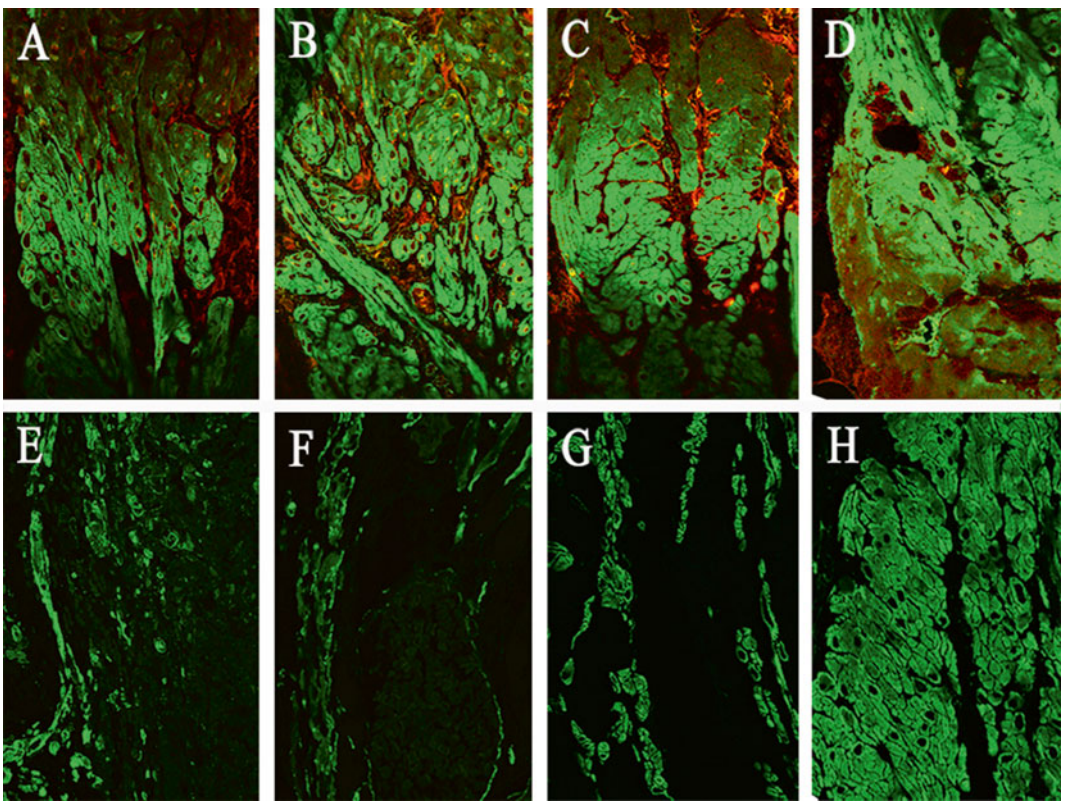


Fig. 18.2 The implanted hAMSCs within the frozen-injured urinary bladders. Representative cell-implanted (*upper*) or control-injected (*bottom*) urinary bladders are shown in merged images of HuNu (*red*) and SMA (*green*), at 1, 2, 4, and 6 weeks (**a** and **e**, **b** and **f**, **c** and **g**, and **d** and **h**, respectively). ×640 [48]

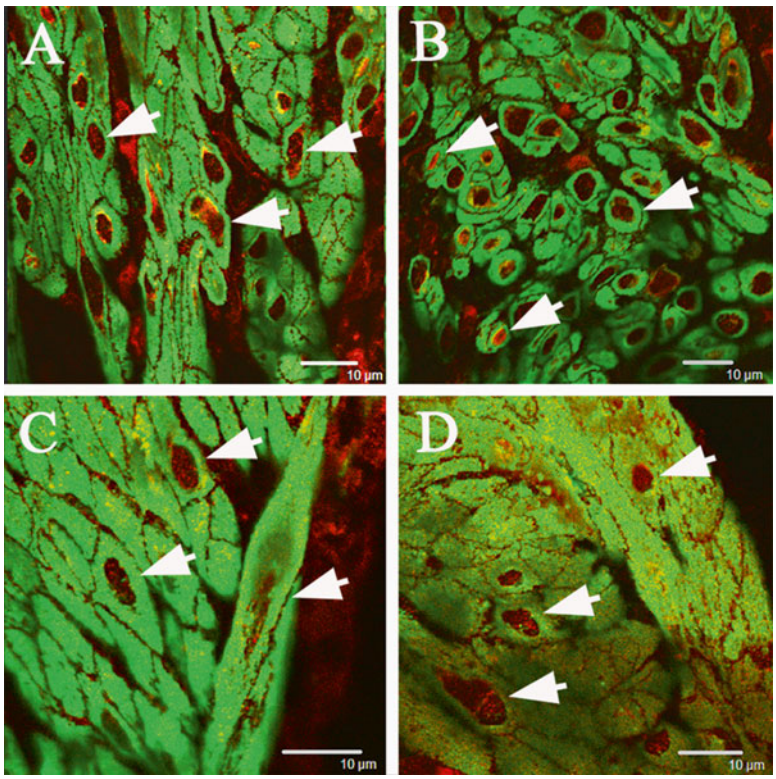


Fig. 18.3 Differentiation of hAMSCs into smooth muscle cells. The hAMSCs that are positive for HuNu (*red*, *arrows*) are simultaneously positive for SMA-positive cells (*green*) at 1, 2, 4, and 6 weeks after implantation (**a**, **b**, **c**, and **d**, respectively). ×640 [48]

positive for HuNu (Fig. 18.3a, b). These cells were round in shape and interspersed among the other cells in the wounded region. At 4 and 6 weeks, both SMA and HuNu-positive cells were spindle-shaped similar to typical smooth muscle cells (Fig. 18.3c, d). These differentiated cells formed components of the layered smooth muscle structures.

11 Functional Recovery by Implantation of hAMSCs

The high K^+-Krebs solution and the muscarinic cholinergic agonist, carbachol evoked a sustained contraction in normal mice bladder strips. However, the contractions evoked by the high K^+-Krebs solution and carbachol in the both posterior and anterior sides of frozen-injured urinary bladders were diminished (Fig. 18.4a, b), and the percentage (% potassium) of the contractile responses to carbachol was not different between the normal bladders and the freeze-injured bladders (Fig. 18.5). Therefore, these results suggest that the contractile dysfunction of frozen-injured bladders might be related with a decrease in the number of smooth muscle cells.

At 1 week, the contractile responses to potassium of the cell-implanted bladders were significantly higher than those of the control-injected ones (Fig. 18.4a, b). Although the control-injected bladders were recovered by 6 weeks, the rate of recovery is slower compared to the hAMSCs-implanted bladders (Fig. 18.4a, b). There were no significant differences of the percentage of the carbachol-induced maximum contraction for the high K^+-Krebs solution-induced ones between both the groups. These results show that the implantation of hAMSCs recovers functional smooth muscle layers.

12 Summary

Regenerative medicine based on tissue engineering methods has been vigorously investigated to provide effective treatments for severe and/or irreversibly injured urinary tracts. The regenerative medicine needs the development of biomaterials and the establishment of cell sources to reconstruct urinary tracts. We believe that a hAMSC has a great advantage as one of the cell sources. In this chapter, we show that the hAMSCs implanted into the frozen-injured

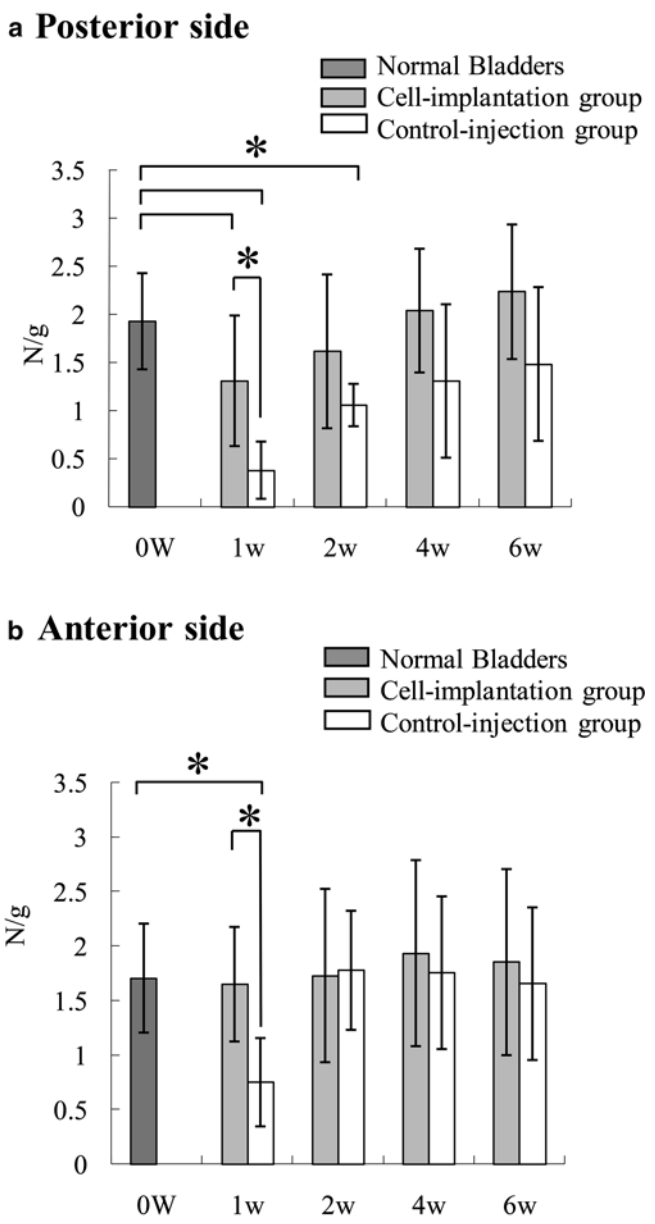


Fig. 18.4 Contractile responses of muscle strip preparations to high K^+-Krebs solution: (**a**) posterior bladder wall, (**b**) anterior bladder wall. *N/g* Newton/gram, $*p<0.05$ [48]

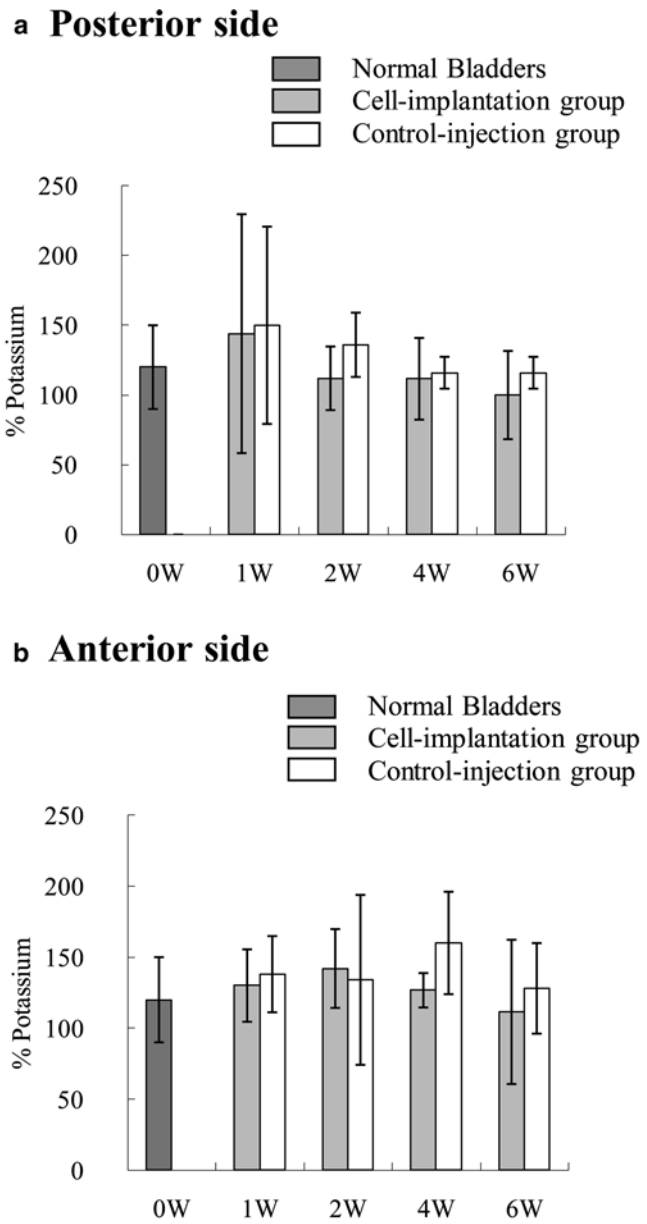


Fig. 18.5 Maximum contractile response to carbachol. The maximum contractile response to the muscarinic cholinergic agonist carbachol is determined as a percent of the maximum contraction to the response induced by the high-K solution. (**a**) Posterior bladder wall, (**b**) anterior bladder wall [48]

bladders survive and differentiate into smooth muscle cells. The differentiated cells form smooth muscle layers. In addition, the reconstructed tissues show contractile responses to potassium and carbachol. These results suggest that hAMSCs implanted into freeze-injured bladders promote morphologic and functional recovery. Therefore, hAMSCs might be a useful cell source to treat for damaged urinary bladders.

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Amnion Epithelial Cells for Lung Diseases

19

Euan M. Wallace, Jean L. Tan, Atul Malhotra, Graham Jenkin, and Rebecca Lim

Abstract

Amnion epithelial cells, derived from the amnion fetal membranes, are pluripotent and immunomodulatory. They have been shown to both prevent and repair acute lung injury in a number of diverse experimental models. They are readily isolated from term placentae in sufficient numbers suitable for clinical application. They also have a number of features that make them particularly attractive as a future cell therapy including being immune-privileged and nontumorigenic. This chapter reviews the use of amnion epithelial cells as a cell therapy for lung injury, addressing likely mechanisms of action and future clinical trials.

1 Introduction

Chronic respiratory disease accounts for nearly one in ten deaths worldwide, making it the third leading cause of mortality globally [1]. While the majority of this burden of disease lies in resource poor countries, and relates primarily to environmental pollution and tobacco smoking, the burden of chronic lung disease is still significant in advanced economies with about 6 % of the adult population in both the United States and Australia suffering from chronic obstructive pulmonary disease (COPD), rates that are stable or rising [2–4]. Indeed, COPD is the third most common cause of death in the United States and has an estimated annual total healthcare cost of over US$150 billion [5] with direct costs not dissimilar to either stroke or hypertension [6]. Very similar rates of COPD are present in Australia [2].

In addition to the considerable adult lung disease burden, advances in perinatal medicine over the past 30 years are leading to a growing burden of lung disease with its origins in very early life. The survival of increasing numbers of extreme preterm infants at increasingly preterm gestations, infants who in previous generations would not have survived, while a great success story of modern medicine, has led to an increasing number of children who are growing up with chronic lung disease [7, 8]. In particular, one in three infants born before 30 weeks gestation will go on to develop a chronic lung disease called bronchopulmonary dysplasia (BPD) [7, 9]. This is a condition of lung dysmaturation characterized by arrested alveolar development, vascular dysregulation, and chronic inflammatory sequelae [7, 10]. BPD is the commonest chronic lung disease in infants [7], second only to asthma as a cause of chronic childhood lung disease and far exceeding the health burden imposed by cystic fibrosis [11]. Importantly, we are still to learn what the lung function of these children will be like in late adulthood. At this time it is clear that many of them have ongoing respiratory compromise throughout childhood [12] and that normal lung ageing

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will likely reveal a new wave of adult COPD unrelated to pollution or smoking [7]. Thus, chronic lung disease is set to continue to be a major burden of disease long into the future.

Common to both BPD and COPD is the lack of any effective treatment. Instead, current ongoing management is largely supportive, including home oxygen, corticosteroids and bronchodilators, is of limited effectiveness and may be deleterious in the long term. It is not surprising then that cell therapies have been suggested for both of these diseases and other chronic lung diseases [13–16]. While a diverse range of cells have been suggested for a diverse range of lung conditions [14, 16], in this chapter we will focus on amnion epithelial cells, their potential role(s), and mechanism(s) of action with reference to other cell types where appropriate.

2 Amnion Epithelial Cells

The other chapters in this book describe the variety of stem cells or stem cell-like cells that may be derived from the placenta and its associated tissues and their diverse applications in regenerative medicine. What is common to all these cell types, whether derived from the placenta proper, the umbilical cord or cord blood, the fetal membranes or the amniotic fluid, is that they all possess some degree of plasticity and immunomodulatory capability [17], properties that are fundamental to their regenerative medicine applications. Human amnion epithelial cells (hAECs) are no exception. Importantly, hAECs are not to be confused with amniotic fluid cells, described in the chapters elsewhere in this book. The amniotic fluid is a rich source of mixed stem cell populations including mesenchymal stem cells (MSCs), epithelial cells, hematopoietic stem cells (HSC), and trophoblast cells [18]. In contrast, hAECs are a heterogeneous population of epithelial cells derived from the amniotic membrane proper. While MSCs may also be isolated from the amniotic membrane stroma—so-called human amniotic mesenchymal stromal cells (hAMSC)—[17], hAECs are epithelial cells and express neither MSC nor HSC markers [19]. Thus, the regenerative properties and applications of hAECs will likely differ from both amniotic fluid cells and from hAMSCs.

Unlike the other fetal membrane layer, the chorion, which is trophoblast-derived, the amnion originates from the embryonic epiblast immediately prior to gastrulation [20]. This is thought to be important in regard to stem cell pluripotency because cells derived from the epiblast prior to gastrulation remain multipotent, reflecting the capability of the epiblast itself to differentiate into all three layers of the definitive embryo: the ectoderm, endoderm, and mesoderm [19]. It was not surprising then that hAECs were shown to express a number of early stem cell markers such as the POU domain, class 5, transcription factor, Nanog homeobox, SRY-2 box, the stage-specific embryonic antigen-4 (SSEA4), Oct4, Nanog, and Sox2 [19, 21–23]. Consistent with the expression of such early lineage markers hAECs have been successfully differentiated into mesodermal lineages (cardiomyocytes, myocytes, osteocytes, adipocytes), endodermal lineages (pancreatic cells, hepatic cells, lung cells), and ectodermal lineages (neural cells and astrocytes [21, 23, 24]).

Reassuringly, despite their pluripotency hAECs do not appear to either form tumors or promote tumor development in vivo [21, 23]. This is important because one of the major limitations of other stem cells, including embryonic stem cells, induced pluripotent stem cells and MSCs, is their ability either to transform into tumors following transplantation or to promote tumor development in the recipient [15, 25, 26].

Furthermore, hAECs are also immune-privileged, expressing no, or very little, class IA and class II human leukocyte antigens (HLAs) [21, 27]. This property likely underlies their ability to escape immune rejection after allogeneic and xenogenic transplantation. Following xenotransplantation hAECs have been shown to survive for prolonged periods in immune competent monkeys, rabbits, guinea pigs, rats, and pigs without immunorejection [28–31] and hAECs injected into healthy human volunteers did not elicit either acute rejection or the production of anti-HLA antibodies [32]. However, all of these experiments utilized undifferentiated hAECs. This is important because following differentiation down some lineages, hAECs start to express class IA HLA [21]. While not yet tested this may have implications for transplantation of differentiated rather than primary hAECs. In addition to being immune-privileged, hAECs are immunosuppressive, reducing the presence of antigen-presenting cells such as macrophages and B cells at sites of injury [33, 34]. It is likely that this function is key to their ability to prevent and or repair tissue damage, particularly in the lung [35].

In summary, hAECs are epithelial cells derived from amniotic membrane. They are pluripotent, immune-privileged and immunomodulatory and promise to be an effective cell therapy for diverse diseases [17]. With regard to likely safety profile as a cell therapy hAECs display karyotypic stability in vitro and do not appear to present tumorigenic risks [19, 21].

3 Lung Injury and hAECs

The first reports of amnion cells being capable of reparative effects in the injured lung were of amniotic fluid stem cells [36], see also Section I of this book, and of a mixed population of epithelial and mesenchymal cells from the amnion and chorion [37]. Both of these studies demonstrated that the mixed stem cell populations used were able to mitigate acute lung injury. However, while hAECs were certainly present in the cell preparations used in both of those studies the presence of other cells, such as MSCs and/or trophoblast cells, made it impossible to discern whether hAECs themselves

were able to mitigate and/or prevent lung injury. This changed with the report that hAECs could significantly reduce bleomycin-induced lung injury in immune-deficient (SCID) mice [38]. In that study, the administration of hAECs immediately after the administration of the cytotoxic bleomycin significantly mitigated bleomycin-induced inflammation and subsequent fibrosis, protecting normal lung architecture [38]. Over the past few years a number of subsequent studies have confirmed the ability of hAECs to both prevent and repair acute lung injury following diverse insults such as bleomycin [39, 40], ventilation [41, 42], infection [43], and hyperoxia [44]. Critically, while the original study undertaken by Moodley and colleagues [38] was in an immune-deficient animal model all of the subsequent studies were undertaken using either immune competent rodents [39, 40, 44] or sheep [41–43], confirming the efficacy of xenotransplantation consistent with immune-privileged cells [37]. This contrasts with MSCs that, while effective in preventing acute lung injury in immune-deficient mice, are not effective in immune-competent animals [45]. Indeed, bone marrow-derived MSCs have been shown to accentuate lung fibrosis in immune-competent neonatal mice following hyperoxia—an effect thought to be secondary to integration of the MSCs into chimeric macrophages [46]. Clearly, an assessment of individual stem cell types in each therapeutic setting will be critical to safe and effective cell therapies. At this stage, it would appear that hAECs are likely to be an effective cell type for successful allogeneic transplantation in lung injury.

3.1 Acute Lung Injury

While there are many causes of acute lung injury [47], several modeled in the various experimental animal studies assessing cell therapies [16], the end mechanisms of acute inflammation, loss of epithelial and endothelial integrity, lung architectural disruption, and fibrosis are all broadly similar. In particular, a key trigger event for the injury, whether clinical [48] or experimentally induced by bleomycin [37–40], barotrauma [41, 42], hyperoxia [44] or infection [43], is the acute inflammatory reaction with an influx of neutrophils, likely driven by resident macrophages and possibly by lymphocytes. Accordingly, assessment of *effectiveness* of any cell therapy, including hAECs, in acute lung injury is the impact of the therapy on lung inflammation. In this regard, when administered at the time of the injury, hAECs are highly effective at reducing acute inflammation including reducing neutrophil, macrophage, and lymphocyte influx [37–39, 44]. Not surprisingly, this reduction in inflammatory influx is associated with a reduction in lung content of several pro-inflammatory cytokines such as interleukin-1β (IL-β), IL-2, IL-6, transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α), and interferon gamma (IFN-γ) [38, 39, 41, 42, 44] and with an increase in some anti-inflammatory cytokines [38].

However, this has not been a universal finding. In a LPS-induced model of acute in utero infection in fetal lambs, administration of hAECs to the fetus increased total lung leukocyte populations, as evidenced by CD45-positive cells, rather than decreased them [43]. The reason why hAECs increased total leukocyte number in the lung following LPS-induced injury, and yet suppressed pro-inflammatory cytokine expression, was not clear. It is possible that the ratio of hAECs to inflammatory cell was too low. Certainly, the hAEC:inflammatory cell ratio appears important such that at higher ratios hAECs suppress leukocyte proliferation while at lower ratios, that is relatively more leukocytes, hAECs stimulate white cell proliferation [49]. This has been shown previously for MSCs [50, 51]. As discussed below, unraveling the relationship and interactions between hAECs and host immune cells is likely to be key to optimizing their application as a cell therapy.

Importantly, the reduced host inflammatory response to acute injury effected by hAECs is associated with reduced lung injury, as evidenced by maintaining a high airspace:tissue ratio, a low Ashcroft score and, subsequently, low levels of collagen, elastin and smooth muscle actin (SMA) deposition. In short, hAECs maintain normal, or near normal, lung architecture and prevent fibrosis following an acute injury [38, 39, 41, 42, 44]. This appears true irrespective of the nature of the insult inducing the injury. Not surprisingly, the maintenance of normal architecture by hAECs is accompanied by preservation of lung function [39]. In that study, plethysmography was used to assess inspiration and expiration phases of breathing in bleomycin-treated adult mice. Bleomycin significantly increased expiration time and reduced minute volume ventilation, effects that were rescued by the administration of hAECs. Similarly, in a fetal sheep model of in utero infection, administration of hAECs following LPS-induced inflammation reduced the increased lung compliance induced by LPS, normalizing the relatively "stiff" lungs typical of the immature fetus [43]. Unfortunately, these are the only two studies to date that have made any assessment of the effects of hAECs on lung function following injury. While the results from these two studies are certainly very promising it would be reassuring if future studies, particularly those using other models of lung injury, could confirm a protective effect on lung function.

Of course, all of the experimental models are just that—models of lung injury, undertaken to inform and direct future clinical studies and, eventually, therapies. In this regard, the two acute lung injuries that would most greatly benefit from a cell-based therapy are acute respiratory distress syndrome (ARDS) in adults [52] and, albeit more of an acute and chronic injury, BPD in preterm neonates [10]. While there

are now clinical trials in progress assessing the utility of MSCs as a therapy for ARDS, there are no clinical trials of hAECs in ARDS yet registered. Indeed, it is likely that any clinical trial using hAECs for ARDS will await the outcomes of the trials using MSCs. In contrast, hAECs are a particularly attractive therapy for BPD, partly because the experimental fetal and neonatal studies reported to date have been designed to specifically mimic the various injurious mechanisms leading that are known to cause BPD. Specifically, the effects of hAECs in acute lung injury secondary to infection [43], ventilation-induced injury/barotrauma [41, 42], and hyperoxia [44] have all been modeled with a view to using hAECs as a future therapy for BPD. In each case, hAECs were able to reduce inflammation, mitigate injury, and preserve lung structure and function [41–44]. Indeed, in a recent review of the various possible cell therapies for BPD, hAECs were identified as a particularly attractive cell therapy [15].

In summary, when administered at the time of, or shortly after, acute lung injury hAECs appear to be highly effective at reducing acute inflammation and structural disruption, preserving lung function and preventing lung fibrosis. As such, hAECs appear a most promising cell therapy for acute injury in both adults, such as in ARDS, and in neonates, such as in BPD.

3.2 Chronic Lung Injury

In contrast to acute lung injury, to date there has been only scant consideration of whether amnion cells may be useful as a therapy in chronic lung injury [41, 42]. This is a pity because the greatest burden of disease is actually in chronic, not acute, lung disease [1, 5] and there are now a large number of clinical trials underway assessing the utility of MSCs.

The first evidence that hAECs may be able to resolve chronic lung injury, rather than simply prevent or modulate acute injury, was the observation that the administration of hAECs 2 weeks after bleomycin-induced lung injury—a time point when lung fibrosis is well established and the acute inflammation has subsided [40]—reduced collagen deposition, increased active matrix metalloproteinase 2 (MMP-2) levels, and decreased levels of tissue inhibitor of metalloproteinases (TIMPs) [38]. Increasing lung MMP-2 activity and decreasing TIMPs are known to be important changes in the resolution of lung fibrosis [53]. That hAECs were able to effect such changes was most promising, particularly when MSCs decrease MMP activity following the same injury [54]. However, the studies of Moodley et al. [38] had been undertaken in immune-deficient mice whereas the prior work of [54] had been performed in immune-competent mice. It was possible that apparent differences in effects of hAECs and MSCs simply related to the immune status of the host animal. Accordingly, a detailed study of the timing of hAEC administration following bleomycin injury in C57Bl6 (immune competent) mice was recently reported [40]. This showed that when administered 2 weeks after bleomycin, at a time of maximum lung fibrosis, hAECs were able to effect repair as evidenced by reduced collagen content and α-SMA expression, and by improved airspace:tissue ratio. Not surprisingly, and in accord with the acute studies, hAECs reduced lung leukocyte numbers [40]. Interestingly, when hAECs were given 1 week after bleomycin, at a time of maximum inflammation, they had no effect on either leukocytosis or lung injury [40]. That observation merits further study to explore whether the lack of effect was simply related to the ratio of hAECs:leukocytes, as discussed previously, or whether timing of administration relative to injury is critical to the effectiveness of hAECs. In any event, hAECs can clearly modify established lung injury, reducing fibrosis and accelerating repair. This is most promising for future clinical trials of hAECs in conditions such as idiopathic pulmonary fibrosis (IPF) and COPD.

4 Mechanisms of Action: Differentiation or Immunomodulation

While it has been known for many years that amnion cells had wound healing and reparative properties, particularly in the fields of burns [55, 56] and ophthalmic surgery [57, 58], it was the more recent observation that the cells possessed pluripotency [17, 21, 23, 59] that stimulated renewed interest in hAECs as a cell with broader regenerative potential. As discussed earlier, hAECs have been shown to be able to differentiate in vitro down ectodermal, mesodermal, and endodermal lineages [21, 23, 24]. In particular, when cultured in small airway growth media (SAGM) hAECs have the ability to differentiate into lung epithelial-like cells, expressing thyroid transcription factor Nkx2.1 and surfactant proteins A, B, C, and D [21, 38]. Indeed, the cells alter their ultrastructure, developing lamellar bodies typical of type II alveolar cells producing surfactant [38]. Further, with extended culture in SAGM it is possible to induce the expression of functional cystic fibrosis transmembrane conductance regulator (CFTR) protein in hAECs [60], a protein unique to alveolar cells.

Thus, the original studies addressing whether hAECs had lung reparative properties were undertaken with the view that they would home to injured lung, integrate into the damaged lung epithelium, differentiate into lung cell lineages, and repair lung injury. The first evidence that amnion cells could home to injured lung, integrate into lung epithelium, and differentiate into lung cells in vivo was provided by the studies of Carraro and colleagues [36]. These studies were undertaken using amniotic fluid cells and so whether the cells integrating into the lung were epithelial or mesenchymal, or both, is uncertain. Nonetheless, it was apparent that human AF cells could traffic to the injured lung preferentially and take up residence there differentiating into lung cells. This work was followed by that of Moodley and his

colleagues who used hAECs, demonstrating that these epithelial cells specifically were able to target injured lung and differentiate into lung cells as evidenced by the de novo production of surfactant proteins [38]. Similarly, when administered to fetal lambs following ventilation-induced injury, hAECs integrated into the lamb lung epithelium and expressed surfactant [41, 42]. The shape of the hAECs also appeared to change in vivo, taking on an elongated appearance suggestive of type II alveolar cells [41, 42]. Intriguingly, while it took 2–4 weeks of culture in SAGM in vitro to induce surfactant protein expression by hAECs, when administered in vivo hAECs had immunostaining for surfactant with a week [41, 42]. This more rapid in vivo differentiation suggests that the in vivo niche provides differentiation signals to hAECs that are superior to those in SAGM. This has possible application to future in vitro studies.

Irrespective of the rapidity of in vivo differentiation of hAECs into alveolar cells, the level of hAEC engraftment in all of the studies was extremely low. Dual labeling of hAECs for surfactant and a passive tracking dye showed that the majority of alveolar epithelial cells were not hAECs but rather host cells [41, 42] such that less than 0.1 % of total lungs cells were thought to be hAECs. This level of engraftment was thought to be too low for engraftment and differentiation to be the primary mechanism of lung repair [41, 42]. Indeed, when it was noted that hAECs could prevent bleomycin-induced lung injury in immune-competent mice without any apparent engraftment [39], other mechanisms of action were considered as the likely primary reparative mechanisms.

4.1 hAECs as Immunomodulators

The ability of amnion epithelium to avoid immune recognition by either allogeneic or xenogeneic T lymphocytes is thought to be due to their lack of, or very low, MHC class I and class II expression [21, 27, 61, 62]. This immune privilege is likely to be important for their role(s) in pregnancy. It is also a most useful property in regenerative medicine, allowing allogeneic transplantation without stirring rejection [32]. However, it would appear that there is more to hAECs than simply avoiding lymphocyte detection. They also possess the ability to modulate immune cell function.

A number of studies have now shown that hAECs suppress lymphocyte proliferation in both allogeneic and xenogeneic mixed lymphocyte reactions (MLRs) [29, 63]. This is due to the release of soluble factors that inhibit T- and B-cell proliferation and induce lymphocyte apoptosis [34]. Further, hAEC-conditioned media has been shown to reduce both neutrophil and macrophage migration in vitro [34, 64]. Given these properties, it is most likely that hAECs exert their effects by modulation of the host immune response to injury. Indeed, in almost all studies where the administration of hAECs reduced or prevented acute injury this effect was in association with a reduction in lung leukocyte recruitment, including neutrophils, macrophages, and lymphocytes [37–39, 41, 42]. While hAECs can clearly modify recruitment, proliferation, and survival of neutrophils, macrophages, and lymphocytes [34] it was not clear until recently whether their in vivo effects were exerted on all three leukocyte populations or preferentially on one or other. The observation that hAECs were unable to prevent bleomycin-induced injury in a surfactant protein C knock-out (*Sftpc*$^{-/-}$) mouse provided some additional insights [35]. The *Sftpc*$^{-/-}$ mouse has deficient macrophage function [65], expressing markers of alternative activation. As such, while hAECs prevented neutrophil recruitment to lung following injury, they did not mitigate macrophage recruitment in the *Sftpc*$^{-/-}$ mouse and, as a result, lung injury was not prevented. It is known that bleomycin-induced lung injury is macrophage-dependent [66]. That the mitigation of lung injury by hAECs depends on inhibiting macrophage recruitment to the lung following injury is in keeping with that and the known ability of hAECs to inhibit macrophage migration [34, 64]. Furthermore, bleomycin polarizes lung macrophages to the injurious M1 phenotype [67, 68], away from the pro-reparative M2 phenotype [64], an effect prevented by hAECs [64]. Indeed, hAECs convert M1 macrophages to M2 and thereby increase phagocytosis [64], an effect that is mediated via lipoxins and likely to assist with lung repair (Fig. 19.1). Importantly, as with hAECs' effects on lymphocytes [34], their effects on macrophage migration and polarization do not require cell-to-cell contact but rather can be exerted by hAEC-conditioned media [64, 69] suggesting that hAECs secrete immunomodulatory factor(s). Indeed, the delivery of amnion-conditioned media, rather than cells, is sufficient to prevent bleomycin-induced lung injury [70, 71] suggesting that all the preventative and/or reparative effects of hAECs may be via soluble factors. Precisely what those factors are remain to be elucidated.

4.2 Soluble Mediators

Insights into the likely mediators of the reparative properties of hAECs may arise from the observation that while term hAECs are highly effective at preventing bleomycin lung injury, hAECs derived from preterm amnion are not [72]. Preterm hAECs are much more proliferative than term hAECs but, despite extended culture in SAGM, do not switch on surfactant protein expression [45]. Importantly, while term hAECs reduce lung inflammation and fibrosis following bleomycin preterm hAECs do not [45]. Surprisingly, while preterm hAECs were not as effective as term hAECs at inhibiting macrophage recruitment to the injured lung, they did mitigate recruitment somewhat. Whether preterm hAECs can modulate macrophage polarization has not yet been reported. There are likely to be many differences in the factors secreted by preterm and term hAECs that might underlie their differential

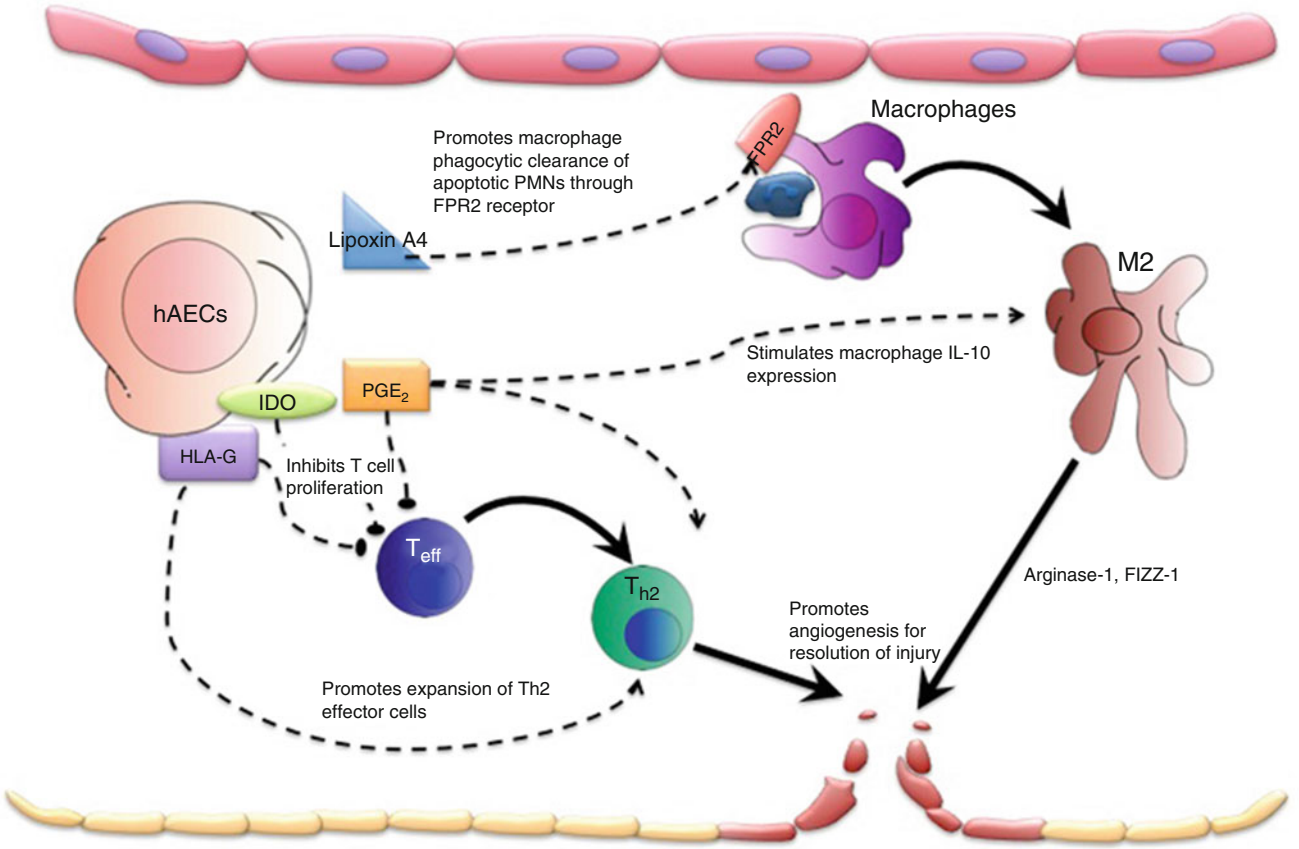


Fig. 19.1 Human amnion epithelial cells (hAECs) promote tissue repair via the modulation of host immune cells. Through the secretion of lipoxins and other mediators, hAECs polarize M1 macrophages to M2 phenotype thereby increasing phagocytosis and promoting resolution of injury. Via numerous soluble mediators such as HLA-G, prostaglandins and IDO, hAECs inhibit T-cell proliferation and likely convert effector T cells to Th2 cells, thereby promoting repair

reparative properties. One possible candidate is HLA-G. HLA-G is a nonclassical immunosuppressive HLA class I molecule [73]. It inhibits the functions of both NK and cytotoxic T cells and induces T regulatory cells (Tregs) [74, 75] (Fig. 19.1). While there are no reports yet that HLA-G mediates the effects of amnion cells, amnion cells do secrete HLA-G [76] and other placental cells are able to modulate immune cell function via HLA-G [77]. Importantly, preterm hAECs secrete less HLA-G than term hAECs [45]. Whether HLA-G is a key mediator awaits further study.

Amnion epithelial cells secrete many other candidate mediators including prostaglandins, indolamine (IDO), and numerous cytokines [34] (Fig. 19.1). Much detailed work lies ahead to determine which of these are required for the immunomodulatory properties of the cells.

4.3 Other Mechanisms

In addition to their immunosuppressive activities, hAECs are likely to exert their reparative effects via other mechanisms. For example, hAECs can directly reduce fibroblast proliferation and activation [40] and amnion stromal cells are able to reverse the myofibroblasts away from profibrotic phenotype [78]. Given the central role that fibroblast play in lung fibrosis these direct effects are likely to be highly beneficial.

It is also possible that hAECs may induce the proliferation of endogenous lung progenitor cells, as has been observed for MSCs [79]. Thus, through the recruitment of host lung progenitor cells hAECs could accelerate repair following injury. Whether hAECs possess such an ability also awaits examination.

5 Summary

hAECs are an abundant and safe source of cells for regenerative medicine. They have proven roles in the prevention and repair of experimental lung injury in both adult and neonatal models and in both acute and chronic injury. While hAECs are certainly able to integrate into injured lung epithelium and differentiate into lung cells, it would appear that they

most likely exert their reparative effects via the modulation of host immune cells, particularly host macrophages. There is much yet to be learned about the properties of hAECs and how they might be best used. Given their abundance and safety record, clinical trials of hAECs in lung diseases appear to be relatively straightforward. Hopefully, the next 5 years or so will reveal whether these cells provide us with a whole new therapeutic armamentarium.

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Potential Efficacy of Amnion Epithelial Cells to Treat Post-stroke Inflammation

20

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Abstract

Ischemic stroke is a crippling disease with few treatment options available. Thus there is an urgent need for novel therapies that can prevent or reverse the damaging effects of stroke. A promising experimental approach is stem cell therapy. Stem cells derived from human tissue, including embryonic, induced pluripotent, neural, and mesenchymal cells, are capable of rescuing injured brain tissue and improving functional recovery in experimental models of stroke. However, ethical issues, concerns regarding tumorigenicity, and difficulty harvesting sufficient cells may hamper their suitability as a widely available cell therapy for stroke patients. In contrast, placental-derived stem cells, particularly human amnion epithelial cells (hAECs), are abundant, non-immunogenic, non-tumorigenic and pose no ethical challenges. Surprisingly, hAECs have received little attention as a potential stroke therapy. This chapter will firstly describe the immune cell response and brain inflammation after stroke, and then consider the potential for hAECs to improve stroke outcome given their unique properties. Protective actions of hAECs could involve modulation of the immune response, differentiation into neural tissue, re-innervation of lost connections, and secretion of important cytokines, growth factors, hormones and/or neurotransmitters to restore cellular function.

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1 Introduction

Stroke is the world's second leading cause of mortality, after only ischemic heart disease, accounting for about 11 % of all deaths, and over six million deaths each year [1]. By 2030, the World Health Organization (WHO) estimates that stroke will account for eight million deaths per annum [1]. In addition, of the 70 % of patients who survive a stroke, over a third suffer from severe irreversible neurological deficits, making them dependent on care for the rest of their lives and shortening their life. Alarmingly, the incidence of stroke more than doubles each decade for people over the age of 55. With the annual number of strokes increasing due to the ageing population, a growing financial and social burden will be imposed on survivors and on the community. Therefore, major advances to prevent and treat stroke will be critical.

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2 Treatment of Ischemic Stroke

There are two major forms of stroke: (1) hemorrhagic and (2) ischemic stroke. Hemorrhagic stroke occurs with the rupture of a cerebral blood vessel, resulting in intracerebral bleeding and reduced blood flow to the brain. Ischemic stroke occurs when the blood supply to the brain is obstructed by an embolus or a thrombus. Ischemic stroke is the most common form of stroke, accounting for approximately 85 % of all cases [1]. It is most disappointing that still the only available "pharmacological" intervention to reduce brain damage after ischemic stroke is the "clot-buster," tissue plasminogen activator (t-PA). Moreover, t-PA has a number of limitations as a therapy. First, it must be administered within 4.5 h of the onset of ischemia and, for optimal results, within 1.5 h [2]. This is challenging in many health settings. Further, it can only be given after a CT scan has confirmed that the stroke is thrombotic rather than hemorrhagic. In the latter case, t-PA would risk worsening the bleed and the injury, hence the need for the precautionary brain CT [3]. These restrictions mean that rt-PA therapy is available to only 2–8 % of stroke patients (based on United States statistics) [4, 5]. Even then, t-PA therapy only benefits about ~30 % of patients who receive it. This is because while it promotes the restoration of blood flow, t-PA cannot target mechanisms of cellular injury nor promote healing. In fact, in human brain endothelium and cultured mouse neurons in vitro, and in an in vivo mouse model, t-PA is reported to be toxic [6]. Other treatment options for ischemic stroke include anti-coagulants such as heparin, that inhibits clot formation, and anti-platelet agents such as aspirin, to reduce the risk of platelet aggregation. However, these treatments have no effect on stroke outcome and are mainly used in the prevention of a secondary stroke. Although a plethora of neuroprotective compounds have shown promise in animal models of stroke, no other treatment has achieved efficacy in clinical trials [7]. Thus in order to achieve further advances in clinical outcomes, it is essential that future therapies target other aspects of stroke pathology. An important step in this process will be to understand the key mechanisms that contribute to injury following stroke.

3 Activation of the Immune System and Brain Inflammation After Stroke

It is now well established that the immune system plays a central role in the pathogenesis of ischemic stroke [8]. In the post-stroke acute phase (minutes to hours), microglia and cerebral endothelial cells within the affected zone are activated by hypoxia, shear stress, and the production of reactive oxygen species (ROS) [9]. This causes the expression of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecules (VCAMs), selectins (in particular, P-selectin and E-selectin), and integrins (in particular, Mac-1 and LFA-1) on endothelial cells, leukocytes and platelets [10]. Simultaneously, oxidative stress and locally derived pro-inflammatory mediators (cytokines and chemokines) produced by the damaged tissue alter the permeability of the blood–brain barrier (BBB) and attract leukocytes to the area of injury. As the ischemic cascade progresses, cell death leads to an inflammatory response phase. Dying and dead cells release "danger signals" such as the nucleotides ATP and UTP, and HMGB1 [8]. These signals engage with receptors on immune cells that result in the release of pro-inflammatory cytokines, thereby augmenting the state of inflammation. These initial inflammatory processes result in activation of local microglia as well as a time-dependent infiltration of immune cells into the ischemic hemisphere (Fig. 20.1). These cells include those of the innate immune system, such as neutrophils, macrophages and dendritic cells, together with cells of the adaptive immune system, such as T and B lymphocytes [9, 11, 12].

Neutrophils are one of the most abundant immune cells to infiltrate the brain following stroke. Once in the brain, neutrophils undergo granule exocytosis to release a variety of pro-inflammatory molecules, including large quantities of nitric oxide (NO) derived from inducible NO synthase, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-derived ROS, and matrix metalloproteinases, which subsequently contribute to neuronal damage and BBB permeability [10]. Interestingly, recent evidence suggests that pro-inflammatory cytokines derived from infiltrating T lymphocytes may attract neutrophils to the ischemic area following stroke [13, 14].

Both $CD4^+$ and $CD8^+$ T lymphocytes are major contributors to brain inflammation following cerebral ischemia. $CD4^+$ T cells can be further subdivided into type 1 helper cells (Th1), Th2 cells, Th17 cells and T regulatory cells, based on their functional and cytokine profiles [8, 15]. Th1 cells are pro-inflammatory and release a variety of cytokines such as interferon-γ (IFN-γ), interleukin-1 β (IL-1 β), and tumor necrosis factor (TNF), which can promote inflammation and cell death [15]. By contrast, Th2 cells are anti-inflammatory and release cytokines such as IL-4, IL-5 and IL-10, which can suppress inflammation and the immune response following stroke [8]. Importantly, mice deficient in Th1 cells have reduced stroke-induced infarct damage whereas mice deficient in Th2 cells have exacerbated injury after cerebral ischemia [14]. Furthermore, cytokines released from Th1 and Th2 cells are capable of driving macrophage polarization to either an activated "M1" or an alternately activated "M2" state, respectively [16]. It is important to note that macrophages and/or microglia that assume an M1 phenotype produce a host of pro-inflammatory cytokines,

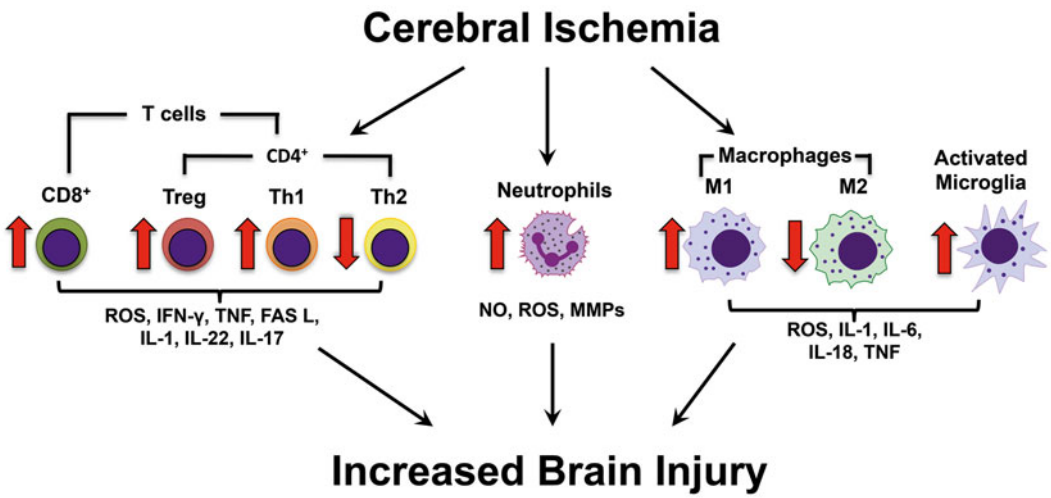


Fig. 20.1 Schematic diagram of immune cells involved in post-stroke inflammation in the brain. Immune cells infiltrate the ischemic hemisphere and are thought to promote cell injury and death via the release of various cytokines and effector molecules. *Upward arrow* increased; *downward arrow* decreased

whereas M2 macrophages and/or microglia probably play a more protective role via the release of anti-inflammatory mediators. It is therefore conceivable that future therapies that alter the balance away from the Th1/M1 and toward the Th2/M2 immune profile may be beneficial for stroke.

The role that $CD4^+$ T regulatory cells (Tregs) play in stroke outcome has received growing interest. Whilst there is some evidence to suggest that Tregs may be cerebroprotective following stroke [17], others indicate they play a minimal [14, 18] or even a detrimental role [19]. Discrepancies between such studies may be explained by specific differences in experimental models or acute versus chronic differences in the role of Tregs after stroke. Further investigations are needed to more fully elucidate the role of Tregs following stroke, particularly to examine both acute and chronic time points after cerebral ischemia.

It is important to highlight that $CD4^+$ T cells are not the only T cell subset thought to influence stroke outcome. $CD8^+$ cytotoxic T lymphocytes are reported to contribute to infarct formation following stroke [14, 20] and, moreover, have the ability to induce apoptosis in already compromised neuronal cells via release of cytotoxins and activation of the Fas receptor [15, 21]. By contrast, the influence that B lymphocytes have on stroke outcome remains controversial. A number of studies have found no evidence that B cells affect infarct formation post-ischemia [20, 22], despite B cells being part of the cellular infiltrate into the brain following stroke [23]. On the other hand, Ren et al. [24] reported that stroke injury is augmented in B-cell-deficient mice compared to wild-type animals. The protective effect mediated by B cells appears to be dependent upon IL-10 secretion, as adoptive transfer of B cells from IL-10-deficient mice had no influence on stroke outcomes [24] while transfer of IL-10 producing B cells to B-cell-deficient mice was found to be protective [25].

4 The Systemic Immune Response Following Stroke

The immune response following stroke is not limited to the brain, with profound effects on immune function also seen in the periphery. These effects include an early activation phase that lasts for 24–48 h, followed by a severe systemic immunosuppression. It is during the early phase that the spleen plays a primary role in the influx of immune cells. For example, Ajmo and colleagues showed that splenectomy prior to experimental stroke reduces infarct size [26]. Moreover, splenectomy-induced neuroprotection following stroke correlated with a decrease in activated microglia, macrophages and neutrophils in the ischemic hemisphere, indicating that the spleen may be a source of detrimental immune cells following stroke [26]. In addition, Offner et al. reported that T lymphocytes, derived from blood and natural killer (NK) lymph nodes, secreted increased levels of pro-inflammatory mediators and expressed elevated levels of chemokine receptors following stroke [27]. More recently, it was shown that the spleen contributes to neurodegeneration through IFN-γ signaling following stroke [28].

Following the initial response, a profound systemic immunodepression or "stroke-induced immunodeficiency syndrome" occurs [15, 29, 30]. This phenomenon is generated by hyperactivity of the sympathetic nervous system and the hypothalamic–pituitary–adrenal axis due to post-stroke brain damage, which leads to decreased numbers of T and B lymphocytes and also of natural killer (NK) cells within the spleen, thymus, bone marrow and lymph nodes [17, 27, 29, 30]. This results in increased apoptosis and increased release of immune cells from these primary and secondary lymphoid organs, resulting in tissue atrophy, which predisposes

patients to infection (e.g., commonly resulting in pneumonia or sepsis), a major determinant of stroke morbidity and mortality.

Although the liver is not a secondary lymphoid organ, an important recent study investigated the effect stroke had on invariant natural killer T (iNKT) cells within the liver [31]. Wong and co-authors showed that cerebral ischemia–reperfusion slowed the migration of resident hepatic iNKT cells in the liver and increased the expression of the immunosuppressive cytokine, IL-10, in association with an increased susceptibility to bacterial infection. In support of these findings, the onset of bacterial infection occurred much earlier in iNKT cell-deficient mice following stroke, whereas cerebral infarct damage was unchanged. This indicated that iNKT cells play an important role in systemic protection and ultimately survival following infection after stroke. Furthermore, the authors found that the increased release of noradrenergic neurotransmitters from sympathetic nerves innervating the liver following stroke can undermine systemic immunity by a direct inhibitory effect on hepatic iNKT cells. Hence, the authors suggested that blockade of stress pathways could improve outcomes in stroke patients by helping to protect systemic immune function and thus preventing infections. Collectively, evidence indicates that various components of the immune system in the brain as well as in secondary lymphoid and visceral organs may play vital roles in the development of post-stroke outcomes.

5 The Potential for Stem Cell Therapy in Stroke

Given the complex, time-dependent molecular mechanisms of injury following cerebral ischemia, as well as the numerous cell types apparently involved, ultimately successful approaches could include cell-based therapies that have the potential to target various injury mechanisms and cell types when administered at an appropriate time(s) after the stroke event. Hence, there is now considerable interest in stem cell therapy as a potential treatment for stroke. Stem cells are undifferentiated cells capable of self-renewal and are broadly classified as being of embryonic, fetal or adult origin [32]. Embryonic stem cells (ESCs) are pluripotent, meaning they can give rise to all cell types of an organism, whereas fetal and adult stem cells are multipotent, such that they can give rise to cells of multiple, but limited numbers of lineages. A number of stem cells, including embryonic, bone marrow, neural and induced pluripotent stem cells (iPSCs) have been shown to improve experimental stroke outcome [33–36]. As a result of such promising preclinical findings, the first fully regulated clinical trial (PISCES study—Pilot Investigation of Stem Cells in Stroke) using a manufactured neural stem cell line to treat stroke patients is underway in Glasgow. Unfortunately, however, there tends to be major limitations with the use of most stem cell types, which may offset their use as a feasible routine treatment for stroke patients.

6 Limitations and Benefits of Stem Cell Lineages for Transplantation

Initially, ESCs were expected to have broad therapeutic potential due to their pluripotent capabilities, and transplantation of human ESC neural derivatives into a rodent model of stroke has been reported to improve functional outcome [33, 35]. Nevertheless, multiple problems exist regarding human ESCs, including ethical/political issues (i.e., due to the destruction of human embryos), immune rejection, and their fetal "age" (i.e., they lack key functional characteristics of adult cells) [37]. Moreover, ESCs may form teratomas (developmental tumors) following transplantation [38].

iPSCs were first derived in 2006 [39] by re-programming mouse and human fibroblasts into pluripotent ESC-like cells. Since then, many types of iPSCs have been created using diverse cell types [40]. iPSCs possess most of the important properties of ESCs but avoid the ethically controversial issues surrounding embryo destruction. These cells have been used to treat central nervous system (CNS) injuries such as spinal cord injury and stroke in rodents [36, 41], but in both cases tumor formation from iPSCs was discovered.

Fetal neural stem cells (NSCs) are isolated from human fetal brains (derived from aborted material) and are capable of differentiating into neurons, astrocytes, or oligodendrocytes [42]. Due to the invasive nature of extracting autologous adult human neural cells, fetal NSCs are considered as an alternative expandable source of neural cells. Although NSCs also involve ethical issues, these cells were thought to be safer than human ESCs regarding tumor formation after transplantation, however brain and spinal cord tumors have been shown to develop following fetal NSC treatment [43–45].

Mesenchymal stem cells (MSCs), derived from bone marrow or umbilical cord blood, can differentiate into neuronal-like cells, astrocytes or endothelial cells, and their administration can decrease infarct size and improve functional outcome in preclinical models of stroke [46, 47]. Transplantation of MSCs can also reduce apoptosis and promote endogenous cellular proliferation after stroke, and long-term follow-up data have revealed improved survival in patients that received bone marrow MSCs compared with controls [48]. Similar to ESCs, however, concerns and limitations associated with MSC use in stroke include poor cell survival and engraftment after transplantation, no direct evidence of functional neuronal differentiation, limited sources, and the fact that their removal from bone marrow requires

invasive procedures, although they do not appear to form tumors after transplantation [49].

7 Human Amnion Epithelial Cells

7.1 hAEC Characteristics

While the above stem cell lineages may have therapeutic potential if their respective issues can be addressed, currently those limitations seriously offset their likely routine use in clinical stroke. An alternative stem cell type that is gaining recognition as a potential therapeutic are human amnion epithelial cells (hAECs). hAECs are derived from the amniotic sac, a thin avascular tissue that encloses the fetus and is attached to the placenta. The innermost layer of the amnion comprises epithelial cells that are in direct contact with the amniotic fluid. It is this amniotic epithelial layer that plays a critical role in providing the embryo with an optimal amniotic environment in which the embryo can develop.

It has been known for more than 30 years that hAECs are safe to transplant into humans [50] and they have a number of properties that make them ideal as a potential therapeutic. For example, they are anti-inflammatory [51], able to modulate host immune cell responses to injury [52], and are anti-fibrotic [53]. Indeed, amnion cells or strips of amnion membrane have long been used to reduce scarring and promote healing in burn victims [54] or following corneal surgery [55]. It is thought that the ability of hAECs to promote "scar-free" healing underlies the ability of the fetus to heal without scarring [56, 57]. hAECs are also pluripotent, including being able to differentiate down neural lineages [58]. More specifically, hAECs may express markers of glial and neuronal progenitor cells, and display multiple neuronal functions, such as synthesis and release of acetylcholine, catecholamines, and neurotrophic factors [59, 60]. Important for any future clinical therapy, and unlike ESCs, hAECs do not form tumors in vivo [50, 61], nor do they differentiate into fibroblasts as MSCs can [62], suggesting that hAECs are an inherently safer cell therapy than these other cells. Further, likely reflecting their role in maintaining maternal immune tolerance of the allograft that is the fetus in pregnancy, hAECs are immunologically inert. Primary hAECs express and release the non-polymorphic, non-classical human leukocyte antigen G (HLA-G) and lack surface expression of the polymorphic antigens HLA-A, HLA-B and HLA-C (class IA) and HLA-DR (class II) [50]. Indeed, when hAECs were administered to healthy volunteers there was no evidence of immune rejection [50]. This is in keeping with the burns and ophthalmology literature where extensive allogeneic use of amnion has occurred without rejection. Moreover, hAECs do not express telomerase and have telomere lengths more in keeping with ESCs than adult bone marrow MSCs [63]. Lastly, with approximately four million births a year (US statistics) and an average yield of 150–200 million cells per placenta [63], hAECs are abundant and relatively easy and cheap to harvest. As such, hAECs are readily available, they require no invasive procedure for harvesting, and they lack major ethical barriers to their use [32].

7.2 hAECs in the Treatment of Stroke

From the information above, it is not surprising that interest in hAECs as a source of allogeneic cells for regenerative therapies is growing [64]. In animal models of CNS disorders, accumulating evidence suggests that hAECs can exert neuroprotection and facilitate neuroregeneration [65–73]. Conversely, only one published study has tested the effect of hAECs on ischemic stroke outcome [74]. It found that direct intra-cerebral (i.c.) injection of hAECs, 24 h after middle cerebral artery occlusion (MCAO) in rats, resulted in a reduced infarct volume and improved behavioral and neurological outcomes at 16 days post-stroke. Furthermore, expression of the key marker of apoptosis, cleaved caspase-3, was reduced in the vicinity of the transplanted cells. The same research group has also reported that i.c. injection of human amnion mesenchymal cells can similarly improve stroke outcome in rats [75]. In analogous studies to those in ischemic stroke, intraventricular injection of hAECs has been reported to reduce brain edema and to improve motor deficit in a rat model of intra-cerebral hemorrhage [76]. Moreover, intra-cerebroventricular transplantation of amniotic fluid-derived stem cells at 3 days post-MCAO resulted in the attenuation of stroke-associated cognitive deficits [77]. Despite these very promising early experimental findings, i.c. injection of stem cells is not attractive as a clinical therapy for stroke patients. This is because i.c. injections would require ready access to suitable imaging facilities and surgical expertise, and could even involve significant adverse effects, such as the breakdown of the BBB and a heightened inflammatory response within the brain [78]. Furthermore, any protective effects from i.c. injection of stem cells would presumably be localized to the immediate region of brain and would not target any of the additionally detrimental stroke-induced effects occurring in the periphery. Therefore, future studies would ideally employ a less invasive and more clinically amenable delivery route of stem cells, such as intravenous (i.v.) injection.

As i.v. injection is a minimally invasive procedure, it poses a substantially lower risk of adverse clinical events when compared to i.c. transplantation and, due to the acute nature of stroke onset, an i.v. injection is ideal so that therapeutics can be administered quickly after the event. However, i.v. administration of stem cells has two initial obstacles that must be overcome: (1) the ability of the cell to pass

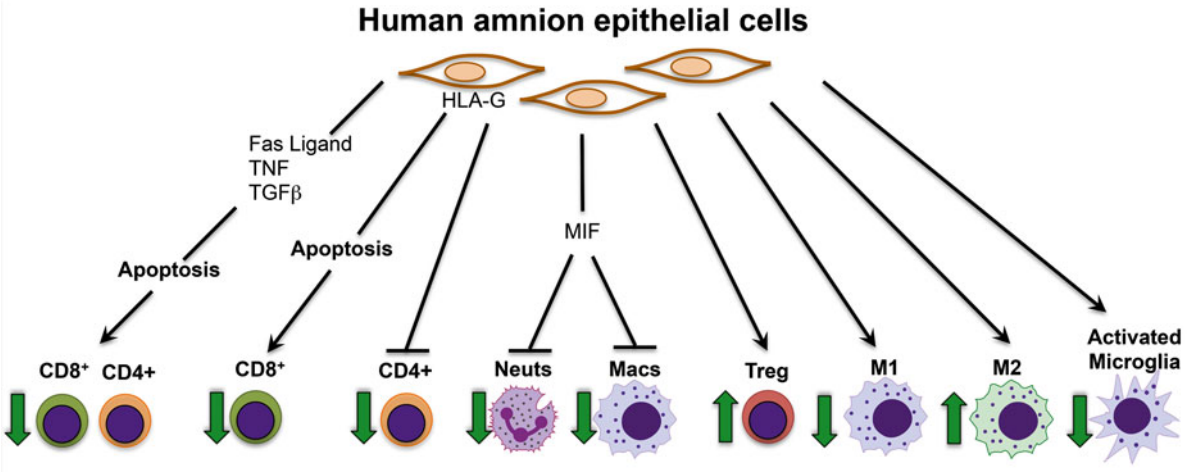


Fig. 20.2 Schematic diagram depicting the various immunomodulatory actions of human amnion epithelial cells (hAECs). It is via these mechanisms that hAECs may improve stroke outcome. *Upward arrow* increased, *downward arrow* decreased, *neuts* neutrophils, *macs* macrophages

through the extensive capillary network of the lungs; and (2) whether the cells can effectively home to stroke-affected regions of tissue in sufficient numbers to provide efficacy. Whether this may occur remains to be tested, but the relatively small diameter of hAECs approximately 15 μm) probably increases the likelihood of these cells passing through the lungs, compared with larger stem cell lineages, such as MSCs, which do not easily passage across the lungs [79]. Indeed, we have reported that only a minor percentage of i.v.-injected hAECs persist in the lungs, even if lung injury has been induced using bleomycin in mice [53]. Thus, it is conceivable that i.v.-administered hAECs may have minimal impact on lung function and that a substantial proportion of these cells can pass into the systemic circulation. In fact, Tarjiri and colleagues have reported that i.v. administration of amniotic fluid-derived stem cells at 35 days post-stroke significantly reduces infarct damage and behavioral deficits assessed at 60–63 days [80]. Hence there is considerable scope to further explore the ability of hAECs to limit injury and/or promote tissue repair and functional recovery when administered systemically following stroke.

7.3 Potential Mechanisms of Action by hAECs in the Treatment of Stroke

There are numerous possible mechanisms by which hAECs could exert therapeutic effects following stroke. Firstly, hAECs could secrete neurotrophic factors that promote recovery of damaged neurons [74, 81]. Such factors could also stimulate synaptogenesis to re-innervate lost connections. Secondly, as hAECs have pluripotent properties, they could differentiate into a neuronal phenotype and replace injured or dead cells [74, 81]. Thirdly, hAECs could act as a "mobile pharmacy," secreting necessary cytokines, growth factors, hormones and/or neurotransmitters to restore cellular function. Lastly, hAECs could potentially improve stroke outcome by modulating the immune response that contributes to brain injury [81, 82]. Included in this mechanism is the protection of neurons from immune cell-mediated apoptosis.

7.4 Immunomodulatory Properties of hAECs

While it was initially proposed that stem cells exerted their reparative effects by integration and differentiation in vivo, growing evidence suggests that it is more likely that they work by modulating the host immune response to cease inflammation and coordinate resolution and repair (see Fig. 20.2). hAECs can exert immunomodulatory actions by actively suppressing T and B lymphocyte proliferation [83] reducing the expression of the potent pro-inflammatory cytokines IL-1α and IL-1β [84], and via producing inhibitors of MMPs and proteolytic enzymes associated with inflammatory reactions. In addition, although expression of HLA-G on hAECs enables their evasion of the immune system, this protein has also been shown to be anti-inflammatory by inducing apoptosis of activated $CD8^+$ T lymphocytes and inhibiting $CD4^+$ T lymphocyte proliferation [85]. Furthermore, hAECs transplanted to the ocular surface can create a local environment that reduces the surrounding inflammatory response [86]. This effect is thought to be due to hAECs reducing infiltration of major histocompatibility complex class II antigen-presenting cells into the inflamed cornea. Furthermore, our group has demonstrated that hAECs transplanted into a bleomycin-induced lung injury model reduces the immune response, preventing lung fibrosis and loss of function [53,

87]. These results were associated with an in vivo reduction in the pro-inflammatory cytokines, TNF, IFNγ and IL-6, and an increase in the anti-inflammatory cytokine, IL-10 [87]. As a consequence of these actions of hAECs on the immune system, there was a reduction in the infiltration of immune cells to the area of damage. In a follow-up study, we found that hAECs reduce macrophage infiltration into the bleomycin-injured lungs, reducing M1 (CD86+) macrophages but increasing M2 (CD206+) macrophages [52]. This is likely to be important to injury repair processes because, as described earlier, M1 macrophages are pro-inflammatory and are involved in the acute inflammatory response, whereas M2 macrophages are immunosuppressive and pro-reparative, coordinating the termination of inflammation, debris scavenging and angiogenesis [88]. Consistent with this, we have also shown that hAECs reduce macrophage migration and stimulate macrophage phagocytosis in vitro—features of an M2 phenotype [52].

With regard to the interactions with T cells, we postulate that hAECs could drive naïve T cells into FoxP3$^+$ Tregs, consistent with a reparative response to injury, analogous to what occurs in pregnancy where the placental/fetal cells manipulate maternal immune function to prevent fetal rejection by biasing maternal immunity away from Th1 and toward Th2 activity—the so-called Th1–Th2 tilt [89]—and where the resultant Tregs drive macrophage polarity to M2 [89]. Thus, if hAECs ultimately influence macrophages to take on an M2 phenotype, and T cells to a Treg phenotype, such actions would be expected to be central to their reparative and protective effects, irrespective of where the injury is. Whether hAECs can provide neuroprotection via similar mechanisms following stroke remains to be established. Tregs certainly enter the brain following stroke where they appear to modulate tissue injury [15]. This is likely to involve the direction of non-Treg CD4$^+$ T cells (T helper cells) toward a Th2-type anti-inflammatory response and away from a Th1 pro-inflammatory response [15]. Whilst there is good evidence for significant macrophage infiltration into the post-stroke brain [90], the extent of damage caused by macrophages, the identity of their phenotype/s (i.e., M1 or M2), or whether their phenotype can be manipulated to alter injury repair remains to be determined.

hAECs are thought to secrete a number of immunomodulatory factors. In fact, supernatant from hAEC culture can inhibit both innate and adaptive immune cells [83]. For example, hAECs produce alpha-fetoprotein, a protein that reduces immune cell reactivity and suppresses neuroinflammation in a mouse model of multiple sclerosis [91]. Furthermore, hAECs secrete macrophage inhibitory factor, which inhibits neutrophil and macrophage migration and natural killer cell-mediated cytolysis [83]. Fas ligand and TNF-related apoptosis-inducing ligand are both members of the TNF family that are produced by hAECs, and can regulate the immune response through apoptosis of lymphocytes [83]. Moreover, hAECs express transforming growth factor-β, which suppresses immune cell numbers through apoptosis [83]. Overall, the immunomodulatory properties of hAECs lead us to speculate that these stem cells may be able to limit the inflammatory response that contributes to infarct formation following stroke.

7.5 Migration of Intravenously Injected hAECs After Stroke

Stem cells communicate with each other and their environment via paracrine signaling [92]. In order to understand why and how cells migrate to their target organs, the relevant chemotactic signal(s) must be identified. Very little is so far known about the chemotaxis response involved in hAEC migration following administration. However, several studies have identified the mechanisms that attract other lineages of stem cells to injured sites following stroke. For example, there is an increase in stromal cell-derived factor-1α (SDF-1α) in brains of experimental animal models of stroke [93, 94] and a subsequent decrease in stem cell migration after the addition of an antagonist of the chemokine receptor type 4 (CXCR4) [94, 95]. SDF-1α is a growth factor produced by multiple types of mouse and human neural cells, and which functions as a chemokine that is thought to be important for neural progenitor migration during development. It is well established that the chemokine interaction between SDF-1α and CXCR4, its cognate receptor that is commonly expressed on the surface of stem cells, plays a major role in stem cell migration [94, 95]. Further research is required to clarify whether CXCR4 and/or other factors play a role in hAEC homing and signaling pathways.

7.6 Ability of hAECs to Engraft and Differentiate and/or Promote Neuronal Repair

Stem cell therapy was originally thought to be an opportunity to treat stroke patients in a manner that ultimately replaced dead neurons with new neurons in the infarcted region of brain. As indicated, hAECs can indeed differentiate toward a neural lineage, which may add to their potential for post-stroke therapy [32, 59, 60]. In fact, i.c. injections of hAECs in rats at 24 h after MCAO were found to migrate to ischemic areas and to then express astrocyte (glial fibrillary acidic protein) and neuronal markers (microtubule-associated protein 2 and nestin) [74]. Correlating with these observations, hAEC-treated rats showed improved behavioral and neurological outcomes, as well as reduced infarct. Thus, the authors suggested that the functional improvement following

hAEC treatment may have been partially due to the newly differentiated neuron-like cells re-establishing connections with surviving host neurons. Similarly, in a hemorrhagic stroke model, hAECs transplanted into the brain were found to express neuron-specific markers and to improve motor deficits after 4 weeks [76]. As a further example, i.v. administration of amniotic fluid-derived stem cells resulted in an increased number of cells expressing microtubule-associated protein 2, and the cell proliferation marker, Ki67, in the dentate gyrus and in the subventricular zone of animals subjected to stroke, indicating increased neurogenesis [80]. Collectively, the existing evidence supports the concept that hAECs can undergo neural differentiation in vivo. Future studies must determine if these newly formed neurons are functional and able to integrate within the existing network of cells to substantially replace dead tissue.

As indicated above, secreted paracrine factors may also play a critical role in hAEC-mediated recovery after stroke. If administered as a delayed post-stroke therapy, hAECs could improve long-term stroke outcome via the release of factors that promote re-innervation, thus restoring synaptic transmitter release to stimulate plastic responses, orchestrating rescue and repair processes, and improving or preserving survival and function of existing neurons. Indeed, it has been shown that hAECs can release trophic factors such as brain-derived neurotrophic factor, neurotrophin-3, nerve growth factor [96, 97] and novel epidermal growth-like factors [98]. Whilst repair of damaged neurons following stroke has yet to be described, hAECs have shown an ability to promote recovery of injured tissue and facilitate functional plasticity in other CNS diseases. For example, transplanted hAECs produce neurotrophic substances and stimulate repair and regeneration of host neurons in a primate model of spinal cord injury [69]. In addition, hAECs transplanted into the cerebral lateral ventricle of a transgenic mouse model of Alzheimer's disease were found to rescue damaged cholinergic neurons [99]. The authors reported that hAEC treatment increased the number of cholinergic neurons, as well as the level of acetylcholine produced by these cells, which was suggested to be largely responsible for the reversal of cognitive decline in this animal model. Therefore, hAECs may possess the ability to both repair and replace lost neuronal tissue and, together with their other anti-inflammatory characteristics, they may represent a very promising cell-based clinical therapy for neurodegenerative diseases, including stroke.

8 Conclusions

In summary, hAECs appear to have key advantages over other stem cell lineages as a cell-based therapy, particularly their non-immunogenic and non-tumorigenic properties. There is evidence that hAECs can cross the BBB where they can engraft, survive for up to 60 days, differentiate into neurons, decrease inflammation, and promote regeneration of damaged CNS tissue in animal models of neurological diseases. We suggest that a concerted focus to characterize the efficacy of post-stroke hAEC therapy in experimental studies may yield valuable information that could be translated to the routine post-stroke use of hAECs in the clinical setting.

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The Potential of Human Amnion Epithelial Cells as an Immunomodulatory and Neuroregenerative Treatment for Multiple Sclerosis

21

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Abstract

Multiple sclerosis (MS) is a chronic autoimmune disease that involves inflammation, axonal damage, and demyelination of the brain and spinal cord. Whilst there are a number of treatments currently available for this often debilitating disease, they all target the immune system with variable efficacy and are often associated with potentially serious side effects. Most compellingly is that whilst these drugs can prevent relapses and minimize long-term disability, they are not curative, do not aid in the regeneration of damaged neural tissue or protect the CNS from neurodegeneration associated with progressive forms of MS. As such there is an urgent need for the development of new therapies that are effective for patients with relapsing–remitting as well as progressive MS and can not only prevent CNS injury but assist with repair. One such potential therapy is stem cell transplantation. A number of different stem cell types have been shown to be efficacious in murine models of MS, and thus clinical trials are being undertaken to investigate the efficacy in the clinic. A novel stem cell source that is gaining attention is human amnion epithelial cells (hAECs). These cells have been shown to suppress inflammation, migrate to inflamed sites within the CNS, engraft and differentiate toward neural lineages. Given their immunosuppressive and neuroregenerative potential, hAECs are attractive candidates for the treatment of MS and other neurodegenerative disorders that affect the CNS.

1 Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system (CNS) [1] that affects more than 2.5 million people worldwide. The disease most commonly occurs in young adults aged 20–40 years and affects women more commonly than men. Symptoms of MS are variable, most commonly including sensory loss, blurred vision, bladder dysfunction, fatigue, memory loss, and paralysis [1]. The cardinal pathological features of the disease include the breakdown of the blood–brain barrier (BBB) and the formation of demarcated plaques within the white matter of the CNS, which are composed of infiltrating immune cells, localized myelin destruction, loss of oligodendrocytes, axonal degeneration, and reactive gliosis [2–4]. Despite these common features, MS is exceedingly recognized as a heterogeneous disease both clinically and pathologically, with a large degree of variability with respect to clinical presentation and clinical course [5]. MS can be divided into four clinical categories, which include, relapsing–remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR). It has been suggested that the course of MS proceeds in two phases, an initial inflammatory

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phase and a degenerative phase [6]. During the initial inflammatory phase of disease, which predominantly presents in the clinic as RR-MS, acute inflammatory plaques of demyelination are formed within the white matter containing lymphocytes, pro-inflammatory cytokines, chemokines and activated macrophages/microglia [7]. RR-MS is the most common form of MS occurring in approximately 70 % of patients and is characterized by sudden attacks causing neurological dysfunction, followed by periods of remission, sometimes with full recovery [2]. Approximately 50 % of RR-MS patients go on to develop SP-MS after their initial presentation with RR-MS. The SP-MS phase is characterized by a steady increase in the level of disability, with few or no recovery periods [8]. A decrease in inflammatory and demyelinating processes with an increase in neurodegeneration is associated with the transition from RR-MS to SP-MS. It is believed that once compensatory mechanisms have been breached, transition to the neurodegenerative phase of disease occurs and neurological deficits become permanent [9]. PP-MS affects approximately 10 % of all MS patients and is characterized by continual neurological deterioration from the onset of disease with no obvious remitting periods [10]. Finally, PR-MS occurs in nearly 5 % of cases, exhibits disease progression from onset, but involves clear acute relapses, with or without recovery [10].

2 Genetic and Environmental Risk Factors

The development of an autoimmune disease is believed to involve several factors, including the presence of self-reactive T cells, that have escaped negative selection during T-cell development in the thymus [11], as well as the inheritance of disease susceptibility genes, which interact with environmental triggers to promote the activation of these auto-reactive T cells [12]. While MS is a multi-factorial disease of unknown cause [4], it is now apparent that genetic background and exposure to certain environmental cues increase the chance of developing MS [2, 4, 13]. Numerous studies have provided strong evidence that there is a genetic component to MS susceptibility, with a high aggregation of susceptibility variants within multi-case MS families [14]. It is well known that for MS, there is a very strong genetic association with human leukocyte antigen (HLA)-DRB1*1501 in the Caucasian population [15]. In other geographic regions, genetic associations have been found in different major histocompatibility complex (MHC) class II alleles, for example, HLA-DRB1*0301, HLA-DRB1*0405, and HLA-DRB1*1303 in the Sardinian population [16]. Although the MHC is considered the major susceptibility locus [17], large genome-wide association studies have identified a number of new genes as having modest associations with MS susceptibility. Interestingly, the majority of the genes identified are involved in immune functions including cytokine pathways (interleukin (IL) 7RA, IL2RA, CXCR5, IL12A, tumor-necrosis-factor receptor superfamily member 1A, and interferon regulatory factor 8), co-stimulation (CD58, CD80, and CD86), and signal transduction (STAT3, CBLB, and MALT1) factors [18, 19].

Despite solid evidence supporting a genetic component, epidemiological studies have shown that other factors are also involved in MS susceptibility. Studies of monozygotic twins have been unable to identify genetic, epigenetic or transcriptome differences that explain disease disconcordance [20], implying that environmental factors are also involved. It has been well established that within regions of temperate climates, the incidence and prevalence of MS increases with increasing latitude [21, 22]. The reason for this difference is still not clear, but it has been speculated that deficiencies in vitamin D levels may be one contributing environmental factor [23]. A recent study has also shown that transcription of the HLA-DRB1*15 allele is regulated by the vitamin D/vitamin D receptor complex [24], offering for the first time, a model linking the interaction between the major genetic and environmental factors that are associated with MS onset.

Another environmental trigger that has been linked to MS is infection with Epstein–Barr virus (EBV) [25]. The mechanism by which EBV may trigger MS is believed to be molecular mimicry [12, 26]. According to this model, self-reactive T cells are activated by cross-reacting with virus-derived products that can mimic host antigens and this results in targeting of self-peptides. In MS patients, an increase in the frequency of activated myelin reactive T cells was found in the circulation as well as in the cerebral spinal fluid (CSF), which is consistent with the hypothesis that the initiating event is linked to an infection in a genetically susceptible host. This may consequently lead to autoimmune-mediated demyelination and axonal destruction [26].

3 Involvement of the Immune System in MS

It is clear that the immune system is a major initiator and currently the predominant therapeutic target for MS. The immune system consists of two arms, adaptive and innate, both of which are comprised of cell-mediated and humoral components [27]. T cells form part of the cell-mediated immune response and during T-cell development a process known as central tolerance ensures auto-reactive T cells are eliminated via apoptosis [12, 28]. Errors in central tolerance lead to the escape of auto-reactive T cells into the periphery.

Failing central tolerance, auto-reactive T cells that do escape into the peripheral lymphoid organs remain tightly regulated by peripheral regulatory mechanisms, which may lead to these auto-reactive T cells becoming anergic or deleted [29]. Furthermore, B cells, which are part of the humoral adaptive immune response, are now increasingly viewed as multi-potent players in CNS inflammation and demyelination. B cells can promote the differentiation of naïve T cells into Th1 and Th17 pathogenic T cells and this was reversible by B-cell depletion [30]. Furthermore, a study examining rMOG-induced EAE in B-cell-deficient mice showed that reconstitution with MOG-primed B cells or MOG-primed serum was necessary for restoration of EAE susceptibility [31].

3.1 T-cell Activation and Their Entry into the CNS

Peripheral myelin-reactive T cells are believed to play a key role in the development of MS. However, the reasons for their initial activation are not well understood. During inflammatory responses, naïve T cells are primed by professional antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages, which engulf and process antigens into fragments that can be recognized by other immune cells. Failure of these cells to maintain tolerance has been shown to lead to autoimmune diseases including MS [12]. Primary T-cell activation requires interaction of the peptide–MHC complex with the T-cell receptor (TCR), as well as with other co-stimulatory signals. Once activated, T cells (CD4+ T cells) can then breach the BBB and cross over into the CNS. Within the CNS, CD4+ T cells are re-activated through binding of the TCR to its cognate CNS antigen presented by local APCs, leading to T-cell proliferation and secretion of pro-inflammatory cytokines. Other effector cells, such as monocytes and macrophages, are then recruited to sites of inflammation, where macrophages secrete cytokines, chemokines and cytotoxic molecules such as glutamate and nitric oxide [32–34] and facilitate the destruction of myelin and axonal damage. While CD4+ T cells have also been identified as the primary disease inducing component in EAE [26], the pathogenesis of the disease is also dependant on many of these other cell types. For example, myeloid DCs were recently shown to drive relapses in EAE by preferentially promoting the differentiation of naïve CD4+ T cells into Th17 cells [35]. Astrocytes and microglia express negligible levels of MHC molecules in healthy CNS tissue, however, during inflammation, these cells upregulate MHC II and co-stimulatory molecules and produce cytokines such as IL-12 and IL-23, which further promote T-cell responses [36–40].

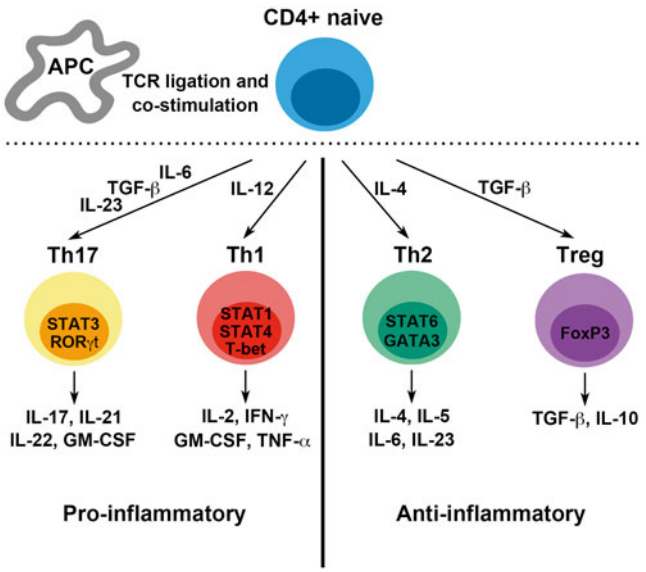


Fig. 21.1 CD4+ T-cell differentiation, cytokine production and their implications in EAE and MS. Naïve CD4+ T cells exit the thymus and upon interactions with antigen-presenting cells they differentiate into different CD4+ T helper lineages depending on the cytokine milieu present. These lineages include Th1, Th2, Th17 and Tregs, which are naturally derived from the thymus or induced in the periphery. Each lineage is under the control of different transcription factors and defined by the particular cytokines they produce. Th1 and Th17 cells are known to be pro-inflammatory T cells that initiate and potentiate EAE and MS, while Th2 and Treg cells are anti-inflammatory and help suppress and reverse disease severity

3.2 CD4+ T-Cell Differentiation and Cytokine Secretion

For many years it was believed that CD4+ naïve T cells could be driven down two pathways, producing pro-inflammatory T helper 1 (Th1) cells or anti-inflammatory T helper 2 (Th2) cells. Th1 cells differentiate from naïve CD4+ T cells under the control of IL-12, which is predominantly expressed by activated DCs [41], while IL-4 promotes the differentiation of naïve CD4+ T cells into Th2 cells [42] (Fig. 21.1). Each lineage is under the control of different transcription factors and is defined by the particular cytokines they produce. Th1 cells predominantly secrete IL-2, interferon-γ (IFN-γ) and TNF-α, whereas Th2 cells secrete IL-4, IL-6 and IL-13 [42]. The main effector T-cell lineage responsible for the pathogenesis of EAE and MS was believed to be Th1 cells, due to the fact that both diseases correlate with increased expression of IFN-γ and IL-12 expression in the CNS [43]. Thus, IFN-γ became one of the primary targets for immune modulation and protection in EAE research [44]. However, it was soon recognized that IFN-γ and IL-12 were not the only cytokines involved in the pathogenesis of EAE, as mice

deficient in IFN-γ and IL-12 displayed more severe disease progression than wild-type littermates [37, 44–46].

A new CD4+ T-cell lineage involving IL-23 and IL-17 has now been implicated as playing a prominent role in the pathogenesis of EAE [47]. This new subset, termed T helper 17 (Th17) cells, secrete IL-17, IL-21 and IL-22 and their differentiation is under the control of transforming growth factor-beta (TGF-β), IL-6 and IL-23 [47–49] (Fig. 21.1). Th17 cells are able to potentiate autoimmune responses in EAE [50] and induce EAE when adoptively transferred into mice [48]. In line with these results, IL-17-deficient mice develop less severe EAE compared to wild-type littermates [51] and neutralizing antibodies directed against IL-17 ameliorates EAE [52]. IL-17 has also been implicated in MS, with increased numbers of IL-17 transcripts detected in chronic MS lesions compared with acute lesions or control tissue from patients [53]. Interestingly, similar to Th17 cells, TGF-β is also required for the development of T regulatory (Treg) cells [54] (Fig. 21.1), suggesting some developmental plasticity between Th17/Treg lineages. The induction of a transcription factor from the forkhead box family, FoxP3, by TGF-β is critical for Treg development [55], but the presence of IL-6 can inhibit FoxP3 upregulation and skew T-cell differentiation toward a Th17 phenotype [49, 54]. Therefore, it was proposed that IL-6 production may be important in controlling the extent of inflammation in EAE, via the Th17/Treg balance [56]. Tregs are generally classified as being CD4+ CD25+ T cells, however in recent years it has become apparent that FoxP3 is necessary for the development and function of naturally occurring Treg cells [57–59]. It is believed that during T-cell development in the thymus, naturally occurring Tregs positive for FoxP3 are delivered into the periphery, where they make up 5–10 % of CD4+ T cells [60]. To add to the complexity of these peripheral tolerance mechanisms, naturally occurring FoxP3+ Tregs are not the only regulatory T cells present. Many other regulatory T cells have been described including TGF-β producing T helper 3 (Th3) cells, IL-10 producing type 1 T regulatory (Tr1) cells, CD8+ CD28− T cells, γδ T cells and NKT cells [61–63].

4 Neurodegeneration in MS

It is now recognized that in addition to demyelination, progression of MS leads to considerable damage to neurons, which results in brain atrophy and cumulative disability. Furthermore, axonal degeneration has been shown to directly correlate with both permanent disability and brain atrophy in advanced MS [64]. However, it is well documented that in RR-MS, demyelination in early MS is not permanent and remyelination by oligodendrocytes occurs [64]. This suggests that the brain does possess endogenous repair mechanisms, however, over time these mechanisms either fail or are no longer sufficient to repair the ongoing damage.

There are many factors that contribute to the failure of the CNS to repair itself, these include the physical barrier created by the astroglial scar and the release of growth inhibitory factors that inhibit the migration of progenitor cells and not only prevent myelination, but also axonal regeneration. These inhibitory factors can be produced by a number of cells including oligodendrocyte precursor cells (OPCs), microglia and meningeal fibroblasts [65]. Furthermore, myelin debris found in lesioned areas also contributes to the inhibition of axonal regeneration [66]. Five myelin proteins have so far been characterized with inhibitory activities, these include: Nogo A, oligodendrocyte myelin glycoprotein (OMgp), myelin-associated glycoprotein (MAG), semaphorin 4D, and ephrin B3 [67]. Signaling by these proteins results in growth cone collapse, which usually drives the extension of the growing axon toward its target.

It is well known that the brain has limited plasticity, however, endogenous OPCs have been identified in the adult brain [68]. Moreover, OPCs have been identified in chronic MS lesions and they appeared to interact with dystrophic axons but were unable to remyelinate these damaged axons [69]. The mechanisms behind this failure of endogenous oligodendrocyte repair are not fully understood, but are believed to be due to the presence of myelin inhibitory proteins as well as the presence of the astroglial scar, the insufficient recruitment of OPCs and deficiencies in their ability to differentiate into myelinating oligodendrocytes. Multipotent progenitor cells, including OPCs and neural progenitor cells (NPCs), reside in specific areas of the CNS including the subventricular zone. The migration of OPCs is regulated by a number of chemoattractants and chemorepellants. Cues from growth factors such as platelet-derived growth factor-a (PDGF-a) recruit OPCs to areas of damage in the CNS where further stimuli are required for proliferation, differentiation, and myelination. The breakdown of the BBB in MS results in exposure of progenitor cells in the SVZ to additional stimulatory molecules that direct their migration, such as nerve growth factor (NGF) [70]. NGF, while playing an important role in differentiation and survival of neurons, also stimulates oligodendrocyte differentiation. Treatment paradigms that can enhance this already endogenous reparative process will therefore be important in improving the clinical outcome for MS patients.

5 Current MS Therapies

Significant efforts are being invested into developing novel therapies for MS and understanding the mechanisms by which such therapies have their effects, as there is at present no cure for this disease. Nonetheless, most of the current drugs that are used in the clinic act in an immunomodulatory or immunosuppressive manner [5, 71, 72], with the primary aim of disabling the immune system. This can be achieved in

a number of ways, including targeting whole populations of immune cells involved in the disease pathology (i.e. activated T cells), blocking migration of peripheral lymphocytes into the CNS or "resetting" the immune system by deleting and replacing the existing immune cell repertoire [72]. Currently available treatments used for RR-MS in Australia include interferon-beta (IFN-β) preparations (Avonex, Betaferon and Rebif), glatiramer acetate (Copaxone), natalizumab (Tysabri), Fingolimod (Gilenya) and Teriflunomide (Aubagio) with dimethyl fumarate (Tecfidera) currently under review by the Therapeutic Goods Administration. Although IFN-β preparations and glatiramer acetate treatments have proven to be safe with long-term application and lead to a reduced number of relapses [73], nearly 70 % of RR-MS patients do not respond and little effectiveness has been shown in patients with progressive forms of MS [72, 74, 75]. Natalizumab is a more potent therapy but this is tempered by a risk of progressive multifocal leukoencephalopathy (PML) [72]. Other humanized monoclonal antibodies that can specifically block molecules relevant to the pathogenesis of MS are completing clinical trials: these include Daclizumab (Zenapax) [76–78], Rituximab (Rituxan) [79, 80], and Alemtuzumab (Campath) [81–83]. Fingoloimod and Teriflunomide specifically target lymphocytes, known to play a role in disease pathogenesis, and have also shown moderate efficacy [84–88]. However, a fundamental issue with these newer treatments is that while they deplete or functionally inhibit pathogenic immune cells, they also have the same effect on the normal immune response. This consequently leaves the patient immunocompromised, as has been demonstrated with deaths from disseminated herpes and varicella. For this reason there is a clear need for improved therapies that are aimed at providing more specific "targeted" treatments, as well as incorporating neuroprotective strategies to prevent the progression of disease to chronic disability. The remainder of this review will be exploring the potential of a new treatment strategy that may help in overcoming some of the pitfalls we face with current therapeutic approaches for MS, stem cell therapy holds the promise of not only offering immune suppression, but also potential neuroprotection and neuroregeneration.

6 Potential of Stem Cell Therapy for MS

Stem cells are characterized by their multipotentiality and their capacity for self-renewal, which is purported to be essential for their role in the development, maintenance, and repair within specific tissue niches [89]. Given that stem cells are also highly immunosuppressive [90] they are, in principle, ideal for targeting the inflammatory process in MS, in addition to potentially promoting and/or enhancing spontaneous remyelination [13]. Transplantation of human and murine-derived mesenchymal stem cells (MSCs), as well as neural precursor cells (NPCs), has been shown to ameliorate clinical signs of EAE as well as to reduce CNS pathology [13, 71, 90–95]. It has also been shown that when MSCs are injected intraventricularly into EAE mice they traffic to areas of CNS inflammation [94]. A large international multicenter clinical trial is currently underway to assess the safety and efficacy of MSC in treating active RR-MS [96].

However, there are limitations in the therapeutic usage of MSCs. These include the requirement of invasive procedures to acquire these cells by bone marrow biopsy, as well as the low number of adult stem cells that can be isolated from adult tissues, necessitating extensive culture expansion. This restricts their use for autologous transplantation in adults. A novel type of stem cell that is gaining interest among the stem cell research community as a potential alternative are human amnion epithelial cells (hAECs). The advantage of these cells is they are easily obtained in large numbers from discarded term placenta and have been reported to possess properties similar to both embryonic stem cells (ESCs) and MSCs [97]. Given that there are approximately 300,000 births each year in Australia alone, amnions provide an abundant potential source of regenerative cellular material and do not possess ethical constraints associated with sourcing ESCs from discarded embryos. Similar to ESCs, hAECs are pluripotent and have the ability to be expanded in culture [98], although the large number of cells that can be obtained from a single amnion may preclude the necessity for expansion in a clinical setting. Importantly and in contrast to ESCs, hAECs do not form teratomas in vivo [99, 100]. Significantly, hAECs display strong immunomodulatory and immunosuppressive properties and thus offer significant practical advantages for potential clinical applications in autoimmune disease therapy [95].

While stem cell therapy has shown promise in a number of disease models, the question of optimal administration is still unresolved and will most likely be disease-specific. However, the appropriate dosage and administration route are very important to the successful treatment of diseases with such therapies. With regards to stem cell therapy in neurodegenerative diseases, currently numerous pre-clinical studies have explored different administration routes, including local delivery into the CNS, intravenous and intraperitoneal injections. The rationale for local administration was to allow stem cells to engraft into the injured tissue and potentially differentiate into neuronal cells. Unfortunately, while some animal studies have demonstrated stem cell engraftment and potential neural differentiation, it is now widely believed that stem cells poorly engraft into the CNS tissue, despite their clinical effectiveness [101]. Moreover, clinical improvement has been found to be comparable following systemic or local stem cell delivery, which suggests that stem cells are most likely having their effect through the release of soluble factors [102]. Thus, the current consensus is that

stem cell therapy is most likely acting by modulating the immune system and potentially increasing neuroregeneration, not through differentiation, but through the release of neurotrophic factors that support host neuroregeneration.

7 Immunomodulatory Capacity of hAECs

The immunomodulatory potential of hAECs has been studied in numerous in vitro assays and in vivo disease models. hAECs are believed to exert their immunomodulatory functions on several immune cells including T cells, B cells, and macrophages (Fig. 21.2). One of the mechanisms suggested is through the secretion of immunomodulatory factors. As shown by Li et al., conditioned media from hAEC cultures has the ability to inhibit cells of both the humoral and cell-mediated adaptive immune system, as shown by the reduction in T- and B-cell proliferation [103]. Various soluble factors have been implicated; these include alpha-fetoprotein (AFP), macrophage inhibitory factor (MIF), Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), TGF-β and human leukocyte antigen-G (HLA-G) [103] (Fig. 21.2). Studies have revealed that hAECs produce AFP [104], which has been shown to reduce lymphocyte activity and decrease neuroinflammation in an animal model of MS [105]. Additionally, hAECs express HLA-G on their surface and in soluble form [106]. HLA-G is a non-classical HLA class I antigen that is believed to provide protection for the developing fetus against the maternal immune system [107]. HLA-G has also been shown to induce apoptosis of activated CD8+ T cells and inhibits the proliferation of CD4+ T cells [108]. Furthermore, in vitro co-cultures have shown that hAECs can significantly suppress antigen-specific, allogeneic, and mitogen-stimulated T-cell responses [109, 110].

Currently available data suggest that the immunomodulatory and reparative actions of hAECs are intrinsically linked with macrophage activity. In an inflammatory lung injury model, where damage was induced by administration of bleomycin, a potent stimulator of lung fibrosis, hAECs were found to modulate the host inflammatory response, reduced lung fibrosis, and prevented the loss of lung function [111]. A subsequent study was performed using surfactant protein C knockout mice, which are highly susceptible to lung injury due to impaired macrophage function. It was found that following the bleomycin insult, administration of hAECs was unable to decrease lung fibrosis or improve lung function [112]. The authors postulated that this failure of hAECs to exert their reparative effect was directly due to impairment of the normal host macrophage function. Furthermore, hAECs were shown to directly influence macrophage behavior, in vitro and in vivo, and polarized macrophages toward a pro-reparative M2 phenotype [113] and this polarization was

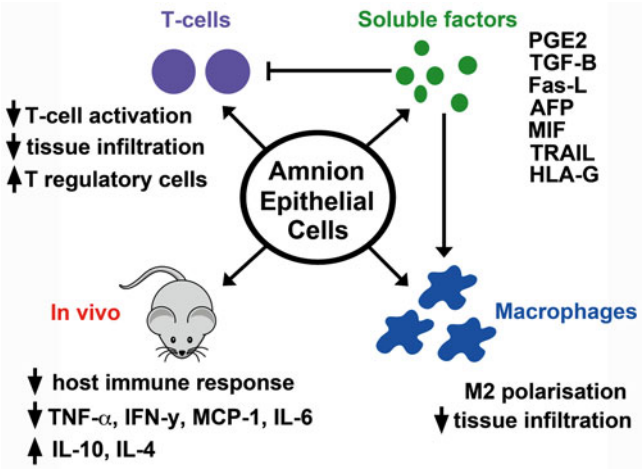


Fig. 21.2 Immunomodulatory properties of hAECs. hAECs are known to suppress the function of many immune cell types. hAECs can suppress T-cell activation, infiltration into inflamed tissue and can increase the number of anti-inflammatory Treg cells. hAECs can also decrease macrophage infiltration into inflamed areas and promotes polarization of macrophages to a pro-reparative M2 phenotype. These immunomodulatory actions are believed to be due to the release of soluble factors including PGE2, TGF-β, Fas-L, AFP, MIF, TRAIL, and HLA-G. hAECs have been shown to modulate the host immune system in vivo, by decreasing the production of TNF-α, IFN-γ, MCP-1 and IL-6 and increasing the production of anti-inflammatory cytokines IL-10 and IL-4

suggested to be critical for the hAECs reparative effect observed in this lung fibrosis model. Similarly, in a model of liver fibrosis [114], transplanted hAECs have been associated with polarization of macrophages to an M2 phenotype associated with an increase in IL-10 and reduction in fibrosis. Macrophages are understood to have a dual role in the pathophysiology of MS. In active MS lesions associated with high levels of pro-inflammatory cytokines, classically activated or M1 macrophages mediate significant cell injury. However, an alternatively activated M2 type of macrophage can be generated through exposure to cytokines such as IL-4 and IL-10 and are involved with cell repair [115]. Although the influence of hAEC on the macrophage polarization in EAE or MS has not been directly examined, macrophage infiltration has been shown to be reduced in chronic EAE [116]. Moreover, studies in spinal cord transection [117] and intracerebral hemorrhage [118] have found reduced gliosis and microglia in animals treated with hAEC.

In addition, hAEC administration has been shown to directly suppress the host immune response. In the bleomycin lung fibrosis model, hAEC administration significantly decreased the local pro-inflammatory response in the lungs by decreasing the expression of TNF-α and IFN-γ [111]. Interestingly, hAEC treatment also significantly reduced the expression of IL-6 and TGF-β [111], both of which are known to drive the differentiation of pro-inflammatory Th17 cells [49], which play an important role in EAE and MS pathogenesis. In a fetal sheep model of intrauterine

inflammation, hAECs were found to attenuate local lung inflammation with a decrease in TNF-α, IL-1β, and IL-6 observed [119]. Moreover, hAECs reduced local brain inflammation and subsequently decreased white and grey matter injury [120]. Emerging data from our lab [109] and others [116] have also suggested that hAECs can modulate the autoimmune response in EAE, an animal model of MS. It was reported that intravenously administered hAECs reduced immune cell infiltration into the CNS and consequently demyelination was reduced in these animals. Furthermore, the authors postulated that this effect was due to a Th2 bias that may in part be mediated by TGF-β and PGE2 [116]. The exact mechanisms and contribution of other immune cells to this observed immunomodulation is yet to be elucidated, however results thus far are promising and warrant further investigation.

8 Neuroprotective/Regenerative Ability of hAECs

Stem cells have the capacity to work by means of paracrine neuroprotective, angiogenic and anti-inflammatory mechanisms, along with the expression of neurotrophic and survival factors that have the potential to stimulate endogenous progenitor cells [121]. Given the lack of sufficient endogenous repair within the CNS there is tremendous interest in cell therapies with stem cell-like properties that could potentially replace and repair injury to the CNS. hAECs are formed from aminoblasts derived from the embryonic epiblast, 8 days after fertilization and prior to gastrulation and thus share many features of ESCs. Moreover, hAECs have been found to express characteristic ESC genes including POU5F1, SOX2, CFC1, NANOG, DPPA3, PROM1, and PAX6 and surface antigens SSEA-4 and GCTM2 [99], suggesting potential for multipotency. Furthermore, mRNA expression and positive immunocytology evidence of immature and mature neural antigens such as nestin and GFAP in hAECs have suggested that these cells are predisposed to differentiate into mature neural cells. In fact, high-density cultures consisting of hAEC spheroid structures have been compared with neurospheres due to the heterogeneity of immature neuronal and glial cell surface protein expression [122, 123]. Following culture in neural differentiating medium, most hAECs appear to express GFAP with astrocyte morphology [99] while a minority express NES or MAP2 neuronal markers.

In vivo studies using hAECs have demonstrated cells can survive and function for at least 4 weeks, some studies up to 8 weeks, following xenogenic transplantation with no evidence of recognition or rejection by the host immune system [124]. A number of studies have demonstrated the therapeutic efficacy of hAEC administration in different neurodegenerative diseases (summarized in Table 21.1). Furthermore, these studies have shown hAECs possess differentiation potential in vivo with the expression of neural markers nestin, MAP2 and GFAP after transplantation of hAECs into rodents [118, 125, 126]. AECs have also been isolated from rats and shown to survive and express MAP2 in a gerbil cerebral ischemia model [127]. Furthermore, significant repair, remyelination and improved spinal cord electrophysiology was observed in a rat spinal cord injury model following transplantation of hAEC within a muscle graft [128]. However, the authors could not identify any neuronal differentiation to account for these findings. In another neurodegenerative model, Kakishita et al. [129] found improved apomorphine-induced rotational asymmetry in the dopamine-depleted striatum of immunosuppressed rats as a result of hAEC transplantation, but found the cells retained their round or oval morphology rather than obtaining neuronal morphology.

Table 21.1 Pre-clinical studies investigating the therapeutic efficacy of hAECs in neurological disorders

Neurological disorder	Study outcome	References
Parkinson's disease	hAECs decreased rotational asymmetry and grafts expressed nestin and vimentin	Yang et al. [126]
Spinal cord injury	hAECs promoted growth and sprouting of host axons and prevented glial scar formation	Sankar et al. [117]
	Overexpression of growth factors in hAECs improved NSCs survival and differentiation in vitro and promoted NSCs differentiation in vivo	Meng et al. [131]
	hAECs promoted regeneration and sprouting of axons and improved hindlimb motor function	Wu et al. [130]
Stroke	hAECs expressed vimentin and nestin in vivo, reduced activated microglia at the injury site and brain edema and improved the motor deficits	Dong et al. [118]
	hAECs were readily transduced with lentiviral vectors. hAECs administration rescued the neurological deficits and decreased neuronal cell death and reduced lesion volume	Liu et al. [125]
Multiple sclerosis	hAECs potently attenuated disease, reduced T-cell and macrophage infiltration and reduced demyelination	Liu et al. [116]
Neonatal brain injury	hAECs reduced CNS inflammation and neuronal cell death	Yawno et al. [120]

hAEC transplantation has been associated with improved clinical recovery in a number of CNS injury models including intracerebral hemorrhage [118], cerebral ischemia [125], spinal cord transection [130, 131], and MPTP and 6-OHDA-induced toxicity. These effects do not appear to be explained solely by the differentiation of hAECs into neurons, but instead suggest the hAECs produce neurotrophic factors that may protect neurons and promote endogenous repair. This observation is supported by in vitro work that clearly demonstrates that the conditioned media of hAECs can improve neuron survival and increase neurite outgrowth [132]. AECs are known to produce a number of different growth factors with the potential to facilitate neuroregeneration. Uchida et al. [132] demonstrated that hAEC produce BDNF and NT3 and that these factors were partially responsible for the improved neuron survival and neurite outgrowth in embryonic rat neuronal cell culture. Similarly, BDNF and NT-3 were identified in the conditioned media of hAEC cultures and it was shown that these factors could improve neural stem cell survival [133]. However, these specific factors alone were not as effective as total hAEC-conditioned media, suggesting that other important factors are yet to be identified.

Sakuragawa et al. [134] observed an increase in survival of E18 cortical neurons with hAEC-conditioned media and concluded that the responsible neurotrophic factor is unknown but may have properties similar to EGF since this cell type only responds to EGF but hAEC did not appear to express EGF mRNA. Correspondingly, Venkatachalam et al. found that hAEC-conditioned media improved the survival and differentiation of chicken neural retinal cells which are known to be highly responsive to FGF-2 and therefore the authors concluded that the responsible neurotrophic factor from the conditioned media was possibly related to FGF-2 [135]. However, examination of hAEC-conditioned media for known isoforms of EGF and FGF-2 by Western blot was negative. Recently, the neurotrophic factor pleiotrophin was identified in the conditioned media of hAECs, and furthermore pleiotrophin was demonstrated to promote the differentiation of UCB-MSC into dopaminergic (DA) neurons in vitro [136]. Multiple roles for pleiotrophin in neurite outgrowth and migration, repair and differentiation of progenitor cells have been described [137, 138]. It is likely that the neurotrophic effect of hAECs is the result of multiple growth factors acting synergistically rather than in isolation. hAECs have also been shown to produce catecholamines including dopamine and norepinephrine through tyrosine hydroxylase [139]. This discovery led to a large number of studies investigating the beneficial role of hAEC transplantation in animal models of Parkinson's disease, a neurodegenerative condition characterized by loss of DA neurons. Collectively these studies demonstrated improved survival of DA neurons associated with improved clinical scores in MPTP and 6-OHDA rat models, following intracerebral hAEC transplantation [123, 126, 129, 140]. The mechanism for these observations remains controversial. While hAECs can differentiate into DA neurons in vitro, the observed in vivo improvements appear greater than would be expected given the limited survival of hAEC-derived DA neurons [129]. The production of neurotrophic factors is a more plausible explanation, which is supported by improved survival of DA neurons when cultured in hAEC-conditioned media [141]. Although high doses of catecholamines are clearly neurotoxic, low doses have been shown to protect neurons against free radical-induced oxidative stress [142]. Furthermore, Noh et al. also demonstrated a synergistic action of dopamine in prolonging the neuroprotective effects of BDNF on neuronal cell death [142].

9 Conclusions

Given the lack of successful treatments available for many relapsing–remitting and progressive MS patients, there is an urgent need to develop therapies that can treat the multitude of pathologies that are involved in MS. As outlined in this review the involvement of the immune system is very important and has thus far been the primary target for current clinical treatments. However, as we have highlighted, neurodegeneration plays an equally important role in disease pathogenesis, particularly in the progressive stages. Furthermore, these two pathways do not act independently and by minimizing peripheral and central (directly within the CNS) inflammation, this can indirectly protect the CNS from neurodegeneration. Therefore, future treatments need to address both of these components. As highlighted in Table 21.2, stem cells, particularly hAECs, offer the ability to target and reverse multiple pathological pathways that contribute to the development and progression of MS. For instance, pathological consequences of MS include increased T-cell activation, increased generation of pathogenic Th1 and Th17 cells and little or no neuroregeneration. Conversely, hAECs have been shown to inhibit T-cell activation, suppress the development and pro-inflammatory actions of Th1 and Th17 cells and in addition can secrete neurotrophic factors such as BDNF, NGF, and pleiotrophin that promote neuroregeneration.

While hAECs promise to be an adjunct for the treatment of inflammatory and neurodegenerative diseases, an important question that still remains is their safety once transplanted into patients. Studies have shown that hAECs are immunoprivileged and immunosuppressive and therefore appear to be safe to use in an allogeneic setting. However, the requirement of HLA matching is still to be determined. In this context it is worth noting that amniotic membrane and its epithelial layer (from which these hAECs originate) have been used for over 30 years to treat several clinical conditions [143]. These include: dural defects [144], ocular surface reconstruction in conditions such as keratopathy [145] and conjunctival

Table 21.2 Potential of hAECs to reverse pathological consequences of MS

Pathological consequences of MS	Beneficial effects of hAECs
Increased T-cell activation	Inhibits T-cell activation
Increased number of Th1/Th17 cells	Suppresses actions of Th1/Th17 cells
Decreased number of Th2/Treg cells	Promotes generation of Th2/Treg cells
Little/no neuroregeneration	Secrete neurotrophic factors to aid in neuroregeneration (e.g. BDNF, NGF, pleiotrophin)
Formation of glial scar tissue	Promotes M2 macrophage polarization which aids in tissue remodeling and decreases fibrosis

Bowens disease [146], as well as for the treatment of burn lesions [147]. In these conditions, hAECs have been shown to be well tolerated, did not form tumors and did not result in graft rejection. Given the current safety profile of the amnion, it is likely that hAECs could be investigated in phase I clinical trials. However, issues associated with their immunogenicity and other factors will need to be carefully assessed before trials are commenced. This aspect is particularly pertinent, given that hAEC graft survival can be significantly reduced in pre-immunized animals [148]. This was illustrated in a study by Gabler and Lohmann, when serial transplantation of amniotic membrane into the eye (which is immune privileged) generated an inflammatory response after the second and third transplantation [149]. These results suggest that the continuous immunosuppressive and regenerative effects of hAECs in a transplantation setting will require several immunological barriers to be overcome.

Nonetheless, results from pre-clinical animal studies suggest that hAECs are strongly immunosuppressive and can potentially induce peripheral immune tolerance and reverse ongoing inflammatory responses. Furthermore, hAECs retain the capacity to differentiate into neural cells in vivo and it is now believed these cells may enhance endogenous neuroregeneration through the secretion of a vast array of neurotrophic factors. Encouragingly, amniotic tissue has already been shown to be effective and safe in the clinic. As such, the transition of hAECs from pre-clinical stages to the clinical setting may be hastened. However, further research is required to understand the underlying mechanisms that contribute to their therapeutic potential. In conclusion, hAECs offer a new promising cellular alternative that is easily obtained in the large quantities required for therapeutic transplantation and have been shown to be immunomodulatory, neuroprotective and these attributes make them attractive potential cellular therapies for neurodegenerative diseases such as MS.

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Human Amniotic Epithelial Cells Transplantation for Contusive Spinal Cord Injury Repair

22

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Abstract

Adult tissues were discovered to possess resident stem cell populations and were termed as mesenchymal stem cells (MSCs). These stem cells are considered to be the best alternatives to overcome certain limitations associated with embryonic stem cells. Epithelial cells of amnion were reported to exhibit characteristic features of stem cells and neural cells. This led to speculation of using them for treating various pathological conditions including spinal cord injury (SCI). Studies conducted elsewhere and our laboratory found beneficial aspects of amniotic epithelial cell (AEC) transplantation for certain neurological conditions. Studies on the usefulness of these cells in SCI appear to be limited. In our lab, we have observed beneficial effects of human amniotic epithelial cells (hAECs) when transplanted in contusive SCI model in rats. This study was found to be unique as there was no other study in which these cells were tested for contusive SCI repair. In our experience, these cells may not possess the two important criteria to be qualified for stem cells viz. self-renewal and multilineage differentiation capacity. Notwithstanding to be qualified for the stem cell tag, amniotic epithelial cells may have the potentials for contusive SCI repair.

Abbreviations

AEC	Amniotic epithelial cells
CNS	Central nervous system
EGF	Epidermal growth factor
FGF-2	Fibroblast growth factor
hAEC	Human amniotic epithelial cells
MSC	Mesenchymal stem cells
SCI	Spinal cord injury/spinal cord injured

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Spinal cord injury (SCI) is a devastating neurological condition, which renders its victims wheel chair bound to the rest of their life. Spinal cord injuries are classified as complete and incomplete (*Editorial*, *J Spinal Cord Med. 2013*). In complete (lacerative) injury, the spinal cord is anatomically severed which is a grave condition with no scope of recovery. Incomplete injuries are due to either compression or contusion. Compression types of injuries are often due to other pathologies such as tumors, which are slow in onset. When the primary cause of compression is effectively controlled, often complete recovery is possible. Contusion injuries due to trauma are the most common types of human SCI in civil life. Incidences of contusive traumatic SCI are due to vehicle accidents, fall from heights, sports injuries, etc. (*Source*: *National Spinal Cord Injury Statistical Center*, *University of Alabama at Birmingham*—https://www.nscisc.uab.edu/). It is no wonder much research works are focused in finding a cure for contusive SCI as it is not only the major type of human injuries but also gives scope for intervention.

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1 Therapeutic Strategies Tested for SCI

Different therapeutic strategies have been tested for SCI repair in experimental studies [1]. Table 22.1 gives a list of some of them.

Detailed descriptions of SCI pathology would be beyond the scope of this chapter and it may be pertinent just to state that it is a complex interplay of several factors such as ischemia, excitotoxicity, oxidative stress, delayed cell death due to necrosis and apoptosis, axon inhibitory molecular expression, poor regeneration of axons due to lack of proper *milieu*, failure of axon path finding and synapse formation, etc. [23]. Due to multifaceted pathology of SCI, a single therapy is extremely unlikely to counter all of them. However, combining many different strategies is not only practically difficult but also reported to be counterproductive under certain combinations [24, 25]. Under these circumstances, transplantation of cells was considered as a single method which can tackle different pathomechanisms in one shot [26].

2 Cell Transplantation as a Treatment for SCI

Renaissance caused by embryonic stem cells discovery in biomedical research raised hopes about their usage in SCI also [27]. Despite positive reports from experimental studies [17, 28], clinical usage of them is being delayed. Several reasons, such as tumorigenic potential in vivo [29] and legal and ethical issues [30] associated with embryonic stem cells, stalled their migration from bench to bedside [31]. This prompted a search for alternate sources of stem cells.

3 Mesenchymal Stem (Stromal) Cells, Alternate Source of Stem Cells

In a pursuit to find alternatives to embryonic stem cells, cells present in the connective tissue component of several organs were thought to be resident stem cells. These stromal cells were named somewhat inappropriately as mesenchymal stem cells (MSCs) as the word "mesenchyme" refers to embryonic connective tissue, whereas adult connective tissue should be referred as "stroma."

Mesenchymal stem cells were discovered in various adult tissues such as skin [32, 33], bone marrow [34], Wharton's Jelly [35], amniotic fluid [36], dental pulp [37], etc. Woodbury et al. [34] had shown MSCs could be induced to adopt neural lineage and that kindled more interest in using these cells for SCI repair. MSCs derived from different sources have been tested in SCI [38] and some of them were found to be beneficial.

Discovery of MSC did not conclude therapy for SCI as there were negative reports about MSC usage for SCI [39]. MSCs were also not free from immunological rejection [40]. This would mean undesirable need for immunosuppressive therapy if MSCs are used. Thus, it would be better to identify a prospective cell type for SCI repair which can be procured in plenty, immunologically naïve, and biologically safe. In this context, placenta-derived cells find their

Table 22.1 Therapeutic strategies for SCI in experimental studies

Nature of therapy	Examples
Pharmacological agents	Methylprednisolone [2, 3], naloxone [4], ibuprofen [5]
Physical/chemical agents	Hypothermia [6], hyperbaric oxygen [7]
Biological materials	Omentum [8], iris, mitral valve, peripheral nerves, embryonic spinal cords, brain tissue, cell seeded scaffolds [9]
Biocompatible nonbiological materials	Scaffolds [10]
Surgical interventions	Dorsal myelotomy [11], vertebral resection [12]
Novel therapies	Energy restriction [13], antibody to NoGo [14, 15], gene therapy [16]
Cellular transplantations	Embryonic stem cells [17], mesenchymal stem cells [18], placental cells [19], cord blood cells [20], genetically modified cells [21]

Despite this impressive list of strategies tested, cure for contusive spinal cord injury is yet to be realized [22]

place as they can be obtained in large quantities without ethical and legal concerns and are found to be immunologically nonantigenic.

4 Placenta as a Source of Stem Cells and Status of Amniotic Epithelial Cells

Placenta was found to harbor MSCs at various parts. Stromal cells derived from amniotic stroma [41, 42], decidua basalis and decidua parietalis [41], and umbilical cord blood cells [43] were shown to possess characteristic features of MSC. Through a series of publication, Sakuragawa et al. [44], reported neural and glial nature exhibited by amniotic epithelial cells which lead to the speculation of using these cells for treating neurological conditions. Later, amniotic epithelial cells were found to exhibit stem cell features [45] although amniotic epithelial cells do not have properties similar to MSCs.

5 Objective and Scope of This Chapter

It was intended to review the status of human amniotic epithelial cells (hAECs) for contusive SCI repair in the light of our own experiments conducted using hAEC in rat model of contusive SCI (unpublished work). We present here our observations which indicate high potentials of these cells in treating SCI. Scope of the phrase "amniotic epithelial cells" was limited to those epithelial cells derived directly from amniotic membrane and amniotic fluid cells are excluded. As amniotic fluid might contain not only epithelial cells shed off from amniotic membrane but also cells derived from the developing fetus, nature of amniotic fluid cells is ambiguous. Similarly, stromal cells obtainable from amnion (amniotic stromal cells) are also excluded as these cells express MSC features and not epithelial type features.

6 Our Experiments Using hAEC for Contusive SCI Repair in Rats

6.1 SCI Model in Rats and hAEC Transplantation

From full-term placenta obtained after elective caesarian deliveries, hAEC were isolated and cultured as per the procedures described elsewhere [46]. Cells were labeled with CellTracker Green (Invitrogen, USA) fluorescent label as per the manufacturer's recommendations. Female Wistar albino rats (*Rattus norvegicus*) of 90 days of age (150–180 g of body weight) were used in the study. Animal housing and maintenance were as per recommendations given by Indian and International organizations such as Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA) and Canadian Council on Animal Care [47]. Protocol of the study was approved by Institutional Animal Ethical Committee, which was formed and functioning as per the guidelines of CPCSEA, Government of India.

Under surgical anesthesia, spinal cords were exposed by standard laminectomy at T9-T10 level. Contusion SCI was created by dropping a 10-g weight steel rod of 3 mm diameter from 12.5 mm height [2]. During weight drop, vertebral column was stabilized with clamps. Twenty minutes after creating injury, CellTracker Green labeled hAEC transplantation was carried out. Using microliter syringe, stereotaxically 4 µl of cell suspension at a concentration of 50,000 cells/µl was intraparenchymally injected.

Prophylactic antibiotics (amoxicillin + cloxacillin 20 mg/kg i.m.) and analgesic Pentazocine (1 mg/kg subcutaneous) were given for appropriate duration [48, 49]. Animals were cared for complications of SCI such as urinary tract infections, pressure sores, etc. In general, postoperative periods in all the animals were uneventful.

Animals were grouped as follows:

Sham group	Sham operated animals ($n = 5$)
Lesion group	SCI by contusion as described above ($n = 15$)
hAEC group	SCI followed by hAEC transplantation as described above ($n = 15$)

In each group other than control, there were 15 animals out of which, 5 animals each were euthanized at the end of 2, 4, and 8 weeks of postoperative periods.

6.2 Evaluation of hAEC Transplantation Effects

Effects of hAEC transplantation for contusive SCI were evaluated using behavioral and histological/histochemical parameters. Motor recovery in animals was assessed using BBB scoring method [50]. Animals were euthanized by overdose of anesthesia and tissues were fixed using 4 % paraformaldehyde in phosphate-buffered saline. Both paraffin and cryosections were carried out at 10–15 µm thickness. Sections were used to visualize CellTracker Green labeled cells and for other staining such as antibody-based ED1, silver and CFV staining methods [51]. In long-term animals (>8 weeks), quantitative histology was carried out using standard principles of morphometric procedures [52] to

count number of neurons present at C1, T1, T6, T12, and L1 levels. Neuronal density (i.e., number of neurons per cu.mm) was calculated using the formula as:

$$N_V = N_A / A(D+t)$$

Where

N_V is the numerical density or number of cells per cubic millimeter

N_A is the average number of cells in the reference area

A is the area of the reference area in millimeter

t is the thickness of the section in millimeters

D is the mean diameter of the cells in millimeters

6.3 Statistical Analysis

All the numerical data were analyzed using SPSS software. Differences between mean values of the groups were compared for significance using one-way ANOVA with post hoc analysis applying Tukey's test. Significance was tested at 0.05 level. Histograms represent mean values of the groups with±standard error of mean values.

7 Results

Postoperative period of the animals in general was uneventful. BBB scores of the hAEC transplanted animals in comparison with lesion group animals were given in Fig. 22.1. BBB score comprise of 21-point scale in which 0 represents complete paralysis and 21 indicates normal motor function in hind limbs. Spinal cord injured animals were with score zero immediately after lesions. However, there was a gradual recovery and by 8 weeks, hAEC transplanted animals reached a score of 15 which is about two points higher than lesion group. The difference was significant at 8 weeks postinjury/transplantation.

CellTracker Green was readily taken up by hAEC in culture and showed bright green fluorescence. hAEC was having either a round or slightly irregular morphology with a granular cytoplasmic appearance in vitro (Fig. 22.2). To discriminate between autofluorescing macrophage and CellTracker Green labeled hAECs, immunofluorescence study for macrophages was carried out using ED1 antibody. Also, hAEC transplantation carried out in uninjured animals served as control for such counter staining study. In uninjured cord, transplanted hAECs were seen to survive up to 2 weeks and there were no ED1 positive macrophages. In injury group, all the macrophages seen in green channel due to autofluorescence were positive for ED1. Interestingly, in hAEC group, there were cells seen in both green and red channels, i.e., macrophages and cells seen only in green channel which were negative for ED1. These cells were presumably the transplanted hAECs. These results are illustrated in Fig. 22.3.

Such green fluorescing ED1 negative cells were rare by 4 weeks and were not seen beyond that. Cresyl fast violet staining and silver staining showed cells with round morphology among host axons which were absent in lesion group animals (Fig. 22.4). These cells might be the transplanted hAECs. It was not known whether the cells lost their fluorescent label by 4 weeks of postinjury/transplantation period.

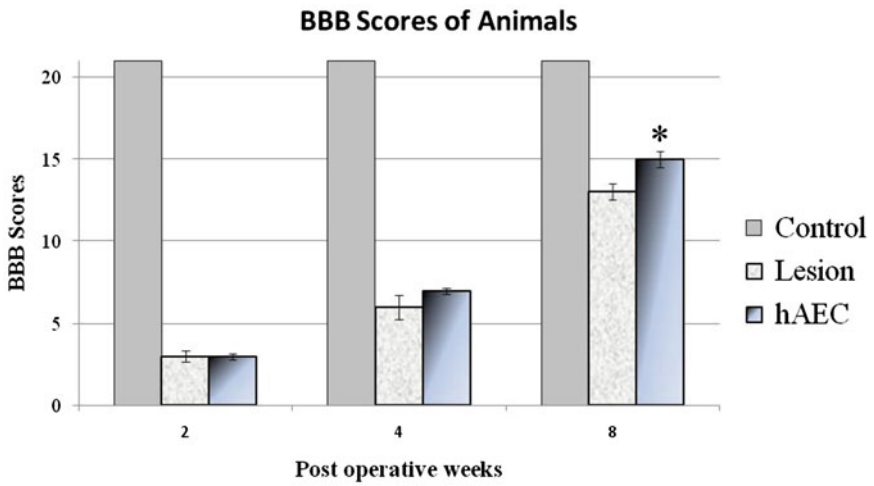


Fig. 22.1 BBB scores of control/sham operated animals remain at the maximum score in the scale, i.e., 21 points. Lesion and hAEC group animals initially were zero (totally paraplegic) in scale and showed gradual recovery over the period. From 4th week, hAEC group animals showed improved performance when compared with lesion group animals. However, the differences were statistically significant only at 8th week. *Note*: Lesion group values were from laboratory's data source and were used in other presentations/publications

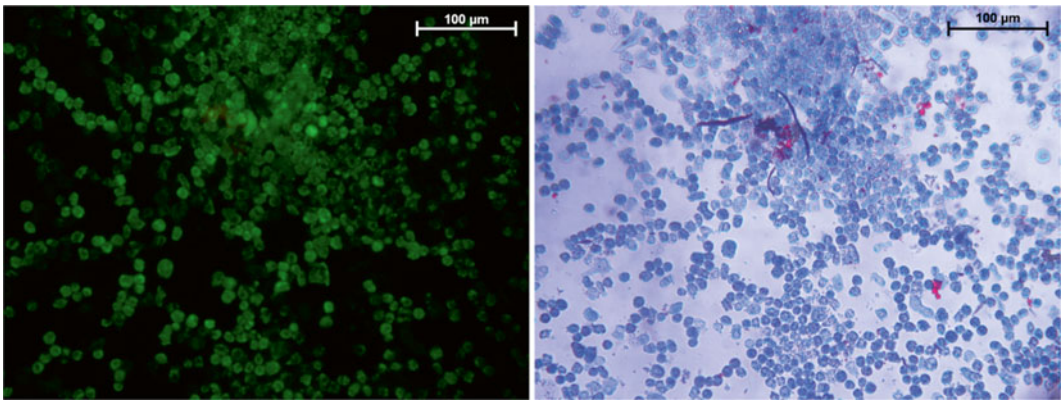


Fig. 22.2 Smear of hAEC labeled with CellTracker Green fluorescent dye fixed with 4 % paraformaldehyde after being used for transplantation. *Panel 1* shows fluorescent view of the labeled cells and *Panel 2* showed trypan blue stained cells. hAEC appeared as brightly fluorescing cells with more or less round morphology and granular cytoplasm

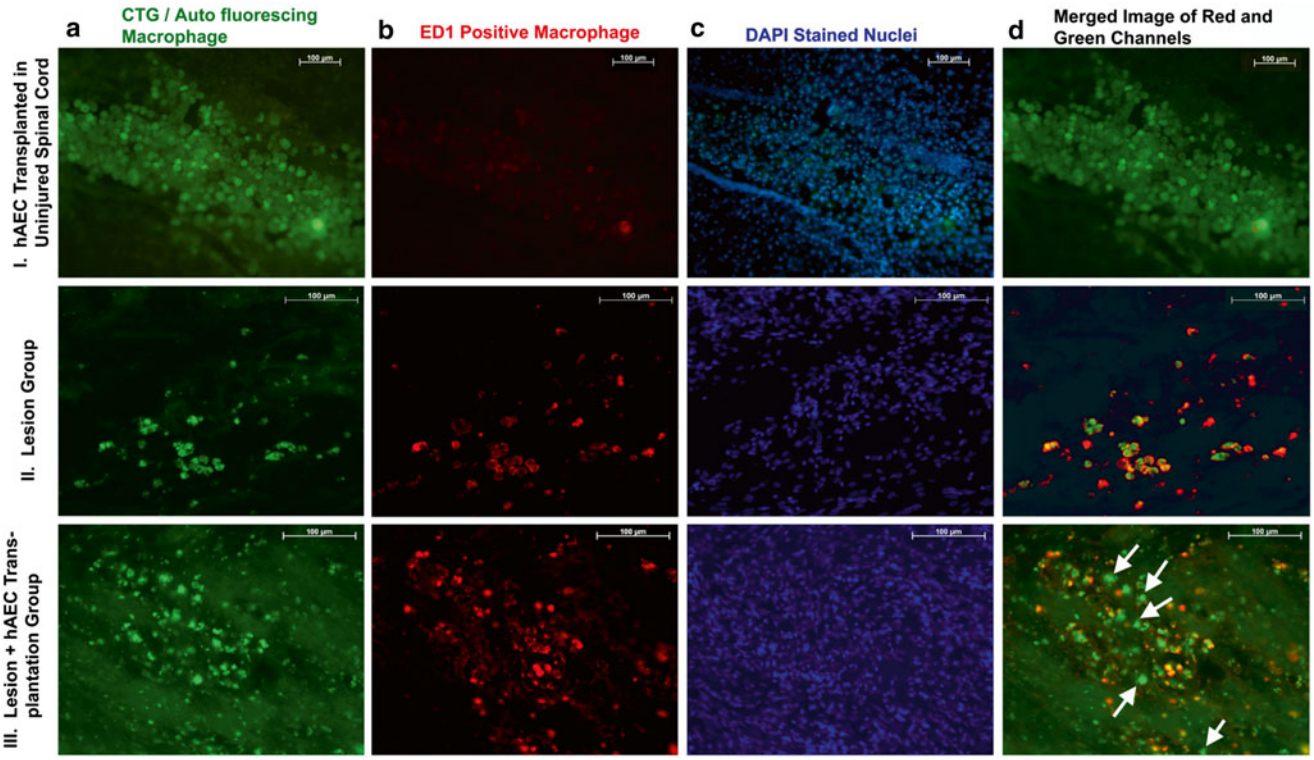


Fig. 22.3 Immunofluorescence study for macrophages. CellTracker labeled hAECs were observed in uninjured spinal cord up to 2 weeks. In lesion group, autofluorescing macrophages seen in green channel were positive for ED1 as visualized through red channel. In hAEC group, in addition to cells seen in both green and red channels (autofluorescing macrophages which are positive for ED1), there were cells seen only in green channel (indicated by *arrows* in panel III.D). Since these green fluorescing cells are negative for ED1, they are presumed to be the CellTracker labeled transplanted hAECs. See text for details (Columns **a**, **b**, **c** represents *green*, *red* and *blue* channel views respectively. Column **d** represents merged view of *green* and *red* channel. Row labels are self explanatory)

Numerical densities of neurons at various spinal segments were given in Fig. 22.5. Lesion group animals showed significant reduction in neurons when compared with control animals. In hAEC group, neuronal densities were less when compared with control animals; nevertheless, they were significantly higher than lesion group animals.

8 Discussion

8.1 About the Use of hAEC for SCI Repair

Concept of using hAEC for CNS disorders was probably inspired by the discovery of neural and glial markers

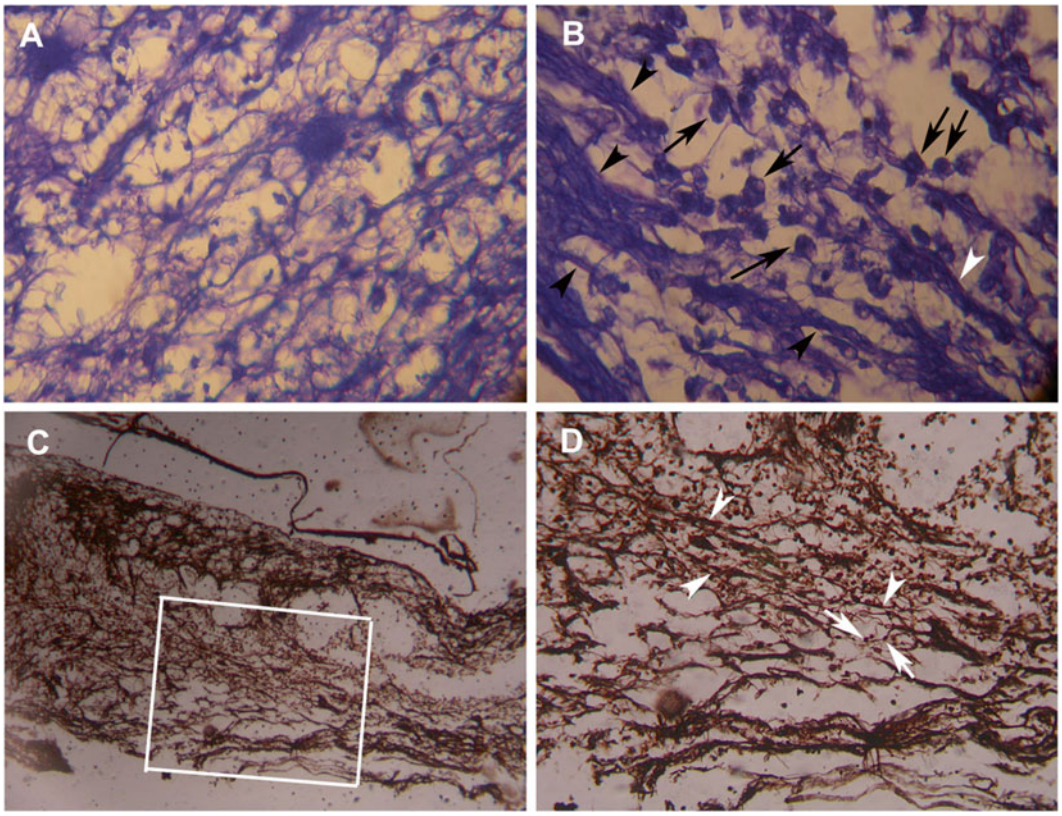


Fig. 22.4 Cresyl fast violet and silver staining carried out at 8 weeks of postoperative period showed cells appears to be hAECs in the transplantation group. (**a**) CFV staining of injury site in lesion group showing vacuolation and fibrous glial scar formation. (**b**) CFV staining of injury plus transplantation site in hAEC group showing cells with round morphology which were absent in lesion group at the comparable postoperative period. These cells present in hAEC group only were presumed to be the transplanted hAECs. Silver impregnation as per Glees and Marshland's modification of Belchowsky's method to stain axons showed extension of neurites among such cells seen in hAEC group (Panel **c**). Panel **d** illustration represents enlarged view of *boxed area* in Panel **c** showing traversing axons (*arrow heads*) and cells with outgrowths (*arrows*). Such appearance was not seen in lesion group animals

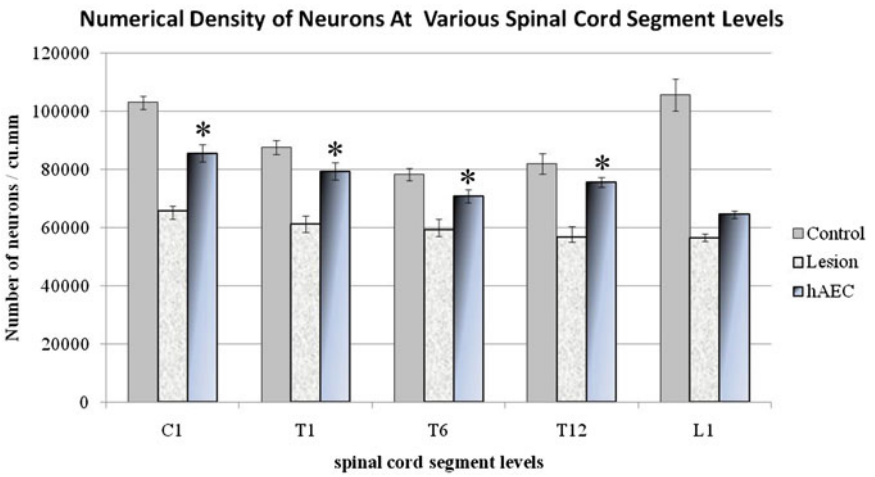


Fig. 22.5 Neuronal densities at various levels of spinal cords during long-term period (>8 weeks). There was significant loss of neurons not only at segments close to lesion viz. T6 and T12 but also at distant segments as far as C1. hAEC transplantation was found to rescue neurons from death with restoration to near normal levels at T1, T6, and T12 levels. However at L1 level, hAEC transplantation did not show rescue effect on neurons. See text for details

expression by amniotic epithelial cells [44]. They were suggested to be a potential cell type for intracerebral transplantation purposes based on their surface expression of neural tissue-specific proteins and their capability to secrete neurotransmitters [53, 54]. Miki et al. [45] report about stem cell properties of hAEC justified pilot animal studies of using hAEC in different neurological disease models.

AEC transplantation in the Parkinson model of rats was found to reverse the condition and prevent death in neurons [55–57]. When transplanted into ischemic hippocampus, they were observed to differentiate into "neuron-like" cells [58].

There were only a few experimental studies in which hAEC was used to treat SCI. Earliest study was from our lab [59] in which, hAEC transplantation after spinal cord transection in monkeys was found to offer neuroprotection and helped in host regeneration. Later, Zhi-yuan et al. [60] reported improved locomotor function in spinal cord transected rats after hAEC transplantation. Cotransplantation of bFGF-expressing amniotic epithelial cells and neural stem cells were reported to promote recovery in spinal cord injured rats [61]. Recently, Xue et al. [62] reported combination of a cellular muscle scaffold with amniotic epithelial cells promote axonal regeneration, better myelination and reduced astrocytic scar formation. Amniotic epithelial cells were found to alleviate mechanical allodynia but not thermal hyperalgesia in spinal cord injured rats.

Surprisingly, except for the above-mentioned studies, there was no other study in which amniotic epithelial cells were tested for SCI repair. Except for our unpublished data presented here, and the work of Meng et al. [61], there were no studies in which amniotic epithelial cells were used in contusive SCI models. Given that Meng et al. [61] had used genetically modified AEC, except for our data presented here, there was no study available about the efficacy of AEC in treating contusive SCI.

8.2 Effect of hAEC Transplantation in Locomotor Function Recovery After SCI

In our study, we have observed improved BBB scores in transplantation group animals when compared with lesion group by 2–4 weeks albeit the differences were statistically insignificant. Nevertheless, by 8 weeks hAEC transplanted animals were showing better recovery and the difference was statistically significant.

Earlier improvement in BBB scores after cell transplantations was considered as the neuroprotective effect of the transplanted cells [63]. Therefore, it was logical to speculate the neuroprotective effect of hAEC transplantation *albeit* lack of statistical significance in short term. Continued improvement in BBB scores during late phase was reported to be associated with regeneration and improved function of long tracts [64]. Given that beneficial effects were seen in both early and late phases of recovery, it appears both neuroprotection (in short term) and regeneration of axons (in long term) might be the cause for the improved locomotor function in hAEC transplanted animals.

8.3 Effects of hAEC Transplantation at Tissue Level After SCI

Qualitative histology using different stains showed less vacuolation in hAEC group indicating reduction in necrosis and tissue loss. In hAEC group animals, axons were seen traversing through cells at the transplantation site and such scenario was not observed in lesion group animals. This corroborate with our earlier report [59] about possible host regeneration stimulated by transplanted hAECs. Numerical density of neurons is considered as one of the indications of neuroprotection [65–68]. In our quantitative histological studies, increased numerical density of neurons at various levels observed in hAEC group animals shall be a clear indication of neuroprotection offered by these cells. Zhi-yuan et al. [60] also reported similar findings of improved neuronal count after hAEC transplantation in SCI rats.

While hAEC rescue neurons from death at various levels, it had no effect at L1 spinal segment level as differences were not significant at this level. This might be because of enhanced vulnerability of L1 segment to circulatory disturbances [69] and thus hAEC might have no effect in countering trauma-induced ischemic insult.

8.4 Possible Mechanism Behind Beneficial Effects Exerted by hAEC

Apart from providing a suitable "neuronal milieu" due to their surface expression of neural/glial markers, hAEC might exert their neurotrophic effects by way of secreting diffusible neurotrophic factors [70, 71]. hAEC-conditioned medium was found to be neurotrophic for E18 rat cortical cells. E18 cortical cells were known to respond to no growth factor other than EGF. As EGF was not detected in the conditioned medium using assay methods, Sakuragawa et al. [72] concluded that an EGF-like "novel neurotrophic factor" might be secreted by AEC. Studies conducted in our lab ushered the possibility of an "FGF-2 like" factor [19, 46].

With Koizumi et al. [73] observations of mRNA and protein expressions for various neurotrophic and growth factors by hAEC, these cells are interesting candidates to be considered for neurological lesion repairs. In general, the exact mechanism of cell transplantation mediated beneficial effects were yet to be identified even in the case of much accredited cell

types such as embryonic stem cells and bone marrow-derived stem cells. Although different mechanisms were proposed, the most convincing speculation is about paracrine effects of secretions by stem cells being responsible for the beneficial effects observed [74]. Corroborating that phenomenon, evidences suggest that diffusible factors secreted by hAEC may be responsible for the beneficial effects observed. At present, it is not known what is the exact nature of the factor and only further studies may reveal its nature.

8.5 hAEC as Stem Cell and Its Application for SCI Repair

From our observations, we could not tag these cells as "stem cells" as these cells lack proliferation ability in culture. Because of this, clonal analyses could not be done for their multidifferentiation potentials. Without demonstrating self-renewal and capacity to differentiate multilineage potential, it would be incorrect to consider these cells as stem cells.

Pratama et al. [75] showed prolonged culture of these epithelial cells resulted in morphological changes and loss of epithelial markers. They have reported that such attempts to expand these cells in vivo resulted in change of morphology to mesenchymal type with increased expression of MSC markers. This creates ambiguity as whether the amniotic epithelial cells transform or inconspicuous contamination with mesenchymal (amniotic stromal) cells overpower culture in long run.

Therefore, we consider while hAECs not withstanding to qualify for stem cells, still may have beneficial effects in SCI repair. However, this requires extensive studies as at present, only in our study, the functional benefit of these cells were observed in contusive SCI as the few other studies quoted above used either transection [60] or hemisection models [62] of SCI or used genetically modified amniotic epithelial cells [61]. In a nutshell, there were no studies (except for our observations presented here) which established the functional benefit of these cells in contusive SCI. Probably because of this, amniotic epithelial cells and their application for SCI was left out in the systematic review of cellular transplantation therapies for SCI [76] which included all studies up to the year 2008.

Identification of transplanted cells in vivo is a crucial factor in all cell transplantation studies. Different types of labels were used and in the case of hAEC, it appears no study has objectively proved its survival including our study in which we have shown its presence by differential diagnosis only. Thus, better stable labeling methods, such as transfection with GFP gene, may be required to prove their survival; however, such methods are not without risk of altering gene expression patterns in such transplanted cells. Therefore, proving functional efficacy and demonstrating fate of transplanted cells in parallel may remain as a challenge.

Functional improvement shall be the key feature to identify a prospective treatment strategy for SCI. Some early proof of concept for potential functional benefits would be required to serve as an rationale to further expand research in that direction. Our data on the usefulness of these cells in contusive injury and other works about the usefulness of these cells in other lesion conditions shall serve as the rationale to further explore the usage of hAEC for SCI treatment. Only through further studies coupled with strategies for stable labeling of the cells used, it might be possible to address the issue of fate of these cells in long term after transplantation.

8.6 Conclusion

hAECs when transplanted after lower thoracic contusion spinal cord injuries in rats enhance functional recovery. Transplanted hAEC cells might survive up to 2 weeks in the spinal cords of rats without any host immune suppression. Survival of these cells beyond 2 weeks at the transplantation site needs verification through certain stable labeling techniques such as vector-based GFP labeling. Notwithstanding to be qualified as "stem cells," these cells are found to be a promising candidate for repairing contusive SCI.

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Therapeutic Potential of Amnion Epithelial Cells for Diabetes

23

Chika Koike, Motonori Okabe, Toshiko Yoshida, and Toshio Nikaido

Abstract

Whole pancreas, islet, or β-cell transplantation has been a treatment option for diabetes mellitus. However, the shortage of transplantation materials limits this transplantation therapy and new sources of insulin-producing cells are required. The amniotic membrane is a part of the fetal membrane and is composed of amniotic epithelium and mesenchymal cells that are derived from the inner cell mass in the blastocyst. It has been shown that human amniotic epithelial cells and mesenchymal cells have the potential to differentiate into various organs, have less immunologic activity, and are supposed to be cell sources for allogeneic transplantation. Several researchers have reported that amnion-derived cells differentiate into insulin-producing cells in vitro and/or the transplantation of amnion-derived cells normalized the blood glucose level in diabetes mice. Amnion-derived cells may be a good cell source for the transplantation therapy of diabetes.

Abbreviations

DM	Diabetes mellitus
HAE cells	Human amniotic epithelial cells
HAM cells	Human amniotic mesenchymal cells
Ngn3	Neurogenin 3
Pdx1	Pancreatic and duodenal homeobox 1
STZ	Streptozotocin
SCID	Severe combined immunodeficient

1 Diabetes Mellitus

Diabetes mellitus (DM) is as devastating disease that affects millions of people around the world. It is estimated that 380 million individuals will have this disease by 2025, because of aging and changing lifestyles. Notable increases of DM patients will occur in Asia and South Africa [1, 2]. There are two major forms of diabetes. Type 1 diabetes mellitus is a debilitating disease caused by an absence of insulin production from pancreatic β-cells and is characterized by autoimmune destruction of pancreatic β-cells in the islets of Langerhans. Type 1 DM accounts for 5–10% of DM, often begins at a young age and is also called insulin-dependent diabetes or juvenile-onset diabetes. Type 2 DM, the more common form, is caused by insufficiency in insulin production or resistance to insulin. It has been suggested that type 2 DM is associated with genetic factors and obesity and is promoted by patients' environment and behavior such as high-calorie diet and a life style with less exercise. Insulin, a hormone produced by pancreatic β-cells, regulates blood glucose concentration. Acute release of insulin occurs within few minutes after an increase of blood glucose caused by meal or other factors and is followed by a more sustained release which lasts for several hours. A lack or insufficiency of insulin production leads to hyperglycemia and serious long-term complications, such as cardiovascular disorders, kidney diseases, and blindness. It is thus a lifelong and often disabling disease that can severely impact the quality of life of patients. At present, there is no cure for DM, though it can be managed. Type 1 DM patients require daily insulin administration.

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Type 2 DM patients need to control their blood glucose levels by adjusting their diet, exercise, antihyperglycemic drugs, and/or insulin injections. The pancreas is a complex organ composed of many types of cells such as exocrine cells that secrete digestive enzymes and endocrine cells which produce hormones for homeostasis. The insulin-producing β-cell is one type of endocrine cell in the islets. Current treatment other than multiple insulin injections relies on insulin pump placement, or β-cell or whole pancreatic transplantation. Allogeneic islet transplantation has also been shown to be a useful treatment for type 1 DM [3, 4]. However, it is limited by a scarcity of transplantation materials due to the shortage of transplantable islets (shortage of donors), difficulty of islet isolation, and the long-term side effects of immunosuppressive therapy. Pancreatic duct cells and endocrine progenitor cells are considered to be potential alternative sources of β-cells [5–7].

2 ES Cells and iPS Cells

Other possible sources of insulin-producing cells are stem cells such as embryonic stem cells (ES cells), induced pluripotent stem cells (iPS cells), or somatic stem cells. ES cells and iPS cells have been induced to become pancreatic cells or insulin-producing cells [8–10]. Stepwise induction using combinations of different growth factors and small molecules was used to induce ES cells or iPS cells to differentiate into insulin-producing cells in vitro, which mimicked the mechanism of in vivo pancreas development, endoderm differentiation, and then pancreatic specific differentiation and maturation [11]. The induced cells produced insulin and C-peptide, which is a short protein cleaved from preproinsulin in the insulin synthesis pathway, in response to glucose level. The implantation of pancreatic-induced ES cells and iPS cells in diabetic model mice resulted in secretion of insulin and C-peptide in the blood and normalized the blood glucose level and function, similar to human adult islets [12]. But so far the differentiation efficiency of insulin-producing cells is not very high. Using ES cells raises ethical problems and a potential risk of immune rejection, and iPS cells have potential tumorigenicity with introduced genes for reprogramming.

3 Somatic Stem Cells

Another potential source for cell therapy is somatic stem cells. It has been reported that somatic stem cells and progenitor cells isolated from the pancreas [6], bone-marrow [13], liver [14, 15], salivary gland [16], adipose [17], endocrine progenitor cells [18], nerve system [19], placenta [20], umbilical cord blood [21] are capable of differentiation into insulin producing cells, as well as amnion derived cells [22–24].

Recent studies have reported pancreatic direct reprogramming. Bone marrow mesenchymal stem cells, pancreatic exocrine cells, or Sox 9+ cells in the liver were induced to become insulin-secreting cells or pancreatic cells by introducing pancreas and duodenum homeobox-1 (Pdx1), neurogenin 3 (Ngn3), and v-maf avian masculoaponeurotic fibrosarcoma oncogene homolog A (MafA) genes with adenoviral or lentiviral vectors [25–27]. Direct reprogramming by gene introduction using these genes can be applied to amnion derived cells in the case where insulin production is not sufficient by inducing with culture conditions, chemicals, and growth factors.

Using autogenic somatic stem cells is the ideal cell source for cell therapy as it presents no risk of immune rejection and less risk of other infections. However, expansion and differentiation take time because of the small number of cells. Allogeneic cell transplantation may have to be applied, which has the same risks of immune rejection and long-term side effects as immunosuppressive therapy. Amnion-derived cells offer a ready-made cell source which can be applied to allogeneic cell transplantation therapy, since the amnion has less immunoreactivity as described below.

4 Amnion Membrane

The amnion is a fetal-origin tissue deriving from the inner cell mass in the blastocyst and is composed of a single layer of epithelial cells (human amniotic epithelial cells, HAE cells) on a thicker basement membrane and spongy collagen layer containing mesenchymal cells (human amniotic mesenchymal cells, HAM cells). At day 8–9 after fertilization, the inner cell mass differentiates into two layers: the epiblast and the hypoblast. From the epiblast, small cells that later constitute the amniotic epithelium appear between the trophoblast and the embryonic disc. The epiblast gives rise to the amnion as well as to all of the germ layers of the embryo. Thus, HAE cells and HAM cells maintain the plasticity of pregastrulation embryo cells and have the potential to differentiate into various tissues [28–30]. Their use would overcome the ethical issues associated with the use of embryonic stem cells; because the amnion membrane is discarded after parturition, it is easy to obtain without harming mothers or babies. As both HAE cells and HAM cells do not express HLA class II, and also express immune suppressing factors such as HLA-G and CD59 [31], they are considered less likely to be rejected, to provide anti-inflammatory functions, and accordingly to be suitable to be cell sources for allogeneic cell transplant therapy [32]. All those facts support the use of amnion-derived cells as a cell resource for regenerative medicine.

5 Characteristics of Amnion-Derived Cells

The human amnion was mechanically peeled from the chorion of a placenta obtained from an uncomplicated cesarean section with informed consent. HAE cells were isolated by

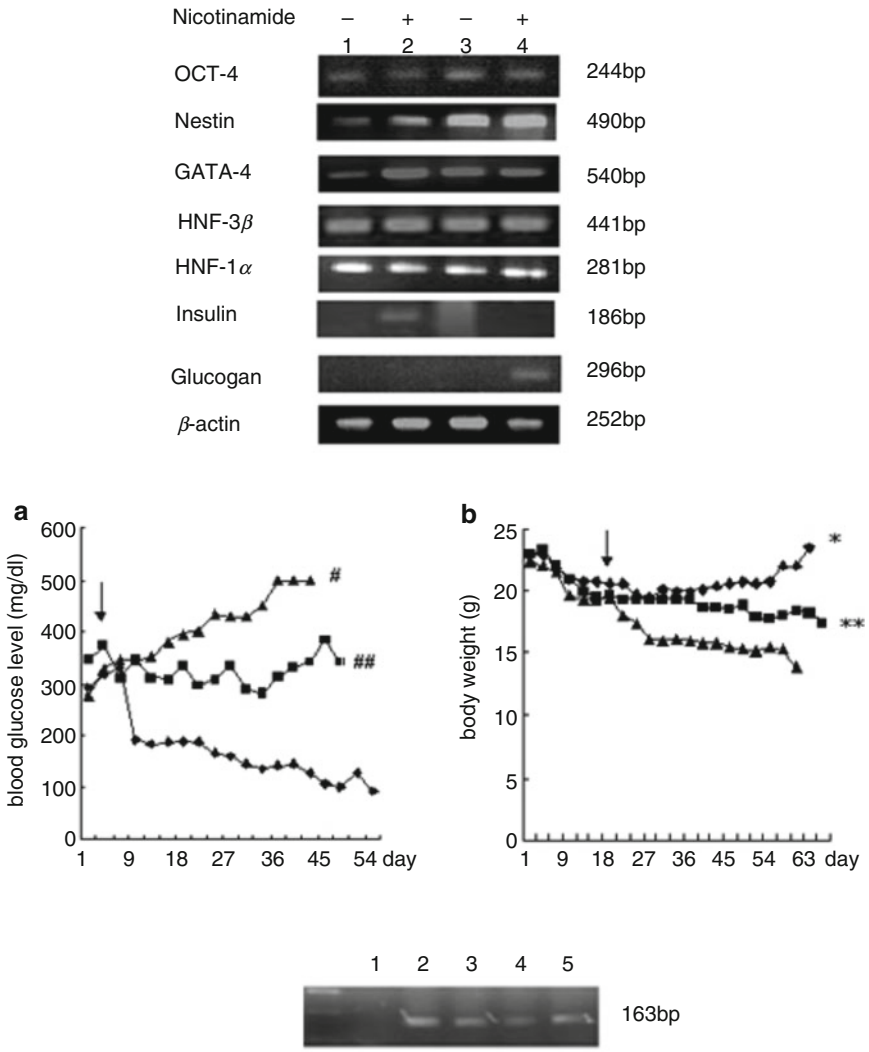


Fig. 23.1 *Upper panel*: expression of markers in HAE cells and HAM cells. *Lane 1*: HAE cells, *lane 2*: pancreatic induced HAE cells, *lane 3*: HAM cells, lane 4: pancreatic induced HAM cells, *middle panel*: changes in blood glucose level (**a**) and body weight (**b**) of mice. Sham-operated diabetic mice (*filled triangle*, $n=6$), HAE transplanted mice (*filled* circle, $n=11$), and HAM transplanted mice (*filled* square, $n=9$). *Lower panel*: detection of human-specific β-2 microglobulin. *Lane 1*: mouse genomic DNA, *lane 2*: human genomic DNA, *lane 3*: HAE transplanted mice spleen, *lane 4*: pancreas, and *lane 5*: liver. The figure is modified from Wei JP et al. [22]

trypsin treatment and HAM cells were isolated by collagenase digestion from the amniotic pieces after the removal of epithelial cells as described previously [22, 28, 33–36]. It has been reported that HAE cells expressed Oct4, Sox2, Klf4, c-Myc, Nanog, nestin, Musashi1, FDF4, Rex1, CD13, CD29, CD44, CD73, CD90, CD105, CD166, CD117, SSEA3, SSEA4, Tra1-60, and Tra1-81, and HAM cells expressed Oct4, Sox2, Klf4, c-Myc, Nanog, nestin, Musashi1, FDF4, Rex1, CD10, CD13, CD29, CD44, CD73, CD90, CD105, CD133, CD166, CD117, SSEA3, SSEA4, Tra1-60, and Tra1-81 shown by RT-PCR, immunostaining, and flowcytometric analysis [29, 36–44]. Figure 23.1 shows some of the gene expressions by RT-PCR. Both HAE cells and HAM cells expressed Oct4, a transcription factor required to maintain the stemness; GATA-4, a marker of definitive (embryonic) and visceral (extra-embryonic) endoderm; hepatocyte nuclear factor-3b (HNF-3b), a marker of definitive endoderm; and nestin, which is an intermediate protein and a neural stem cell marker [22]. Amnion derived cells do not express hematopoietic markers CD 14, CD 34, CD 45, and HLA-DR [28]. They also express some differentiated markers, hepatocyte markers, osteocyte markers, chondrocyte markers, and neural markers [28, 34, 45–48]. These results suggest that amnion-derived cells contain stem cells.

6 Diabetes Treatment Using Amnion Cells

Several studies have reported the possibility of diabetes treatment using amnion-derived cells. Amnion-derived cells were induced into insulin-producing cells in vitro by treating with nicotinamide and other supplements. The expression level of pancreatic β-cell related genes, insulin, glucagon, somatostatin, Pdx-1, pancreas and duodenum homeobox-4 (Pdx-4), paired box gene 6 (Pax-6), NK2 transcription factor-related locus 2 (Nkx-2.2), NK6 transcription factor-related locus1 (Nkx6.1), Islet-1(Isl-1), glucokinase (GCK), glucose transporter-2 (Glut2), and Neuro D were all increased after the pancreatic induction in vitro (Fig. 23.1 upper panel, Fig. 23.2, [22–24]).

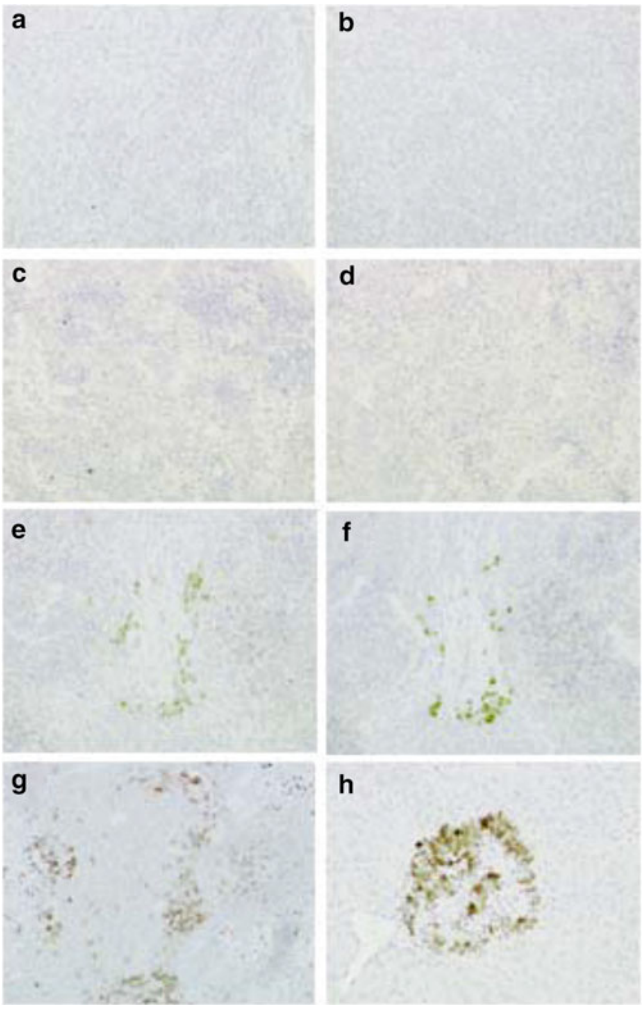


Fig. 23.2 Histological analysis of implanted mice. (**a**, **c**, **e**, **g**) anti-human-β-2 microglobulin, (**b**, **d**, **f**, **h**) anti-human insulin. (**a**, **b**) Normal mouse spleen (**c**, **d**), diabetic mouse spleen (**e**, **f**), HAE-transplanted mouse spleen (**g**), human lung, and (**h**) human pancreas. Original magnification: ×200. The figure is modified from Wei JP et al. [21]

To see the effect of amnion derived cells in vivo, a diabetic mouse model was made by a single IP injection of streptozotocin (STZ) into severe combined immunodeficient (SCID) mice. The body weight of STZ-injected mice was lower than that of normal mice and the blood glucose level increased after injection. HAE cells or HAM cells were implanted into the spleens of diabetic mice. Body weight of HAE cell-implanted mice increased gradually and was significantly higher than that of sham-operated mice. The blood glucose level of HAE cell-implanted mice gradually decreased to a normal level, which was significantly lower than that of sham-operated mice (Fig. 23.1 middle panel, [22]).

The body weight of HAM cell-implanted mice decreased but was significantly higher than that of sham-operated diabetic mice. The blood glucose level of HAM-implanted mice was also significantly lower than that of sham-operated diabetic mice (Fig. 23.1 middle panel, [22, 23]). Transplanted human cells were detected 1 month after the transplantation by immunohistochemistry and RT-PCR (Fig. 23.1 lower panel and Fig. 23.2, [22]). The results indicated that the implanted human cells had been integrated into the spleen and migrated into the liver and pancreas. Also, they had differentiated into insulin-secreting cells within 1 month. However, more efficient induction methods will be investigated for clinical use, such as gene introduction.

7 Conclusion

The therapeutic potential of both amnion epithelial cells and mesenchymal cells for diabetes has been reported. They appear to be cell sources for cell therapy and to be more useful than ES cells or iPS cells or other somatic stem cells as amnion cells could be used for allogeneic transplantation. Amnion-derived cells may be a good cell source for the transplantation therapy of diabetes.

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Part III

Umbilical Cord Cells

Collection, Processing, and Banking of Umbilical Cord Blood

24

David T. Harris

Abstract

The blood in the umbilical cord and placenta after the birth of a child is comparable to bone marrow for use in hematopoietic stem cell transplantation and offered a number of advantages. In the past 20 years, more than 30,000 cord blood transplants have been performed worldwide. Stem cell transplantation for hematological malignancies and genetic disorders however, is an uncommon occurrence. Research performed by several independent laboratories has demonstrated that cord blood also contains a mixture of pluripotent stem cells capable of giving rise to cells derived from the endodermal, mesodermal, and ectodermal lineages. Thus, CB is a readily available stem cell source for use in tissue engineering and regenerative medicine applications, which are hypothesized to be more frequent events than the need for hematopoietic stem cell transplant. This chapter will review the methodologies for collection, processing, and banking these cells for these future clinical uses.

1 Introduction

Research in the early 1980s revealed that the blood in the umbilical cord and placenta after the birth of a child was comparable to bone marrow for use in hematopoietic stem cell transplantation and offered a number of advantages over bone marrow, including a lower incidence of GVHD and less strict HLA-matching requirements [1–9]. In 1989, my laboratory began a series of studies examining the use of cord blood (CB) stem cells for transplantation [10–14]. During this and subsequent work we established the methodologies that were needed for efficient and reproducible cord blood collection, processing, and banking for clinical use [15–19].

In the past 20 years, more than 30,000 cord blood transplants have been performed worldwide [9]. Stem cell transplantation for hematological malignancies and genetic disorders however, is an uncommon occurrence. Research performed by several independent laboratories [20–26] demonstrated that cord blood also contains a mixture of pluripotent stem cells capable of giving rise to cells derived from the endodermal, mesodermal, and ectodermal lineages. In addition, mesenchymal stem cells (MSC), although rare in cord blood, can be easily isolated from the cord tissue (CT) and preserved for later use [27], prompting us to develop methods for the collection and cryopreservation of cord tissue [20]. Thus, CB and CT are readily available for use in tissue engineering and regenerative medicine applications, which are hypothesized to be more frequent events than the need for hematopoietic stem cell transplant. It is estimated that almost one in three individuals in the United States, or 128 million people, could benefit over their lifetime from regenerative medicine, including therapies for cardiovascular, neurological, and orthopedic diseases [28]. Diseases such as myocardial infarction, stroke, and spinal cord injury might possibly be treated with greater efficacy using cell therapy based approaches rather than the current limited treatment options. Translation of these potential therapies from the laboratory to the clinic requires that the stem cells are medically available and reasonably economic. Political and

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ethical controversy surrounds the use of embryonic stem cells, and significant biological and regulatory concerns limit their clinical use. However, CB and CT are non-controversial and if preserved at birth, will provide a source of autologous stem cells for an individual's use in regenerative medicine.

The results presented in this chapter will primarily summarize our cord blood banking efforts, highlighting the methodologies needed for cord blood stem cell collection, processing, and storage. For those individuals interested in the applications of cord blood stem cells in regenerative medicine, the reader is referred to a recent review by Harris [29].

2 Cord Blood Biology

Many of the advantages of CB for traditional transplantation (and some emerging) applications are attributable to its collection at birth and the resultant immunological naivety of the neonatal tissue compared with bone marrow and peripheral blood, which are generally collected much later in adult life. CB is primarily composed of monocytes and lymphocytes, which reside in the mononuclear cell (MNC) fraction of the tissue. The lymphocyte population of CB is immunologically immature compared with bone marrow and peripheral blood. CB has a higher ratio of naïve versus memory $CD4^+$ and $CD8^{high}$ T cells, a higher concentration of natural killer (NK) cells, and a lower concentration of highly reactive NKT cells compared with bone marrow and peripheral blood [30]. Cytokine expression by CB cells is lower than found in peripheral blood and bone marrow [31], and CB cells secrete greater amounts of anti-inflammatory cytokines compared with pro-inflammatory cytokines. The immunologically immature characteristics and the anti-inflammatory properties of CB are responsible for the low frequency and reduced severity of graft-versus-host disease (GvHD) after use in allogeneic transplants, and may facilitate indirect cellular therapeutic benefits for regenerative medicine applications [30, 32]. The largest stem cell population in CB is of the hematopoietic lineage expressing the cell surface glycoprotein CD34, with approximately 1 % of the MNC expressing the CD34 molecule [33]. The $CD34^+$ cell population is heterogeneous, containing primitive cells as well as more mature cells further differentiated along the hematopoietic lineage. CB has a higher percentage of primitive $CD34^+$ cells such as the pluripotent $CD34^+CD38^-$ and $CD34^+CD33^-$ cells than either bone marrow or peripheral blood [30]. These primitive $CD34^+$ cells are believed to be primarily responsible for long-term hematopoietic reconstitution after transplantation [30, 34]. CB stem cells have a higher proliferative potential with reports of up to eightfold greater proliferation rates than observed with bone marrow stem cells [35], which may be due to longer chromosomal telomeres in the stem cells of this young tissue [36]. In addition to hematopoietic stem cells, CB has also been reported to contain endothelial cell precursors, mesenchymal cell progenitors, and multipotent/pluripotent stem cells, which may prove to be of high utility for regenerative medicine applications [33]. An average cord blood collection is generally 70-80 mL of blood from a typical full-term, live birth, containing an average of slightly more (or less) than 882×10^6 total nucleated cells (Personal Experience).

3 Cord Blood Collection, Processing and Banking

Prior to CB collection all patients are required to sign informed consent forms. If possible, informed written consent should be obtained from both the mother and the father (or significant other). In addition, all mothers are tested for infectious diseases as is typically done with blood donors (e.g., reactivity for HIV, hepatitis B and C, etc.) either at the time of cord blood collection, or alternatively up to 7 days before or 7 days after birth of the child. The mother's test results are used as a surrogate indicator for the health status of the cord blood donor. Furthermore, the collected CB units are tested for (aerobic, anaerobic, and fungal) microbial sterility, which can be performed using manual or automated systems (e.g., bioMerieux, Hazelwood, MO; [37]). When CB is collected for unrelated allogeneic use in a public cord blood bank, positive infectious disease testing or microbial contamination may influence whether or not the unit is included in the bank repository. CB units are generally collected with the assistance of the patient's caregiver (i.e., physician or midwife). It is also possible however, to obtain collections using dedicated "collectors" in which case the scope of the CB banking operations will be limited in size with an associated increase in costs. In the majority of cases, the collections are made after delivery of the infant and ligation of the cord, prior to expulsion of the placenta. Prior to collection of CB, the cord is wiped with an antiseptic (e.g., alcohol, betadine or chloraprep), particularly at the needle insertion site, to ensure sterility of the collection [38].

There are a variety of methods that can be used to collect cord blood. Most CB banks have adopted the use of small (or pediatric) blood bags (approximately 250 cc in size). Bag collections must not be left unattended in order to prevent unintended contamination or loss of blood flow from occurring. Routinely, collections are completed within 5 min (prior to placental expulsion) by accessing the umbilical vein. In our experience, less than 2% of all patients were unable to have CB collected, generally due to the rapid birth of the child. CB collection kits should be provided either to the expectant mother or to the hospital, in a sterile state to be amenable for use during surgical deliveries, as well as being preloaded with anticoagulant, and containing all necessary shipping materials. These collection kits must meet all regulatory requirements for shipping blood, including double

containment and a crush-resistant container. Furthermore, the collection kits must be temperature-insulated and padded for safety during transport [29, 39] by couriers, preferably medical couriers, or other personnel. In my personal experience the collected CB should be received and processed within 24 h of harvest to maximize viability and cell recovery.

Whole CB is composed of red blood cells (RBC), white blood cells (containing the MNC fraction), and plasma. In reality only the MNC fraction, which contains the stem cell population, is needed for banking. CB has a very high hematocrit and RBC can make up more than half of the collection by volume. Thus, to facilitate the banking procedure, the vast majority of CB collections are RBC-depleted or reduced prior to cryopreservation. Several methods are in use to accomplish this goal including Hespan sedimentation to obtain a modified buffy coat [39], density gradient centrifugation (Ficoll method) to obtain enriched MNCs [38], and two automated processes (Sepax® from Biosafe SA, Eysins, Switzerland and the AutoXpress Platform® (AXP) from Thermogenesis, Rancho Cordova, CA; [40, 41]) that result in a buffy coat product. The Hespan, Sepax, and AXP processing methods result in cord blood products containing all nucleated cell populations found in the original collection (MNC, neutrophils, some normal and nucleated RBC), while the Ficoll method enriches for the stem cell-containing MNC subpopulation (generally greater than 85% MNC with a few neutrophils and nucleated RBC). Total cell counts obtained in the final Ficoll product are generally 50% or less of the cell counts found in the other processes for this reason, although absolute stem cell recovery is similar.

The processing facility of CBR Systems, Inc. (CBR) has extensive experience with the use of the automated AXP process. This method reproducibly recovers greater than 95–99% of the total nucleated cells and the CB $CD34^+$ stem cells found in a typical collection and results in a reduced final volume of approximately 22 mL, facilitating the banking process. The AXP method allows for greater throughput with fixed personnel numbers (increasing the economy of operations) than other manual methods, is an FDA-cleared, and is a functionally closed system (which is recommended under the current regulatory guidelines; [42]). RBC reduction (by whatever method) leads to much higher cell recoveries and viabilities upon thawing. Furthermore, RBC reduction reduces the amount of DMSO used in cryopreservation and decreases the risk of complications associated with hemoglobin overload due to RBC lysis upon thawing of a cord blood unit for transplant or infusion. In addition, by performing RBC reduction, the unit is amenable to immediate use in gene therapy, cell expansion, as well as storage in multiple aliquots for later multiple uses. Finally, AXP processing does not introduce a significant risk of microbial contamination when performed using a closed system, as shown by an overall microbial contamination rate approaching 0% due to processing.

CB units at CBR are cryopreserved using an automated, microprocessor-controlled cell freezer (e.g., Custom Biogenic Systems, Romeo, MI). The processed CB unit is resuspended in autologous plasma at densities up to 300×10^6 cells/mL. Autologous plasma is used for cryopreservation to avoid exposure to heterologous and/or animal proteins (and the inherent infectious disease risks associated with such use). Cryopreservative solution containing the cryoprotectant DMSO is added slowly over the course of approximately 10–20 min. The cryopreservation protocol utilizes a controlled rate freezing process to slowly reduce the temperature to −180°C. Other cryopreservation methods such as methanol immersion could be used, but these approaches do not provide controlled and documented cryopreservation temperature curves, ideal for meeting regulatory guidelines. At the end of the freezing procedure, the cells are stored in liquid nitrogen dewars, which allow for vapor storage of the units at liquid nitrogen temperatures [43]. The longest study to date has shown that under proper cryogenic conditions, no significant loss of cord blood stem cell biologic activity was evident after 21 years of storage [44].

Vapor phase liquid nitrogen storage of the frozen unit should prevent cross-unit contamination. However, certain viruses, such as hepatitis and papilloma viruses, are known to survive exposure to liquid nitrogen and have been shown to cross-contaminate bags of bone marrow during liquid nitrogen storage [45, 46]. As units will be banked for extended periods of time this aspect becomes a major concern, and efforts must be made to minimize if not eliminate completely this potential problem. Therefore we take extra precautions by overwrapping each freezing bag with an impermeable cover prior to cryogenic storage to provide an additional barrier to contamination.

The majority of CB banks currently store CB units in multiple aliquots. Generally, this task is accomplished by the use of a freezing bag divided into multiple compartments. Multiple aliquots allow for future use of the stem cells in cell expansion, gene therapy, or for regenerative medicine uses, which may only require a fraction of the frozen unit. Thus, it is not necessary to thaw the entire unit unless absolutely needed, avoiding the damaging effects of repeated episodes of freezing/thawing. Multiple aliquots also allow for potency testing and confirmation of identify of the unit. Commercially available freezing bags now routinely provide for at least two unit aliquots comprising a 20% and an 80% fraction of the processed unit in separate compartments. Larger cord blood collections may be distributed into two processing bags to ensure maximal cell recovery during processing, resulting in multiple, independent storage bags.

CB units are stored in commercially available liquid nitrogen dewars (e.g., model MVE 1830, Pacific Science,

Laguna Beach, CA). In the absence of continuous liquid nitrogen availability, these devices will maintain their -196°C temperature for more than 1 week. It is preferable and consistent with regulatory requirements to construct the storage facility such that the storage dewars are hard-piped to a large liquid nitrogen container on-site at the facility, which helps to eliminate the concern of liquid nitrogen depletion. Each dewar should be monitored continuously for liquid nitrogen levels and temperature both to meet regulatory requirements as well as a precaution. Of course, there should be multiple back-up and fail-safe systems in place, including the immediate availability of empty, spare dewars in case of catastrophic failure. The entire storage facility should be alarmed and monitored, to guard against any unforeseen events.

An additional source of stem cells that can be simultaneously obtained at birth is the cord tissue (CT) which is an abundant source of MSCs. CT can be collected and banked as a future source of stem cells for regenerative medicine and tissue engineering. In addition to MSCs, CT also contains endothelial and epithelial precursor cells that may be useful for these applications [47]. It is estimated that the cord tissue of more than 100,000 donors has been banked worldwide over the past 5 years based on potential future uses. Enzymatic and non-enzymatic protocols for the isolation of MSCs from CT, both before and after cryopreservation, have been described. At CBR the CT is processed, placed in multiple aliquots with a cryoprotectant, labeled, and cryopreserved in the vapor phase of liquid nitrogen dewars. We have shown that cryopreserved CT can be thawed at a later date and MSCs, pericytes, and other cell populations isolated without loss of cell viability or function [20]. This methodology allows for access to the entire population of cells found in the tissue. Furthermore, cryopreserving cord tissue allows for isolation of MSCs at the point of care when the specific clinical application is known. This may be advantageous as MSC isolation protocols continue to be optimized dependent on intended use. CT is just now making its way into the clinic [47–53].

4 Cord Blood Shipping, Thawing and Clinical Use

Cord blood is a widely used source of hematopoietic stem cells for use in transplant medicine, and clinical trials using cord blood stem cells to treat cerebral palsy, traumatic brain injury, Autism as well as other indications have been ongoing for several years (Table 24.1) [54–63]. In order to use these cells the units must be transported to the clinic, evaluated and thawed before infusion. All regulatory requirements must be met in order for a CB unit to be released to a physician for transplant or infusion including completing the necessary paperwork, verifying the unit identity and testing an aliquot of the CB unit. Generally, CB units are sent to the "end-user" in liquid nitrogen dry shippers which can maintain acceptable temperatures (below −150 °C) for at least 1 week. Individual dry shippers are validated by the cord blood bank prior to each shipment to ensure they will maintain acceptable temperatures for the expected duration of transit. Data loggers are included with the CB unit to record temperatures inside the dry shipper. In the event that the internal temperature rises above −150 °C, which may compromise the quality of the CB unit, the treating institution can be notified. Dedicated medical couriers are recommended for transport of this "one of a kind" biological unit.

Each cord blood bank is responsible for establishing its own recommendations for thawing of the CB unit. Broadly speaking CB is generally recommended to be thawed by a

Table 24.1 US clinical trials evaluating autologous cord blood in regenerative medicine applications for pediatric patients

Indication	Phase	Institution	Enrollment	Clinical trials indicator
Autism	Phase II	Sutter Health	30	NCT01638819
Cerebral palsy	Phase I/II	Georgia Health Sciences University	40	NCT01072370
Cerebral palsy	Phase II	Duke University Medical Center	120	NCT01147653
Hypoplastic left heart syndrome (HLHS)[a]	Phase I	Duke University Medical Center	20	NCT01445041
Neonatal hypoxic ischemic encephalopathy	Phase I	Duke University Medical Center	25	NCT00593242
Pediatric stroke	Phase I	Children's Memorial Hermann Hospital; University of Texas Health Science Center—Houston	10	NCT01700166
Traumatic brain injury	Phase I/II	University of Texas Health Science Center, Houston	10	NCT01251003
Type I Diabetes[b]	Phase I	University of Florida	15	NCT00873925
Type I Diabetes[b]	Phase I/II	University of Florida	23	NCT00305344

[a]Cord blood stem cells are being evaluated for their potential to ameliorate neural injury resulting from HLHS as well as their potential to contribute to enhancing cardiac function

[b]Both studies evaluating the use of cord blood in the treatment of Type 1 Diabetes demonstrated safety and feasibility, although they failed to show significant preservation of C-peptide levels in participants

modification of the method described by Rubinstein et al. [46] with a single wash, followed by immediate infusion or injection at the bedside. It is interesting to note that recent guidelines from the FACT (Foundation for the Accreditation of Cellular Therapy) regulatory body now recommend additional washing steps for those units that are not RBC-depleted or -reduced to minimize DMSO toxicity in the recipient as well as to prevent hemoglobin overload due to RBC lysis upon thawing (as found in the 2013 AABB SmartBriefs). FACT now requires additional planning to accommodate this possibility in younger patients. We have found that it requires more skill and technical expertise to thaw and infuse a RBC-replete unit than it does an RBC-depleted unit.

5 Conclusions

As described above, the collection, processing, and banking of CB for immediate or future clinical use can be reproducibly performed with the proper methodology. It is important that all procedures used in the banking endeavor pass regulatory scrutiny. Regulatory compliance may increase operational costs but is essential in providing the assurance to clients and the treating physician that the cord blood unit is collected, processed, and cryopreserved under proper conditions, ensuring that the quality of the CB unit will be unchanged during long-term storage.

CB is being used with increasing frequency in hematopoietic stem cell transplants [64] facilitated in part by the establishment of large repositories of CB collected and cryopreserved for future use. CB donation to public banks has been integral to the over 30,000 estimated worldwide unrelated allogeneic cord blood transplants [9] while private (family) banks provide storage of cord blood for related allogeneic autologous use. Efforts are being made to extend the use of CB in HSC transplants including double cord blood transplants and expansion technologies which aim to overcome cell dose limitations in adult recipients [9].

Although many individuals over the past 20 years have elected to collect and bank cord blood for its potential use in the treatment of hematopoietic cancers and genetic blood and immune disorders, more and more, individuals are now taking advantage of the ability to bank cord blood for uses that are only now being realized or have yet to be discovered. Primarily, these stem cell applications are in tissue engineering and regenerative medicine. Regenerative medicine has the potential to treat many of the most common diseases by replacing or repairing malfunctioning tissues and organs. Because regenerative medicine focuses on functional restoration of damaged tissues, not just the abatement or moderation of symptoms, this field has the potential to cut healthcare costs significantly. However, in order for the promise of regenerative medicine to be realized, it is necessary to identify optimal stem cell sources for particular disease states, and make efforts to inform the lay and medical communities as to their options.

Already the therapeutic potential of CB stem cells to treat patients with neurologic and orthopedic issues, autoimmune diseases, and those suffering from the side effects of transplantation is being evaluated in clinical trials (Personal Communication from CBR and [65, 66]; see Table 24.1). Other trials will surely rapidly follow, including therapies for the eye, joints, and wound healing. In the next decade there will likely be additional uses that are not yet anticipated. The key to these advances is thought to lie in the paracrine effects of CB stem cells, and their ability to be used in many cases under the practice of medicine, since it appears in many instances that it is possible to merely infuse the stem cells directly without timely and costly in vitro culture and differentiation. That is why, in my opinion, CB stem cells are likely to become the preferred source of stem cells for many regenerative medicine applications, now and in the future.

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Conflict of Interest The author is a consultant to CBR Systems, Inc. and Chief Science Officer for Adicyte, Inc.

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Umbilical Cord Blood Cells in the Repair of Central Nervous System Diseases

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Abstract

Umbilical cord blood is composed of a heterogeneous population of cells including epithelial progenitors, mesenchymal stem cells, lymphocytes, and monocytes. These cells are immune-immature and have the ability to modify inflammation, promote angiogenesis and neurogenesis, secrete numerous cytokines and neurotrophic factors, and differentiate into cells of the neural lineage. The latter effect is not believed to be their primary mode of action in bringing benefit in numerous animal models for diseases, especially since the cells do not necessarily need to enter the CNS to be effective. In this way, umbilical cord blood has great translational potential from the animal models to the clinic for treatment of a multitude of CNS disorders including Alzheimer's disease, amyotrophic lateral sclerosis, cerebral palsy, spinal cord injury, and stroke. This potential is being explored in both animal models and also in a few clinical trials.

1 Introduction

Cell transplantation is a promising avenue of therapy for the repair of central nervous system (CNS) diseases. The cells found in umbilical cord blood (UCB) have shown promise in a number of animal models of disorders of the CNS and in this chapter we will be discussing the encouraging results so far observed.

Since its first use in 1972 to treat a male with acute lymphoblastic leukemia [1], UCB has been used over 6,000 times to treat children suffering from numerous hematological disorders [2–4]. UCB can be obtained relatively easily and noninvasively compared with most other possible stem cell sources. The cells are immature and result in less of an immune response, graft-versus-host disease (GvHD) or rejection despite being allogeneic in origin compared to bone marrow cells [4, 5]. However, unlike a bone marrow transplant, the US FDA judge UCB cells (UCBCs) to be a drug or biologic due to their heterogeneity (e.g., from different storage sources—public vs. private, as well as consisting of multiple cell populations) and depending on how they are prepared may not fulfill the "minimal manipulation" requirement. The latter specifies that "For cells or nonstructural tissue, minimal manipulation is processing that does not alter the relevant biological characteristics of cells or tissues." (http://www.fda.gov/downloads/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/Guidances/Tissue/UCM085439.pdf). This means that they are more heavily regulated than a bone marrow transplant and so are currently only approved for specific hematopoietic disorders. They show great promise for the treatment of a number of different neurological disorders from animal studies but will need to undergo further rigorous study and eventually limited clinical trials before becoming an approved treatment. In this chapter, we will be discussing the studies that suggest that it may be worth pursuing these rigorous procedures.

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2 The Characteristics of UCB Cells

Human umbilical cord blood (hUCB) is a heterogeneous mixture of different cell types including red blood cells and mononuclear cells (MNCs). Most studies involve isolating the MNCs by density gradient separation (e.g., Ficoll) and either using this fraction or purifying further to obtain a more specific cell population by cell culture, and/or fluorescent or magnetic-activated cell sorting (FACS or MACS, respectively). The mononuclear fraction includes hematopoietic (and nonhematopoietic) progenitors, lymphocytes, and monocytes. The hematopoietic progenitors have been shown to be able to reconstitute blood lineages long term and to a greater extent than bone marrow stem cells [6–8]. Bone marrow and UCB share a similar number of myeloid progenitors [6], but UCB possesses a greater colony-forming ability with longer telomeres than adult cells [9, 10]. The lymphocytes and monocytes of UCB resemble that of peripheral blood with respect to the B cell population but tend to have a lower number of total T cells (cluster of differentiation 3 positive [$CD3^+$]) and a higher helper T cell ($CD4^+$)/Natural killer cell (NK; $CD8^+$) ratio [11, 12]. The immunological immaturity of UCB is typified by a higher proportion of immature T cells ($CD45RA^+$) but fewer mature memory ($CD45RO^+$) and cytotoxic killer ($CD56^+$) cells [11, 13]. The cells also appear to preferentially express the anti-inflammatory cytokine interleukin 10 (IL-10) [14] and lower levels of IL-2, IL-6, IL-7, tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) [15]. The elevated IL-10 could prevent activation of the T-cell-mediated immune response [16, 17]. The monocytes found in hUCB do not respond to hepatocyte growth factor (HGF) unlike adult monocytes and express less human leukocyte antigen (HLA)-DR supporting their immaturity [18, 19]. In addition, dendritic cells (DCs) from hUCB tend to be more lymphoid rather than the myeloid form seen in peripheral blood [20]. These cells promote anti-inflammatory effects and all these factors appear to combine so that a prolonged immuno-deficient state is observed following hUCB transplantation [21, 22], which may mean that donor–recipient matching is less strict [20].

Culturing of the MNC fraction of hUCB has revealed two distinct cell populations: one adherent and one floating [23]. The adherent population was primarily lymphocytes and positive for hematopoietic markers, whereas the floating population expressed progenitor and neural cell antigens suggesting a nonhematopoietic origin, which could become neural. 1 % of the MNC population is $CD34^+$ suggesting that they are early hematopoietic cells, though they are more immature than those found in bone marrow [24]. Approximately 80 % of the $CD34^+$ cells are also positive for another hematopoietic marker $CD133^+$ which maybe an even more primitive population [25, 26]. Interestingly, $CD133^+$ cells have also been identified within fetal brain and so may also be a marker for neural stem cells (NSCs) [27, 28].

A recent study suggests that the means used to isolate the MNCs from fresh samples can dictate the makeup of the fraction [29]. Sepax processing (Biosafe, Switzerland) was found to result in significantly greater (10×) numbers of a nonhematopoietic, multipotent, lineage-negative stem cell population than other procedures. These cells expressed multipotent markers such as octamer binding transcription protein 4 (OCT-4), sex determining region Y box 2 (SOX-2), Nanog, TRA-1-60/TRA-1-81 antigen, stage-specific embryonic antigen-3 (SSEA-3), and SSEA-4 and they could then be cultured in a differentiation medium to generate neural cells. In a separate study, this group demonstrated generation of an in vitro developing cerebral neocortex that could be used to model neurogenesis in the developing brain from UCBCs [30].

As with most tissues, a minor proportion of hUCBCs have been identified as mesenchymal stem cells (MSCs) [31, 32], which have shown significant plasticity and multipotency by differentiating into cells from all three germ lines [32–35]. These cells also exhibit immunomodulatory properties as shown by their coculture with murine splenocytes [36] and lymphocytes [37].

There have been reports of an unrestricted somatic stem cell (USSC) within cord blood that can be expanded by adherent culturing with MyeloCult medium and supplemented low glucose Dulbecco's modified Eagle's medium from the mononuclear fraction [38]. While these cells have been defined as pluripotent, it is unclear whether that is really the case. A second stem cell type that has been described as pluripotent, the very small embryonic-like stem cells (VSELs) have been identified by some researchers in fresh cord blood and it is said that these cells are lost during MNC isolation and so the full potential of UCB (and bone marrow) may not be fully realized [39]. Further study is required to determine if this is the case.

These properties mean that cells derived from hUCB have the potential to be efficacious against a number of different disorders of the CNS which will be discussed in turn in the next part of this chapter.

3 Disease-Specific Studies

A recent study was published demonstrating that allogeneic non-HLA matched UCB transplantation by the intrathecal and intravenous route was safe for a number of different diseases, though no data on efficacy was provided [40]. One hundred and fourteen patients were treated with an age range of 15–68 and a sex ratio of 1.6:1 (male:female) and received four or five infusions in as many weeks. The patients were followed for only 4–5 weeks, but these initials results are promising, suggesting that HLA matching may not be necessary

for treating nonhematological disorders, even when administered intravenously. Diseases included paraplegia, ataxia, multiple sclerosis, amyotrophic lateral sclerosis, multiple system atrophy, cerebral palsy, traumatic brain injury, and hypoxia–ischemia. Other studies for the use of UCB as a therapy for some of these disorders will be discussed below. Since stroke studies probably provide the most detail about possible mechanisms they will be discussed first, but all other diseases are discussed in alphabetical order. Many of the mechanisms discussed for stroke are likely to be true for the other disorders.

3.1 Stroke

Strokes arise due to either a blood clot occluding a blood vessel or by hemorrhaging resulting in brain tissue being starved of oxygen and "food." The current therapy for stroke is the use of the thrombolytic tissue plasminogen activator (tPA) to dissolve the clot, which must be administered within 3–4 h of the onset of stroke to be effective. There are a number of different animal models for stroke currently in use and one of the most common involves either a permanent or temporary occlusion of the middle cerebral artery (MCAo). Numerous studies using human umbilical cord blood cells (hUCBCs) as a therapy for stroke have been performed and there is strong evidence that the mode of benefit is primarily via paracrine effects rather than cell replacement. In the other diseases mentioned here, the paracrine effects are also probably the most relevant, though some studies do seem to suggest that some cell replacement (such as remyelination of axons) may occur. The majority of UCB-stroke studies do not show significant migration of the cells into the brain following either intravenous or intra-arterial infusion [41–43], but they do show functional improvement and reduced infarct size, though there are exceptions [44, 45]. One possible explanation for the discrepancies is a species difference, since they used 3–4-month old Wistar rats while most successful studies use 2-month-old Sprague Dawley rats. Other explanations may relate to how the cells were obtained and cultured. Since the cells do not need to be in the brain to exert a beneficial effect, this provides support for a paracrine effect. The cells can modify the innate inflammation and prolong cell survival [46] and protect oligodendrocytes [47] but reduce microglial survival [48] and proinflammatory cell recruitment [49].

In a permanent MCAo Sprague Dawley rat model, the optimal route of administration for the hUCBCs would appear to be intravenously as striatal transplants did not show the long-term benefits at 2 months of the intravenous route [42]—though a neonatal stroke model does show improvement following intracerebroventricular treatment 6 h after injury for up to 28 days [50]—and an optimal dose is 1×10^6 or greater [51]. The optimal time of cell delivery would appear to be 48 h [52], which may be related to this being around the time of maximal migratory activity for the cells as demonstrated by an in vitro essay using brain tissue extracts from the stroked animals [53]. Increased levels of cytokine-induced neutrophil chemoattractant-1 (CINC-1) and monocyte chemoattractant protein 1(MCP-1) were observed suggesting they may be involved in the migratory response. However, since relatively few cells enter the brain and they do not need to survive long term, it is unclear of the importance of this. In an earlier study using Wistar rats and a transient MCAo, Chen et al. showed that 1-day and 7-day treatment appeared to be effective, though the 1-day treatment was more effective and 35 days later showed migration of the cells into the brain and the cells expressed astrocytic and neuronal markers [54]. In another study using male spontaneously hypertensive rats exposed to a permanent MCAo, the therapeutic time window was expanded to 72 h [55]. These studies all suggest that an early treatment will be more effective than late but have increased the size of the current therapeutic window. A possible explanation for the limited time window is that the glial response peaks around 72 h after a stroke [56, 57] and generates a glial scar that may inhibit repair [58, 59]. Since hUCBCs modify the activation of glia and reduce the infarct size if administered within the 3 day timeframe, they may prevent the inhibition of repair (as well as promoting repair in other ways). The therapeutic effects of UCBCs appear to be multifaceted as they have anti-inflammatory, neurotrophic, angiogenic, and neurogenic actions which probably arise due to their paracrine abilities [41, 60–63].

The anti-inflammatory actions of hUCBCs include the downregulation of microglia and macrophages and reduction of proinflammatory cytokines such as TNF-α and IL-1β as well as the secretion of anti-inflammatory factors [49, 64, 65], effects which are likely to be important in the treatment of other disorders also. The importance of the spleen to the body's response is currently being revealed along with the ability of hUCBCs to influence it. Spleen size is normally reduced along with $CD8^+$ T cell counts in the first 24–48 h following a stroke, but hUCBC treatment restored splenic weight and T cell count [66]. Splenectomy was shown to greatly reduce the detrimental effects of stroke, showing that much of the damaging inflammatory response (activated microglia, macrophages, and neutrophils) is mediated by the spleen [67]. The reduction in size may relate to the spleen releasing natural killer cells, monocytes, and T cells into the circulation where they then target the vasculature around the infarct [68] and the secretion of the proinflammatory cytokine IFN-γ [69].

Since hUCB is a heterogeneous mixture of cells, studies to determine whether a specific cell type is more effective are ongoing. However, a recent study examining the contribution

of the spleen to the damaging inflammatory response suggests that it is the combination of cells that exerts the best effect rather than an individual cell population [70].

Promotion of neovascularization and endogenous neurogenesis has been demonstrated by hUCBC transplants. Vascular endothelial growth factor (VEGF) secretion is increased and endothelial progenitor cells (or other cell populations that can become endothelial progenitor cells) exist in UCB meaning that new vasculature is a likely response [61, 71–74]. This was confirmed in a study by Taguchi et al. where they showed induction of angiogenesis and subsequent neurogenesis following $CD34^+$ cell transplantation in a stroke model [75]. hUCBCs have previously been shown to promote neurogenesis in aged rats [62].

Two recent studies by Cui et al. revealed that combined statin and UCB therapy exerted better effects on stroke than either treatment alone [76, 77]. They observed upregulation of brain-derived neurotrophic factor (BDNF) and TrkB (BDNF receptor), which play an important role in neurogenesis [78, 79], as well as an increase in migrating neuroblasts, axon and myelin growth and synaptogenesis, in addition to an increased presence of the hUCBCs within the brain [76]. Endogenous angiogenesis, arteriogenesis, and vascular remodeling were also enhanced [77]. Another combined therapy that promoted cell survival, reduced brain infarct and enhanced functional recovery compared with cells alone, was the use of gene therapy—the $CD34^+$ cells derived from UCB were transfected with glial cell line-derived neurotrophic factor (GDNF) and the cells were then transplanted into spontaneously hypertensive rats after a stroke had been induced [80].

3.2 Aging

As we age, the ability of the body to repair itself declines due to a buildup of oxidative stress and DNA damage, proinflammatory processes, cellular senescence, telomere shortening, and other factors [81, 82]. The importance of aging cannot be overlooked as many neurodegenerative disorders are age dependent (e.g., Alzheimer's and Parkinson's disease). Since UCB is taken at the beginning of life, the cells have not been exposed to aging factors, which can make them more effective than autologous cells taken later in life. One example of this is their possession of longer telomeres than cells from other sources such as peripheral blood [83]. However, if you overculture the cells (100 % confluence so cell contact occurs) then replicate senescence is induced [84].

NSCs can be found within the subventricular zone and dentate gyrus of the hippocampus. With aging there is clear evidence for hippocampal neurodegeneration, as typified by a reduction in the number of proliferating stem cells, but not in the total number of NSCs in rodents, meaning that more of the cells are quiescent [85], though a study in primates suggests that proliferation is not affected but cell number and morphology is [86]. hUCBCs have been shown to promote neurogenesis in aged animals for at least 15 days and suppress microglial activation (due to aging) [62]. In a more detailed analysis, the different cell populations found in UCB were tested to explore their effects on aging [87]. They observed that only the T-cell populations ($CD2^+$, $CD4^+$, and $CD8^+$) or whole UCB were able to induce NSC proliferation and survival in vitro. The monocyte ($CD14^+$) and $CD133^+$ (stem cell) populations were ineffective. However, in vivo, only the $CD4^+$ cells were able to significantly increase neurogenesis. Dendritic spine density was also increased at 1 week and inflammation (based on microglial staining) decreased by 2 weeks. The ability of these cells to secrete a number of different neurotrophic factors and cytokines that can alter the neurogenic niche to make it more favorable for neurogenesis is a likely mode of action.

Since aging is a major comorbidity factor of many CNS diseases, the ability of UCBCs to create a more favorable environment (for neurogenesis and cell survival) is of great importance.

3.3 Alzheimer's Disease

Alzheimer's disease (AD) is typified by the presence of extracellular plaques composed of beta-amyloid and intracellular neurofibrillary tangles comprised of hyperphosphorylated tau, as well as significant neuronal cell loss and synaptic connectivity, brain atrophy and cognitive impairments, and dementia. Beta-amyloid is a possible cleavage product of amyloid precursor protein (APP) proteolysis and animal models for AD are based on overexpression of human APP or human mutations of APP that maximize the generation of beta-amyloid. Inflammation is also believed to be an important characteristic of the disease [88]. It is known that the deposition of beta-amyloid activates microglia and astrocytes resulting in a proinflammatory state and neurotoxicity [89–91] and the interaction between CD40 and CD40 ligand (CD40L) is a key component of this cascade [91]. These two components are overexpressed in AD patients and animal models [92–94] and blockade of the interaction between CD40 and CD40L improved AD symptoms such as amyloid accumulation and cognitive deficiencies in animal models [95]. Since hUCB has previously been shown to modify inflammation, it is likely that it could be beneficial in AD.

In 2001, Ende et al. [96] transplanted high numbers of hUCBCs (1.1×10^8) into the eyeballs of mice transgenic for the Swedish mutation of human APP (tg2576), an animal model of AD, and they observed a significant extension in lifespan, thus demonstrating that hUCBCs may be able to exert some benefit. Lee et al. have performed a number of

studies where they show that a single hippocampal transplantation of hUCB-derived MSCs is able to modify inflammation and other AD characteristics in both an acutely induced and the APP-presenilin 1 (APP/PS1 or PSAPP) mouse models of AD [97–99]. However, injections directly into the brain are highly invasive and so an intravenous injection would be preferred if possible. Studies by the Tan lab suggest that this route will also work. Since AD is a chronic disorder, they also performed multiple intravenous injections of the mononuclear fraction of UCB for 6 months into two different AD mouse models (Tg2576 and PSAPP) and observed beneficial effects [100]. They also confirmed that the benefit was related to CD40–CD40L interactions, since decreased plasma beta-amyloid levels was not observed in a CD40 knockout PSAPP mouse. In vitro studies suggested that hUCBCs may secrete a factor that inhibited the CD40–CD40L interaction [100]. In other in vitro studies, they previously showed that the CD40–CD40L interaction shifted APP processing toward amyloidogenesis [101] and that inhibition led to reduced astrocytosis and microgliosis and also reduced abnormal tau formation [102]. Interestingly, Nikolic et al. showed that while microgliosis was reduced, the microglia present were more prone to phagocytosis of beta-amyloid following hUCB suggesting that the cells may work by promoting beta-amyloid clearance as well as by inhibiting the CD40–CD40L interaction and promoting anti-inflammatory effects [100]. Levels of IL-4 were also elevated, which could be a sign that the microglia had been "alternatively activated" toward an anti-inflammatory response [103]. This alternative microglial activation has also been shown by others to increase beta-amyloid clearance as well as reducing plaque size and promoting neurogenesis [103]. The studies of Jiminez et al. suggest that at 6 months, the microglia in AD transgenic mice are alternatively activated, but with time this changes to the classical proinflammatory response [104]. The Tan group has performed additional studies to determine if behavioral benefits are also observed following multiple doses of hUCBCs and they detected an improvement in cognitive impairment along with the previously reported changes in APP processing, beta-amyloid levels and microglial activation [105]. Microglia are normally neuroprotective but excessive activation can lead to neurotoxicity [62, 106]. Excessive microglial activation is suppressed by the interaction between the neuronal protein fractalkine and the microglial chemokine (C-X3-C motif) receptor 1 (CX3CR1) [107]. However, with age, the levels of fractalkine decrease, meaning that excessive microglial activation is more likely to occur [108]. Since aging is a major risk factor for AD (and many other disorders), decreased fractalkine-CX3CR signaling has also been demonstrated in AD transgenic mice and AD patients [109, 110]. This means that excessive microglial activation is likely to play an important role in these disorders, which can be modified by hUCB transplants. Another important aspect of this pathway is it has been shown to regulate neurogenesis, and exogenous fractalkine can restore the age-dependent decrease in neurogenesis [108]. It will therefore be interesting to see whether hUCB can also influence this pathway and whether this could be another means by which they exert a beneficial effect in AD and other diseases.

In the Tan studies, they did not observe any hUCBCs within the mouse brains, suggesting that their mode of action is again likely to be more of a paracrine effect [100]. The lack of a need to cross the blood–brain barrier to be effective, supports a secretory mode of action which modifies the inflammatory status and suggests that the intravenous route of administration may suffice. A recent investigation by Kim et al. revealed that UCB-MSCs secrete intracellular adhesion molecule 1 (ICAM-1) and promote microglial expression of neprilysin (NEP), the degrading enzyme for beta-amyloid in vitro. Following intrahippocampal transplantation, NEP expression was increased in the brain and the cells were observed migrating toward amyloid plaques [111].

These studies show that UCB has great potential for the future treatment of AD.

3.4 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder typified by loss of the spinal motor neurons. Animal models are generally based on the expression of human mutated copper zinc superoxide dismutase (SOD1), which is linked to the familial form of the disease. Again, there is evidence for an inflammatory component in both animal models and patients [112–115], which could be treated with hUCB. Using large doses of cells implanted via an intraocular injection, Ende's group demonstrated a significant extension in the life span of ALS mice [116, 117]. The more favorable route of intravenously has been explored in several studies using much smaller doses by the Garbuzova-Davis group [118–120]. In presymptomatic ALS mice (G93A SOD), they observed a delayed symptom onset and increased survival [120]. In a second study, an optimal single dose of 25×10^6 cells was determined and a shift toward an anti-inflammatory profile and a reduced number of microglia was observed [119]. Peripheral lymphocytes increased while neutrophils decreased suggesting that the immune defenses may have been potentiated. In a third study, multiple small doses (1×10^6) were used and were again found to extend life span, delay disease onset, and reduce astrocyte and microglia numbers [118]. In contrast to the AD studies, transplanted cells were observed in the spinal cord, but were nestin positive, suggesting that they had not differentiated and so were again functioning primarily by growth factor secretion and modulation of inflammation rather than cell replacement [119].

It is possible that the presence of cells within the spinal cord may relate to impairments in the blood–brain barrier and blood–spinal cord barrier, as these impairments have been demonstrated in animal models of ALS, as well as in ALS patients [121–124]. Since the MNC fraction of UCB also contains endothelial progenitors, restoration of the impaired blood–brain barrier is an additional possible mechanism of action [125]. This is highlighted by studies in which gene therapy was used to promote the secretion of growth factors. Rizvanov et al. first performed retro-orbital infusion of cells transfected with human VEGF and mouse neural L(1) cell adhesion molecule (mL(1)CAM) into presymptomatic mice [126]. They observed cells engrafting into the spinal cord as endothelial cells, creating new blood vessels, which occurred to a greater extent with the transfected cells, suggesting that L(1)CAM may potentiate cell migration while VEGF may direct the cells toward an endothelial phenotype. However, when they repeated this with cells transfected with VEGF and fibroblast growth factor 2 (FGF2), they found that the cells tended to become astrocytes rather than endothelial cells [127]. Due to the neurovascular aspects of ALS [128], generation of new endothelial cells could prove to be an effective treatment for ALS.

A single intracerebroventricular injection of UCBCs was also found to prolong the lifespan of G93A and wobbler mice [129]. In addition, the cells remained in the ventricles, providing support for a paracrine rather than cell replacement mechanism of action. An anti-inflammatory cytokine and growth factor profile was observed. Another group performed an intraspinal injection into presymptomatic and symptomatic mice and observed increased survival, reduced motor neuron loss and astrogliosis [130]. Interestingly, they observed better effects in females than males, and the contribution of the sex of the recipient and donor should be explored further, as it is well known that male and female cells respond differently to numerous stimuli [131, 132].

Along with loss of motor neurons, motor nerve function is also affected in ALS and so measurement of the probability of neuromuscular transmission could be used as an indicator of motor nerve function. Souyah et al. demonstrated that UCB transplantation into ALS mice promoted probability of neuromuscular transmission, thus restoring motor nerve function [133].

This demonstrates that there is significant therapeutic potential from UCBCs to treat ALS.

3.5 Cerebellar Ataxias

Cerebellar ataxias are typified by a nonspecific symptom (ataxia) that arises due to dysfunction in the movement coordinating centers of the brain such as the cerebellum. A number of these are hereditary including spinocerebellar ataxia, Friedreich's ataxia, and ataxia telangiectasia. In 1975, an HLA-matched bone marrow transplant was shown to improve cellular immune responses in a male patient suffering from ataxia telangiectasia [134]. An NSC transplant used to treat a child with ataxia telangiectasia was reported to have caused tumors [135]. However, a more detailed examination of the procedure revealed that a nonspecific mixture of cells had been transplanted and therefore the cell-specific origin of the donor-derived tumors is unknown [136, 137], thus highlighting the need to know what you are transplanting. There have been no reports of UCB transplants causing tumors and so this maybe a more effective treatment (though again a heterogeneous mixture of cells is frequently used). For instance, female mice intraperitoneally injected with 3-acetylpyridine, which selectively lesions calbindin-expressing neurons in the cerebellum, as a model of cerebellar ataxia, showed improvement when treated intravenously with hUCBCs 3 days later [138]. Cells homed to the cerebellum and brain stem, reduced neuronal loss and glial reactivity and improved motor coordination. The survival factors phospho-Akt and B-cell CLL/lymphoma 2 (Bcl-2) were found to be upregulated in these areas. In an open-label treatment study of 30 hereditary ataxia patients (18 male, 12 female; average disease duration of 10 years), HUCBCs were administered intravenously and intrathecally 4–6 times within a 5–7-day interval [139]. Reductions in both pathological symptoms and signs of ataxia were observed. The treatment was combined with rehabilitation therapy for 6 weeks, though it is not clear how long after this the patients were followed for. This study was performed in China, with authors from Stanford University and Medistem Inc. helping with data interpretation. The results of this study bode well for the next step which should be a double-blind, placebo-controlled study, so that the efficacy of hUCBCs against cerebellar ataxias can be determined. It is worth pointing out that no mention of immunosuppression or HLA matching was made with respect to this study.

3.6 Cerebral Palsy

Cerebral palsy (CP) is a collection of perinatal disorders characterized by nonprogressive, noncontagious disturbances in the developing brain that lead to cognitive and movement deficits. White matter lesions are common. Models of the disorders include excitotoxic lesions and hypoxic–ischemic damage. An animal model of excitotoxic brain injury using intracranial ibotenate was used to model CP and the authors of this study treated the animals with an intraperitoneal or intravenous injection of UCBCs [140]. Exacerbation of the white matter damage was observed from an intraperitoneal injection along with elevated inflammation, while the intravenous injection had little effect,

suggesting that UCBCs were not effective in this model. Conversely, intraperitoneal injection into a model of cerebral hypoxic–ischemic damage was shown to improve spastic paresis [141]. The cells were found to target the lesion, but did not differentiate into a neuronal phenotype. Intravenous administration in a rat model of neonatal hypoxia–ischemia 24 h after the insult demonstrated a temporary increase in microglia, neocortical neuronal protection, and significant behavioral improvements after up to 10 weeks follow-up, although the cells were not observed 3 weeks later [142]. This again highlights the paracrine effects of the cells. Use of an autologous UCB intravenous transplant into 20 children with CP also had a favorable result as 9 of the children (who were diplegic or hemiplegic rather than quadriplegic), showed neurological improvements [143]. A two patient study of autologous hUCB and subcutaneous granulocyte colony stimulating factor (G-CSF) also revealed some improvements and appeared to be safe [144].

3.7 Huntington's Disease

Huntington's disease (HD) is a genetic disorder characterized by progressive cognitive decline caused by a multiple CAG repeat mutation in the huntingtin gene. Animal models are based on either use of a neurotoxin or expression of a mutated form of huntingtin. Decreased neurogenesis has been shown in a transgenic mouse model of HD [145], and UCB has been shown to promote neurogenesis in aged animals [62]. Therefore, UCB therapy could prove effective in correcting this error and possibly treating the disease. Huge doses of UCB have been shown to extend the life span of HD transgenic animals [146], but little further study has, as yet, been performed.

3.8 Metabolic Diseases

There are a number of inherited metabolic diseases that result in progressive degeneration of the CNS and patients frequently die in childhood. Many of these disorders arise due to a single mutation in a specific enzyme associated with lysosomal storage, which results in accumulation of a toxic substrate (lysosomal storage disorders) or a defect in a membrane transporter protein (peroxisomal storage disorders). These disorders normally impact multiple tissues including the CNS and encompass disorders such as mucopolysaccharidosis Type I (Hurler syndrome) and mucopolysaccharidosis Type III B (Sanfilippo syndrome Type B). Prasad and Kurtzberg [147] provide a recent review of the use of UCB to treat these disorders including clinical data and so we will not be discussing the studies mentioned therein.

Sanfilippo syndrome Type B is caused by α-*N*-acetylglucosaminidase (Naglu) deficiency and this leads to heparin sulfate accumulation within lysomes, culminating in multiple systemic organ abnormalities, including the CNS. A common animal model for this disorder is the Naglu knockout mouse and this has been extensively studied by the Garbuzova-Davis group [148–150]. They first determined that intracerebral transplantation into Naglu knockout mice led to long-term survival of the transplanted cells and their integration into the brain parenchyma and other regions where they could restore the deficient enzyme [150]. They subsequently showed that intravenous transplantation of hUCB MNCs into pregnant heterozygote females led to transmigration of cells into the embryos which corrected Naglu enzyme activity [148]. In a third study, intravenous administration into presymptomatic and symptomatic animals also improved behavioral outcomes, exerted an anti-inflammatory effect in the brain and led to widespread distribution of the cells within the CNS and other organs of the body, where heparin sulfate accumulation was reduced [149]. Preliminary studies have also shown that the monocyte/macrophage fraction of cord blood may be the most effective [151] as well as the presence of an impaired blood–brain barrier in the animal model [152], similar to that observed for ALS. Multiple hUCB injections may also be more effective than a single injection at restoring the deficient enzyme and maintaining an anti-inflammatory status in the long term [153].

3.9 Multiple Sclerosis

Multiple sclerosis (MS) is an inflammatory disorder which leads to demyelination of the axons of the brain and spinal cord and can have neurological and cognitive symptoms. Since UCBCs can differentiate into oligodendrocytes or promote the differentiation of endogenous NSCs to oligodendrocytes, they may be able to restore damaged myelin. The ability of UCBCs to modify the inflammatory response is another reason why they could be effective. A typical model of MS is the experimental autoimmune encephalomyelitis (EAE) model, which involves immunization with myelin oligodendrocyte glycoprotein (MOG), a component of the myelin sheath. A number of studies have demonstrated some efficacy against EAE with bone marrow-derived MSCs [154, 155], as well as a few cases in humans against MS [156, 157], while allogeneic hematopoietic stem cells were not effective [158]. Umbilical cord-derived MSCs also appear to be effective against EAE as they were able to restore behavioral function and attenuate long-term pathology [159]. Currently, no UCB studies appear to have been performed, which is surprising based on their advantages over bone marrow stem cells. A possible explanation for this maybe a

study in 1993 that suggested that cord blood contains higher levels of autoimmune T cells for myelin proteins than peripheral blood [160].

3.10 Neonatal Hypoxic–Ischemic Injury

During birth or early in life, the brain can be starved of oxygen and/or blood due to complications, including cardiac arrest or occlusion of the umbilical cord, and this is known as neonatal (or perinatal) hypoxic/ischemic injury. Animal models normally consist of common carotid artery occlusion and exposure to a hypoxic environment around day 7. Striatal neurogenesis was demonstrated to aid recovery in adult animals from a model of neonatal hypoxia–ischemia [161]. This was dependent on the presence of BDNF and epidermal growth factor (EGF). Both of these factors can be secreted by hUCBCs suggesting that they could be effective against this disorder and the neonatal nature means that autologous UCBCs could theoretically be used shortly after injury. One study looked to see if UCB-MSCs obtained during the birth of hypoxic babies differed from that of nonhypoxic babies. While they found no difference in cell viability or number, the hypoxic-derived cells did appear to be more likely to undergo neural differentiation, providing an additional reason why autologous cells maybe the optimal treatment [162]. Intracerebral transplantation of UCB-MSCs was shown to alleviate some of the detrimental effects of hypoxia–ischemia and some of the cells were observed to differentiate into astrocytes [163]. Intraperitoneal injection of hUCBCs has been shown to reduce the sensorimotor deficits observed in rodent models of the disorder [141, 164] and help reduce the injury-induced impairment of neural processing [165]. A recent study attempted to elucidate possible mechanisms for this effect [166]. hUCBCs were administered intraperitoneally into animals 24 hrs after lesioning and they were followed for 2 or 14 days. The lesion-induced increase in proinflammatory cytokines IL-1α, IL-1β, and TNF-α was reduced by the hUCBC transplant as well as a marker for activated microglia/macrophages (CD68). This again provides support for a paracrine action of the cells for generating a favorable environment for repair. However, one study of intravenous delivery showed no significant benefit and no entry of the brain by the cells [167]. However, the same group recently published a later study whereby they show a dose-dependent improvement by intravenous injection of 1×10^6 to 1×10^8 HUCBCs and observed significant spatial memory recovery and less brain atrophy at 8 weeks [168]. The authors did not specify the sex of the animals in the first study (male in the second), followed up for a shorter time (3 vs. 8 weeks) and only used the 1×10^7 dose.

A recent clinical case was reported in which a 16-month-old child who had suffered a heart attack-induced global hypoxic/ischemic brain injury and was in a permanent vegetative state, was transplanted with multiple intracerebroventricular injections of their own UCBCs over several months [169]. Prior to transplant, the cells had been predifferentiated to a neural state and were labeled with superparamagnetic iron oxide particles (SPIOs) so that they could be traced. After 6-month follow-up some minor, but significant improvement in his neurological status was observed and the cells were seen to survive for at least 4 of the 6 months follow-up, demonstrating that this procedure was feasible, safe and has limited benefit, though with some tweaking of the procedure (and perhaps longer follow-up), more benefit may be evident.

An in vitro study compared three different populations of UCBCs; one normal, one enriched with $CD133^+$ stem cells and one depleted of the aforementioned cells. In each case, the cells were found to be able to reduce cell death of a post-hypoxic population of neuronal cells suggesting that the $CD133^+$ cells were not necessary for benefit [170], though Tanaka et al. [171] demonstrated that these cells could be beneficial with respect to reducing cortical damage and neuronal projections in hypoxic rat perinatal tissue cultures.

The homing of stem cells to the site of injury is a potentially important factor in the benefit of cell transplants, though it is worth remembering that entry into the brain from peripheral transplants is not normally necessary for efficacy. Rosenkranz et al. [172] demonstrated that cell homing in neonatal hypoxia–ischemia was stromal cell-derived factor (SDF) dependent. In further studies, they showed that the cells increased angiogenesis, decreased apoptosis, and promoted cell survival [173]. BDNF and VEGF expression was also elevated which may contribute to the increased cell survival and angiogenesis, respectively. Following systemic infusion of cells, along with mannitol to promote crossing of the blood–brain barrier, very few cells were observed in the brain, but a robust upregulation of neurotrophic factors was observed providing support for a paracrine rather than a cell replacement effect of the cells [174]. Behavioral recovery was also observed.

A recent study suggests that an additional mode of action for UCBCs in the treatment of neonatal hypoxia–ischemia is potentiation of endogenous neurogenesis [175]. An increased number of NSCs were found in the subventricular zone along with elevated expression of sonic hedgehog and glioma-associated oncogene family zinc finger 1 (Gli1) following transplant. UCBC transplantation was also found to modify the inflammatory response induced by perinatal hypoxia–ischemia [176]. Acute inflammation characterized by microglial and macrophage activation, reactive astrogliosis and upregulation of the astrocytic gap junction protein connexin 43 are initially observed in response to ischemia, which slowly decline. UCBCs hastened the decline in connexin 43 and reactive astrogliosis thus reducing the detrimental effect and potentiating postischemic plasticity.

3.11 Parkinson's Disease

Parkinson's disease (PD) is a movement disorder typified by loss of the dopaminergic nigrostriatal neurons and the presence of intracellular inclusions of alpha synuclein, known as Lewy bodies. Animal models either use a toxin such as 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or transgenic models overexpressing either mutated or normal human alpha synuclein. A high intravenous dose of UCBCs was shown to delay symptom onset and prolong life expectancy in an animal model of PD [177]. The previously mentioned USSCs have been tested in vitro to determine if they can be differentiated into dopamine neurons, which could be used as a cell replacement therapy for PD [178]. Serum withdrawal induction medium led to neuronal-like cells expressing dopaminergic cell markers. Using hUCB-derived multipotent stem cells, which maybe the same cell as described above, though the reported culture media are different, Li et al. demonstrated that all transretinoic acid could be used to differentiate the cells into neurons with a dopaminergic phenotype, as shown by the presence of the dopamine transporter, tyrosine hydroxylase expression and the ability to secrete dopamine [179]. A recent study showed that incubation of UCB-MSCs in the conditioned medium of amniotic epithelial cells also led to formation of a dopaminergic neuron-like cell [180]. This was shown to be partly dependent on the presence of BDNF and nerve growth factor (NGF) in the medium, since use of a trk inhibitor reduced the occurrence of these cells. In a further study, they showed that pleiotrophin may also contribute to this differentiation [181].

3.12 Spinal Cord Injury

Spinal cord injuries (SCI) arise primarily from trauma resulting in crushing or severing of the spinal cord and cell therapy maybe a relevant treatment. It can generally be divided into an acute phase whereby the initial cell death occurs and a chronic phase by which time glial scarring has occurred which will inhibit reinnervation. Most studies focus on the acute phase. hUCBCs infused intravenously 5-day postinjury in an SCI rat model were more effective than cells infused 1-day postinjury and were found to migrate to the site of injury [182].

Bromodeoxyuridine (BrdU)-labeled hUCBCs were transplanted into the spinal cord injury site of rats, in some cases with BDNF [183]. After 8 weeks, functional recovery was greater in animals transplanted with cells and BDNF compared with those transplanted with cells alone, which in turn were better than those just given media. The cells were seen to differentiate into cells that were positive for microtubule-associated protein-2 (MAP-2) and glial fibrillary acidic protein (GFAP), and so could be neurons and astrocytes. The BDNF may have promoted axonal regeneration at the early injury stage. In another study, hUCBCs were infused intravenously immediately after injury [184]. Seven days later, significant improvement in hindlimb dysfunction, elevated levels of IL-10, but decreased levels of TNF-α were observed. VEGF and GDNF were also detected within the spinal cord. Cells transplanted at the site of injury, 7 days after injury were seen to survive for at least 2 weeks and to differentiate into neurons, astrocytes, and oligodendrocytes and improve function [185]. The cells were shown to myelinate axons and the cells were able to secrete neurotrophin-3 (NT-3) and BDNF. Myelin basic protein and proteolipid protein were also expressed by the cells which aid remyelination. Continuing studies demonstrated cell survival for 5 weeks and downregulation of Fas protein which reduced apoptotic protein levels. Antiapoptotic effects were also promoted by the phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway, suggesting that UCBCs can reduce programmed cell death [186, 187]. Transplantation of allogeneic UCBCs embedded in alginate gel into an acute SCI rat model demonstrated significant astroglial proliferation and improvements in motor function [188]. Immunosuppressed female rats transplanted with male hUCBCs 1 day and 9 days after injury demonstrated cell survival for up to 6 weeks and improved motor recovery, but the cells did not show any signs of differentiation [189]. In one study, fresh hUCB was transplanted at 0 or 4 days after injury with no immunosuppression in a female aged rat model of SCI and no differences based on the time of transplant were detected after an 8-week follow-up, but clinical and neurophysical improvement was observed [190].

Predifferentiation or expansion of specific cell lines found within UCB could also prove effective. In one study, the UCBCs were predifferentiated into neural progenitor cells before transplantation directly into the injury site, 7 days after injury with immunosuppression, in a male rat model [191]. Improvements in locomotor activity and recovery of somatosensory-evoked potentials were observed. The cells were also seen to take on an oligodendrocytic phenotype that surrounded the lesion. A multipotent stem cell isolated and expanded from UCB was transplanted into immunosuppressed female rats immediately after SCI [192]. The cells were no longer present at 6 weeks, but a significant improvement in locomotor activity was observed, with a nonsignificant decrease in lesion size. The cells were capable of releasing angiogenic, anti-inflammatory, and neurotrophic factors into the media in vitro and so these may have caused the beneficial effect since the cells did not survive. Transplantation of HLA-matched multipotent stem cells derived from UCB were found to have beneficial effects when administered without immunosuppression at the site of injury to a 37-year-old female, who had suffered a spinal

cord injury 19 years previously [193]. Magnetic resonance imaging suggested that regeneration of the spinal cord had occurred.

Transplantation of MSCs derived from hUCB into female rats near the lesion site, 24 h after injury, resulted in significant recovery of hindlimb motor function, as well as evidence of neurofilament fibers and growth cone-like structures [194]. The cells survived, migrated, and secreted GDNF and NT-3. The number of reactive astrocytes was also decreased. hUCB-MSC transplantation along with a 7-day BrdU treatment to determine proliferation of new ependymal cells, revealed that locomotor function was restored, cavity volume and apoptosis were reduced, and proliferation of new cells was significantly enhanced [195]. The new cells expressed astrocytic markers, suggesting that they were adopting an astrocytic phenotype. Matrix metalloproteinase 2 (MMP-2) can promote functional recovery after SCI by inhibiting glial scar formation and in one study, UCB-MSCs were shown to upregulate MMP-2, reducing glial scar formation and chondroitin sulfate proteoglycans immunoreactivity [196]. Inhibition of MMP-2 prevented the protective effects of the cells. tPA is upregulated following spinal cord injury and its expression is reduced by UCB treatment, suggesting it may be detrimental as its expression inversely relates to that of myelin basic protein [197].

$CD34^+$ cells derived from hUCB have also been shown to be beneficial in the treatment of spinal cord injury in a rat model when administered directly after injury [198]. This included reduction of spinal cord infarction and apoptosis, and hindlimb dysfunction, which coincided with the release of VEGF and GDNF. In another study, the $CD34^+$ cells were transplanted into rats on the first day and on the sixth day after injury and the earlier transplanted animals showed a greater degree of recovery with a reduction in infarct size and increased blood vessel density [199]. The cells survived for at least 3 weeks, but were not seen to differentiate. A separate study had the cells transplanted 1 week after injury and increased residual white matter volume, regeneration, and sparing of axons [200], but the cells had disappeared by 5 weeks and at 3 weeks the cells did not show signs of differentiation. A comparison study of UCB-derived $CD34^+$ cells and bone marrow stromal cells in a rodent model of SCI revealed that the $CD34^+$ cells achieved greater functional improvement 7 and 14 days after transplantation than the bone marrow stromal cells [201]. The cells migrated to the site of injury and expressed GFAP and neuronal nuclei protein (NeuN).

In a comparison transplantation study of UCB-MSCs and amniotic epithelial stem cells (AESCs) to determine their effects on mechanical allodynia and thermal hyperalgesia, 2 weeks after SCI [202], it was observed that neither treatment affected thermal hyperalgesia while there was a trend toward improvement with UCB-MSCs and a significant improvement of mechanical allodynia with the AESCs. The AESCs also significantly reduced N-methyl D-aspartate (NMDA) receptor NR1 subunit phosphorylation. Both cells decreased expression of the microglial marker F4/80, but not GFAP or induced nitric oxide synthase. Transplantation of UCB-derived USSCs into an immune-suppressed rat model of SCI close to the site of injury led to HGF-mediated migration to the site of injury, reduced lesion size, enhanced axon regrowth, and improved locomotor activity [203]. Neural differentiation and cell survival were not necessary for benefit suggesting that the paracrine function is more important.

Combination therapies may also prove to be useful. For instance, a 29-year-old male was transplanted intrathecally with allogeneic $CD34^+$ cells expanded from UCB, and MSCs expanded from the umbilical cord matrix 5, 8, and 14 months after injury [204]. Neuropathic pain was significantly reduced as well as some recovery of bowel, sexual, and muscle function. It is unclear what contribution the different cell types made to the recovery effort. The anticancer drug, taxol has been shown to inhibit scar formation, promote axonal elongation, and improve locomotor activity in SCI rats. Therefore Zhilai et al. combined 4 weeks of intrathecal taxol with an immediate transplantation into the injury site of hUCB-MSCs [205]. They showed that the combined treatment was more effective than either individual treatment by greater enhancement of anti-inflammatory, antiapoptotic, reduced astrogliosis and greater axonal preservation.

While the majority of animal studies are performed in rodents, there have been a few using more complex animals. For example, Lim et al. used a canine model of SCI [206] and transplanted allogeneic UCB-MSCs and/or recombinant methionyl human G-CSF 1 week after injury directly into the injury site. Stable recovery of nerve function was observed for at least 8 weeks in the groups treated with cells and no additional benefit was observed as a result of the G-CSF. Exploration of dosing time in canines demonstrated that 1 week after injury appeared to be optimal as less fibrosis was observed, though neuronal and astrocyte markers were also elevated after transplantation at 2 weeks [207]. Lee et al. transplanted hUCB-MSCs into a canine SCI model [208]. The number of animals was low in this instance ($n=2$ per group) and hUCB-MSCs were transplanted directly into the spinal cord and followed for 2 or 4 weeks. Benefit tended to be seen by 3 weeks with an improvement in hindlimb locomotion. Cyst and injury size were significantly reduced at 4 weeks and the cells were positive for neuronal, astrocytic, and blood vessel markers (NeuN, GFAP, and von Willebrand factor, respectively). In a follow-up study with more animals, they saw long-term functional recovery over 3 years and axonal remyelination [209]. Ryu et al. compared the ability of fat, bone marrow, Wharton's jelly, and

UCB-MSCs to treat SCI in dogs [210]. In each case, the cells survived for 8 weeks and showed improvements that were not significantly different from each other, but the UCB-MSCs did show more nerve regeneration and anti-inflammatory activity, suggesting that they may be a better therapy than the other cells in this regard.

3.13 Traumatic Brain Injury

Traumatic brain injury (TBI) includes concussive injury that arises due to an impact to the head with either a solid object or possibly even sound or force waves (such as from an explosion) and so is a frequent problem for soldiers returning from war zones. Lu et al. showed that intravenous hUCBCs could improve the neurological function following such an injury in an animal model [211]. The cells migrated into the brain and some were incorporated into the vascular walls surrounding the lesion. The cells were shown to express neuronal and astrocytic markers suggesting that they may be differentiating into neural cells. Contralateral intracerebroventricular injection of UCB-MSCs into TBI-damaged mice with immunosuppression led to long-lasting improvements in sensorimotor function [212]. The cells were observed to migrate to the injured side within a week and cause a non-phagocytic activation of microglia, which over time led to a decrease in GFAP expression within the glial scar. Intravenous transplantation of the $CD34^+$ UCBC population immediately after TBI in a rat model revealed significant angiogenesis, neurogenesis as well as improvements in motor dysfunction, apoptosis, and inflammation compared with vehicle-treated animals [213]. They also observed migration of endothelial progenitor cells originating from the bone marrow.

The favorable results from animal models have resulted in attempts to treat humans. Three patients were treated intravascularly with allogenic UCB and recombinant erythropoietin (EPO) [214]. EPO has been shown to have neuroprotective and reparative properties, which maybe mediated via the Janus kinase 2 (Jak2)-PI3K-Akt pathway [215, 216]. Improvements in motor and cognition were observed suggesting that this type of therapy should be pursued as a treatment for TBI.

It is worth pointing out that none of the therapies described have cured any of the mentioned diseases, but many of them clearly suggest promise for treatments if they can be translated to the clinic. It is however likely that a multifactorial therapy will provide the best recovery and this will need to be explored in future studies, once more is known how the single therapies work.

4 Clinical Trials Using hUCB for CNS Repair

Nineteen clinical trials are currently listed at clinicaltrials.gov (as of the end of May 2013) that use hUCB for the treatment of CNS disorders (search criteria "umbilical cord blood and CNS") (see Table 25.1). Many of the studies are primarily safety studies with some or little exploration of efficacy and without a placebo or randomization, which will make their interpretation difficult. The majority of the studies are also based in Asia, with only 5 originating in the USA. Most of the studies are also allogeneic (13/19) and several do not specify a dose or route of administration. Four of the allogeneic studies also specify immunosuppression, though it is unclear whether the others neglected to mention it or are not performing it. Some of the allogeneic studies also mention HLA-matching (or mismatching), though studies without matching (as mentioned earlier) suggest that this may not be as relevant for UCB transplants for neurological treatments [40].

While cerebral palsy appears to be the most popular disease (6/19), four of the studies originate from the same institution and three of them appear to be Phase or type of trial variants. The other study was recently published with favorable results [217] and also uses EPO as a cotreatment. Unfortunately, there was no UCB alone group in this study, which makes it difficult to evaluate the contribution of the EPO.

Except for spinal cord injury, which is exclusively by spinal injection, in most cases, the cells will be injected intravenously. The AD studies and two of the stroke studies also involve direct injection into the brain.

While some of the studies may not be as rigorously performed as would be ideal to maximize interpretation of their effectiveness, it will be interesting to see how the trials fare with respect to treating the disease of interest. The animal studies suggest that many more clinical trials could be on the horizon.

5 Conclusions

UCB contains a heterogeneous mixture of immature cells that have shown potential for the treatment of many diseases of the CNS. This relates to their ability to modify the inflammatory response, promote angiogenesis and neurogenesis, and secrete a number of cell survival cytokines and neurotrophic factors. There are several clinical trials exploring their value in the clinic and as our understanding of these cells progress, many more trials should be performed.

Table 25.1 The clinical trials using umbilical cord blood to treat disorders of the central nervous system

Disease	NCT ID	Source	Dose (/kg)	Route	Age	Follow-up	Type of study	Location
Cerebral Palsy	01072370	Autologous (MNC)	1×10^7	iv	2–12 years old	1 year	Phase I/II (placebo, double blinded)	Georgia Regents University
	01147653	Autologous	1×10^7	iv	1–6 years old	2 years	Phase II (randomized double blinded)	Duke University, NC
	01193660	Allogeneic (immuno-suppression)	3×10^7	iv	10 m-10 years	6 months	Randomized/double blind + erythropoietin	Bundang CHA Hospital, Korea
	01486732	Allogeneic (immuno-suppression)	NS	iv/ia	6 m-20 years	3 months	Phase II (Randomized, placebo + rehabilitation)	Bundang CHA Hospital, Korea
	01528436	Allogeneic (immuno-suppression)	D/K	iv/ia	6 m-20 years	3 months	Phase II (randomized, double-blind, placebo + rehabilitation)	Bundang CHA Hospital, Korea
	01639404	Allogeneic (immuno-suppression)	D/K	iv/ia	6 m-20 years	3 months	Open-label + rehabilitation	Bundang CHA Hospital, Korea
SCI	01046786	Allogeneic (HLA matched)	$1.6–6.4\times10^6$ (total)	Spinal	18–60 years old (>12 months after SCI)	1 year	Phase I/II (open-label, dose escalation)	University of Hong Kong
	01354483	Allogeneic (HLA matched)	$1.6–6.4\times10^6$ (total)	Spinal	18–60 (>12 months after SCI)	1 year	Phase I/II (open-label, dose escalation)	Chengdu Army Kunming General Hospital, China
	01393977	Allogeneic (MSC)	D/K	spinal	20–50	3 months	Phase II (Open-label + rehabilitation)	Chinese People's Armed Police Force, China
	01471613	Allogeneic (StemCyte)	6.4×10^6 (total)	spinal	18–65 (<4 weeks since SCI)	1 year	Phase I/11 (Randomized, double-blind, placebo-controlled + lithium)	Chinese PLA Chengdu Army Kunming General Hospital
Stroke	01438593	CD34+ (StemCyte; HLA mismatch 5/6)	5×10^6 (total)	ic	35–75 years old (6–60 months post stroke)	6 months	Phase I (open label)	China Medical University, Taiwan
	01673932	UCB-MNCs (HLA-matched)	$1–4\times10^7$ (total)	ic	35–65 years old (6–60 months post stroke)	12–36 months	Phase I (open-label randomized control)	The University of Hong Kong
	01700166	Autologous	D/K	iv	6 weeks–6 years old	2 year	Phase I (open label)	The University of Texas Health Science Center, Houston
AD	01297218	Allogenic (NeuroStem-AD; MSC)	$3–6\times10^6$ (total)	ic	50–75 years old	12 weeks	Phase I (Open label)	Medipost Co., Korea
	01696591	Allogenic (NeuroStem-AD; MSC)	$3–6\times10^6$ (total)	ic	50–75 years old	24 months	Phase I (Open-label; follow-up)	Medipost Co., Korea
Neonatal hypoxia–ischemia	00593242	Autologous	5×10^7	iv	14 days old	26 months	Phase I (open label)	Duke University, NC
	01649648	Autologous	D/K	iv	1–3 days old	2 years	Phase I (open label)	National University Hospital, Singapore
TBI	01251003	Autologous	$<10\times10^9$	iv	6–18 months post TBI (Children)	2 years	Phase I/II (open label)	University of Texas, Houston
	01451528	Allogeneic	D/K	iv/ia	>18 yearsold (>6 months with TBI)	6 months	Open label	CHA University

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Umbilical Cord Blood for Cardiovascular Cell Therapy

26

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Abstract

Cardiovascular diseases, including atherosclerosis, hypertension, and heart failure, remain major causes of morbidity and mortality with substantial economic cost worldwide. Unlike other vertebrate animals, it was postulated that heart cells in adult mammals did not have self-regenerative potential. However, the basic foundations of cardiac regeneration changed radically in the 1990s owing to pioneering studies with significant biological and clinical implications. Since then, most efforts have been directed to repair myocardial tissue and the supportive vascular system using cells with regenerative potential. In this context, to highlight the great potential of UCB in human regenerative medicine, we first provide a broad overview of current advances, pitfalls, and future goals, focusing on cardiac regeneration. We then show UCB, in the context of other existing potential cell sources, as a rich reservoir of both hematopoietic and nonhematopoietic cells with regenerative capacity. We finally recount ongoing research using UCB-derived cells, focused on cardiovascular cell therapy.

1 Introduction

Beyond certain unresolved issues, the outlook regarding the use of umbilical cord blood (UCB)-based therapies for emerging clinical applications is more than a promise. This assumption has been reinforced by cumulative knowledge during approximately the past four decades since Knudtzon found relatively mature progenitor cells circulating in human UCB. To highlight the great potential of UCB in human regenerative medicine, we first provide a broad overview of current advances, pitfalls, and future goals, focusing in the field of cardiac regeneration. We then show UCB, in the context of other existing potential cell sources, as a rich reservoir of both hematopoietic and nonhematopoietic cells with regenerative capacity. Finally, we recount ongoing research using UCB-derived cells, focused on the cardiovascular field. We hope that this chapter will serve as an introductory summary for those who wish to further investigate the role of UCB in cardiovascular cell therapy.

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2 Cardiac Regeneration: Advances and Future Goals

Cardiovascular diseases, including atherosclerosis, hypertension, and heart failure, remain major causes of morbidity and mortality with substantial economic cost worldwide. In particular, the incidence of heart disease, most notably myocardial infarction (MI), has increased dramatically in recent decades [1]. The number of pharmacological and surgical treating options is currently limited. Severe cardiac dysfunction

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Table 26.1 Major cell types for cellular cardiomyoplasty: advantages vs disadvantages

Cell type	Advantages	Disadvantages
MNC	Multipotent and autologous	Limited expansion and high cell heterogeneity
Skeletal myoblast	Autologous, easy isolation and expansion	Arrhythmogenic and lack of electromechanical coupling
MSC	Multipotent, autologous, lack of immunogenicity, and cryopreservable	Invasive and painful isolation, risk of infection (bone marrow), require expansion, and sometimes adverse events following cell implantation (e.g., calcification and bone formation)
CPC	Autologous, high expansion, cardiomyocyte phenotype, and electromechanical compatible	Limited availability and survival
ESC	Totipotent and high expansion	Teratoma formation following cell implantation, host immune response, and both legal and ethical refusal
iPSC	Pluripotent, autologous, epigenetically and functionally indistinguishable from ESCs, acceptable availability, and neither legal nor ethical constraints	Teratoma formation

Note: *MNC* mononuclear cell, *MSC* mesenchymal stem cell, *CPC* cardiac progenitor cell, *ESC* embryonic stem cell, *iPSC* induced pluripotent stem cell

can only be resolved by complete replacement of the injured heart (cardiac transplantation), a medical practice that is restricted owing to both the scarcity of donors and the risk of organ rejection. Moreover, to maintain overarching homeostasis, the tissues of multicellular organisms are critically endowed with a vascular system, which is responsible for moving nutrients, gases, and waste products to and from cells. Maintenance of this extremely paramount function is dependent on endothelial integrity. Disruption of vascular endothelial homeostasis contributes to the origin and/or progression of numerous cardiovascular disorders, including atherosclerosis, hypertension, and heart failure [2].

Vertebrate organisms exhibit a body pattern that is usually established on a small scale and then grows larger. During the growth stage, the majority of cells normally replicate while preserving both phenotype and function. In contrast, as cells die, new cells with the same tissue-specific characteristics and turnover rate replace them. For many years, this widely accepted behavior has been the foundation of comparing the adult organism with a perfectly stable ecosystem, in which each generation of cells can be replaced without altering the performance of the entire system. However, few cell types are generated in sufficient quantity during embryonic development to be maintained throughout the adult lifespan. Although there was no reasonable explanation for these nonrenewable cells, such as cardiac muscle cells (cardiomyocytes), researchers and clinicians shared the understanding that such cells never divided to provide replacement cells postinjury. Hence, it was postulated that some organs in the adult, such as the heart, were terminally differentiated and did not have self-regenerative potential [3].

However, the basic foundations of cardiac regeneration changed radically in the 1990s owing to pioneering studies with significant biological and clinical implications. These paramount studies demonstrated cardiomyocyte turnover in the postinfarct myocardium [4–6], cardiac chimerism as a mechanism of self-repair [7–12], and the existence of resident populations of cardiac "stem" and/or progenitor cells (CPCs) with the capacity to replicate and differentiate into the cardiomyogenic lineage [13–15]. Nevertheless, there is little disagreement among cardiac experts regarding the extremely low occurrence of these phenomena, which shows that they are collectively insufficient to counteract the constant loss of cardiomyocytes that leads to irreversible cardiac dysfunction.

Since the pioneering observations mentioned earlier, most efforts in the field of cardiac regeneration have been directed, together with cytokine and gene therapies [16, 17], to repair myocardial tissue and the supportive vascular system using cells with regenerative potential (cellular cardiomyoplasty) postinjury. In this context, researchers agree that the ideal source of cells with cardiac regeneration potential should: (1) be autologous (in order to reduce immune rejection and disease transmission); (2) expand on a large scale ex vivo; (3) integrate with damaged tissue; and (4) differentiate either into new cardiomyocytes electromechanically coupled with the host tissue or functional vascular endothelial cells. Additionally, its application should not be restricted by ethics concerns [18].

A variety of "stem" and/or progenitor cells have been used in cardiac cell therapy (Table 26.1). Researchers have explored the cardiomyogenic and vascular potential of unfractionated adult bone marrow mononuclear cells (BMMNCs); skeletal myoblasts; hematopoietic progenitor cells (HPCs) derived from both adult bone marrow and peripheral blood; and mesenchymal stem cells (MSCs) from adult and perinatal sources, such as bone marrow, subcutaneous and cardiac adipose tissue, and (more recently) placenta and umbilical cord blood (UCB) [15, 18, 19]. BMMNCs, HPCs, and MSCs have already been administered clinically using mostly intracoronary, myocardial injection, and endomyocardial transplantation delivery routes, with generally modest outcomes [15, 20]. As previously mentioned, postnatal heart is known to contain distinct CPC populations; intensive efforts have been made to locally activate their functions in injured hearts without cell administration [15, 21, 22].

In addition, totipotent embryonic stem cells (ESCs) derived from the inner cell mass of blastocysts are nonautologous but can differentiate toward cardiomyocyte-like cells that are electrically connected to the surrounding myocardium without triggering arrhythmias [23]. Although the use of ESCs is at present restricted in many countries because of both legal and social barriers, the implementation of advanced genetic reprogramming techniques to generate cytoplasmic hybrids, or cyhibrids, which are created by taking an egg from a nonhuman animal and removing the nuclear DNA, leaving only the cytoplasm or ooplasm of the animal egg, should help create a more ethical source of "stem" or progenitor cells [24]. However, the undifferentiated state of ESCs is currently associated with a risk of teratoma formation [25]. In contrast to ESCs, there have been no ethical objections raised in relation to the use of induced pluripotent stem cells (iPSCs), which are artificially derived from adult somatic cells through the induction of an embryonic gene profile and are thought to be equivalent to ESCs with regard to doubling time, differentiability, and (unfortunately) teratoma formation [15].

In the clinical arena, despite being safe and technologically feasible, cellular cardiomyoplasty cannot be used successfully (e.g., after MI) because of the formation of a noncontractile scar and extreme ischemia [26–29]. Taken together, clinical advances are not as solid or conclusive as advances in animal models (generally rodents and pigs), which have provided the proof of concept for the regeneration of damaged cardiac tissue [30]. For instance, data reported from acute and chronic MI patients infused with bone marrow-derived cells through intracoronary or intramyocardial delivery routes regarding improvement of the left ventricular ejection fraction, local contractility, and reduction of infarct size have been modest at best, mainly because of low cell engraftment and survival within the fibrous myocardium [31–52]. Thus, addressing paramount issues, such as optimal dose and delivery system, as well as increasing cell homogeneity and functionality (e.g., declining to harvest cells from patients that exhibit cardiovascular risk), remain major challenges to increasing the efficacy of future cell infusion studies. For this purpose, additional details about both clinical and functional outcomes of ongoing trials, such as NCT00462774 (Cardio 133), NCT00529932 (SELECT-AMI), NCT01625949 (COAT), and NCT01414452 (R^2ACE), are awaited with great interest [www.clinicaltrials.org].

Novel therapeutic strategies have recently been developed to deliver therapeutic cells in combination with biomaterials, growth factors, and advanced monitoring devices ("tissue engineering") [18, 53]. Although the results using these techniques, which aim to re-establish electromechanical coupling, stable contractile function, and functional vascularization, are clearly promising, the challenges are enormous. A majority of these experimental procedures have only been tested in small animal models because the major disadvantage is the difficulty of extrapolating to the porcine model (more similar to humans) and, consequently, to the clinical context. The size of the human heart makes these procedures unviable because of the limited matrix dimensions and number of cells that can actually be implanted. Thus, there are few trials currently employing tissue engineering for cardiac regeneration in the clinical context; such trials include the intramyocardial application of alginate (NCT01311791) and the intracoronary delivery of a combination of sodium alginate and calcium gluconate (NCT01226563).

As previously mentioned, therapeutic procedures that are able to strongly support blood supply to injured tissues, including infarcted myocardium, will be more useful for human regenerative medicine. Thus, the possibility of applying vascular precursor cells belonging to the endothelial lineage draws great deal of attention. However, the identification and isolation of genuine vascular precursors from different biological sources (e.g., bone marrow, peripheral blood, UCB, and fetal liver [54–57]) has been the subject of recurring debate since the key findings published in the 1990s by Asahara and colleagues [58–61]. For that reason, and prior to the obtaining of more homogeneous, clonogenic, and proliferative hierarchies of progenitor cells committed to the endothelial lineage, alternative cell sources have been investigated. Basically, these include ESCs [62] and both HPCs and MSCs derived from bone marrow [63–65], adipose tissue [66], and (more recently) UCB [67–69]. The most relevant questions about the role and application of UCB-derived progenitor cells in multiple human disorders are described below.

3 Umbilical Cord Blood as Source of Regenerative Cells for Multiple Clinical Settings

The continuous growth of the global population, which is expected to remain at a constant level of more than 130 million annual births according to an estimate from the United States Census Bureau, is one of the numerous reasons why UCB is currently recognized as the richest source of "stem" or progenitor cells for a vast number of regenerative applications in humans [70]. Compared with other unrelated donor cell sources, UCB can also be obtained safely and painlessly, can be long-term cryopreserved without loss of cell viability, has low risk of transmitting viral infections and somatic mutations, and its application is not subject to the ethical restrictions that complicate the use of other sources [71]. Moreover, there is considerable support for UCB collection, donation, and storage from the major world religions [72].

Since Broxmeyer, Gluckman, and colleagues reported convincing evidence in the 1980s, there has been an increasing consensus that UCB could be used in clinical settings for hematopoietic cell transplantation [73]. In particular, Broxmeyer et al. were the first to firmly demonstrate that UCB is a rich source of HPCs [74]; in the same year, Gluckman et al. documented the first hematopoietic cell transplant to use UCB instead of bone marrow as the source of hematopoietic cells. Remarkably, they were able to reconstitute the hematopoietic system of a child with Fanconi anemia using UCB from an HLA-identical sibling [75]. However, the earliest evidence dates from 1974, when Knudtzon demonstrated the presence of relatively mature HPCs in human UCB [76]. About 10 years later, Ogawa and colleagues reported the presence of more primitive subpopulations of HPCs in UCB [77, 78]. Since then, evidence has continued to accumulate and a global network of UCB banks has been established worldwide; UCB has become one of the most commonly used sources for allogeneic transplant of HPCs for adults and children afflicted by hematological disorders, with an estimated 600,000 samples collected and more than 20,000 UCB units distributed around the world [70].

The spectrum of diseases against which UCB-derived hematopoietic progenitors are effective has been expanded to include bone marrow failure, hemoglobinopathies, and inborn errors of metabolism, among other human disorders. A variety of possible new applications are being investigated as emerging cell therapies, many which are being tested in multiple clinical trials. This active field of investigation includes ex vivo expansion of UCB-derived hematological progenitors (NCT00343798, NCT01175785, and NCT00498316), neurological improvements in cerebral palsy (NCT01072370, NCT01147653, and NCT01528436), neonatal ischemic encephalopathy (NCT00593242), orthopedic cartilage repair (NCT01041001), and treatment of metabolic diseases such as type I diabetes (NCT00305344 and NCT00873925) [19]. Furthermore, owing to its enrichment in vascular progenitors [79], angiogenesis was induced in a 27-year-old woman with Behçet's multisystemic disease [80] and in autistic children [81] receiving cells derived from UCB.

In addition to current clinical trials, UCB (and particularly UCB-derived MSCs) are undergoing powerful preclinical and clinical research in the cardiovascular area ranging from MI to stroke [68, 82–85]. For instance, Leor and coworkers showed that intravenous infusion of human UCB-derived $CD133^+$ cells promotes functional recovery by preventing scar thinning and left ventricular systolic dilatation after an extensive MI in rats [83]. In 2008, Ichim et al. presented a case report of a patient suffering dilated cardiomyopathy who was treated with intravenous allogeneic MSCs and expanded UCB $CD34^+$ cells and experienced profound clinical improvement [85].

4 Past and Current Investigations Using UCB-Derived Cells in the Cardiovascular Area

In addition to being a source of HPCs, UCB also contains MSCs at a lower frequency similar to that of other human tissues, including bone marrow [86], adipose tissue [87], placenta [88], dermal tissues [89], and umbilical cord [90]. These cells are recognized by their capability to differentiate into osteogenic, chondrogenic, and adipogenic lineages in vitro, typical mesenchymal-like morphology, plastic-adherence when maintained in standard culture conditions, and exhibition of a nonhematopoietic cell surface pattern, according to the International Society of Cellular Therapy criteria [91]. However, distinct populations of mesenchymal-like stem cells with similar adhesion properties and antigen surface expression patterns, but different pluripotencies, have been isolated from UCB [19]. Briefly, Kogler et al. described intrinsically pluripotent or unrestricted somatic "stem" cells with the potential to reprogram into mesodermal, endodermal, and ectodermal fates [92]. Subsequently, other investigators used MSCs with more restricted pluripotency [93, 94].

A decade ago, Erices and colleagues investigated the ability of transplanted human UCB-derived MSCs to home in the bone marrow of unconditioned nude mice. As a result, after systemic infusion, they detected human DNA in cardiac muscle, as well as in other recipient tissues [95]. Since then, one central unanswered question is whether these cells have cardiomyogenic potential [19]. Although several investigators have reported the differentiation of UCB-derived MSCs into the cardiomyogenic lineage in vitro [96, 97], others have failed to demonstrate such differentiation [98, 99] using a broad range of pro-cardiogenic stimuli, including 5-azacytidine [96, 100], dimethyl sulfoxide [101], a combination of growth factors involved in early cardiomyogenesis [102], activation of Wnt signaling pathways [101, 103], and coculture with neonatal rat cardiomyocytes [104, 105]. A coculture system using rat primary cardiomyocytes had been effective in inducing a cardiomyocyte-like phenotype in UCB-derived $CD133^+$ hematopoietic progenitors that were first selected from UCB by immunomagnetic separation and then expanded by stimulation with platelet-derived and epidermal growth factors [106]. When cultured for up to 4 weeks in a cardiac differentiation-promoting medium, this same cell population gained the expression of certain cardiac-specific markers (e.g., α-actin, myosin heavy chain, and troponin I) in association with the upregulation of the cardiac-specific transcription factors GATA-4 and MEF2C and the nuclear receptor transcription factors PPARα, PPARγ, RXRα, and RXRβ [107]. However, other authors did not find that direct contact with neonatal rat cardiomyocytes

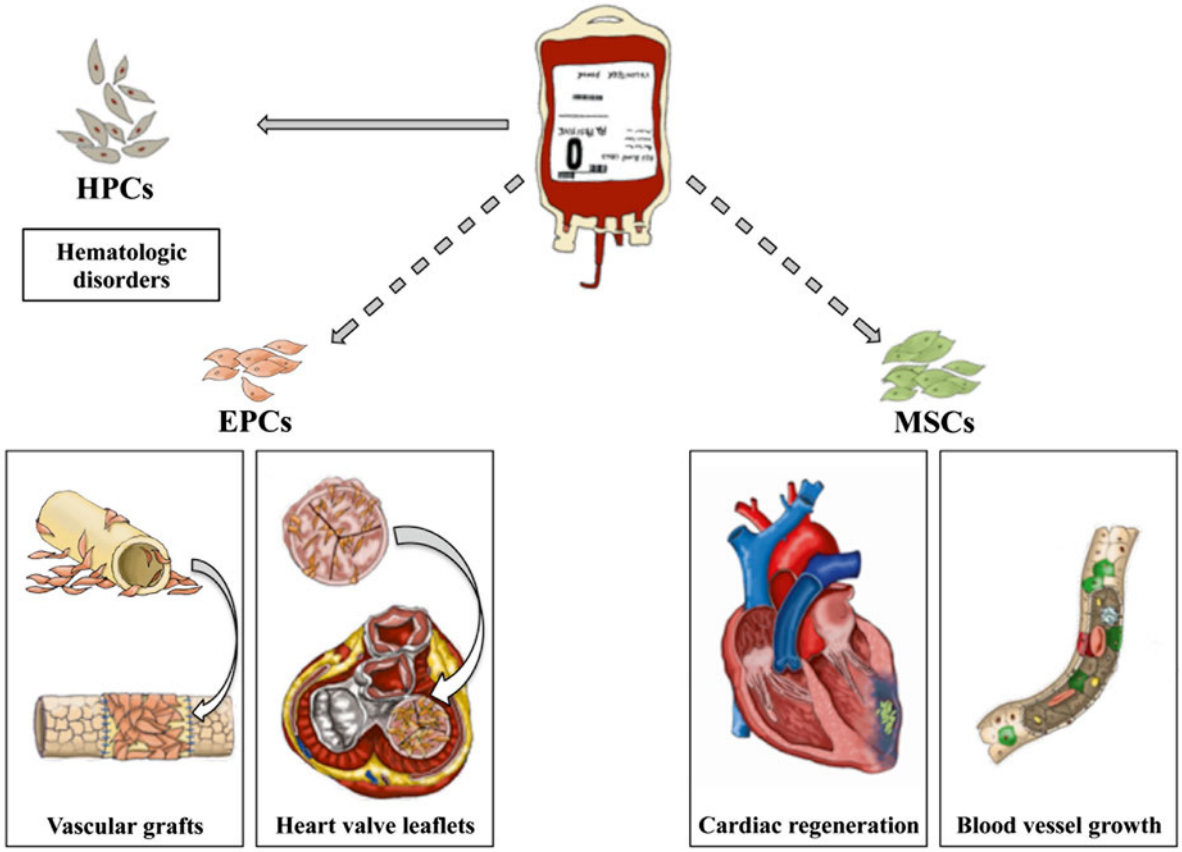


Fig. 26.1 Potential of UCB-derived "stem" or progenitor cells for human regenerative medicine. Since Gluckman et al. reconstituted the hematopoietic system of a child afflicted by Fanconi anemia in 1989, UCB has become one of the most used source for allogeneic transplant of HPCs against multiple hematological disorders. In addition, there is increasing evidences from animal models that point out UCB as a promising source of nonhematopoietic progenitor cells (endothelial progenitors and MSCs) for blood vessel growth and to develop vascular grafts and heart valve leaflets. For instance, they could be useful for cardiac regeneration following MI

promoted either the expression of cardiomyocyte-specific proteins or the presence of rhythmic calcium oscillations and potential-dependent fluorescence emission in UCB-derived MSCs [99]. Thus, they concluded that alternative strategies, regulatory factors, or signaling clues might be better suited to recruit UCB-derived MSCs into the cardiac cell lineage. Surprisingly, a nonhematopoietic cellular subpopulation within the mononuclear cell fraction isolated from UCB was differentiated toward the cardiomyogenic lineage after these cells were cocultured with brown adipose tissue-derived cells [108]. Additional details about the cardiomyogenic potential of UCB-derived MSCs from ongoing investigations in both preclinical and clinical scenarios will definitively answer this crucial question.

The vascular potential of both HPCs and MSCs derived from UCB has garnered a great deal of attention for therapeutic purposes (Fig. 26.1). In 2005, Ma et al. described hematopoietic precursors isolated from UCB that were responsible for increasing capillary density after MI in mice [67]. These authors observed that mononuclear cells (containing 0.11–1.1 % $CD34^+$ cells) injected into the tail vein of recipient animals migrated to the infarcted myocardium, where they engrafted, participated in neoangiogenesis, and beneficially influenced remodeling processes. Intramyocardially administered $CD105^+$ MSCs also exhibited favorable survival in infarcted mouse hearts, which translates into better capillary density in both border and remote zones 6 weeks postinfarction and more robust preservation of cardiac function [109]. Lee et al. have recently found that N-cadherin determines individual variations in the therapeutic efficacy of human UCB-derived MSCs in a rat MI model, and that variations in capillary density are correlated with therapeutic efficacy in improving left ventricular function after MI [110].

Nondestructive monitoring techniques like bioluminescence imaging (BLI), which is based on the application of natural reactants with light-emitting capabilities (photoproteins and luciferases), currently provide valuable information about the location and functional status of regenerative cells implanted into numerous animal models of cardiovascular

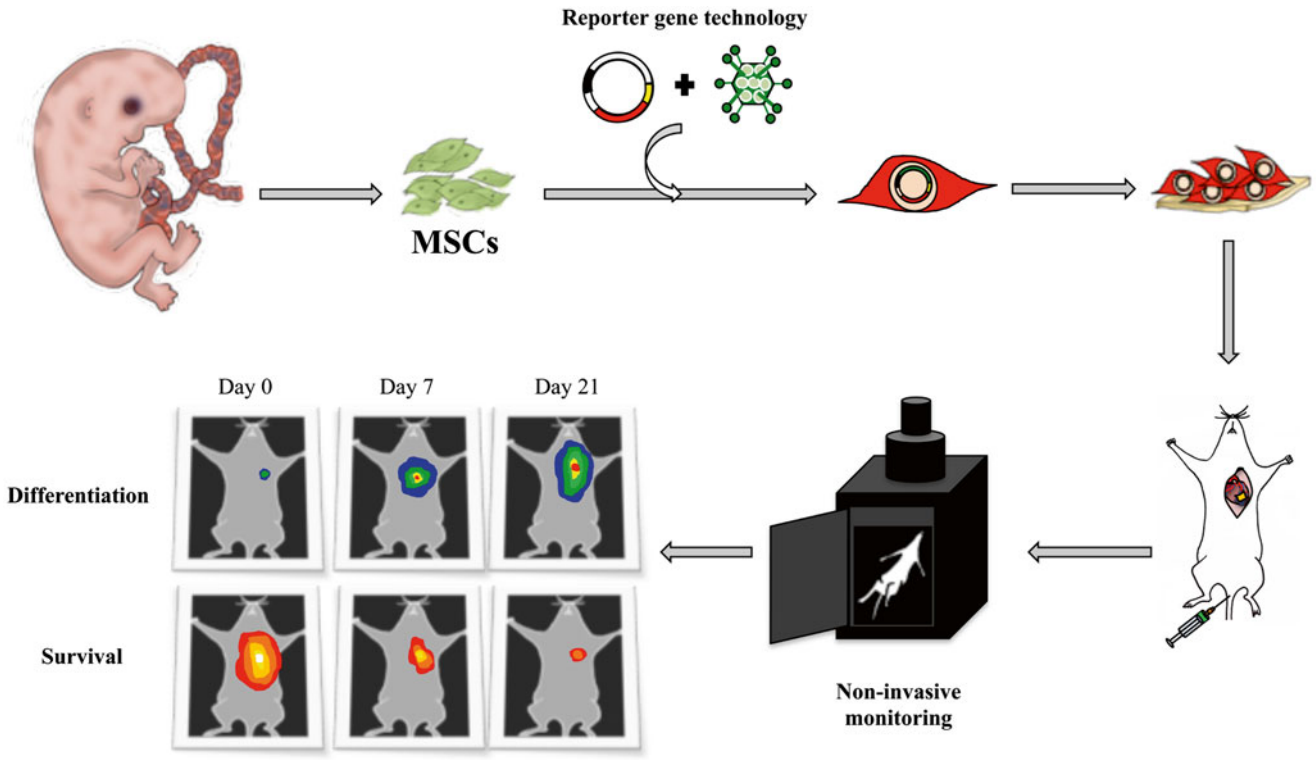


Fig. 26.2 Use of UCB-derived MSCs to validate efforts in cardiac regeneration. By using advanced reporter gene technology and BLI, researchers can first label UCB-derived MSCs and subsequently perform a noninvasive monitoring of cell survival and differentiation in the context of cell-based therapies. Accordingly, implanted cell retention and therapeutic outcomes can be correlated and the most suitable cell type and dose determined

disease [111]. Notably, BLI was used by Roura et al. to follow the expression of chimeric luciferase/fluorescent proteins by human UCB-derived MSCs in a mouse model of angiogenesis [19]. Their results revealed that the promoter of endothelial cell marker CD31 was highly activated in the injected human cells, suggesting efficient cell differentiation; the injected cells also self-organized into new functional blood vessels connected with the circulatory system in the recipient animals. In this study, the administered cells also carried another chimeric reporter of "cell number" to evaluate cell survival over time. Once again, BLI in combination with advanced reporter gene technology allowed basic researchers to illuminate current efforts in cardiac regeneration, where the future is bright (Fig. 26.2). More recently, these same authors employed these genetically modified UCB-derived MSCs embedded in a fibrin patch to assess the effect of their implantation after acute MI in mice [69]. They have demonstrated that UCB-derived MSCs of the engineered construct survived for 4 weeks postimplantation and organized into $CD31^+$ network structures over the infarcted myocardium. MI-treated animals also exhibited a reduced infarct scar and a larger vessel density in comparison with MI-control animals.

Numerous authors have also proposed using UCB-derived cells and biodegradable scaffold materials for cardiovascular tissue engineering [112, 113]. For instance, UCB-derived endothelial progenitors exhibited exceptional growth characteristics appropriate to the development of heart valve leaflets and vascular grafts [114, 115].

5 Conclusions and Future Perspectives

Unlike other vertebrate animals (e.g., salamanders, zebra fish and axolotl), the mammalian heart has limited regenerative potential. A growing body of evidence indicates that cell therapy, together with endogenous stimulation by selected micro RNAs [116–118], is an important regime to repair myocardium postinjury. This regenerative procedure, which has garnered expanding interest in the clinical setting, is based on the implantation of cells that are able to promote myocardial revascularization, reduce ventricular remodeling and cardiomyocyte apoptosis, and improve global left ventricular function. Although ESCs are capable of unlimited self-renewal and have the potential to give rise to all tissue types in the body, ethical difficulties and technical hurdles

may limit their application. Meanwhile, adult and perinatal "stem" or progenitor cells, which have been widely used to reconstitute the hematopoietic system in humans, might represent a valuable alternative.

Although numerous clinical trials have already demonstrated the indisputable safety and feasibility of cardiac cell therapy, it is necessary to address many extremely important issues, such as the optimal cell type, route of delivery, and time point of administration. Thus, additional multicenter studies are ongoing in an attempt to resolve these questions and to reveal true benefit in clinical outcomes. Notably, increasing the number of introduced cells that are retained in the injured myocardium is the key challenge for developing more effective treatments. However, it is mandatory to understand that "stem" or progenitor cells are not an alternative to cardiac transplantation; ideal patients should be at an early stage of heart dysfunction, as the overarching aim of this regenerative approach is to avoid or delay organ transplantation. Because providing a proper niche for implanted cell survival and function is of paramount importance, the most realistic outlook includes the use of tissue-engineered scaffolds or matrices to evolve from an inert cell delivery approach to a more dynamic and efficient regenerative product. Increasing both experimental and clinical experience using these approaches to deliver cells with cardiac potential, including UCB-derived cells, are emerging as novel strategies to treat an old problem, as Friedrich and Böhm envisioned 8 years ago [119].

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Bone Regeneration Using Wharton's Jelly Mesenchymal Stem Cells

27

Kyoko Baba, Yasuharu Yamazaki, Akira Takeda, and Eiju Uchinuma

Abstract

Wharton's jelly (WJ) is one of tissues where mesenchymal stem cells (MSCs) are present. WJ, whose abundant tissue volume is noninvasively available from the neonate at birth, has good clinical applicability to the patient him/herself if allowed to be used as autologous tissue. We confirmed WJ-MSCs' self-renewal capability and multilineage differentiation potential in vitro. Furthermore, the differentiation of WJ-MSCs into osteoblasts was verified in vivo. Our experiences to date give us the impression that Bone marrow (BM)-MSCs have better osteogenic potential compared with WJ-MSCs. On the other hand, another group of researchers reported better osteogenic potential for WJ-MCSs than for BM-MSCs. It is possible that osteogenic potential differs between WJ-MSCs and BM-MSCs. It is not easy to determine the superiority of osteogenic potential between WJ-MSCs and BM-MSCs. Regardless of their superiority, nevertheless, the clinical applications of WJ-MSCs are promising because of their nature of autologous tissue with osteogenic potential that can be attained without surgical invasion. Safety issues (e.g., infection and risk for canceration) should also be resolved. In addition, we consider that a long-term investigation is required to examine time-course changes in implanted and surrounding tissues.

1 Introduction

1.1 Background of Bone Regeneration Using Wharton's Jelly Mesenchymal Stem Cells (WJ-MSCs)

Cleft lip and cleft palate, which are congenital anomalies of the maxillofacial area, are found in any races in the world. However, the incidences of the anomalies differ among races and are as high as one in every 1,000–1,800, 800–1,000, and 500–700 live births in the Negroid, Caucasoid, and Mongoloid races, respectively [1]. Cleft lip, as well as cleft lip and palate are considered to occur due to the abnormal formation of the primary palate at about 5–7 weeks of gestation, while cleft palate is considered to develop due to the abnormal formation of the secondary palate at about 8 weeks of gestation [2]. These congenital anomalies often occur in combination. Both environmental and genetic factors are deemed to be involved in the pathogenesis thereof [2]. Varieties of impairments are provoked by the anomalies, including not only cosmetic abnormalities of the lips and external nose but also sorts of drawbacks (e.g., impaired sucking caused by nasopharynx closure deficiency, swallowing disturbance, dysarthria, propensity to otitis media, hypoplasia of the maxillary bones, and malocclusion caused by impaired bone growth) [2]. The medical care for patients with the anomalies requires a complex, lengthy treatment plan that ranges from birth to adulthood and involves numerous specialties (e.g., obstetrics, pediatrics, plastic surgery, oral surgery, otorhinolaryngology, orthodontic dentistry,

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pediatric dentistry, general density, and speech therapy). In general, chiloplasty and palatoplasty are conducted at about 3 months and 1 year after birth, respectively [3]. Also thereafter, treatment requires diversities of surgeries (e.g., bone grafting to the site of alveolar cleft, correction of the external nose, and osteotomy when hypoplasia of the maxilla and/or mandible is present). Most patients need to undergo surgery several times before reaching adulthood.

The acquisition of normal occlusion is an important objective among a series of treatments. For this objective, bone should be formed at the site of alveolar cleft bone defect to accelerate the adequate eruption of teeth. Nevertheless, it is difficult to form bone at the site where bone is lacked congenitally. In the past, numerous experts have investigated therapeutic modalities to form bone at the site of alveolar cleft. In the 1950s, primary bone grafting at the time of chiloplasty initiated to be conducted [4]. In consideration of drawbacks caused by surgical invasion (e.g., bone growth impairment), secondary bone grafting to be conducted secondarily after chiloplasty during the permanent dentition period commenced thereafter in the 1960s [5]. In the 1970s, secondary bone grafting, which induce the eruption of permanent teeth at the site of alveolar cleft, was initiated. And it was conducted during the mixed dentition period (about 8–12 years of age) [6]. In the 1990s, the usefulness of cancellous bone as grafted bone went on to be recognized. Millard et al., who turned their attention to the environments for osteogenesis, reported the usefulness of alveolus and periosteum formation aiming at the arrangement of environments for osteogenesis [7]. We also conduct alveoloperiosteoplasty for patients who are eligible for the surgical procedure. However, bone mass results to be insufficient in not a few patients. Therefore, we currently conduct the grafting of cancellous bone of the ilium at the site of alveolar cleft during the mixed dentition period. Despite these efforts, some pediatric patients suffer from greater burdens because of requiring several sessions of bone grafting due to drawbacks (e.g., resorption of grafted bone and bone deformation in association with bone growth). The alleviation of surgical invasion in such patients constitutes an important challenge to be addressed.

We think that the insufficient number of osteogenic cells may be considered as one of causes for deficient bone mass at the site of alveolar cleft and that an improvement in osteogenesis can be expected if supplementing a given amount of osteogenic cells. Attention has been given to the multilineage potential of mesenchymal stem cells in recent years, which elevates an expectation for their clinical applications. With the objective to reduce surgical burdens of patients with cheilognathopalatoschisis, our department is using the osteogenic potential of MSCs in an effort to investigate the methods to improve osteogenesis at the site of alveolar cleft.

The usefulness of MSCs in regenerative medicine is becoming widely recognized. Bone marrow is the most common supply source of MSCs for osteogenesis. In recent years, the clinical applications of BM-MSCs in the field of craniomaxillofacial surgery have been sporadically reported [8, 9]. On the other hand, recent studies revealed the presence of MSCs in sorts of tissues. MSCs have been isolated from bone marrow [10–12], cartilage [13–15], adipose tissue [16], tendon [17], periodontal ligament [18], synovial membrane [19], skin [20], periosteum [19], muscle [21], and embryonic tissues [22–24]. The umbilical cord (UC) containing WJ is also one of tissues where MSCs are present [25, 26].

Recently, diagnostic ultrasound of fetuses has revealed congenital anomalies involving deficits in the maxillofacial region in routine clinical settings. Consequently, the number of pregnant women visiting our department for the prenatal consultation of their fetus diagnosed with maxillofacial anomalies has increased. The prenatal diagnosis of congenital craniofacial anomalies allowed us to prepare for the collection of biomaterials required for this study at the time of birth. Therefore, we focused on the UC as the autologous supply source of osteogenic cells.

2 Benefits of the UC and UC Blood for Clinical Use in Regenerative Medicine

In general, concerns about medical safety (e.g., immune response to biomedical materials, infection, and adverse events) and ethical issues about the source of biomedical materials arise when applying regenerative medicine to clinical practice [27]. Since the use of autologous tissue can minimize many of these issues that originate from the use of allogenic or xenogenic tissue, we consider that autologous tissue is preferred biomedical material for clinical use in regenerative medicine. The UC, whose abundant tissue volume is noninvasively available from the neonate at birth, has good clinical applicability to the patient him/herself if allowed to be used as autologous tissue that causes less ethical, medical, and safety issues. Currently, BM-MSCs represent the mainstay of osteogenic cells for use in regenerative medicine. In this study, however, we focused on the UC and UC blood (UCB) which have the following advantages: (1) autologous tissue, (2) noninvasive collection from the pediatric patient, and (3) abundant volume of tissue. We intended to maximally purvey materials for regenerative medicine with autologous tissues.

When considering the preparation of a hybrid-type bone substitute that is applicable clinically, fetal bovine serum (FBS) involves various medical issues, e.g., immune response induced by remaining bovine serum proteins, possible presence of unknown viruses, and potential contamination with prions. Serum-free media for MSCs have also been developed

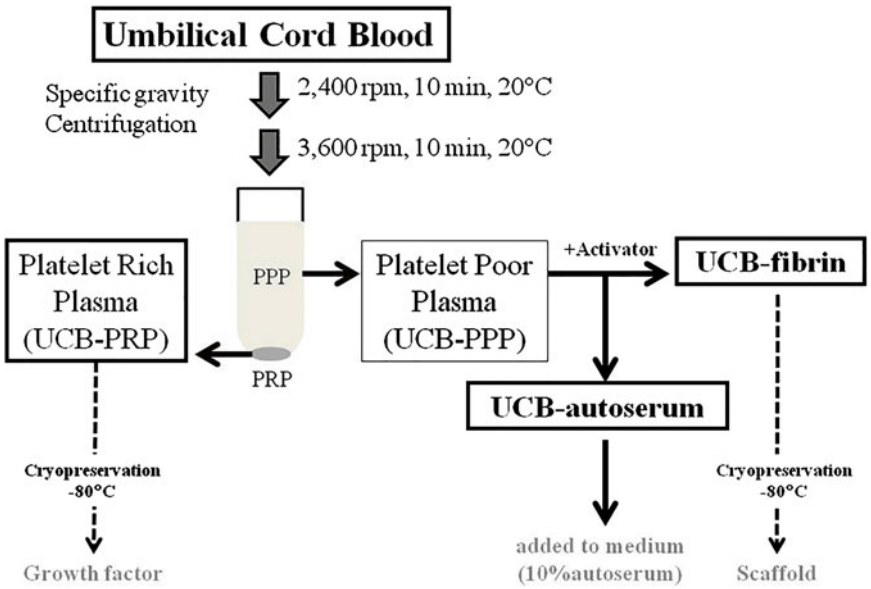


Fig. 27.1 Processing procedures of umbilical cord blood (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013)

as countermeasures for these problems of FBS [28, 29]. However, the long-term safety of all components that serum-free media contain still remains to be established. In consideration of these issues and ethical aspects, the use of autoserum is ideal.

We dealt UCB as described below to maximally utilize autologous tissue as biomaterial for regenerative medicine and made use of autologous serum derived from UCB and of platelet-rich plasma (PRP) containing abundant growth factors to culture cells.

UCB was collected by puncturing the umbilical vein after the expulsion of the placenta. Immediately after collection, UCB was mixed with ACD-A at a volume ratio of 1:1 to perform anticoagulation. Subsequently, the mixture was centrifuged twice (2,400 rpm, 10 min; 3,600 rpm, 10 min) at 20 °C to obtain UCB-derived platelet-rich plasma (UCB-PRP) and UCB-derived platelet-poor plasma (UCB-PPP). Serum was obtained from UCB-PRP before use in the culture of MSCs. UCB-PRP was cryopreserved at –80 °C until immediately before use (Fig. 27.1).

3 Treatment of the UC

The UC has two umbilical arteries and one umbilical vein, and blood is contained in the lumen of the arteries and vein. MSCs are present in the vascular endothelium and UCB as well [24, 30]. When assessing MSCs derived from WJ (WJ-MSCs), it is necessary to deal specimens in order not to allow any contamination of cells that are contained in the vascular wall and blood. WJ-MSCs have higher expansion and engraftment capacities compared to BM-MSCs [31]. On the other hand, the success rate of WJ-MSC isolation was only 63 % from UCB [32].

We used WJ in the culture as described below and cultured WJ-MSCs by using Dulbecco's modified Eagle's medium (DMEM) that are used for the selective culture of MSCs. After the collection of UCB, the UC was washed with phosphate buffered saline up to the moment when no attached blood remained. After the removal of the periumbilical membrane, umbilical arteries, and umbilical vein, WJ was cut into about 5-mm sections. These sections were incubated by using DMEM added with 10 % autoserum derived from UCB in the 25-cm^2 flask under the conditions of 5 % CO_2 and 37 °C. Media were replaced for the first time at 1 week after the onset of the culture and every 3 days thereafter. Following the emergence of adhesive spindle-shaped cells, the WJ sections were removed at about 2 weeks after the onset of the culture (Fig. 27.2). These cells resembled fibroblasts and were adhesive and spindle-shaped, thus demonstrating no discordance with the morphological features of MSCs [33].

4 Features of WJ-MSCs

Stem cells may be categorized to embryonic stem cells (ESCs) and adult stem cells (ASCs), and the latter cells are categorized mainly into hematopoietic stem cells and MSCs [33]. These cells present different features [34]: ESCs are considered to have the potential of differentiating into almost all tissues, while MSCs do not [34]. In 2006, the International Society of Cellular Therapy (ISCT) proposed the minimal criteria for defining MSCs: the cells can adhere to the plastic under standard culture conditions and should have the multipotency to differentiate into osteoblasts, adipocytes, and chondrocytes in an in vitro study as demonstrated by specific staining [33].

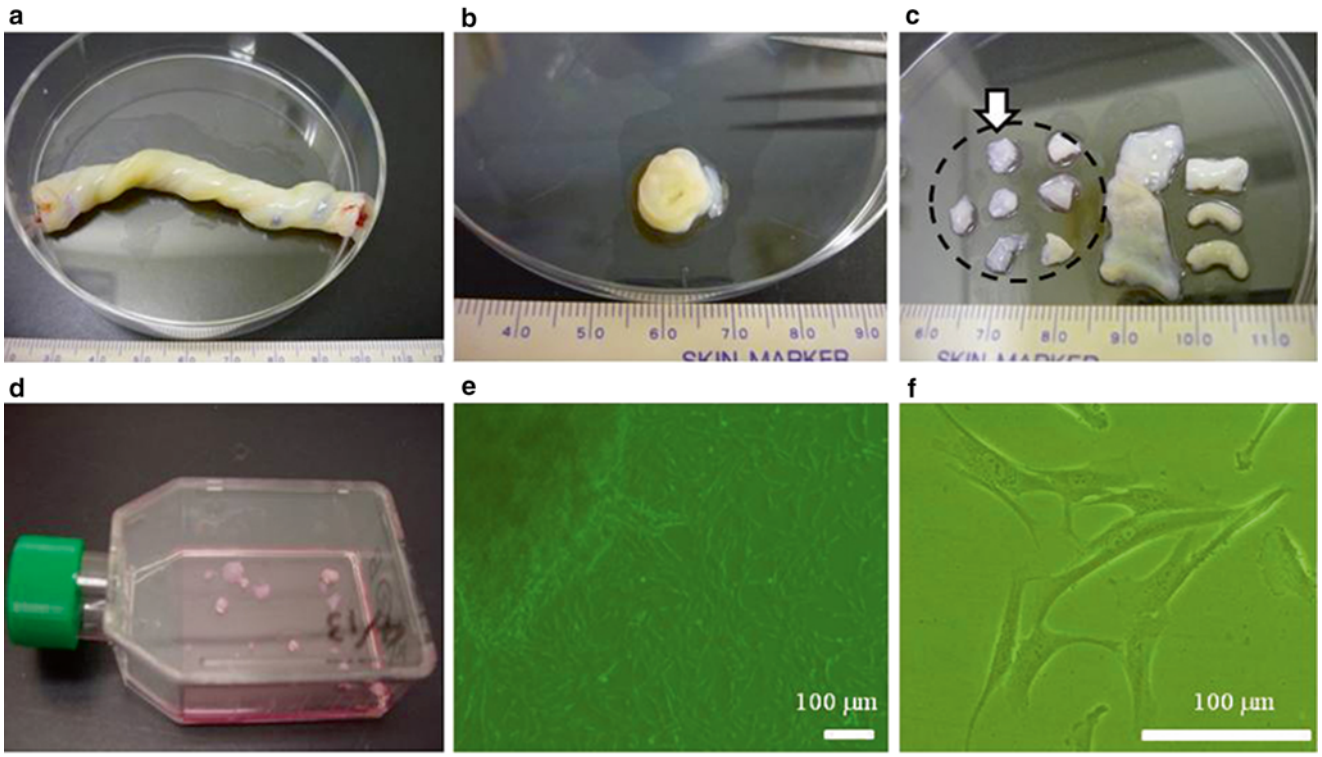


Fig. 27.2 Pretreatment of the umbilical cord (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived autoserum. J Craniomaxillofac. 2012). The umbilical cord was collected after the expulsion of the placenta. (**a**) The umbilical cord was cut into about 10-cm sections and was washed with phosphate buffered saline up to the moment when no attached blood remained. (**b**) Cross section of the umbilical cord. The umbilical cord was washed up to the moment when no attached blood remained also in the vascular lumen. The cross section identified the umbilical vein and arteries. (**c**) After the removal of the periumbilical membrane, umbilical arteries, and umbilical vein, Wharton's jelly was cut into about 5-mm sections (white arrow). (**d**) Wharton's jelly was cultured in Dulbecco's modified Eagle's medium added with 10 % autoserum. (**e**) Outgrowth cells from Wharton's jelly that were observed at 2 weeks after the onset of the culture. (**f**) Outgrowth cells were adhesive spindle-shaped cells

The general features of MSCs are (1) self-renewal capability and (2) plurilineage or multilineage differentiation potential [32–34]. We verified the self-renewal capability of WJ-MSCs based on the cell growth curve. Furthermore, we confirmed WJ-MSCs' multilineage differentiation potential into bone and fat. WJ-MSCs, which had been induced to differentiation into osteoblasts, were stained extracellularly and diffusely with alizarin red, verifying the presence of calcium. WJ-MSCs, which had been induced to differentiation into adipose cells, showed intracellular lipid droplets that were stained with oil red (Fig. 27.3). WJ-MSCs differentiate into cartilage [35, 36], nerve [35, 36], myocardium [37], insulin-producing cell [38], hepatocyte [39], and other tissues. WJ-MSCs possess flexible differentiation potential [32–35].

In recent years, the elucidation of cell surface markers (CSMs) has advanced. According to the ISCT's proposal, MSCs positively express CD73 (SH2), CD90, and CD105 (SH3) and negatively express CD34, CD45, CD14/CD11b, CD79α, CD19, and HLA-DR [32]. CSMs of different tissue origins are expressed differently in MSCs. CD73, CD90, CD105, and other markers are expressed in WJ-MSCs [34]. WJ-MSCs express the typical MSCs markers (CD105, CD73, and CD90) and but do not express CD34, CD45, CD14, CD19, and HLA-DR [35]. Furthermore, WJ-MSCs express a trace amount of CSMs that are common to ESCs (e.g., Oct4, NANOG, DNMT3B, and GABRB3) [26, 40]. Culture has been suggested to possibly modify the expression of CSMs [41], and this modification may influence the results from previous studies.

The specification of CSMs, which are specific to WJ-MSCs with excellent osteogenic potential, in the future will allow the elective collection of the relevant cells and will permit promise for more efficient and stable osteogenesis.

5 Osteogenic Potential of WJ-MSCs Using UCB-Derived Fibrin Net (UCB-FN)

MSCs are featured, depending on their origins, to generate tissues that are prone or less prone to differentiation [34]. Cells to be used as osteogenic cells in clinical settings are

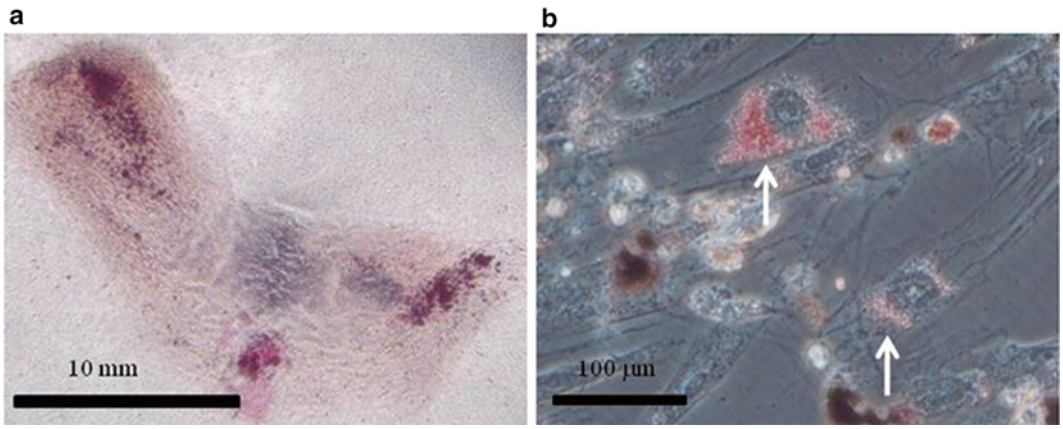


Fig. 27.3 In vitro assessment of the osteoblastic and adipogenic differentiation potential of Wharton's jelly-derived mesenchymal stromal cells (WJ-MSCs) (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived autoserum. J Craniomaxillofac. 2012) WJ-MSCs were induced to differentiate into osteoblasts and adipose cells. (**a**) Staining with alizarin red. The specimens of WJ-MSCs, which had been induced to differentiation into osteoblasts, were stained extracellularly and diffusely with alizarin red. (**b**) Staining with oil red. The specimens of WJ-MSCs, which had been induced to differentiation into adipose cells, showed intracellular lipid droplets that were stained with oil red (*white arrows*)

required to have sufficient potential to differentiate into osteoblasts. WJ-MSCs are believed to have features shared by ESCs and adult MSCs and have been reported to possess flexible differentiation potential [32]. The extent of "differentiation potential flexibility"—a characteristic of WJ-MCSs—may require more restrictive conditions for the induction of differentiation into target tissue. Will WJ-MSCs differentiate into osteoblasts if arranging conditions for culture? A number of authors have reported the osteogenic potential of WJ-MSCs in in vitro studies on cell activity, development of osteoblast marker expression, CMSs, and other study topics [22, 26, 35, 40]. We examined the osteogenic potential of WJ-MSCs in an in vivo study, in which WJ-MSCs were used as osteogenic cells, UCB-derived fibrin (UCB-fibrin) as scaffold, UCB-PRP as growth factor, and UCB-derived serum (UCB-autoserum) as serum for culture for future clinical application. This study was designed to provide all three elements required for regenerative medicine and autoserum for culture by means of autologous tissues. The availability of autologous tissues as biomedical materials for regenerative medicine suggests that these may be safe to use in pediatric patients.

5.1 Assessment of UCB-FN

Fibrin is a physiological material that is produced in tissue damage (e.g., bone fracture) and is very compatible with surrounding tissues and cells. The structures of thawed UCB-fibrin resembled those of peripheral blood from adults in which fibrin components were mixed homogeneously.

The structures of thawed UCB-fibrin were examined by scanning electron microscopy (SEM). UCB-fibrin seeded with WJ-MSCs was also examined by SEM, which revealed the reticulated structures of thawed fibrin in which fibrin fibers were intertangled. The structures were preserved even after cryopreservation and thawing. These three-dimensional structures were relatively homogeneous in both the superficial and deep layers (Fig. 27.4). In recent years, the differentiation of MSCs has been indicated to require three-dimensional cultures [42–45]. SEM verified that the fibrin structures preserved their three-dimensionality to deep layers without any artificial manipulation, leading us to consider that the structures are compatible with the culture of MSCs.

Furthermore, autologous WJ-MSCs naturally infiltrated into UCB-fibrin only by seeding. This result drives us to consider that UCB-fibrin has good compatibility with autologous cells. Although not investigated in detail, one of permissible reasons is that there are some unelucidated actions of several proteins [46–48] that were contained in serum and other fluids present in the surface of or spaces between fibrin fibers. UCB-fibrin was simple to prepare and was easy to handle in the entire process of manipulation. Fitting to the configuration of the implantation site was possible by superimposing multiple sections, indicating the readily adaptable feature of UCB-fibrin to the defect morphology when applied in clinical settings.

5.2 Osteogenic Potential of WJ-MSCs In Vivo

UCB-fibrin, which was cut into pieces of 5 mm in size, was put into the flask, and primary outgrowth cells were seeded (1×10^5 cells/piece). DMEM was used to conduct 1-week

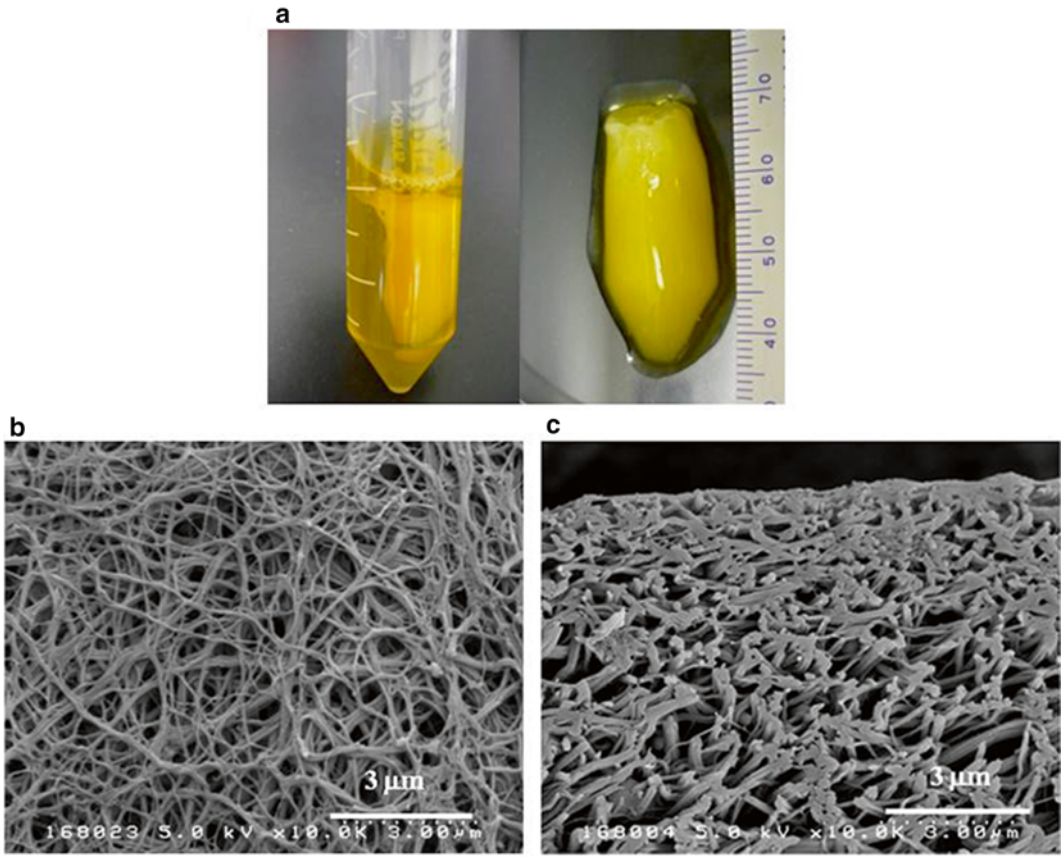


Fig. 27.4 Umbilical cord blood-derived fibrin (UCB-fibrin) immediately after thawing (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013). (**a**) Macroscopic appearance. (**b**) Scanning electron microscopy image (SEM) of the UCB-fibrin surface showing reticular structures. (**c**) SEM image of the UCB-fibrin cross section showing reticular structures that are preserved to deep layers

culture to wait for cell infiltration into UCB-fibrin. Afterwards, the medium was changed to NH OsteoDiff Medium, and this medium was used for differentiation induction to osteoblasts during 3 weeks. When the medium was changed to NH OsteoDiff Medium, UCB-PRP was added to the medium at the ratio of 300 nL/mL only at the first time. UCB-PRP and specimens were implanted subcutaneously into the dorsum of seven male nude mice BALB/Ca Jcl-nu nu/nu aged 5 weeks.

Specimens were removed 6 weeks later. In order to remove specimens, mice were subjected to euthanasia in accordance with our institution's guidelines on methods to kill and dispose of animals. At the time of removal, specimens appeared yellowish white, had slightly elastic rigidity, and showed several capillary vessels on the surface (Fig. 27.5a, b). These removed specimens were assessed as described below.

1. Micro-computed tomography (microCT)
   MicroCT revealed internal radiopaque areas of the specimens that were suggestive of calcification (Fig. 27.5c).
2. Hematoxylin and eosin (HE) staining and alizarin red staining
   The HE-stained specimens included areas that were well stained with hematoxylin which were suggestive of ectopic calcification (Fig. 27.6a). The same areas were positive for alizarin red staining and exhibited ectopic calcium (Fig. 27.6b). Cells with a definite nucleolus were found adjacent to these areas. Several sites of ectopic calcification included a scanty number of cells internally (Fig. 27.6a). Specimens exhibited extremely small eosin-stained parts but included no cells, providing no histological picture suggestive of definite bone tissue.
3. Immunohistochemical staining of human mitochondria
   Cells positive for antihuman mitochondria antibody staining were located in the periphery of ectopic calcification within the fibrin (Fig. 27.6c).
4. SEM with energy dispersive X-ray spectrometry (SEM-EDX) and transmission electron microscopy (TEM)
   The surface of the specimens was covered with cells arrayed in a squamous manner (Fig. 27.7a). A specimen exhibited numbers of spindle-shaped cells and granular

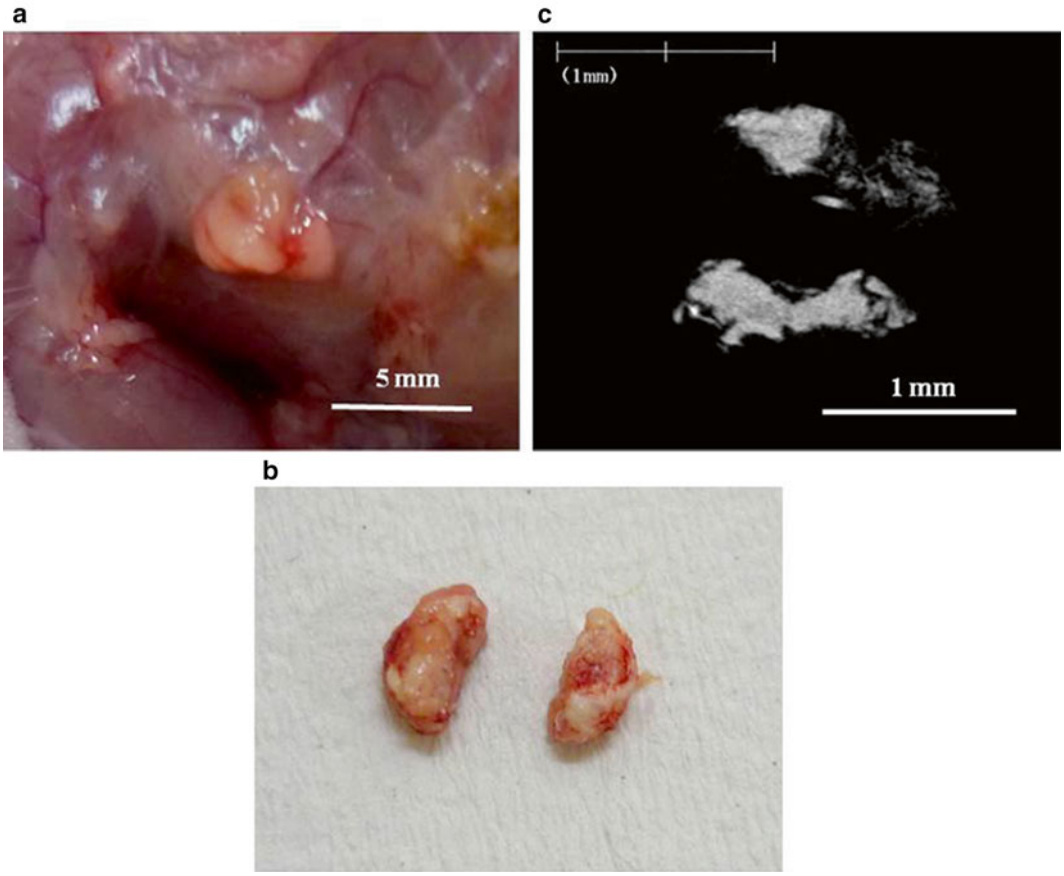


Fig. 27.5 Specimens obtained when combining Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) with umbilical cord blood-derived fibrin (UCB-fibrin) in the in vivo study (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013). (**a**) Macroscopic appearance of specimens found subcutaneously in the mouse dorsum at the time of removal. The specimens showed capillary vessels on their surface. (**b**) The removed specimens had a yellowish white appearance and were hardened at the time of removal. (**c**) Image of micro-computed tomography showing high-density areas

materials (Fig. 27.7b). Granular materials were accumulated in some areas and were continuous with cell processes (Fig. 27.7c). We speculate that these granular materials had been secreted from cells. SEM-EDX of these granular materials revealed calcium and phosphorus, with a Ca/P molar ratio of 1.67 (Fig. 27.7d). We found that the materials have a Ca/P molar ratio close to that of hydroxyapatite. The formation of secretory vesicles was observed in cells by TEM (Fig. 27.8). Analyzed together, we interpret that cells that differentiated from human-derived cells, i.e., WJ-MSCs, caused the accumulation of hydroxyapatite.

5. Expressions of human osteoblast markers with real time reverse transcriptase polymerase chain reaction (RT-PCR) As per the previously reported method [49, 50], RT-PCR was performed to assess the expressions of human osteoblast markers, runt-related gene 2 (Runx2), alkaline phosphatase (ALP), and osteocalcin (OC). All specimens showed the expression of osteoblast markers and exhibited a significant difference (unpaired Student's *t*-test, $P < 0.05$) in ALP expression against controls. No significant difference was found in the expression of Runx2 and OC, although specimens exhibited high expressions of these osteoblast markers (Fig. 27.9a–c). The fact that the expression of osteoblast markers differs depending on the degree of cell differentiation possibly had an effect [51, 52]. However, all specimens indicated a greater expression of osteoblast markers compared with the control.

These results provide basic evidence for the realization of regenerative medicine using autologous tissues derived from patients.

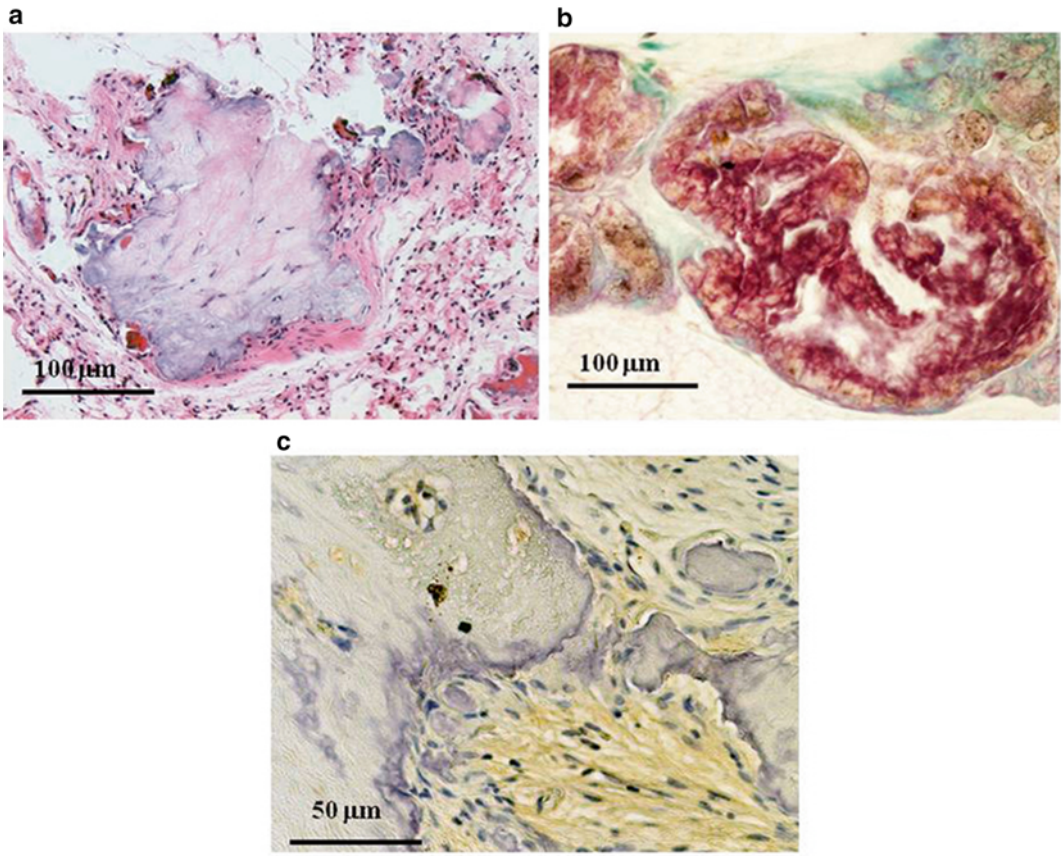


Fig. 27.6 Specimens obtained when combining Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) with umbilical cord blood-derived fibrin (UCB-fibrin) in the in vivo study (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013). (**a**) Image of a specimen stained with hematoxylin and eosin (HE staining), showing areas of ectopic calcification in its interior. (**b**) Image of the specimen shown in Panel a, which was stained with alizarin red, exhibiting positivity in the areas corresponding to ectopic calcification in the image of HE staining. (**c**) Image of the specimen shown in Panel a, which was immunohistochemically stained with the antimitochondrial antibody, exhibiting positive cells in the periphery of the site of ectopic calcification

5.3 Speculated Causes of Failure to Obtain Bone Tissue

We speculate there may be three causes to explain why bone tissue could not be obtained: (1) the number of osteogenic cells that had been seeded was insufficient; and (2) the subcutis—where bone tissue is not formed naturally—was used as the graft bed, leading to afford an environment that was not ideal for osteogenesis.

6 Comparison of UC-MSCs with BM-MSCs with respect to Osteogenic Potential

WJ-MSCs are considered to have greater flexibility of differentiation than adult MSCs [32]. Furthermore, CSMs common to those in BM-MSCs are also expressed in WJ-MSCs [36]. On the other hand, CSMs common to those of embryonic MSCs are expressed in WJ-MSCs [26, 40]. These findings suggest the probably more primitive nature of WJ-MSCs [35]. Namely, a greater number of factors potentially influence osteogenesis in WJ-MSCs than in BM-MSCs that are widely recognized as osteogenic cells at present. Therefore, it is possible that osteogenic potential differs between WJ-MSCs and BM-MSCs.

Our experiences to date give us the impression that BM-MSCs have better osteogenic potential compared with WJ-MSCs [50, 53–55]. The authors successfully obtained mature bone tissue by using BM-MSCs using the subcutaneous implantation procedure [55]. Furthermore, the authors found the in vitro osteogenic potential of BM-MSCs (with autoserum and in the absence of differentiation induction) but did not detect the presence of definite bone tissue [53]. However, the previous study using WJ-MSCs only occasionally indicated the formation of an osteoid-like matrix.

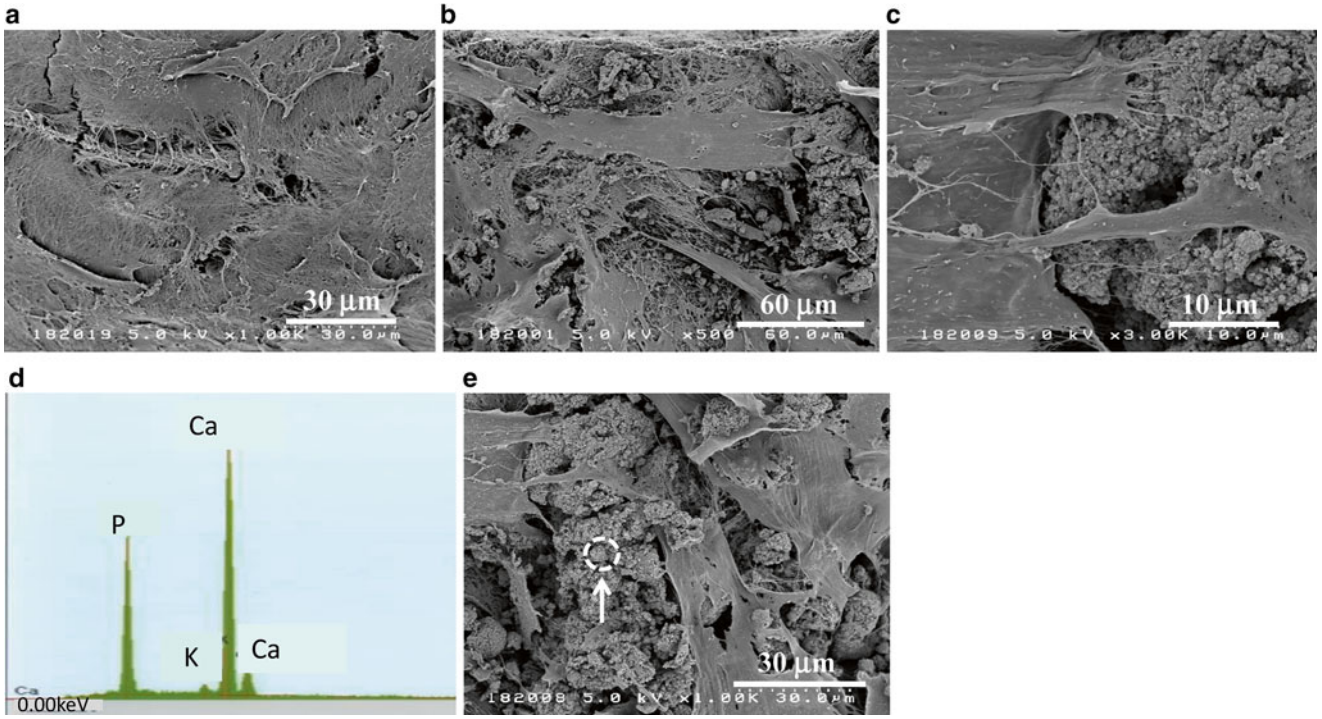


Fig. 27.7 Images of scanning electron microscopy of specimens when combining Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) and umbilical cord blood-derived fibrin (UCB-fibrin) in an in vivo study (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013). (**a**) The surface of a specimen covered with cells that are arrayed in a squamous manner. (**b**) Cross section of a specimen showing spindle-shaped cells and granular materials in its interior. (**c**) Magnified image of the cross section showing the continuation of cell processes and granular materials. (**d**) Analytical electron microgram of granular material showing a Ca/P molar ratio of 1.67. (**e**) The analyzed site is indicated with a *white arrow*

On the other hand, Baksh et al. reported better osteogenic potential for WJ-MCSs than for BM-MSCs [36]. They also described faster differentiation for WJ-MSCs than for BM-MSCs [36]. Our methods for comparison between WJ-MSCs and BM-MSCs are outlined, and discussion is given below.

6.1 Culture, Differentiation Induction to Osteoblasts, and Implantation into Nude Mice

In accordance with a published study [46], the HA ($Ca_{10}(PO_4)_6(OH)_2$) disks of 5 mm in diameter and of 2 mm in thickness with a porosity of 85 % (pore diameter: 100–500 μm) were used as scaffold. The seeding of primary cultured cells (1×10^5 cells/disk) was performed, and DMEM was used to conduct 1-week culture to wait for cell infiltration into HA. Subsequently, the medium was changed to NH OsteoDiff Medium in which 300 ng/mL rhBMP had been added to conduct 3-week culture. Specimens were subcutaneously implanted into the dorsum of male nude mice BALB/Ca Jcl-nu nu/nu.

6.2 Histological Assessment of In Vivo Specimens

Specimens were removed at 6 weeks later and were fixed with 10 % formalin, followed by decalcification with KCX. After washing, the decalcified specimens were embedded in paraffin, cut to about 3-μm sections, stained with HE, and observed with an optical microscope. In the specimens of WJ-MSCs, the formation of immature bone tissue was occasionally found in the pores of the HA disks (Fig. 27.10a). Considering that bone tissue was immature, the osteoblastic differentiation potential of WJ-MSCs was verified by the conduct of immunohistochemical staining and by the investigation of osteoblast marker expression. On the other hand, the formation of mature bone tissue was found in the pores of the HA disks in the specimens of BM-MSCs (Fig. 27.10b). These facts suggest that (1) BM-MSCs possibly have greater osteogenic potential compared with WJ-MSCs, (2) the number of osteogenic cells present in BM-MSCs exceeds that in WJ-MSCs, and (3) MSCs included in WJ-MSCs were probably more primordial, thus requiring further induction to differentiate WJ-MSCs into osteoblasts.

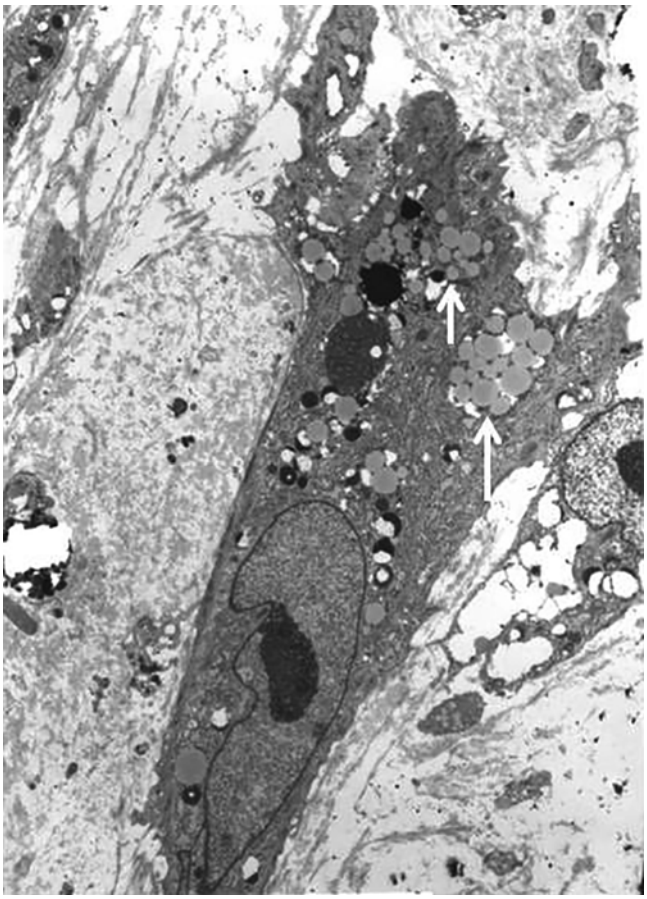


Fig. 27.8 Formation of secretory vesicles (*white arrows*) was observed in cells by transmission electron microscopy (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013)

Another group of researchers [56] showed that cells have an intrinsically limited life span, making it difficult to proliferate cells indefinitely. The UC is a tissue that lasts about 10 months. Therefore, it is possible that the UC presents a life span shorter than that of other cells and also has different tolerance to altering its life span. In addition, stress caused by culture environments other than the living organism (e.g., oxygen concentration) is not negligible, and tolerance to this stress probably differs between WJ-MSCs and BM-MSCs [57]. Therefore, further induction may be required to differentiate WJ-MSCs into osteoblasts.

It is not easy to determine the superiority of osteogenic potential between WJ-MSCs and BM-MSCs. Regardless of their superiority, nevertheless, the clinical applications of WJ-MSCs are promising because of their nature of autologous tissue with osteogenic potential that can be attained without surgical invasion.

7 Safety Issues

The final goal of the present research is clinical application, for which we consider that physiological materials available with the minimal number of manipulations are preferred. Osteogenic cells which had erupted from WJ were used without performing any complex manipulations. We consider that minimal artificial interventions cause less burden to cells and tissues, thus avoiding unknown risk. The challenge of more secure and efficient osteogenesis requires further investigation. Safety issues (e.g., infection and risk for canceration) should also be resolved. In addition, we consider that a long-term investigation is required to examine time-course changes in implanted and surrounding tissues.

Our study provided promising evidence for osteogenesis by culturing WJ-MSCs with UCB-derived autoserum. WJ-MSCs were thus suggested to be a potential material for regenerative medicine. On the other hand, we consider it necessary to further investigate safety concerns (e.g., canceration) prior to the clinical application of WJ-MSCs.

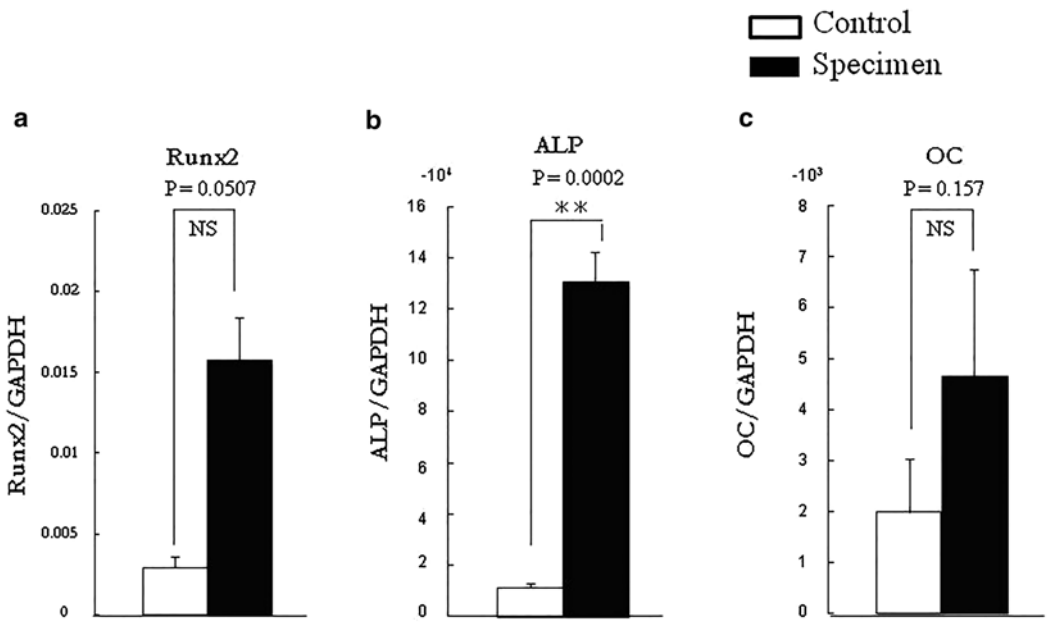


Fig. 27.9 Expressions of osteoblast markers according to reverse transcriptase-polymerase chain reaction in a specimen when combining Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) and umbilical cord blood-derived fibrin (UCB-fibrin) in an in vivo study (Baba K et al. Osteogenic potential of human umbilical cord-derived mesenchymal stromal cells cultured with umbilical cord blood-derived fibrin: a preliminary study. J Craniomaxillofac. 2013). (**a**) Expression of Runx2, showing no significant difference against control. (**b**) Expression of ALP, showing a significant difference against control. (**c**) Expression of OC, showing no significant difference against control. Runx2, runt-related gene 2; ALP, alkaline phosphatase; OC, osteocalcin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

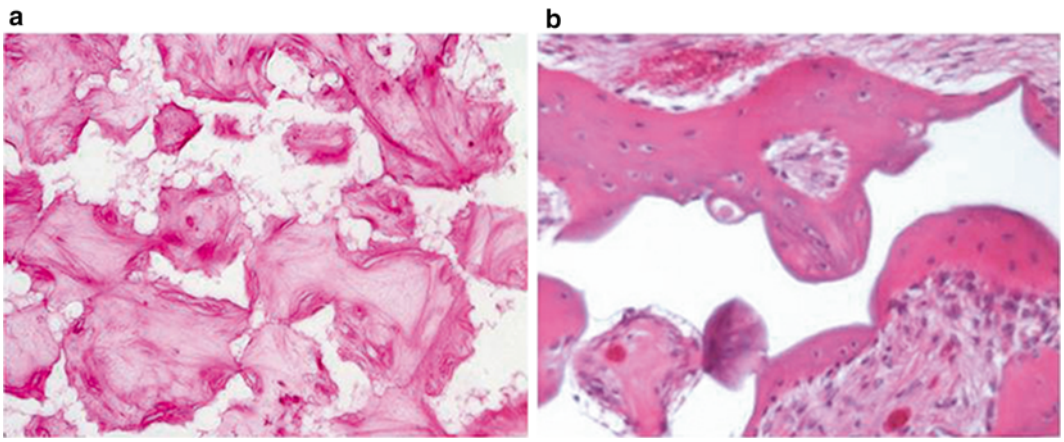


Fig. 27.10 Hematoxylin and eosin (H&E) staining of specimens from nude mice. (**a**) WJ-MSCs: immature bone tissue is found in the pores of the HA disks. (**b**) BM-MSCs: immature bone tissue is found in the pores of the HA disks WJ-MSC, Wharton's jelly-derived mesenchymal stem cells; BM-MSCs, bone marrow-derived mesenchymal stem cells; HA, hydroxyapatite

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Wharton's Jelly Mesenchymal Stem Cells for the Treatment of Type 1 Diabetes

28

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Abstract

Type 1 diabetes is an autoimmune disease caused by the destruction of endocrine pancreas β cells by T lymphocytes, for which genetic and environmental risk factors have been proposed. Patients require daily infusions of recombinant insulin to overcome the reduced production by their own cells, but there is an increasing demand for a permanent and efficient supplementation which could better modulate the need for the hormone during the normal activities. For this reason, transplant-based therapeutic models have been proposed such as whole organ transplantation and Langerhans islets transplantation. These techniques are limited by many factors such as the lack of donors, the risks linked to the surgical practice, and the rejection reactions of the transplanted organ. Further opportunities may come from the research on stem cells. Multiple studies showed that multiple populations of stem cells may be differentiated toward pancreatic endocrine-like ones, which may express insulin in vitro and in vivo and often respond to glucose challenge. Another developing field in stem cells research is that on the immunomodulatory ability of some stem populations, in particular those defined as "perinatal," derived from fetus-associated tissues usually discarded at birth. Wharton's jelly mesenchymal stem cells (WJ-MSCs), which derive from the mature mucous tissue constituting the bulk of the umbilical cord, can also differentiate toward beta cells. Moreover, these cells feature important immunomodulatory activities, which seem to be maintained also in differentiated populations, which should render these cells even more promising for cell therapy applications in type 1 diabetes. This chapter analyzes the literature regarding the features and potential of WJ-MSCs for the therapy of type 1 diabetes, in the light of multiple possible therapeutic approaches, in which cells should be used both undifferentiated and differentiated, and in cotransplantation with islets. We propose that WJ-MSCs transplantation may be useful both to regenerate β cells and also prevent the autoimmune destruction of remnant and neogenetic β cells in patients.

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1 Introduction

The term regenerative medicine refers to a growing number of frontier therapies conceived to treat multiple diseases by means of stem cells. The last 30 years of research ended up with a plethora of cellular populations, obtained from tissue sources ranging from embryonic to fetal to adult tissues, which present the key property of multipotency, being considered able to repopulate diverse human organs in which physiological repair mechanisms are insufficient or dampened by the underlying disease.

The pancreas is a parenchymal organ of the abdominal cavity. It is a major gland associated to the digestive apparatus and features both an endocrine and exocrine compartment. The vast majority of the organ parenchyma is formed by the exocrine glands, organized in acinar structures, connected by branched excretory ducts, tributary of the main pancreatic duct. Acinar cells account for the production of the enzymes, which will serve digestion in the intestine. The endocrine compartment of the organ accounts for 1–2 % of the total organ volume. It consists of about one million insular-type endocrine glands known as islets of Langerhans. These are diffused throughout the organ, with a higher frequency in the tail. Four endocrine cell types have been described in islets, and they synthesize peptide hormones: α cells, β cells, PP cells, and δ cells. β cells are responsible for insulin secretion. Insulin is the main hypoglycemizing hormone, which regulates blood glucose concentration. Its action is counterbalanced by alpha cells' secreted glucagon.

Although pancreatic progenitors have been found in the adult organ, physiological pancreas regeneration occurs in vivo only in rare cases. In fact, exocrine pancreas may regenerate after severe toxin-induced pancreatitis. On the contrary, while beta cells can undergo intense replication in vivo in some physiological conditions (e.g., during pregnancy), they are not able to overcome the injuries which lead to their destruction, and the stem cells compartment in Langerhans islets may be easily damaged by multiple conditions [1, 2].

Diabetes is a leading cause of death in developed countries. In type 1 diabetes, there is an absolute insulin decrease caused by β cells destruction by T cells [3]. Pathological conditions, such as atherosclerosis, kidney failure, blindness and coronary artery disease, are favored by the presence of diabetes. In particular, heart failure commonly coexists with diabetes, making the treatment of each condition more problematic due to the presence of the other disease [4, 5].

Type 1 diabetic patients need insulin injections every day, but this external administration only grossly mimics the physiological insulin secretion process. Although intensive insulin therapy may be effective to delay or prevent the progression of complications, it is difficult to achieve and maintain long term in most subjects. Therefore, whole pancreas or islet transplantation have been explored as therapeutic alternatives.

Islet transplantation has long been favorably viewed, also due to reports showing that human islet transplantation in type 1 diabetic patients achieved insulin independence on the first year, with a subsequent decline [6]. However, this technique is hampered by the need for a concurrent immune suppression protocol and the scarcity of human donor pancreases. Therefore, clinical interest has switched toward cellular therapy, with multiple researches focusing on differentiation of insulin-producing cells (IPCs) from diverse stem and progenitor populations [7–10].

The relative abundance of available sources for stem cells derivation raises the question of how mesenchymal stem cells (MSCs) should be used to treat diabetic patients. The use of autologous cells derived from either bone marrow (BM) or adipose tissue of the same patient should pose no limitations due to host immune reactions. However, subjective and disease-linked factors limit the broad use of autologous stem cells. In fact, as stated previously, the number of MSCs, which are found in BM aspirates, constitutes a minor fraction of nucleated cells, and their numbers decline as function of donor age [11]. More recent data also indicated a possible functional impairment of autologous MSCs due to the underlying disease: this has been demonstrated in diabetic subjects [12, 13]. Between the potential sources of cells with stemness features, perinatal tissues have gained great attention in recent years [14]. These tissues can be accessed without risks for the donor, cells are isolated easily with simple and reproducible lab techniques, and no ethical or safety issues can be raised against the procedures used. Other factors, such as the high cellular yield, the overall percentage of successful isolations, and the ease of culture in vitro, render these cells promising for the vast field of regenerative medicine. In the present chapter, we analyze the potential contribution of perinatal stem cells derived from the umbilical cord, named Wharton's jelly mesenchymal stem cells, in the treatment of type 1 diabetes.

2 Molecular Regulation of Endocrine Pancreas Development and Repopulation

Endocrine pancreas development is a tightly controlled multistep process, which is recapitulated experimentally in most of the published protocols of stem cells differentiation. During organogenesis, multiple transcription factors (TFs) regulate the differentiation of endocrine cells since the initial phase of specification of definitive endoderm toward the generation of mature beta cells. While a detailed description of the molecular regulation of pancreas development is out of

the scope of the present chapter, the readers may be referred to some excellent reviews in the field [15–18]. We will however provide some basic notions, which may be useful to comprehend the rationale behind in vitro differentiation experiments and the choice of markers to monitor the success of these attempts. Most of our knowledge of pancreas development derives from animal (mainly rodent) models [15]. This poses an immediate problem since the well-defined events in rodent pancreas development may be not exactly recapitulated for the human organ. This suggests caution in the interpretation of the results and may further promote the definition of better differentiation strategies.

The human pancreas originates from the fusion of two distinct outgrowths of the foregut endoderm, one dorsal, the other ventral. The first appearance of rare cells expressing insulin, glucagon and somatostatin, has been observed between 7.5 and 8.5 weeks of development [reviewed in 18]. One of the most important factors in the endocrine pancreas developmental process is PDX1 (pancreatic and duodenal homeobox 1) [16]. During development, this TF has been described in both endocrine and exocrine progenitors, whereas in the mature organ its expression is mainly restricted to β cells [19]. Following the first wave of expression of PDX1, cells which will give rise to both acinar and endocrine cells start coexpressing the b-HLH (basic Helix–Loop–Helix) transcription factor Ptf1a (pancreas-specific transcription factor 1a) [17, 20, 21]. Ptf1a null animals result in pancreas agenesis [22]. Another b-HLH factor, namely Ngn-3 (Neurogenin 3) properly initiates the endocrine program. Ngn3-null mice feature islets agenesis and rapidly die due to hyperglycemia [23]. Ngn-3+ cells are the progenitors of all of the endocrine cell types in mature islets [17]. Specification of single hormone positive cells occurs later through the action of other TFs, with either an inhibitory or activatory role such as Pax4 (paired box 4), Arx (aristaless related homeobox), Pax6 (paired box 6), and NeuroD1 [reviewed in 14].

3 Wharton's Jelly Mesenchymal Stem Cells: Phenotype and Immune-Related Properties

The bone marrow is certainly the most common source of MSCs. Friedenstein and colleagues first demonstrated that mesenchymal stem cells from bone marrow (BM-MSCs) may differentiate towards various cellular lineages such as adipocytes [24, 25], osteocytes, chondrocytes, tenocytes, and nervous tissue cells [26]. According to the general consensus, BM-MSCs express typical "core" markers such as CD44, CD73, CD90, CD105, CD166, CD49e, CD51, CD54, CD59, and CD71 [27–31]. Specific markers of the endothelial lineage, such as CD31 and vWF [32–35], are absent in BM-MSCs, as well as hematopoietic markers (CD14, CD34, CD45, CD79, CD86, and glycophorin A-CD235a) [30]. Alternative sources of MSCs are present in other easily accessible tissues such as adipose and perinatal tissues: placenta, amniotic membrane, and umbilical cord [36–39].

Human umbilical cord (hUC) is an extraembryonic formation that arises at day 13 of embryonic development and that connects fetus and mother during pregnancy through the placenta [40]. The umbilical epithelium, an ectoderm-derived structure that continues with amniotic membrane epithelial cells and the tegumentary epithelium of the fetus, covers the entire cord separating it from the amniotic cavity. hUC epithelium covers the subamnion and a mucous connective tissue, the so-called Wharton's jelly (WJ), which surrounds the adventitia and media of the fetal vessels (one vein and two arteries in cord at term) and is involved in the prevention of adverse events such as compression, torsion, and bending of the vascular structures during fetal life. The specialized extracellular matrix of hUC is composed by an amorphous ground substance containing sulfated glycosaminoglycans (GAGs) and proteoglycans. In addition, collagen types I, III, and VI as well as basement membrane molecules, such as collagen type IV, laminin, and heparan sulfate, have been also described [reviewed in 40].

The abundant ECM of umbilical cord stroma contains dispersed stromal cells, now referred to as MSCs. Classical studies, such as that by Takechi and colleagues, identified the majority of stromal cells as myofibroblasts [41]. Even if the stroma can be divided into three different zones (subamnion, Wharton's jelly, and perivascular zone) and data from ex vivo cells have shown differences between cells pertaining to the various zones, the term Wharton's jelly cells (WJCs) is often extended to all umbilical stromal cells. As demonstrated by different groups, MSCs derived from hUC and other fetal/neonatal tissues share common features with MSCs derived from adult tissues (bone marrow, adipose tissue, and peripheral blood) as well as self-renewal capability and differentiative potential toward different types of tissue cells. These cells may be differentiated toward typical mature mesenchymal cell types, such as adipocytes osteoblasts and chondroblasts [39]. In addition, WJ-MSCs have been successfully differentiated toward cells of other mature organs such as neural cells [42], hepatocytes [43], skin follicular cells [44], and cardiomyocytes [45].

In the last years, the interest for perinatal MSCs in regenerative medicine also increased due to their immune modulation and hypoimmunogenicity features [46]. The main proposed mechanisms of in vitro and in vivo immune modulation by MSCs involve secretion of soluble factors such as transforming growth factor-β (TGF-β), hepatocyte growth

factor (HGF), prostaglandin E2 (PGE-2), and indoleamine 2,3 dioxygenase (IDO). This process may be due to the cross-talk between MSCs and T-lymphocytes [47, 48]. Moreover, cell–cell contacts may also have relevant roles for the immunomodulatory activities of MSCs [49]. Further reports suggested that MSCs may modulate T-cell proliferation due to their low expression of classical costimulatory molecules (such as B7-1 and B7-2) and the lack of class II MHC [49–51]. In addition, the immunosuppressive capacity of MSCs may also be mediated by the induction of T-cell anergy and regulatory T cells (Tregs), with significant consequences for postinfusion therapies [51–53].

Several reports indicate that MSCs express nonclassical type I HLAs such as HLA-G (as well as its soluble form HLA-G5) [51, 54, 55], HLA-F, and HLA-E [56]. To date, HLA-E expression has been observed in BM-MSCs and WJ-MSCs, as well as in heart-resident MSCs [56–60]. Interestingly, HLA-E expression has been demonstrated in CL-MSCs and BM-MSC after TNF-α challenge [61]. All of these class Ib MHC molecules are involved in the instauration of tolerance processes in the mother's immune system toward the semiallogeneic embryo and in the induction of tolerance of NK cells toward self-cells [62, 63].

Further recent reports from us and others allowed to better define the immune properties and immunomodulatory markers expressed by perinatal stem cells not only in their naïve state but also after the induction of a differentiation protocol. Tee et al. recently demonstrated that hepatocyte-differentiated hAECs (human amniotic epithelial cells) maintained the expression of key immunomodulatory molecules [64]. In a parallel report, our group demonstrated that also WJ-MSCs, subjected to the standard three-lineage differentiation experiments, maintained the expression of immunomodulatory molecules such as HLA-E and B7-H3 (CD276), both at the mRNA and protein level [60]. Taken together, these data open new possibilities for differentiated cells derived from perinatal ones, in that not only mature functions may be properly achieved but also favorable anti-inflammatory and immunomodulatory activities which may help the engraftment of the infused cells in compromised microenvironments in vivo, as well as contribute to the amelioration of the disease by reactivating local progenitors.

The research on novel markers expressed by hUC stem cells is growingly producing new data, so that other immune-related antigens, such as CD68 and CD14, have been also reported in WJ-MSCs and cord lining (CL)-MSCs [65, 66]. More recently, also CD200 and its receptor, both involved in immune modulation processes, have been demonstrated to be expressed in WJ-MSCs [67]. In addition, CD271, an immunomodulatory molecule initially described in BM-MSCs, has also been shown to be expressed in fresh umbilical cord specimens [68].

4 The Use of WJ-MSCs in Beta Cells Differentiation Experiments In Vitro and In Vivo

WJ-MSCs recently gained much attention in the regenerative medicine panorama due to their easy sourcing, culture and differentiation into several mature cell types. Multiple recent reports indicate that these cells can be successfully differentiated into IPCs. In addition, in vivo experiments also showed that transplanted IPCs may improve the clinical parameters in experimental animals. Moreover, very recent data on the direct transplantation of umbilical cord- or cord blood-derived cells in animal and human models also showed promising results independently from the direct differentiation of stem cells toward IPCs.

The first attempt to differentiate WJ-MSCs into IPCs was performed by Chao and coworkers through a stepwise culture protocol using neuron conditioned medium (see Table 28.1). To assess typical β-cell functions in vivo, the cell clusters of differentiated IPCs were transplanted into the liver of diabetic mice. The authors demonstrated the expression of insulin in response to physiological glucose levels, as well as secretion of C-peptide and expression of pancreas-specific genes PDX1, Nkx2.2, HLXB-9, and Glut-2 [69].

Subsequently, a comparative study carried out by Wu and colleagues compared the differentiation potential of WJ-MSCs and BM-MSCs toward IPCs. Both cellular types were able to form islet-like clusters on the first day of culture in a medium containing nicotinamide, activin A, HGF, exendin-4, and pentagastrin. Notably, the higher expression levels of PDX1 were assessed in differentiated WJ-MSCs with respect to differentiated BM-MSCs. Likewise, secretion of insulin and C-peptide was comparably higher in the differentiated WJ-MSCs [70].

In a more recent paper, Wang and coworkers provided further data derived from in vitro and in vivo experiments using differentiated human WJ cells to treat diabetes in NOD (nonobese diabetic) mice. After transplantation, IPCs were located in the liver and were able to restore physiological glycemia [71]. Authors' results suggest that WJ-MSCs possess the ability, both in vitro and in vivo, to differentiate into IPCs and revert hyperglycemia.

Wang and colleagues, after isolation of MSCs from human umbilical cord, induced differentiation through a proprietary protocol. First they performed gene transfection via plasmid DNA (NeuroD1 and GFP under the control of CMV promoter) and then they performed the last phase of differentiation, which was called reprogramming-induced differentiation. The authors reported that during the first induction steps the cells changed their morphology, from fibroblastoid to islet cluster like. Differentiated cells started expressing human insulin and glucagon (differently from

Table 28.1 Summary of literature reports on the use of umbilical cord-derived stem cells to obtain IPCs for type 1 diabetes management

MSC population	Pancreatic differentiation protocol	Analysis of markers expression	Functional assays	References
WJ-MSCs	4 steps: Step 1 with neuronal conditioned medium (NCM) for 7 days Step 2 H-DMEM/F12 (25 mM glucose), FBS 2 %, nicotinamide, and B27 Step 3 H-DMEM/F12 (25 mM glucose), FBS 2 %, Nicotinamide, B27, and SCM Step 4 maturation of insulin secreting islet-like clusters	I. Expression of insulin, Glut-2, Hlxb9, Nkx6.1, nkx2.2, and Pdx1 by RT-PCR. II. Expression of insulin by IHC, IF staining	In vitro: ICC, RT-PCR, insulin, and C-peptide secretion assay In vivo: STZ-mouse, Insulin ELISA-Kit, IPGT test, IHC, IF staining	[69]
WJ-MSCs in comparison with BM-MSCs	One-step protocol: The cells were seeded in ultra-low attachment culture plates in DMEMF12, with 17.5 mM glucose, 10 mM nicotinamide, 2 nM activin-A 10 nM exendin-4 100 pM HGF and 10 nM pentagastrin	I. Expression of CD29, CD44, CD59, and CD34, by flow cytometry analysis; II. Expression of hC-peptide, h Gcg, h PDX-1 by ICC; and III. expression of insulin by RIA	In vitro: expression of insulin by RIA expression of C-peptide by flow cytometry analysis of viability by cell counting Kit8 and apoptosis by Annexin V-FITC apoptosis detection kit	[70]
WJ-MSCs	3 steps Step 1: CMRL1066 medium with 10 % FBS, 1 %penicillin/streptomycin/amphotericin B, 100 ng/mL of β-nerve growth factor, 4 nM activin-A, 10 mM nicotinamide, and 25 ng/mL of epidermal growth factor (EGF) for 7 days Step 2: new medium DMEM/F12 for 7–10 days. Step 3: 10 mM nicotinamide insulin/transferrin/selenium and 10 ng/mL of basic fibroblastic growth factor were added to medium for 17 days	I. Expression of C-peptide by ICC and immunogold staining on differentiated line II. Expression of *MafA*, *Pax4*, *NeuroD*, *Isl-1*, *Nkx2.2*, *Glut2*, and *insulin* by RT-PCR (differentiated line)	In vitro: ICC, immunogold, SEM, *Glucose Challenge Test,* C-peptide ELISA kit, RT-PCR In vivo: diabetic NOD mice, IHC, Intraperitoneal glucose tolerance test	[71]
WJ-MSCs	High-glucose DMEM (H-DMEM) with 0.1 mmol/L β-mercaptoethanol and 10 μg/L bFGF until formed fibroblast-like cell. Then washed with 0.1 mol/L PBS and incubated with H-DMEM supplemented with 10 mmol/L nicotinamide After gene transfection, WJ-MSC were cultured for 24 h with high-glucose DMEM (H-DMEM, 25 mmol/L glucose) supplemented with 10 % FBS and 10^{-6} mol/L RA. The medium was changed to H-DMEM with 10 % FBS for 2 days and then to low glucose (L-DMEM) with 10 % FBS, 10 mmol/L nicotinamide and 20 ng/mL EGF for 6 days	I. Expression of insulin and glucagon by IFA II. Expression of insulin and Pdx1 by RT-PCR	In vitro: ICC, IF, RT-PCR Dithizone staining Statistical analysis Radioimmunoassay for insulin	[72]
Human umbilical cord blood cells (CB-MSCs)	5 steps Step 1, for 24 h H-DMEM, 25 mmol/L glucose with 10 % FBS and 10^{-6} mol/L retinoic acid, then the medium was changed to H-DMEM with only 10 % FBS for 2 days Step 2, L-DMEM, with 10 % FBS, 10 mmol/L nicotinamide and 20 ng/mL EGF for 6 days Step 3, L-DMEM with 10 % FBS and 10 nmol/L exendin-4 for 6 days. Cellular differentiation was monitored by observation of three-dimensional formation of islet-like cell clusters, the expression of genes related to pancreatic endocrine cell development and insulin production As a control group, cells were cultured in L-DMEM containing only 10 % FBS	I. Expression of GAPDH, insulin, Pdx1, Pax4, Pax6, Ngn3, Isl-1, Nkx6.1, Nkx2.2 and Glut-2 by RT-PCR.	In vitro: IHC, FCA, RT-PCR. In vivo: STZ mouse model HPLC result showed that there was no insulin in the control group, but there was insulin in the IPCs grafted group	[73]
Human umbilical cord cells (UC-MSC)	Three steps protocol with four groups. [The four groups included the MSCs control group, pAdxsi-CMV-PDX1 + induction factor group, vector group + induction factor group, and induction factor group.]	I. Expression of insulin, Pdx1, Ngn3, NKX6.1, and Glut-2 by RT-PCR after differentiation	In vitro: pAdxsi-CMV-PDX1 infection and induction of MSCs. ICC, IFA, RT-PCR, WBA, insulin, and C-peptide secretion detection	[74]

(continued)

Table 28.1 (continued)

MSC population	Pancreatic differentiation protocol	Analysis of markers expression	Functional assays	References
	Step 1: undifferentiated MSCs with pAdxsi-CMV-PDX1 for 7 days. Step 2: 2 % FBS/DMEM/F12 medium and supplemented with 100 ng/mL EGF and 2 % B27 for 3 days. Step 3: 10 ng/mL glucagons-like peptide-1 (GLP-1), 10 ng/mL betacellulin, 10 ng/mL hepatocyte growth factor (HGF), 10 mmol/L nicotinamide, 2 % B27, 0.1 mmol/L β-mercaptoethanol for 7 days	II. Expression of insulin, Pdx1 by ICC, IFA after induced and uninduced		
Human umbilical cord blood cells (CB-MSC)	Protocol I. 6 steps: Step 1: RPMI (without FBS), activin A (100 ng/mL), and Wnt3a (25 ng/mL) for one day. Step 2: RPMI with 0.2 % v/v FBS and activin A (100 ng/mL) for 2 days. Step 3: RPMI with 2 % v/v FBS, fibroblast growth factor 10 (FGF10; 50 ng/mL), and 3-Keto-*N*-(aminoethyl-amino-caproyl-dihydrocinnamoyl) (KAAD)-cyclopamine (CYC, 0.25 μM) for 4 days. Step 4: DMEM with 1 % v/v B27 supplement, all-*trans* retinoic acid (RA, 2 μM), CYC (0.25 μM), and FGF10 (50 ng/mL) for 4 days. Step 5: DMEM with 1 % v/v B27supplement, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-phenylglycine *t*-butyl ester (DAPT; 1 μM) and exendin-4 (50 ng/mL) for 3 days. Step 6: CMRL with 1 % v/v B27 supplement, exendin-4 (50 ng/mL) for 4 days, insulin-like growth factor (IGF-1; 50 ng/mL), and hepatocyte growth factor (HGF; 50 ng/mL) Protocol II. Six steps: Step 1: RPMI (without FBS), activin A (100 ng/mL), Wnt3a (25 ng/mL) and PI3K inhibitor 50 μM for one day. Step 2: RPMI with 0.2 % v/v FBS and activin A (100 ng/mL) for 2 days. Step 3: RPMI with 2 % v/v FBS, fibroblast growth factor 10 (FGF10; 50 ng/mL), and keratinocyte growth factor (KGF, 50 ng/mL) for 4 days. Step 4: DMEM with 1 % v/v B27 supplement, all-*trans* retinoic acid (RA, 2 μM), CYC (0.25 μM), and Nogging (50 ng/mL) for 4 days. Step 5: DMEM with 1 % v/v B27supplement for 3 days. Step 6: CMRL with 1 % v/v B27 supplement, exendin-4 (50 ng/mL), insulin-like growth factor (IGF-1; 50 ng/mL), and hepatocyte growth factor (HGF; 50 ng/mL) for 4 days.		In vitro: IFA, RT-PCR, glucose-stimulated C-peptide (ELISA) In vivo: NOD-SCID. glucose-stimulated C-peptide (ELISA). Statistical analysis	[75]
Wharton's jelly stem cells (WJ-MSCs)	Three steps: Step 1: for 2 days with SFM-A contained DMEM/F12 (1:1) with 17.5 mM glucose, 1 % BSA Cohn fraction V, fatty acid free, 1 % penicillin/streptomycin/amphoteric B, 1× insulin-transferrin selenium-X (ITS-X; 5 mg/L insulin, 5 mg/L transferrin, 5 mg/L selenium), 4 nM activin A, 1 mM sodium butyrate, and 50 μM 2-mercaptoethanol Step 2: on the third day, the culture medium was changed to SFM-B, which contains DMEM/F12 (1:1) with 17.5 mM glucose, 1 % BSA, 1 %PSA, ITS-X, and 0.3 mM taurine Step 3: on the fifth day, for the next 5 days, the cell culture was replaced by SFM-C, which contained DMEM/F12 (1:1) with 17.5 mM glucose, 1.5 % BSA, ITS-X, 1 %PSA, 3 mM taurine, 100 nM (GLP)-1 (amide fragment 7–36), 1 mM nicotinamide, and 1× NEAAs	I. Expression of C-peptide by ICC II. Expression of Pdx1, Pax4, insulin, GPDH by RT-PCR and real-time PCR	In vitro: I. Measurement of spontaneous C-peptide secretion II. Glucose challenge test In vivo: STZ induction in 6- to 8-week old Sprague Dawley rats I. Measurement of blood glucose II. Human nuclei and human C-peptide by IHC III. Statistical analysis	[76]

(continued)

Table 28.1 (continued)

MSC population	Pancreatic differentiation protocol	Analysis of markers expression	Functional assays	References
Human WJ-MSCs		I. Expression of CD40, CD40L, CD80, and CD86 by IF II. Expression of Pdx1, human insulin, human glucagon, HLA-I, and HLA-DR by qRT-PCR	In vitro: I. Lymphocyte proliferation assay by Elisa In vivo: Ad293-EGFP transfected into the HUMSCs and transplanted in STZ induced rats I. Measurement of body weight, blood glucose, and serum insulin levels II. Statistical analysis	[77]
Umbilical cord (UB-MSCs) and cord blood mononuclear cells (CB-MNCs)	Three steps protocol: Step 1 for 2 days, UB-MSC in high glucose DMEM with 1 % β-mercaptoethanol Step 2 for 6 days in DMEM containing 100 ng/mL endothelial growth factor, 10 ng/mL basic fibroblast growth factor and 2 % B27 Step 3 for 6 days in H-DMEM (25 mmol/L) containing 20 mmol/L nicotinamide	I. Expression of α-SMA, desmin human insulin and C-peptide by ICC II. Expression of ALP, OPN, LPL, PPARg, glucagon, somatostatin, insulin, Ngn3, β-actin by RT-PCR III. Expression of CD31, KDR and CD45, CD29, CD90, CD34 by FC	In vivo: different ratio UB-MSCs and CB-MNCs transplanted in STZ-treated C57/BL6 mice 6–8 weeks old I. Measurement of blood glucose II. Intraperitoneal glucose tolerance test III. Expression of human insulin and nuclei antigen by IHC IV. Human Alu polymerase chain reaction assay V. Insulin secretion assay VI. Statistical analysis	[78]

Abbreviations: *Glut-2* glucose transporter-2, *Hlxb9* homeobox protein HB9, *Nkx6.1* homeobox protein NKX6.1, *nkx2.2* homeobox protein NKX2.2, *Pdx11* pancreatic duodenal homeobox-, *STZ* streptozocin, *Gcg* glucagon, *MafA* musculoaponeurotic fibrosarcoma oncogene homolog A, *Pax4* paired gene box-4, *NeuroD* neurogenic differentiation, *Isl-1* insulin gene enhancer protein-1, *Ngn-3* neurogenin-3, *PAX-6* gene paired box-6, *ALP* alkaline phosphatase, *LPL* lipoprotein lipase, *OPN* osteopontin; *PPARg-2* peroxisome proliferator-activated receptor gamma-2

control cells) and were also positive to dithizone staining. At the mRNA level, differentiated cells did express both human insulin and human PDX1 genes [72].

In another report, Phuc and colleagues isolated MSCs from cryopreserved human umbilical cord blood (UCB) and performed differentiation toward IPCs. After formation of clusters, some typical pancreatic genes, such as Pdx-1, Ngn3, Isl-1, Pax6, Pax6, Pax4, Glut-2, Insulin, Nk2.2, and Nkx6.1, were detected by RT-PCR [73].

He's group proved the benefit of PDX1 gene transfection along with the administration of a series of induction factors to UC-MSCs in order to obtain IPCs in vitro. The pancreatic differentiation protocol comprised three steps. In particular, insulin and C-peptide expression, as well as positive dithizone stain, were assessed after the third step of differentiation. In addition, insulin, PDX1, and Nkx6.1 expressions were also confirmed by RT-PCR and western blot analyses in induced MSCs. Interestingly, the expression of such genes was restricted to transfected cells alone, whereas untransfected ones, or cells subjected only to the differentiation protocol, failed in expressing such genes [74].

Prabakar and colleagues investigated the use of CB-MSCs for the treatment of diabetes mellitus through in vitro and in vivo experiments. Subsequently to a pancreatic differentiation protocol, the cells expressed key markers such as PDX1, NKX6.1, and NGN3 by immunofluorescence and RT-PCR, thus confirming that CB-MSCs may be successfully differentiated toward a pancreatic lineage [75].

In a parallel report, Tsai et al. performed differentiation experiments using MSCs from hUC, which were induced with a three steps protocol to obtain IPCs. The features of differentiated cells were assessed by immunocytochemistry, real-time PCR, and ELISA. In vivo experiments were performed by transplanting differentiated cells into the liver of diabetic rats via portal infusion. In vitro data showed that pancreatic β-cell development-related genes (such as PDX1, Pax4, and insulin) were expressed in the differentiated cells. Furthermore, C-peptide release was increased after glucose challenge in vitro. In vivo, human nuclei and C-peptide were detected in the rat livers by immunohistochemistry. In addition, after transplantation of differentiated cells into the diabetic rats, blood sugar level decreased [76].

Wang and coworkers performed in vitro and in vivo experiments aimed to the evaluation of the role of immunomodulatory features of WJ-MSCs for the treatment of diabetes. The authors first investigated the immunological features of WJ-MSCs and their effects on lymphocyte proliferation and interferon (IFN)-γ secretion [77]. WJ-MSCs

were transplanted into diabetic rats to investigate whether these cells could engraft and differentiate in vivo into pancreatic β cells, and whether the hyperglycemia of diabetic rats could be improved. In vitro data from the authors confirmed that WJ-MSCs did not stimulate lymphocyte proliferation and did not induce allogeneic or xenogeneic immune responses. In vivo experiments showed that after transplantation in diabetic rats, WJ-MSCs were detectable in both liver and pancreas. Interestingly, albeit no differentiation protocols were performed prior to infusion, there was an improvement of hyperglycemia of the diabetic rats. More importantly, the destruction of pancreatic cells was partly reversed. Therefore, the authors concluded that hyperglycemia improvement could be related to the immunomodulatory effects of WJ-MSCs in vivo, even if further experiments are needed to elucidate the actual mechanism [77].

Xiao and colleagues performed in vivo experiments aimed to investigate whether cotransplantation of umbilical cord-derived mesenchymal stromal cells (UC-MSCs) and cord blood mononuclear cells (CB-MNCs) could reverse hyperglycemia in type 1 diabetic mice. Authors also aimed to determine the appropriate ratio for cotransplantation [78]. UCMSCs and CB-MNCs were transplanted into type 1 diabetic mice at different ratios and blood glucose concentration was monitored in animals. Histology, immunohistochemistry, and human Alu PCR assays were performed to evaluate for the presence of donor-derived cells and the repair of endogenous islets. In separate experiments, the authors also induced UC-MSCs differentiation toward islet-like cells to determine their differentiation potential. Cotransplantation experiments showed that UC-MSCs and CB-MNCs at a ratio of 1:4 effectively reversed hyperglycemia in diabetic mice. Donor-derived cells were detected into pancreas and kidney of transplanted animals. While cells were able to differentiate in vitro toward islet-like cells, human insulin was not detected in the regenerated pancreases; therefore, suggesting that the mechanism of action of the transplanted cells may involve the reactivation of local pancreatic precursor cells, stimulated by the infused cells [78].

A very recent report from Hu and coworkers showed that WJ-MSCs may be administered to treat type 1 diabetes in human patients, and the treatment is safe and prospectively effective [79]. The authors assessed the long-term effects of the implantation of Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) on patients with newly onset T1DM. Patients were randomly divided into two groups, patients in group I were treated with WJ-MSCs and patients in group II were treated with normal saline based on insulin intensive therapy. The long-term follow-up of patients was up to 21 months. The authors reported no acute or chronic side effects in group I compared with group II. Clinical parameters such as HbA1c and C peptide were significantly better in group I patients when compared either to pretherapy values or parallel values from group II patients during the follow-up period. These data suggested that the implantation of WJ-MSCs for the treatment of newly onset T1DM may safe and effective.

5 Conclusions

The cumulative data on umbilical cord-derived mesenchymal stem cells, which were showed above, highlight the extreme cellular plasticity and differentiation capacity of these cells. This results in their ability to be differentiated in vitro into IPCs, which are able to respond to glucose levels variations and improve clinical parameters in experimental models of diabetes. The novel data coming from more recent studies highlight that also without a prior differentiation step toward IPC, both cells from umbilical cord matrix and umbilical cord blood may be able to rescue the diabetic phenotype in in vivo models and in patients. Therefore, as shown in Fig. 28.1, the regenerative medicine approach using

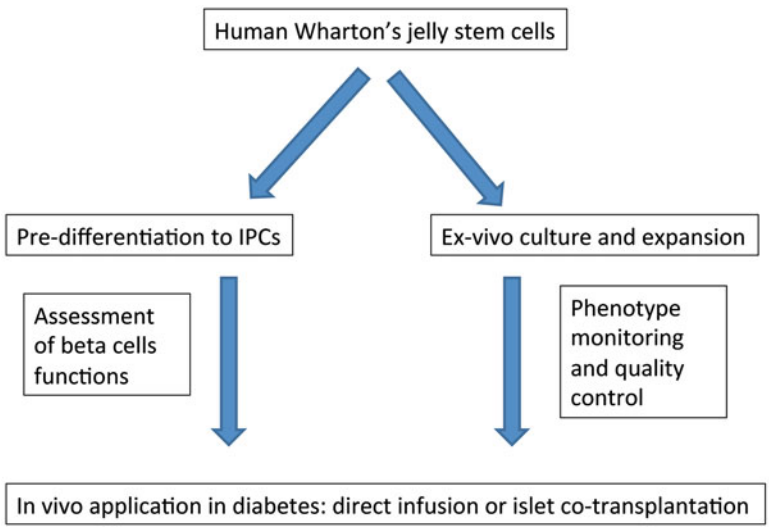


Fig. 28.1 Schematic diagram showing the possible options for the use of Wharton's jelly stem cells in regenerative medicine applications for type 1 diabetes. Either differentiated or naive cells may be used in vivo

WJ-MSCs may be based not only on a classical repopulation model but also on the use of WJ-MSCs as support cells for the organ self-repair. It is probable that the frank immune privilege of undifferentiated WJ-MSCs together with their anti-inflammatory activity may be involved in the rescue of local islet cells and progenitors to reduce the disease progression. Comparative studies have demonstrated that WJ-MSCs can be differentiated better than BM-MSCs toward a mature beta cell phenotype, therefore increasing the prospective usefulness of perinatal stem cells in beta cell replacement therapy. The possibility of umbilical cord cells to be banked in parallel to cord blood units, which is desirable for the future, should render these cells available in high numbers for multiple patients. The co-transplantation experiments of UCB-derived and WJ-derived cells push forward for the need of co-banking these two valuable sources of cells for future uses. The present data will be a strong base to generate more research to better characterize the immune features of these cells and of their differentiated progeny and to increase their engraftment potential and survival in vivo.

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Umbilical Cord Blood-Derived Endothelial Progenitor Cells for Cardiovascular Tissue Engineering

29

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Abstract

Term and preterm human umbilical cord blood (UCB) represents an easily accessible autologous and allogeneic cell source of hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial colony forming cells (ECFCs, or alternatively outgrowing endothelial cells, OECs). All three cell types are regarded as valuable sources for regenerative medicine for pre- or postnatal therapies. In this section we would like to focus on endothelial progenitor cells (EPCs) used for cardiovascular tissue engineering purposes and ECFCs as example to investigate standardized chemically defined cell-culture protocols. Various cells originally identified as EPCs presented in various studies were and are in fact mostly hematopoietic cells (CD45+ progenitor cells, monocytes, platelets) that display angiogenic properties but are clearly distinct to rare circulating ECFCs or OECs (CD31+, CD34+, CD105+, CD146+, but 45−), since only ECFCs have the potential (in contrast to EPCs) of postnatal vasculogenic activity upon transplantation in a matrix scaffold. Because several different types of blood-derived endothelial cells are implicated as pro-angiogenic, future studies will be required to determine the exact role that each endothelial cell type plays in the process of vascular repair or regeneration. Up until now little is known of the in vivo functions of ECFCs in the many preclinical models of human cardiovascular disease and increased studies in this area may be illuminating. Recent studies revealed that infusion of ECFCs into pigs following experimentally induced acute myocardial infarction resulted in significant improvement in myocardial infarct remodeling and heart function via direct incorporation of the cells into the host endothelium. Also studies in murine models of retinal ischaemia showed that human ECFCs directly incorporate into the host murine vasculature, significantly decreasing avascular areas, concomitantly increasing normo-vascular areas and preventing pathologic pre-retinal neovascularization. In the field of cardiovascular regeneration and tissue engineering the endothelialization of constructs before implantation represents a crucial element of the fabrication process of potential implants to ensure lack of thrombogenicity in vivo. Therefore, UCB-EPCs have been intensively investigated in several in vitro studies for the fabrication of tissue engineered patches, vascular grafts and heart valves showing promising initial results. In vitro, the seeded UCB-EPCs showed

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a stable phenotype during static conditioning and the functionality of the cells was similar to mature vascular-derived endothelial cells. However, prior to a clinical translation of the concept, careful preclinical in vivo assessment of the UCB-EPC-based concept seems indispensable.

1 UCB-ECFCs Under Chemically Defined Cell Culture

1.1 Derivation and Definition of Cell Source

Endothelial colony forming cells (ECFCs) can give rise to true progenitors of endothelial cells (ECs). ECFCs display extraordinary proliferative capacity in vitro and by definition structurally contribute to de novo blood vessel formation by the process of vasculogenesis when implanted in vivo [1]. Numerous studies have shown that ECFCs isolated from term human umbilical cord blood (UCB) develop a homogenous population and can be passaged without losing their differentiated EC phenotype and angiogenic potential [2–4]. The expansion capability of UCB-derived ECFCs was found nearly 100-fold higher than those derived from adult peripheral blood (PB) making this cell source specially predestinated for biomedical applications where a therapeutically relevant amount of cell material is desired [5]. Of note, the frequency of circulating ECFCs in UCB increases as the fetus develops towards term gestation. It has been revealed, that gestational age of 33–36 week UCB yielded predominantly ECFC colonies at almost equivalent numbers to term infants. UCB from 24 to 28 week gestational age infants had significantly fewer ECFCs [4]. Due to the high level expression of the anti-oxidant enzyme manganese superoxide dismutase, UCB-derived ECFCs seem further protected against oxidative stress which could give therapeutic ECFC transplants a survival advantage in necrotic or ischemic conditions [2, 6]. A running debate concerns the origin of blood-derived ECFCs. The striking similarities between ECFCs and mature ECs have led to assumptions that culture-expanded ECFCs may not be the progeny of BM-derived, circulating immature endothelial progenitor cells (EPCs) but perhaps a fraction of highly proliferating ECs that became dislodged from the vessel wall into the blood, e.g., at angiogenic sites, during shear stress or even simply through the way of vein puncture during blood collection [7]. This close connection became clear in a previous comparison of protein profiles of ECFCs and human umbilical vein endothelial cells (HUVECs) performed in our research laboratory where we discover only a noticeable difference in the solid expression of the anti-oxidant enzyme manganese superoxide dismutase in ECFCs which makes these cells much more tolerant to oxidative stress than HUVECs [2]. Because collection of UCB in our research laboratory occurs by needle puncture of the umbilical vein in the born placenta, at this point, we cannot exclude the possibility that ECFCs are vessel wall-derived [8] occurring through the performed technique.

1.2 Isolation Methods of UCB-ECFCs

ECFCs can be isolated from the mononuclear cell (MNC) fraction of fresh term UCB units using the classical cell adhesion method on fibronectin-coated tissue culture plastic in presence of EC selection medium containing 2 % fetal bovine serum (FBS) and EC specific cytokines (EGM-2) [1, 2, 5, 9–12].

Applying an ideal cell-culture protocol using a FBS-containing medium in the presence of EGM-2, the MNC fraction of term UCB is a very reliable and rich source of ECFCs resulting in >90 % success rate finding outgrowing colonies. The mean amount of eight to nine outgrowing ECFC colonies/10^8 MNCs outgrowing in a term UCB unit has been reported [4, 12]. Cells derived from such colonies display the phenotypic and functional physiognomies typically used to define in vitro derived ECFCs [1, 11]. ECFCs are positive for CD31, CD34, vWF, CD105, CD 144, CD146, negative for hematopoietic markers, show EC functional characteristics by responding to TNFα stimulation, form capillary networks on matrigel in vitro and establish de novo angiogenesis in vivo.

A yield of 10^{11} ECFCs from a single colony after approximately 60–70 days in culture can be normally obtained due to own experiences [12] and a harvest of up to 10^{14} ECFCs from a clonal stage after only 40 days of culture has been stated [1]. Such extensive serial dissemination or aging of cells in culture may induce chromosomal abnormalities, but also other degenerative cellular fluctuations, including irregular organizations in the cytoplasm, changes in metabolism, replicative efficiency or growth level that results in apoptosis or tumorgenicity [13]. Extended cell culture might result in alteration of the original cellular characteristics and may also drive cells to senescence, chromosomal anomalies or even spontaneous transformation. A current report found an extraordinary incidence of karyotype aberrations in ECFCs during ex vivo expansion [14] whereas others presented stable cell expansion results [15]. In particular, recent findings have raised caution about clinical application of in vitro

expanded ECFCs by pointing out the high risk for karyotype aberrations even at early culture [14]. However, our investigations of the ECFC chromosomal quantity and structure after 35 population doublings within the exponential growth phase of ECFCs, revealed a stable karyotype [12]. It remains uncertain which type of karyotype deviations in cultured ECFCs is challenging for their physiological purpose.

While on one hand UCB seems a source for huge scale amounts of ECFCs, on the other hand anxieties are existing regarding their safety for clinical application after expansion. Additional concerns exist due to the fact that existing procedures for in vitro initiation and expansion of ECFCs utilize FBS to provide growth factors, hormones and nutrients for cell endurance and proliferation. FBS is a mix of several uncharacterized components, which poses the subsequent drawbacks. (1) Variability in FBS composition makes consistent cell preparation, which is a prerequisite for clinical transplants, very multifaceted and not reproducible. For example, it was reported by the Cancer Vaccine Consortium of the Sabin Vaccine Institute (CVC/SVI) that the serum choice among their participants was responsible for suboptimal performance in one of their international enzyme linked immuno spot technique (ELISPOT) proficiency panels [16]. (2) Development of antibodies to FBS components [9, 17, 18]. Metabolic uptake of nonhuman sialic acid Neu5Gc by cultured human cells from FBS, against which many humans have circulating antibodies, might lead to complement activation and killing of cells in vivo subsequently their transplantation [19]. (3) The possibility of transmitting identified or unknown animal pathogens from FBS into the human recipient could prime to, for example, bovine spongiform encephalopathy virus to infect humans with an alternate of Creuzfeld-Jakob disease [20, 21]. For all these aims, cell preparations in the presence of animal sera are under increased scrutiny by regulatory authorities [22]. Moreover, in several countries it is demanding to pass treatment protocols for cellular transplantation therapy that rely on FBS expanded cells. Guidelines have been put in place to reduce the risk of probable transmission from cell-based products into humans and comprise restriction of the sources of FBS, as well as guidelines for production and handling [23]. However, the best approach is to eradicate conceivable harmful sources of contamination for cell-based therapies and products, which means the elimination of animal-derived serum from the complete production procedure.

Human serum has been proposed as substitute for animal serum replacement using e.g., joint human platelet lysate [15, 24–27]. For clinical applications it will be imperative to launch chemically defined and serum-free cell derivation over the whole sequence of in vitro cell processing; from initiating the ECFC cell type in adherent culture and expansion through cryopreservation.

Consequently, alternate, chemically defined culture conditions have to be established. So far our results show that chemically defined media distributed under the promise to maintain or expand human ECs fail to achieve outgrowth of ECFCs, both in the initiation phase, as well as in expansion of ECFCs which were previously established in presence of FBS [12]. Potential protocols are still under investigation. Cell culture in chemically defined media results in a modest ECFC growth but significantly lower compared to the FBS-containing control. Following subculture under these conditions is still ineffective. A further problem is that culture in diverse chemically defined media is associated with altered cell morphology and loss of endothelial cobblestone organization. In addition, attempts to gradually adapt ECFCs to serum-free conditions fail even when we use conditioned culture medium through sequential FBS reduction. ECFCs fail to proliferate and die as soon as the FBS concentration extents beneath 0.5 %. In conclusion, with currently accessible chemically defined media preparations, ECFC expansion to clinically relevant levels is not promising.

Why do ECFCs fail to cultivate in chemically defined, FBS-free settings? Problems of FBS-free cell culture have remained fairly unsolved and even overlooked in the academic community although improvements of serum-free media formulations have been accomplished. Changes in ECFC morphology and organization have been described under chemically defined cell-growth conditions. Recently, we investigated whether these changes are related with genetic modifications and explicitly focused on the expression of 52 designated EC marker genes. We found, that all EC markers were expressed both under the serum- as well as serum-free cell-culture conditions and at the level of gene expression, the specific endothelial signature in ECFCs under serum-free medium appeared to be maintained. Seven of the 52 genes, *CD34*, *KIT*, *TNFSFF4*, *TNS3*, *TEM8*, *CXCR4*, and *KDR* were found to be significantly up-regulated during cell culture in a serum-free medium, whereas only a single gene, *VCAM-1*, became significantly down-regulated compared to ECFCs cultured in FBS-containing media. Carrying out a microarray gene expression study we confirmed, that ECFCs express several tumor endothelial marker (TEMs) such as *TEM1*, *4*, *5*, *6,* and *8*. The TEM family acts as tumor EC markers, and the initial report appealed that TEMs were absent or barely detectable in normal ECs [28]. However, TEM appeared not stringently restricted to tumors but they were also identified in normal vessels such as HUVECs and human microvascular ECs participating in wound healing and *corpus luteum* formation [28]. Moreover, multiple TEM, e.g., *TEM1*, *TEM2*, *TEM4*, *TEM5*, and *TEM8* were also detected in BM-derived $CD45^{-}/CD144^{-}/CD133^{+}/CD34^{+}/KDR^{+}/CD31^{+}$ EC precursors [29]. EPCs and BM-derived cells have previously been demonstrated to

incorporate into malignant blood vessels, at least in some extent. One may speculate that such presence of TEM on ECFCs could be a molecular indicator for their capacity to integrate as cellular component of novel vessels.

1.3 Cryopreservation Methods

Alike for other transplants, an understanding of ideal donor selection, short-term storage settings of whole UCB before handling and long-term loading conditions of cryopreserved UCB is a prerequisite to enhance the efficiency of UCB-ECFCs but also other UCB-derived stem- and progenitor cells for therapeutic applications in the near future [30, 31]. In order to address these demands and to achieve ideal ECFC yields from UCB samples at term deliveries, Coldwell et al. [31] investigated the ensuing topics: (1) how to optimally select UCB donors based on obstetric factors, (2) whether storage temperatures of UCB units using current standards for UCB of 22 ± 2 °C prior to processing is superior to storage at 4 °C, and (3) whether ECFC content is compromised following cryopreservation. Out of 20 obstetric factors examined, e.g., maternal age, parity, gravidity, mode of delivery, gestational age, and neonatal weight, placental weight was the only statistically significant obstetric factor that predicted ECFC frequency in UCB. Studies on the effects of storage revealed that transient storage of fresh UCB at 4 °C reduced ECFC yield compared with storage at 22 °C, while cryopreservation of UCB MNCs significantly reduced ECFC recoveries [31]. However, studies on ECFC recovery after UCB cryopreservation are conflicting, with Lin et al. [32] indicating that the ECFC content is not statistically different between cryopreserved and fresh UCB MNCs although on average fewer were recovered after cryopreservation, while Vanneaux et al. [34] recovered significantly less ECFCs from cryopreserved than from fresh UCB units.

Because of the previously presented advantageous properties of ECFCs and millions of UCB units internationally cryobanked, term UCB is considered to be a valuable source to deliver the therapeutic EC amount required in regenerative medicine approaches. Examples are pre-vascularization of in vitro engineered tissue constructs [34, 35] or in vitro endothelialization of synthetic blood vessel replacements prior to transplantation [36–38].

With the upcoming establishment of cell- and tissue-based therapies, reliable cryopreservation methodologies for biobanking have become progressively imperative [39–42] since a variety of clinical studies already showed the promising therapeutic value of various stem and progenitor cell transplantation protocols [43–45]. While cryopreservation of hematopoietic stem cells (HSCs) for clinical applications has been widely performed for decades [46, 47], cryopreservation protocols for other emerging progenitor cell populations require improvement and still need to demonstrate their practical effectiveness [48, 49]. Although significant developments in cryopreservation of therapeutically relevant cell amounts have been accomplished at the laboratory stage, the translation with respect to the derivation, enumeration, and cryopreservation for clinical applications, e.g., to generate cells in compliance with good manufacturing practices (GMP), is still in the state of infancy [50–52]. One concern is the development and usage of GMP grade reagents free of animal serum for the entire procedure [12, 53, 54]. As previously described in this chapter, in cell-based therapies, the risk of transmitting potentially infectious agents to humans is a real concern. Especially FBS used as supplement in various cryopreservation media is difficult to remove during washing and any residue left can trigger adverse reactions in recipients getting cell infusions or transplants [23, 55]. This issue has prompted the successful development of serum and xeno-free isolation, proliferation and maintenance of cell-culture media in recent years. However, the removal of FBS should not be limited to the cultivation of cells, but should also be applied to the cryopreservation of cell stocks. Many infectious agents like viruses and bacteria are capable of surviving adverse conditions like liquid nitrogen temperatures (−196 °C) that are regularly used for the storage of cell stocks [56, 57]. Vitrification in open straws has been successfully and widely used [58], however, it is problematic to preserve the liquid nitrogen and the cells in this open system sterile. GMP-compliant straws, which are essential for clinical grade vitrification allow cryopreservation only for a limited quantity of cells. In addition, it will be imperative to develop a less or even non-toxic cryoprotective agent (CPA) for cryopreservation and biobanking [59]. The frequently used CPA dimethyl sulfoxide (DMSO) is potentially toxic at the used concentrations, which complicates use of cryopreserved cells in human patients [60–62]. Moreover, addition and elimination of a CPA is a multifaceted process associated with a potentially detrimental osmotic shock to the cells [63]. Several serum-free cryopreservation media and protocols have been established and distributed on the market as GMP-compliant or -amenable products [64–67].

Consequently, we examine chemically full defined cryopreservation media [68] as a next step for the complete defined sequence of in vitro cell processing. Importantly, we investigate the effect of reduced levels of DMSO supplements on cell physiology following subsequent cryopreservation. The straightforward use of a chemically defined cryo-protocols enables freezing of huge magnitudes of ECFCs and other stem and progenitor cells for both research and therapeutic applications. Valuably, it does not require extensive cryo-equipment, e.g., programmed gradient-freezer etc., and it is easy to handle. Even though the internal presently used chemically defined cryo-medium has not yet

available as a GMP-compliant product, all the ingredients are GMP compatible making clinical grade attainable.

UCB-ECFCs demonstrate correspondingly well together in presence of synthetic, containing 5 or 10 % DMSO compared to FBS-containing cryo-media containing 10 % DMSO in respect to cell recovery and cell repopulation post thaw. Functional assays do not reveal any quantitative nor qualitative alterations indicating, that efficient cryopreservation of UCB-ECFCs can also be achieved with a chemically defined freezing protocol under reduced DMSO-concentrations [69].

Monitoring the freezing process of UCB-ECFCs by multiphoton laser scanning microscope (MPLSM-) cryomicroscopy, the effects of cryopreservation can be verified at the single-cell level. With the combination of a MPLSM equipped with a cryostage makes it possible to investigate cells throughout freezing and thawing. With this technique, intra- and inter-cellular ice-crystal creation and loss of membrane integrity can be observed for various cryo-protocols and cryo-media. Using synthetic cryo-media and concentrations of ≥5 % DMSO, we find qualitatively only modest or no loss of membrane integrity post-thaw. Also scanning electron microscope (SEM)-image analysis indicates qualitatively that cryopreservation in the xeno-free cryo-medium containing 5 or 10 % DMSO is comparable to FBS-supplemented cryo-media with 10 % DMSO. In these preparations UCB-ECFCs show undamaged surfaces with features that do not vary from the controls without any sign of shrunken cells afterward cryopreservation due to osmotic stress response to freezing [70, 71]. The cryopreservation of UCB-ECFCs using our current fully defined cryopreservation protocol guarantees high cell recovery, repopulation, membrane integrity, and post-thaw cellular functionality. The used protocol is xeno-free, easy-to-use, does not require extensive laborious equipment, and is GMP amenable. Reduced supplementation of the cell permeating CPA DMSO from 10 to 5 % is feasible and generally supports cell recovery, repopulation, membrane integrity, and maintains cell functionality post-thaw comparable to 10 % DMSO [69]. Based on our presented data, we conclude that chemically defined and DMSO-reduced cryopreservation provides a relevant solution for the cryopreservation of UCB-ECFCs and other progenitor and stem cells for laboratory and clinical applications.

2 UC- and UCB-EPCs and Their Use for Cardiovascular Tissue Engineering

2.1 The General Concept of Cardiovascular Tissue Engineering

Structural cardiovascular disease is responsible for a major global disease load [72, 73]. In particular, structural pathologic changes of heart valves represent a widespread health problem with an age-adjusted prevalence of 2.5 % worldwide [67]. Although currently used heart valve replacement procedures are efficacious and change the otherwise deadly evolution of heart valve disease, currently used heart valve prostheses are suboptimal as they are either prone to thromboembolic complications or progressive calcific deterioration [74]. In addition, in pediatric patients the lack of adaptation to somatic growth is of a particular problem, resulting in repeated reoperations of these young patients, which causes high morbidity and mortality. Therefore, the scientific concept of heart valve tissue engineering has been initiated aiming at growing implants with thromboembolic surfaces as well as remodeling and regeneration potential [75]. In principle, the term tissue engineering has been defined by Vacanti and Langer as an interdisciplinary field applying the principles of engineering to the derivation of biological substitutes for the repair or replacement of diseased tissues [76]. Still in accordance with this rather broad predefinition, cardiovascular tissue engineering can be subdivided into two main strategies: (A) the classical "in vitro tissue engineering" approach (2.2) as well as (B) the "in situ tissue engineering" approach (2.3) [77].

2.2 The In Vitro Tissue Engineering Approach

The classical in vitro tissue engineering approach aims at the full development and maturation of native-analogous tissues ex vivo. As part of this approach, autologous cells are isolated from the patient and seeded onto fully biodegradable, polymeric heart valve matrices. First, these constructs are incubated under static conditions for about 1 week to induce further proliferation and attachment of the cells on the scaffold [75, 78]. Next, these seeded constructs are then placed into a pulsatile bioreactor system where dynamic conditioning is performed [79]. These bioreactors mimic the native situation in temperature, CO_2 levels, oxygenation and flow, which induces the formation of native-analogous tissue in vitro [78, 79]. As part of this "dynamic" phase, which usually lasts for up to 4 weeks, an additional chemical stimulation of the extracellular matrix (hydroxyproline) formation is performed by adding ascorbic acid to the conditioning media [75, 79]. As a final step these in vitro engineered constructs are then endothelialized and again statically incubated for about 1 more week in vitro to ensure sufficient attachment of the endothelial cells to the surface. In spite of the promising concept of having fully matured native-like autologous replacement constructs, the in vitro approach is associated with major logistical hurdles as the ex vivo cell processing procedures require approval by regulatory authorities, which is time and cost-intensive. In the light of these logistical complexities, the less complex in situ tissue engineering approach has evolved as an alternative concept [77].

2.3 The In Situ Tissue Engineering Approach

In the contrary to the in vitro tissue engineering approach, the in situ approach does not aim at the complete formation of native-like tissue in vitro, but relies more on the regenerative potential of the body itself. As part of this concept, autologous cells—such as bone marrow MNCs [77]—are isolated and seeded onto a scaffold starter matrix. This matrix is not dynamically incubated but directly implanted into the patient from whom the cells were taken. The seeded cells themselves secrete cytokines—such as MCP-1, SDF-1α, or IL6 [80]—which induce an inflammation-based remodeling cascade. These factors orchestrate the attraction of host monocytes to the site of implantation, which then also start secreting factors, such as VEGF, that induce further attraction of endothelial cells and smooth muscle cells. Thereby, this inflammatory driven process leads to the formation of a neo-vascular structure in the patient. The main advantage of this approach is the fact that it can be performed as part of a single-step surgical intervention combined with the fact that this approach does not require the use of extensive in vitro conditioning procedures [80]. However, it also relies on the intrinsic regenerative potential of the patient itself and is therefore dependent on the in situ tissue formation and remodeling by the patient, which could be of concern in several groups patients.

2.4 From Animal to Human: The "Evolution" of Bioengineered Heart Valves

Cardiovascular tissue engineered constructs have been successfully fabricated and tested in vitro using different engineering approaches as well as cell sources [81]. In spite of the importance of these initial in vitro studies, the actual thorough in vivo assessment represents an indispensable prerequisite for a safe translation of the results into clinical practice. After early pioneering studies on tissue engineered vascular grafts (TEVGs) and tissue engineered heart valves (THEVs; see Fig. 29.1) [82–84], Schmidt et al. implanted a

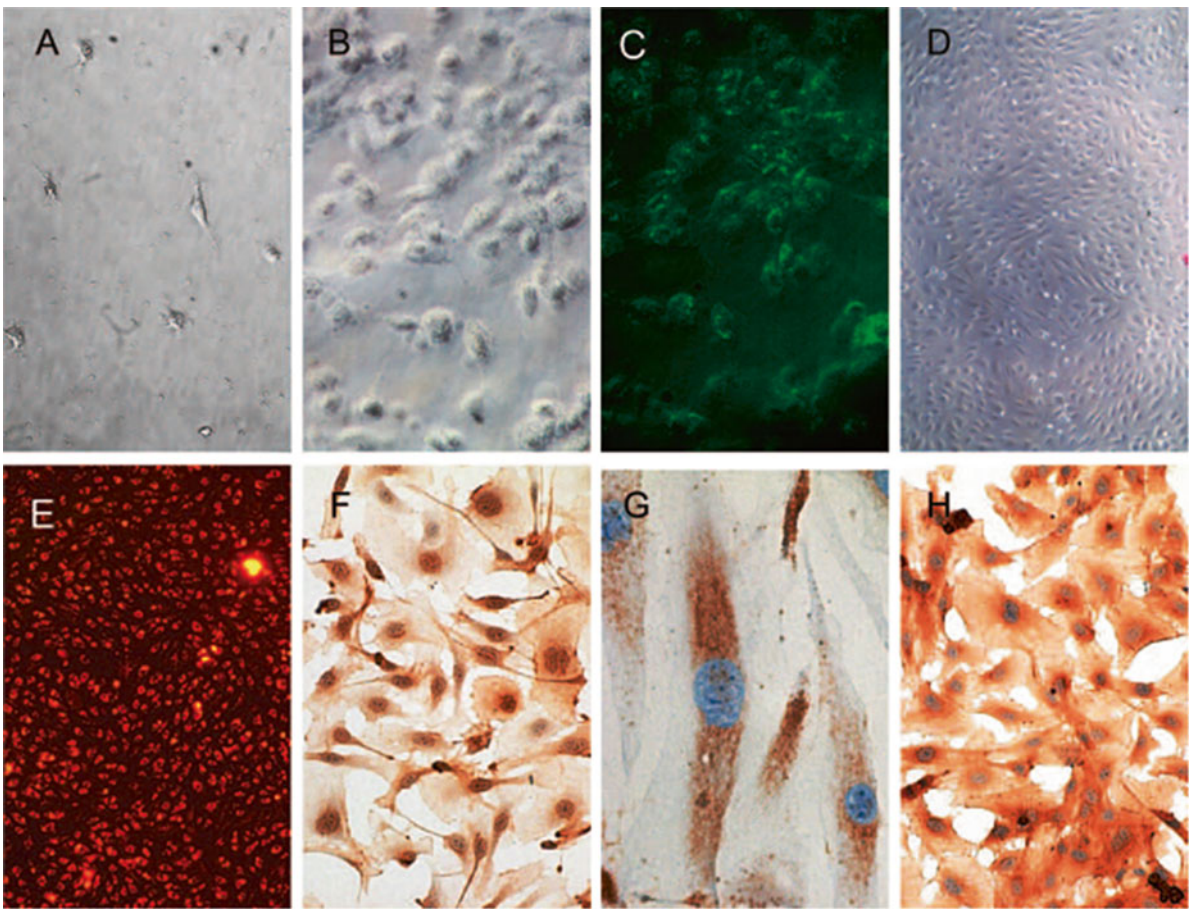


Fig. 29.1 Pre-seeding characterization of endothelial progenitor cells (EPCs). Twelve days after isolation, polymorph EPCs began to proliferate (**a**, magnification: ×100). Outgrowing cells formed colonies (**b**, magnification: ×100) with binding of lectin (Ulex europeus agglutinin-1) (**c**, magnification: ×100). After 3 weeks, EPCs formed a confluent monolayer (**d**, magnification: ×50) with uptake of acetylated low density lipoprotein (**e**, magnification: ×50), expression of CD31 (**f**, magnification: ×200), von Willebrand factor (**g**, magnification: ×400) and endothelial nitric oxide synthase type III (**h**, magnification: ×200). (Schmidt et al., Tissue Engineering. November 2006, 12(11): 3223-3232)

large series ($n=16$) of in vitro engineered heart valves into an ovine in vivo model using a minimally invasive trans-apical approach [85]. After deployment, the autologous constructs showed adequate in vivo functionality; however, as early as after 1 month in vivo the leaflets presented with profound thickening limiting the function and mobility of the cusps [85]. The origin of the thickening could not be fully unraveled, however, the impact of the crimping procedure on the valvular thickening could be ruled out in a further in vivo study [86]. As a next route, the in situ tissue engineering approach was applied to heart valve regeneration investigating bone marrow MNC-based heart valves in a preclinical large animal model [87]. In this study, PGA-P4HB-based heart valve matrices were fabricated and seeded with autologous bone marrow MNCs. After a short incubation, these seeded constructs were delivered minimally invasively into the orthotopic pulmonary position using a trans-apical implantation approach. Although no major thickening phenomena were observed after explantation of the TEHVs following 1 month of in vivo follow-up, the radial length of the leaflets was substantially shortened [87]. A similar result was obtained when using autologous amniotic fluid cells (AFCs) for the in situ engineering of stented heart valves in the fetal model. In spite of the adequate in vivo performance of the valves and functionality up until birth, shortening of the leaflets occurred after 1 week in vivo [88]. Stimulated by first human patients treated with decellularized native valves, human cell-derived decellularized tissue engineered valves were fabricated in vitro [89] also overcoming the limitation of initial cell-mediated leaflet retraction observed in previous studies [85]. These homologous decellularized valves were then implanted into a preclinical large animal model and showed a remarkable initial mobility and durability [90]. However, also in these decellularized human fibroblast-derived constructs some shortening of the radial leaflet extensions was observed after 4- and 8-week of follow-up suggesting that further improvement is necessary before the technology can be translated to clinical practice.

2.5 Umbilical Cord-Derived Cells for Cardiovascular Tissue Engineering

The umbilical cord represents a material which is left-over after every delivery and can be used as a cell source without harming the fetus, which makes this a unique source of different fetal cells. In general, three major cells sources can be isolated from the umbilical cord for the use of cardiovascular tissue engineering: (1) umbilical cord Wharton's jelly (matrix-) derived mesenchmyal (stem) cells, (2) umbilical cord artery/vein-derived (myo-)fibroblastic cells and endothelial cells, (3) umbilical cord blood-derived mesenchymal stem cells (MSCs) and EPCs. In addition, several tissue engineering studies also used mixed cell populations of umbilical cord-derived cells containing cells from both the Wharton's jelly as well as partly from the outer vascular layers [91].

2.5.1 Umbilical Cord Matrix-Derived (Mesenchymal) Stem Cells

The umbilical cord represents an indispensable component of the materno-fetal circulation as it connects the fetal circulatory system with the placenta where the fetal blood is enriched for nutrients and oxygen. The vessels of the umbilical cord are embedded into a mucoid fetal mesenchmyal tissue component, called the Wharton's jelly. In 2004 Wang et al. showed for the first time that this fetal mesenchyme contains a fraction of mulipotent (mesenchymal) stem cells, also demonstrating their multi-lineage differentiation capacity [92]. These initial results suggested that these cells might be of interest for therapeutic applications as to their ease of harvest and high differentiation potential [93, 94]. In several studies the use of these Wharton's jelly-derived cells for cardiovascular tissue engineering applications could be demonstrated. This included the fabrication of cardiovascular patch materials [95] and TEVGs [95], THEV leaflets [96–98] as well as septal occluder membranes [99].

2.5.2 Umbilical Cord Blood-Derived Mesenchymal Stem Cells

Besides the Wharton's jelly, also the fetal blood—accessible by prenatal chordocentesis [100] or postnatal cord blood sampling—represents a source of fetal mesenchymal cells with the characteristic immunophenotype of human mesenchmyal stem cells [83, 101, 102] (see Fig. 29.2).

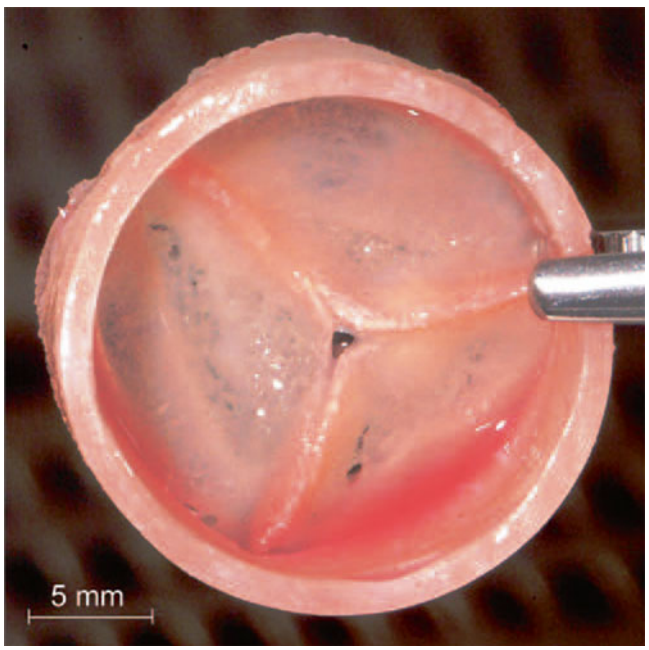


Fig. 29.2 Tissue engineered heart valve leaflets with supporting ring after 21 days in the bioreactor. Leaflets were intact, pliable, and densely covered. (Schmidt et al., Tissue Engineering. November 2006, 12(11): 3223-3232)

The group of Sodian et al. recently demonstrated the successful fabrication of autologous THEVs using CD133+ stem cells isolated via magnetically activated cell sorting as a single cell source [103]. In spite of these promising results, recent studies dampened existing hopes as the outgrowth of adherent cell fractions out of unseparated frozen cord blood has been poor [104] substantially limiting the clinical versatility of existing cryobanked blood specimens.

2.5.3 Umbilical Cord Blood-Derived Endothelial Progenitor Cells

Besides the isolation of MSCs from the UCB, EPCs can be isolated. This is of fundamental importance for cardiovascular tissue engineering approaches as a confluent endothelial surface coverage is a crucial prerequisite for any bioengineered cardiovascular implant [74, 75]. In 2004, Schmidt et al. for the first time isolated UCB-EPC using a Ficoll gradient and used them for the fabrication of TEVGs [105]. One year later, in 2005, Schmidt et al. used fibroblastic as well as EPCs for the fabrication of living tissue engineered patches [95]. In this study, EPCs were isolated from 20 mL of fresh UCB by density gradient centrifugation and myofibroblasts were harvested from umbilical cord tissue (for isolation techniques—see also Chap. 1) and cellular phenotypes were assessed using immuno-histochemistry. Endothelial phenotypes of isolated EPCs were confirmed by Ac-Dil-LDL, CD 31, von Willebrand factor and endothelial nitric oxide synthase (eNOS) stainings. Biodegradable patches fabricated from synthetic polymers (PGA-P4HB) were seeded with myofibroblasts followed by endothelialization with UCB-EPCs. All patches were cultured in a perfusion bioreactor. Microstructural histomorphological analysis of the seeded patches demonstrated layered viable tissue formation in all samples. The cells in the newly formed tissues expressed myofibroblast/smooth muscle cell markers, such as desmin and alpha-SMA. The EPC-derived neo-endothelia showed expression of common endothelial markers (CD 31, vWF). Uniaxial stress–strain analyses of the tissue engineered patches showed features of native-analogous tissues [95].

In a next study Schmidt et al. [96] focused on the fabrication of tissue engineered living blood vessels (TEBVs) based on umbilical cord myofibroblasts and umbilical cord-derived endothelial cells. Also in this study in vitro maturation the TEBVs was performed by exposure of the TEBVs to physiologic conditioning in a flow bioreactor. In addition, control TEBVs were fabricated in parallel and endothelialized with standard vascular endothelial cells. After harvest histology revealed a three-layered tissue organization of the TEBVs comparable to a native vessel and biochemically the major matrix constituents (collagen, proteoglycans) of blood vessels could be measured. The phenotype of isolated EPC lines was assessed by staining for typical endothelial cell markers including CD31, von Willebrand factor, and eNOS and compared to standard vascular endothelial cells. In addition, some biofunctional assessment of the isolated EPCs was performed by stimulation with tumor necrosis factor-alpha, which resulted in physiologic up-regulation of tissue factor (TF) and down-regulation of thrombomodulin (TM) expression also confirming the maturity and endothelial nature of isolated EPC lines [96].

In addition to TEBVs, Schmidt et al. [97] also demonstrated the feasibility of using these cells for the fabrication of in vitro engineering of biologically active heart valve leaflets (see Fig. 29.1). Also in this study Wharton's jelly-derived cells and UCB-derived EPCs were subsequently seeded onto biodegradable scaffolds and cultured in a biomimetic system under native-like pressure and flow conditions. They reported that—depending on the stimulation protocol used—the engineered leaflets revealed more or less matured and layered tissue formation with functional endothelia. In addition, the extracellular matrix production was comparable with that of native heart valves [97].

Taken together, these three studies indicate that umbilical cord cells represent an easily accessible, precious cell source for therapeutic concepts according to the pediatric tissue engineering approach. These studies also clearly show that EPCs isolated from the UCB (of term pregnancies) represent source of functional endothelial cells that can be used for the generation of mature tissue engineered constructs in vitro. In vivo studies are mandatory to confirm these in vitro findings before a potential clinical realization of the concept seems possible.

3 Outlook/Discussion

The umbilical cord represents an easily accessible, versatile cell source. Besides the isolation of MSCs and/or fibroblasts, the isolation of endothelial progenitor and ECFCs from the UCB represents a well-established technique. In addition, UCB is frequently banked in cryopreservation facilities worldwide, thus representing an available cell source for already existing heart defects without prenatal detection. Even in comparison to minimally invasive cell sources, such as bone marrow-derived MSCs (BM-MSCs) or AFCs, UC, or UCB cells have a major advantage: The cells are not harvested directly from the patient, but from an adnexal organ that has no direct postnatal function. That's so crucial, as even in BM-MSCs or AFCs punctures or harvest procedures have to be performed, usually also having a (low) complication rate. In addition, another advantage is the high (practically unlimited) amount of available cells—a factor which is highly limited in other cells sources, e.g., in AFCs. These factors make UC cells, including UCB-derived cells, a highly attractive cell source for potential autologous and allogeneic cell-based therapeutic approaches in general.

Moreover, the studies outlined in this chapter clearly show that these cells can be used for a very specific application: the in vitro fabrication of different cardiovascular tissue engineered constructs including patches, blood vessels, and heart valve leaflets. Importantly, these studies also show evidence that the isolated EPCs hold full biological functionality of mature endothelial cells and are thus suitable for future therapeutic approaches using in vitro engineered constructs. However, so far preclinical in vivo data is missing and previous animal in vivo trials have shown that the overall predictability of the in vivo functionality and fate of in vitro engineered constructs is highly limited. Therefore—for a clinical realization of the tissue engineering concept based on umbilical cord EPCs—a thorough preclinical in vivo assessment seems indispensable in order confirm functionality as well as biocompatibility and also to exclude adverse events and failure. Only based on these careful in vivo assessments a future clinical translation of the presented technologies seems feasible.

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Part IV

Clinical Translation

Manufacturing Perinatal Stem Cells for Clinical Applications

30

Celena F. Heazlewood, Nina Iliac, and Kerry Atkinson

Abstract

Mesenchymal cells are being increasingly explored in clinical trials. They are promising candidates for allogeneic "off-the-shelf" cellular therapy because they preferentially migrate to sites of inflammation and injury and can be transplanted into patients without the need for immune suppression. We used mesenchymal stromal cells derived from term placenta for our clinical trial program exploring the use of these cells. In this chapter we describe the methods that we utilised to harvest and administer these cells according to the Code of Good Manufacturing Practice principles and according to policies and procedures of our internal Quality Management System based on the International Organisation for Standardisation (ISO) requirements. Although hematopoietic stem cells are present in placenta, this chapter focuses solely on mesenchymal cells in the term placenta.

1 Introduction

There are only two types of cells that are currently used therapeutically in mainstream clinical practice. These are hematopoietic stem cells (HSCs) and lymphocytes derived from the individual who donated the HSCs. HSC transplantation has been a routine clinical procedure for the last 40 years [1, 2]. HSCs are adult stem cells and can be derived from three different sources: the bone marrow, mobilised peripheral blood or cryopreserved cord blood units. Bone marrow is generally aspirated from the iliac bones under general anaesthesia. It requires two clinicians to aspirate the marrow (usually between 500 and 1,500 mL) and incurs nursing costs, technician costs and operating theatre costs. This was the standard procedure for procuring HSC until the discovery in 1992 that the hematopoietic growth factor granulocyte colony-stimulating factor (G-CSF) caused cleavage and release of bone marrow HSCs and HSC progenitor cells into the circulation [3], where they could be collected in large numbers and either infused into the HSC recipient on the same day (for allogeneic recipients) or cryopreserved until the pre-transplant high-dose chemotherapy had been completed (in autologous recipients). Autologous peripheral blood stem cells have replaced marrow as a source of HSC in recipients of autologous HSC transplants and in a majority of allogeneic HSC transplants, because of their advantage of accelerating the rate of hematopoietic reconstitution post-transplant. Cord blood units simply have to be ordered from the global network of cord banks.

The point being made here is that mesenchymal cells are logistically much more complicated to obtain in academic medical centres compared to HSCs, and this presumably explains the relatively small number of academic medical institutions that have initiated clinical trials with MSCs—with the exception of those institutions that participate in clinical trials using MSCs in trials run by commercial

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biotechnology companies. However, when a trial is completed, such institutions no longer have access to these cells. More importantly, such institutions are unable to explore the use of MSCs in medical conditions not being addressed by the commercial companies.

Several commercial companies have received marketing approval for their bone marrow-derived MSCs for specific medical indications: Osiris Therapeutics, Inc. (USA) has received marketing approval for their product derived from unrelated allogeneic donors in children with severe graft-versus-host disease after allogeneic HSC transplantation in Canada and New Zealand. Mesoblast Pty Ltd (Australia) has received marketing approval for their product derived from autologous bone marrow.

How, then, can academic medical centres manufacture their own MSCs to supply their own clinical trial program? This will be addressed below, but first some of the properties of MSCs that make them attractive candidates for therapeutic use will be discussed.

2 The Biology of Mesenchymal Cells

Mesenchymal stem cells do exist but are rare cells with a frequency in bone marrow of between 1 in 10^4 and 1 in 10^5 [4]. It is therefore, necessary to expand mesenchymal cells for use in clinical trials. The cells thus produced are not true stem cells in that they divide into daughter stem cells as well as differentiate to more mature cells. Rather, they are mesenchymal stromal cells or mesenchymal progenitor cells. The abbreviation "MSC" used in this chapter refers to these ex vivo expanded mesenchymal stromal cells. MSC populations have been isolated from almost every tissue in the body [5, 6]. Ideally, sources that are easily accessible and contain large amounts of tissue are the most useful [7–11]. Since MSCs were first isolated from first trimester foetal liver, blood and bone marrow (BM) [12], broadly comparable cells have been isolated from a range of foetal and adnexal gestational tissues. MSCs have been isolated from amniotic fluid (AF) [13, 14], umbilical cord blood (UCB) [15], umbilical cord (UC) including Wharton's jelly and perivascular tissue [16, 17], first trimester [18] and second-trimester liver, lung, bone marrow [19] and pancreas [20], term placenta [8] and amniotic and chorionic placental membranes [21].

Gestational sources can be collected at delivery or from prenatal diagnostic specimens such as amniotic fluid. The latter can be sourced by the minimally invasive technique of amniocentesis or obtained peri-partum from otherwise discarded tissue [22]. However, this chapter will focus on the manufacturing of MSCs from term placenta obtained in the operating theatre at the end of an elective Caesarean section operation, as, this is a source of perinatal MSC that is easily accessible, free from ethical issues, and, considered a 'biological waste product' after the mother has given birth. Importantly, we compared human term placenta-derived MSCs with human bone marrow-derived MSCs and could find no difference in their ability to suppress T cell alloproliferation in vitro [23], cell surface phenotype, cytokine receptor display or mesodermal lineage differentiation to osteogenic and cartilage lineages [24]. Placenta-derived MSCs did, however, consistently show less differentiation ability to adipogenesis in vitro compared to marrow MSCs [24].

MSCs have several biological features that can be exploited in a clinical setting. Firstly, they are relatively non-immunogenic with no need to tissue match the MSC donor with the MSC recipient and no need to administer immune suppressive medications to the recipient after MSC administration. MSCs can thus be manufactured in bulk by expansion in tissue culture and cryopreserved until required. They can therefore, be regarded as an "off-the-shelf" product. Secondly, MSCs have tropism for inflammatory sites and will home to such sites preferentially when injected intravenously (after their passage through the lungs).

Haack-Sorensen et al. compared fresh and frozen ex vivo expanded MSC and reported that frozen MSCs expressed the same phenotypic and cellular characteristics as fresh MSCs [25]. This is important, since MSCs used for any clinical trial must be characterised in terms of cell surface phenotype, mesodermal differentiation potential and T cell immunosuppressive function. In vivo toxicity studies of the manufactured human MSCs in animals also need to be performed in a dose-escalation manner and the safety data must be available from the principal investigator's laboratory prior to initiating a trial [24].

3 Writing the Documents Required for Manufacturing Mesenchymal Stromal Cells

Once the Principal Investigator/s have been identified, their initial role is to write the clinical trial protocol and seek funding for the clinical trial. Other documents that (at our Institution) must be sent to the Institutional Ethics Committee for their consideration include the Investigators Brochure (which contains facts about the biology of MSCs and their use in the clinic to date), a maternal Donor Informed Consent Form, a Recipient Informed Consent Form and a Staff Information Form. This is an extensive list and is time consuming but essential.

4 The Importance of Project Team Management in Manufacturing Mesenchymal Stromal Cells

A team of people with a variety of different skills is required to manufacture clinical grade MSCs using current Good Manufacturing Principles (cGMP). These include a team leader, who ideally will have both clinical and scientific

expertise as well as a thorough understanding of cGMP manufacturing of cells. It is also their responsibility to seek funding for the MSC manufacturing. In our experience this is currently approximately $50,000 Australian dollars. A major component of the budget is foetal calf serum (FCS) since the MSCs grow best in 20 % FCS. Three production scientists are required who need to be experienced in aseptic tissue culture techniques, MSC biology and who have received specific training in cGMP manufacturing. A Quality Manager is required who should have scientific knowledge but whose primary expertise is in the principles and practice of cGMP manufacturing. A Research Nurse is required to explain the research aims of the study and to obtain informed consent from the maternal donor of the placenta. This person also checks the extensive inclusion and exclusion criteria for potential maternal donors. This is a long and complicated list since it includes all the criteria used by the Australian Red Cross for blood donor selection, as well as all the criteria set by Auscord (the Australian Cord Blood Bank) for collection of allogeneic cord blood for subsequent cryopreservation. In addition, we added an exclusion criterion of cytomegalovirus seropositivity. MSCs are not known to be used as a host cell for CMV but we preferred to err on the side of caution. A consequence of the latter exclusion criterion is that it excludes approximately 80 % of donors. However, we have a maternity hospital which delivers 10,000 babies/year and approximately 40 % of them are delivered by elective Caesarean section, so we have not found this a major obstacle. An Ethics Committee representative is required for managing the extensive interactions between the Project Team and the Institutional Ethics Committee. This is also time-consuming work since it involves not only the first proposal submission and subsequent questions about it from the Ethics Committee, but annual reports are required on the progress of the trial, if approved.

We have found it best for the Project Management team to meet for 1 h once a week. This provides an excellent way of expediting the clinical trials.

5 Setting up a Data Safety Monitoring Committee

When we started our MSC clinical trial program we took the view that a Data Safety Monitoring Committee (DSMC) was essential, since MSCs derived from placenta had not been previously used in man.

6 Using a cGMP Facility for MSC manufacturing

The Australian Government has provided funding for a cGMP facility for the manufacturing of biological agents in the capital city of each state. Before this became available in Brisbane we used a specially designed cell manufacturing facility in the Mater Adult Hospital, Brisbane. The physical design of this facility was not of cGMP standard but we had very close monitoring of the MSC manufacturing runs by our Quality Manager. Our Quality Management System delineates the organisational structure, procedures, processes and resources that control quality activities within the manufacturing unit. It is defined in a series of policy statements in our Quality Manual and is implemented through standard operating procedures (SOPs). SOPs are used to ensure that work having an effect on service and product quality, either directly or indirectly, is carried out in a consistent and satisfactory manner. Clear and effective procedures are developed and maintained by relevant staff. Procedures are regularly reviewed for accuracy, relevance and consistency with the policies, requirements of the relevant standards, guidelines and best practices.

Since the opening of a cGMP facility in Brisbane, which is approved for cell manufacturing and cryopreservation by the Australian Therapeutics Goods Authority (the Australian equivalent of the FDA in the USA), we have used this facility.

7 Pre-manufacturing Requirements

The national drug and biological agent regulatory bodies of the USA (FDA), Australia (TGA) and Europe (EMA) provide guidelines on the administration of cellular products for human use (reviewed in [26, 27]). These include requirements for current Good Manufacturing Practice (cGMP) manufacturing and release criteria such as cell viability, purity, sterility, mycoplasma and endotoxin presence [28].

As indicated earlier, our laboratory was the first in Australia to use maternal-derived placental MSCs for clinical trial use [29]. Placentas obtained during elective term Caesarean sections were used as our source for MSC manufacture in order to minimise the risk of microbial contamination, a major concern with ex vivo expansion of cells for therapeutic use. As MSCs are considered non-immunogenic cells, there is no need to MHC match the donor to the intended recipient. Therefore, MSCs from a single manufacturing campaign can be utilised in numerous clinical trials and for a number of patients [30]. In accordance with the guidelines provided by the Therapeutics Goods Authority of Australia (TGA), our laboratory has established a large-scale manufacturing method for producing safe and reproducible MSC under current Good Manufacturing Practice (cGMP) conditions. We applied a Code of Good Manufacturing Practice standard (or its close equivalent) across the entirety of our cell manipulation process, including acquisition of the starting cell population (in this case, placental tissue), isolation of cells, processing, storage and

transport [23]. We have also devised a quality management system (QMS) to provide support for the manufacturing process based on International Organization for Standardization (ISO) standard requirements [31]. This includes an extensive documentation system for all relevant procedures (including laboratory protocols) to comply with cGMP requirements [32]. It covers all stages of MSC manufacturing. It also applies, but is not limited to the following: facilities, equipment, materials, staff, monitoring, validation, process change, record generating and record keeping [30].

Again as indicated earlier, together with the study protocol, a Participant Information Sheet and Consent Form (PISCF) and other supporting documents including an Investigators Brochure are submitted for review by the Human Research Ethics Committee (HREC) and is used by study personnel to facilitate their understanding of the key features of the protocol. In particular, the MSC dose and methods of administration of the MSCs. The Human Research Ethics Committee reviewing a new clinical trial protocol utilising MSC must have sufficient experience among committee members in order to effectively review the protocol ensuring that the proposed trial has scientific validity, and that participants' rights and well-being are protected according to the Australian National Health and Medical Research Council's National Statement on Ethical Conduct in Human Research 2007, the Declaration of Helsinki and International Conference on Harmonisation Good Clinical Practice ICH GCP(CPMCP/ICH/135/95).

The TGA is notified of the trial once it has been approved and the trial is registered with the Australian clinical trials registry.

All MSC donors are subject to screening requirements of the Australian Red Cross Blood Transfusion Service and AusCord (the Australian National Network of Umbilical Cord Blood Banks and Cord Blood Collection Centres) Guide to Selection of Mothers and Cord Blood Donors. Prior to the collection of the placenta, the donating mother undergoes screening serology for infectious disease markers and completes an in-depth medical questionnaire (as per AusCord Guidelines).

Infectious disease markers on the maternal donor are repeated at 180 days after placental donation and information is sought about the health of the baby to identify if any medical conditions have been identified that may exclude the donated placenta from use in a clinical trial.

We will now discuss the manufacturing processes that are required for using current Good Manufacturing Practice (cGMP), quality assurance and regulatory considerations when conducting clinical trials with placenta-derived MSCs in Australia.

8 Manufacturing Mesenchymal Cells Using Current Good Manufacturing Principles

The Code of Good Manufacturing Practice (cGMP) standard (or its close equivalent) is applied across the entirety of the MSC manufacturing process, including acquisition of the starting cell population (placental tissue), isolation of cells, processing, storage and transport. As indicated earlier a Quality Management System (QMS) is required to provide support for the manufacturing process based on the International Organization for Standardization (ISO) standard requirements.

A new Biologicals Framework was established recently by the Therapeutic Goods Administration (TGA) in Australia (http://www.tga.gov.au/industry/btb.htm). Its purpose is to improve the regulation of human tissue and cellular therapies and provide improved clarity by applying different levels of pre-market regulation to biological products based on the risks associated with the use of each product. According to the Biologicals Framework, MSC are categorised as Class 3 products because of the ex vivo expansion involved in their manufacturing.

8.1 Manufacturing

Human placental MSC manufacture currently represents a complex, specialised, time-consuming and labour-intensive exercise [28, 29, 32]. At present, MSC manufacture requires an "open" system due to the multiple steps required for isolation and expansion of MSCs from placenta. Although, class II safety cabinets or clean rooms can be utilised, the extended period of expansion (up to 6 weeks in our process) introduces the risk of microbial contamination. We have utilised extensive in-process and end-product testing prior for release of product for clinical use to minimise these risks [32].

To ensure a successful and efficient manufacturing outcome, our production scientists work closely with the quality assurance (QA) staff to ensure safe cellular therapy product. Education of staff is of great importance and adherence to QMS principles is required from all staff, including scientific support and laboratory management staff. Implementation of the QMS often presents significant change in laboratory as well as in many supportive activities. The conventional research scientists' mindset for performing discovery-motivated laboratory-based scientific experiments is not appropriate for the provision of a cellular therapeutic product that is safe for the recipient, staff and the institution. We have established a formal training system, which includes a

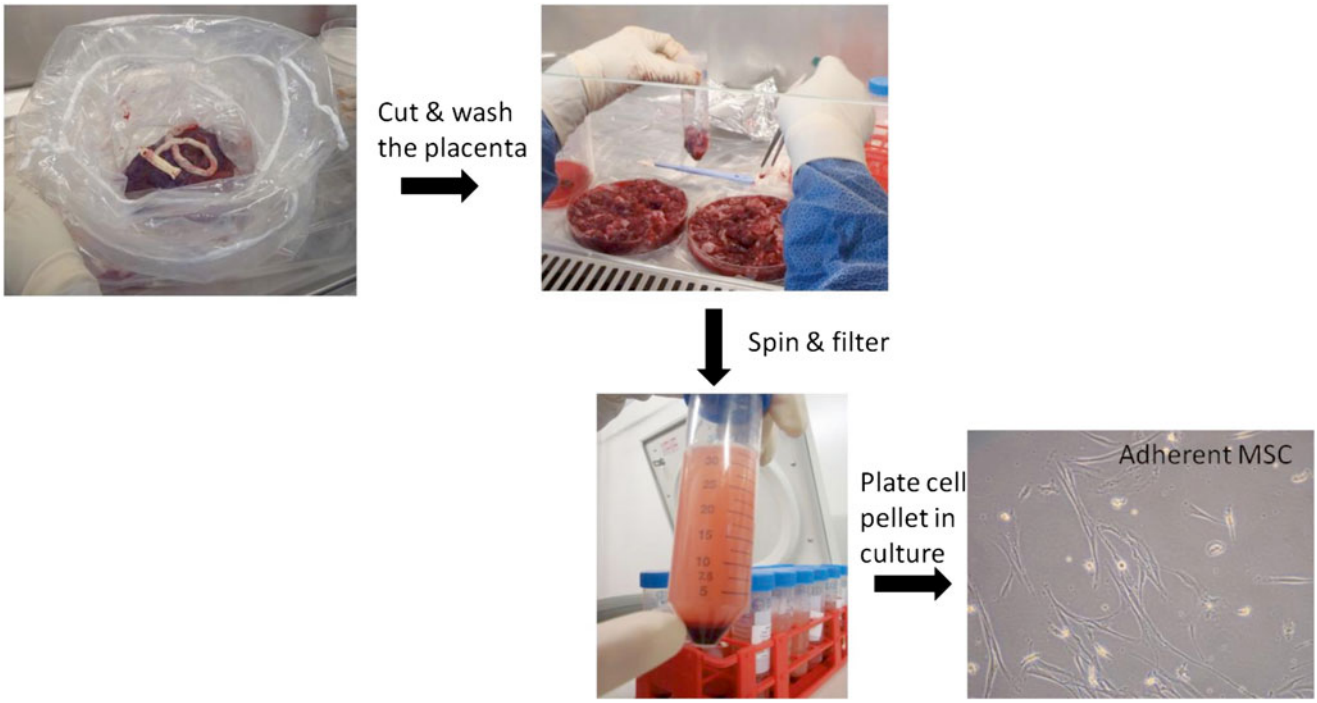


Fig. 30.1 The isolation method of placental MSC

comprehensive training manual, online learning modules and assessments, task-based training as well as continuing education for staff [33].

An efficient and robust system of reporting and addressing of non-conformances (non-compliances with external or internal requirements), improvement suggestions and change control has been established [31]. The QMS staff conduct regular internal audits, while providing relevant reporting to stakeholders. In areas where the QMS interacts with hospital departments and infrastructure, relevant service level agreements are established. The entire process is founded on the following elements: quality program, organisation and personnel, standard operating procedures, facilities, environmental control, equipment monitoring, supplies and reagents, process controls and changes, process validation, labelling design and control, storage requirements, records, tracking, non-conformances and complaints management, risk assessment, and reporting and reviewing [34].

By applying these guidelines, we have been able to successfully generate approximately 2.5 billion MSC from a single placenta that were sufficient to populate a single phase I trial [29].

8.2 The Isolation of Placental MSC

Whole placental MSC were manufactured under cGMP conditions for their use in clinical trials according to the method described by Brooke et al. Briefly, the placenta was obtained from healthy mothers undergoing elective Caesarean sections with their full informed consent prior to delivery. The placenta was subsequently double bagged, placed in a cool box and transferred to our manufacturing facility for processing. Our protocol for the isolation of MSC from placenta utilises a GMP grade collagenase-based digestion of tissue that has been dissected and washed to remove blood before isolation of cells. After digestion, large particulate matter was removed by low speed centrifugation and cell suspensions were collected and filtered into fresh tubes using 70 μm filters. The cells were then pelleted by centrifugation, resuspended and erythrocytes were subjected to rapid lysis with water. The cells were washed with Hank's Balanced Salt Solution (HBSS) and the final cell pellet was resuspended in Dulbecco's Modified Eagle Medium (DMEM-LG), 20 % foetal calf serum (FCS), and 50 μg/mL gentamicin. Cells were initially seeded into eight T175 cm^2 tissue culture flasks and cultured in a humidified incubator at 37 °C, 5 % CO_2 (Fig. 30.1).

When the Brisbane cGMP facility became available (QGen) procedures were performed in clean rooms at Q-Gen, a Therapeutic Goods Administration (TGA) licensed facility (Licence #174586). All cell products were tested according to the TGA's product release criteria which include maternal placental donor exclusion criteria and release criteria at cryopreservation including release from manufacturing (see below). Maternal placental donor guidelines were those of

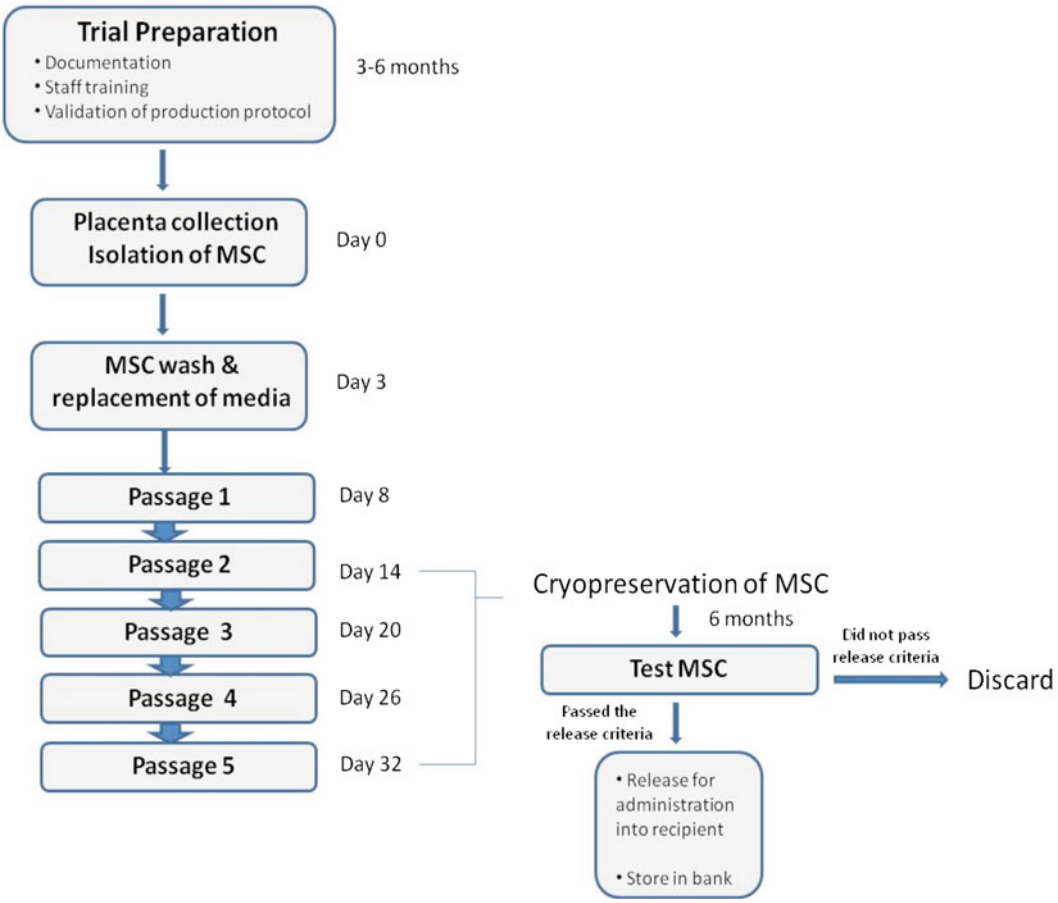


Fig. 30.2 Human placental MSC manufacturing process

the Australian Red Cross Blood Services (ARCBS) and the Australian Cord Blood Registry (AUSCORD). The donors of placentas underwent serological testing for infectious disease markers including Hep A Ab, Hep B sAg, Hep C Ab, HIV I/II Ab, HTLV I/II Ab, Syphilis Ab, EBV Ab, CMV Ab, HSV Ab, VZV Ab and Tozoplasmosis. The results had to be negative prior to placenta donation with the exception of EBV, HSV, VZV and Tozoplasmosis. A health questionnaire was carried out on the maternal donor prior to donation (using the ARCBS and AUSCORD donor guidelines) and had to comply as described earlier before the cells could be released for clinical trial use.

In addition, MSC from each passage was subjected to stringent release criteria at the time of cryopreservation and again 3 weeks prior to clinical administration. At the time of cryopreservation the following release criteria had to be met before the MSC could be released for use in clinical trials: sterility after 14 days microbiology culture; negative on mycoplasma testing, >70 % viable (using Trypan Blue exclusion); negative 16 s rRNA testing, purity defined as >85 % $CD73^+/CD105^+$ and <1 % $CD45^+$, endotoxin testing level of <2 EU/mL and exhibition of a normal karyotype by standard cytogenetic analysis.

A schema of the production schedule is shown in Fig. 30.2.

8.3 Release Criteria at the Time of Cryopreservation (After Harvest of each Passage)

In order for the cryopreserved cells to be available for subsequent use in a clinical trial, the following release criteria must be met: MSC passages (P) zero to five (P0–P5) must be sterile after 14 days microbiology culture; the MSC must be greater than 70 % viable (using Trypan Blue exclusion); MSC purity is determined by flow cytometry, and must be >85 % $CD73^+$, >85 % $CD105^+$ and <1 % $CD45^+$. A normal karyotype analysis must be demonstrated on MSC from P2 to P5; mycoplasma testing (P2–P5) must be negative, 16 s rRNA testing must be negative and endotoxin testing (P2–P5) must show a level of <2 EU/mL.

9 Clinical Trials Using Mesenchymal Stromal Cells

We have completed one phase I trial of placenta-derived MSC administered intravenously in persons with idiopathic pulmonary fibrosis in conjunction with A/Professors Daniel Chambers and Peter Hopkins at Prince Charles Hospital, Brisbane. We have completed two of the three planned dose escalation cohorts with MSCs injected directly intratendinously under ultrasound guidance in persons with treatment-refractory Achilles tendinopathy in conjunction with Dr. Mark Young from Mater Private Hospital, Brisbane. There were no serious adverse events attributable to the MSCs in either trial. Both trials will be reported separately by the Principle Investigators.

10 Conclusions

In summary, we believe that the placenta represents a biologically equivalent and more readily accessible source for manufacturing human MSC for clinical trials compared to other sources. Therapeutic applications of in vitro manipulated cells represent an exciting and growing field. Cell and cell-based gene therapy manufacturing facilities need to be purpose designed and will need to be accredited by their national medicinal regulatory body. Production scientists need to work in close tandem and harmony with quality assurance and ethics committees to absolutely ensure the safety of new cellular products.

Our "open system" of manufacture is labour intensive. The optimal method of manufacturing MSC (from any source) for clinical trials would be an automated, large-scale, closed system bioreactor that could be placed in a biohazard hood and would obviate the need for costly purpose-built cGMP manufacturing suites. Such a system would be cost and time efficient and would provide optimal product safety since it would minimise the use of production personnel and the inevitable infection risk associated with multiple manual manipulations. Pluristem Therapeutics Inc. has reported on large-scale production using their proprietary PluriXTM 3D bioreactor system. This company has been able to expand adherent, placenta-derived MSC with immunomodulatory properties on a routine basis [35].

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Towards Clinical Applications of Umbilical Cord Derived Mesenchymal Stem Cells

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Abstract

Stem cells hold great promise for treating and, ultimately, curing diseases that are currently being managed with pharmaceutical intervention to control the disease progression and/or disease-related manifestations. Stem cells can be used to regenerate normal cells and tissue, while replacing diseased cells and tissue, in order to restore homeostasis and normal tissue function. Although, there is currently great ethical debate concerning embryonic stem cells—specifically in regards to the dilemma of terminating (potential) life, in order to derive stem cells that could be used to treat and potentially cure another ailing individual—no such ethical quandaries exist with adult stem cell sources—with appropriate patient education and consent. In fact, adult stem cell treatments have been in use for more than 50 years, beginning with the first bone marrow transplants spearheaded in the 1950s to organ transplantations of today. As the stem cell field moves forward towards the next generation of cell-based and regenerative medicine therapies, a new paradigm is being explored as a valuable source of primitive stem cells—otherwise known as perinatal stem cells. This chapter explores, in depth, the potential clinical use and benefit of perinatal stem cells and analogous regenerative medicine therapies sourced from the umbilical cord tissue and Wharton's Jelly.

1 Introduction

Over the past several decades, there has been increasing interest in perinatal stem cells for use in the clinic, as a cell-based therapy and as the starting material for regenerative medicine-based products. In the broader sense, perinatal stem cells are stem cells derived from the various tissue sources related to pregnancy. These sources include umbilical cord blood (UCB), umbilical cord (UC) tissue/Wharton's Jelly (WJ), amniotic fluid, amnion, placental tissue, and placental blood. Specifically, perinatal stem cells can refer to stem cells that are derived from the fetus or mother, depending on the genetic origin of the after-birth tissue.

Perinatal stem cells are neither embryonic stem cells (ESCs) nor are they adult (somatic) stem cells (ASCs). Perinatal stem cells represent a new paradigm that offers properties of both embryonic and ASCs. For instance, perinatal stem cells are more pluripotent than ASCs, but are not totipotent like ESCs. Mesenchymal stem cells (MSCs)

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derived from Wharton's Jelly (WJ-MSC), for instance, express genes regulated in human ESCs (e.g., Oct-4, nanog, sox-2), whose expression relates to maintaining pluripotency of human ESCs. Furthermore, these genes are regulated in deriving induced pluripotent stem (iPS) cells from somatic cells. Interestingly, however, these genes are not expressed in ASC sources (e.g., bone marrow, BM). Unlike human ESCs and like ASCs, perinatal stem cells are not tumorigenic. Like human ESCs, perinatal stem cells have long telomeres that allow for greater regenerative potential before reaching senescence. ASCs have shorter telomeres and, therefore, have limited regenerative potential since they reach senescence relatively faster [55, 59]. In turn, perinatal stem cells represent a stem cell source with properties of both human ESC and ASCs, without the misgivings of either source. This chapter focuses on one specific perinatal stem cell source—MSCs derived from the Wharton's Jelly of the umbilical cord.

2 Umbilical Cord Mesenchymal Stem Cells for Use in Hematopoietic Reconstitution

Perinatal MSCs derived from the Wharton's Jelly of the umbilical cord tissue possess widespread clinical applications—from (stem) cell-based to regenerative medicine therapies. In the context of this chapter, (stem) cell-based therapies refers to clinical use of primary cell populations—derived under minimal manipulation (e.g., UCB); regenerative medicine therapies refers to stem cell populations that have been culture expanded and/or genetically or otherwise biologically modified (e.g., Osiris' Prochymal® off-the-shelf bone marrow MSC product).

For (stem) cell-based therapies, umbilical cord tissue derived MSCs have therapeutic benefit in UCB-related transplantations. UCB is a great perinatal source of blood forming stem cells, otherwise known as hematopoietic stem cells (HSCs). Currently, UCB derived HSCs are used in the treatment of a host of blood-related diseases due to the ability of these cells to reconstitute the blood and immune system. A major drawback of UCB and UCB-related transplantations, however, is the finite volume of cord blood that can be collected. The collection of UCB can occur at only one point in time—at delivery. After the fetus has been delivered, the fetal end of the umbilical cord is clamped and cut—thereby, physically detaching the fetus from the mother. If the UCB is to be collected, a needle attached to a blood collection bag with an anticoagulant solution (e.g., citrate phosphate dextrose, CPD) is inserted into the umbilical cord vein—the largest vessel of the three vessels within the umbilical cord. The umbilical cord vein is "milked" until blood flow from the collection has ceased. Due to the finite (and variable) volume of UCB that can be collected at delivery, a finite number of stem cells can be derived from a single UCB collection—as the UCB volume directly correlates with the number of attainable HSCs. The higher the number of HSCs available for a transplant (i.e., dose), the more efficient the UCB unit can engraft when transplanted. Numerous methods are currently under investigation to increase the efficiency of UCB transplantations, including ex vivo HSC expansion technologies, increasing homing capacity to the marrow cavity, direct intra-femoral injections, and multiple unit transplantations. A seemingly more straightforward method takes advantage of the unique properties of another important stem cell population—MSCs—in the setting of co-transplantation with an UCB unit [57].

The rationale for the use of MSCs to enhance engraftment of HSCs is several-fold. First, the current gold standard source in attaining the most-efficient hematopoietic engraftment is bone marrow. If a bone marrow unit is available—either from one's own disease-free bone marrow (autologous) or from a healthy HLA (human leukocyte antigen) matched donor (allogeneic), it is the preferred (stem) cell source for hematopoietic reconstitution. The primary reason for this is the relatively quick time to hematopoietic engraftment—determined by time to absolute neutrophil counts (ANC) above 500 (per microliter blood) for three consecutive days and megakaryocyte counts between 20,000 and 50,000 (per microliter blood) [69]. On average, bone marrow units reach this ANC in 18 days [26, 58]. This is significantly better than current UCB unit transplantations, where time to neutrophil engraftment takes 22–27 days, on average [26, 58]. The reason for this discrepancy is possibly explained by the following concept: bone marrow contains HSCs and MSCs, as well as differentiated progeny of each stem cell population, whereas UCB primarily contains HSCs and its differentiated progeny. When bone marrow is transplanted from cells derived from either an autologous source or from a HLA-matched donor, both HSCs and MSCs are transplanted. This is significant since HSCs and MSCs work in concert to maintain proper blood and immune cell production and function to sustain life and proper homeostasis. UCB, on the other hand, is a rich source of blood forming HSCs, but a poor source of MSCs. MSCs are protein factories that provide support for blood and immune function, by producing growth factors and cytokines that signal and cue HSCs to perform a specific function to maintain proper hematopoietic activity. Due to this interdependent relationship, it has recently been found that MSCs may also play a critical role in engraftment and maintenance of proper blood formation when co-transplanted into patients with diseased and damaged blood [57].

MSCs exist in adult bone marrow (BM-MSCs), as previously described, and adipose (fat) tissue, but also can be found in after-birth tissue—specifically within the Wharton's

Jelly of the umbilical cord tissue. The human umbilical cord contains three vessels—one umbilical vein and two umbilical arteries. The tissue surrounding the vessels is referred to as Wharton's Jelly. Wharton's Jelly is the primitive mucous, connective tissue of the umbilical cord lying between the amniotic epithelium and the umbilical vessels. This gelatinous substance, first observed by Thomas Wharton in 1656, is comprised of proteoglycans and various collagen types. The principle function of the Wharton's Jelly is to prevent the compression, torsion, and bending of the umbilical vessels which provide the bi-directions flow of oxygen, glucose, and amino acids to the developing fetus, while also depleting the fetus and placenta of carbon dioxide and other waste products [6, 59, 61]. Cells found in Wharton's Jelly are a primitive MSC, likely trapped in the connective tissue matrix as they migrated to the AGM (aorta-gonad-mesonephros) region through the developing cord, during embryogenesis (prior to E10.5) [61].

Wharton's Jelly has powerful therapeutic benefits, especially since UC is a rich source of a perinatal, potent population of MSCs. More specifically, WJ derived MSCs have immediate therapeutic benefit in potentially increasing the engraftment efficiency of UCB—to the levels currently observed with bone marrow. This hypothesis rests on the principle that UCB possesses a rich supply of HSCs, but lacks a significant population of MSCs [10, 51, 55]. In the human bone marrow cavity, MSCs produce and secrete various factors that provide support and promote HSC mitotic activity, survival, expansion, differentiation, self-renewal, as well as maintaining multilineage differentiation of mesodermal cells (e.g., osteocytes, adipocytes, chondrocytes). The simple absence of significant numbers of MSCs in UCB may be the biological key in increasing the engraftment efficiency and potential use of UCB derived HSCs without the dependence of any of the aforementioned ex vivo manipulation technologies or need for multiple unit infusions. Although a significant population of MSCs are absent from UCB, the cytokines and factors that are produced and secreted by MSCs are visible to HSCs in the UCB, since MSCs exist in the tissue surrounding the vessels within the umbilical cord (i.e., Wharton's Jelly). It is hypothesized that these factors produced by MSCs in the Wharton's Jelly are available to the UCB in utero, primarily by way of diffusion from the Wharton's Jelly to the blood. Additionally, MSCs exist in the placenta and amnion and provide for further local UCB maintenance [55].

To take full advantage of the paracrine factors secreted by MSCs and their functional effects on UCB HSCs, currently, there is great interest and effort to bank and cryopreserve native MSC populations from the Wharton's Jelly of umbilical cord tissue. Native MSCs are primary MSCs residing within the umbilical cord, minimally procured without ex vivo manipulation and expansion. Native MSCs derived from UC tissue can be thawed and, subsequently, used to increase engraftment potential of UCB units. In fact, several groups have investigated the in vivo engraftment effects of co-transplanting MSCs from the Wharton's Jelly of UC tissue, along with UCB, derived from both native WJ-MSCs [56] and ex vivo culture expanded WJ-MSCs [10]. In pre-clinical trials, where human umbilical cord MSCs are co-transplanted with human UCB into NOD/SCID (non-obese diabetic/severe combined immunodeficient) IL2 receptor (IL2R) gamma null mice, a dose-dependent increase in human HSC engraftment of three- to six-fold is observed [10, 56]. These results suggest that the derived, cryopreserved MSCs maintain post-processing, post-cryopreservation functional activity—for both native MSCs and ex vivo expanded umbilical cord MSCs. Notably, a greater number of transplanted ex vivo expanded Wharton's Jelly derived MSCs were necessary to achieve the same level of engraftment observed with transplanted native WJ-MSCs. This observation suggests that even though ex vivo expansion results in an overall increase in the numbers of MSCs, it does so at the expense of MSC potency [10, 55–57].

Clinical trials have exhibited the safety and potential efficacy of MSCs in increasing hematopoietic engraftment. In one clinical trial, autologous bone marrow was procured from 28 breast cancer patients. MSCs were cultured and expanded ex vivo under GMP (good manufacturing practice) compliance under an FDA (Food & Drug Administration)-approved IND (investigational new drug) application. The autologous BM derived MSCs were infused into 28 breast cancer patients receiving MPB (mobilized peripheral blood) grafts—another source of adult HSCs—in an effort to augment hematopoietic engraftment [21]. No toxicity related to the intravenous infused expanded MSCs was observed. Furthermore, rapid hematopoietic engraftment was observed in all patients, with median neutrophil recovery (>500 μL^{-1}) in 8 (range: 6–11) days and platelet count recovery of between 20,000 and 50,000 μL^{-1} unsupported in 8.5 days (range: 4–19) and 13.5 days (range: 7–44), respectively [21]. This study was not designed to determine the efficacy or mechanism of action of the infused MSCs with respect to hematopoietic engraftment. It did, however, justify further clinical studies to investigate the effect of co-transplanted MSCs on engraftment of hematopoietic grafts, such as UCB, in controlled, randomized, multicenter clinical trials. Additional clinical studies have further established the safety and efficacy of infused MSCs and in the setting of hematopoiesis, have exhibited accelerated engraftment, positive outcomes and potential treatments for graft-versus-host disease (GvHD) [27–29].

More recently, in a pilot study clinical trial, ex vivo expanded MSCs from the Wharton's Jelly of donor umbilical cords were co-transplanted into five patients undergoing

UCB transplantation. Compared with nine patients receiving cord blood transplantation alone, the five patients receiving Wharton's Jelly MSCs, together with UCB had significantly faster engraftment of neutrophils and platelets ($p = 0.02$ and 0.01, respectively). The time to achieve neutrophil and platelet engraftment ranged from 7 to 13 days (median, 11 days) and 22 to 41 days (median, 32 days), respectively, for the five co-transplanted patients. The nine patients receiving cord blood alone achieved neutrophil and platelet recovery in 19–39 days (median, 25 days) and 55–113 days (median, 69 days), respectively. Although no adverse effects were observed in the co-transplanted cohort of five patients, further clinical studies need to be conducted in order to ensure safety and efficacy of Wharton's Jelly derived MSCs [65].

Although the exact mechanism(s) of action of MSCs in accelerating hematopoietic engraftment of UCB is currently unknown, there are several postulations of the potential mode of action of MSCs in this setting. For one, MSCs may be promoting greater survival of HSCs during homing, thus, enabling quicker engraftment. With greater HSC survival, more HSCs can reach the bone marrow and, subsequently, engraft. This is the same mechanism observed in the bone marrow microenvironment and in in vitro co-culture experiments [39, 40, 47]. MSCs may also be increasing the homing capacity of UCB HSCs, directly effecting the sensitivity of the SDF-1 (stromal cell derived factor-1) homing receptors (i.e., CXCR4; C-X-C motif receptor 4), believed to be responsible for HSC homing and engraftment in the BM [66–68]. Alternatively, MSCs may be indirectly increasing homing capacity by suppressing the initial, local immune response by the host, as an initial immune response has been observed with UCB injections intravenously (IV) [45, 46]. Cells that function in innate immunity—specifically natural killer (NK) cells—quickly destroy infused UCB cells transplanted IV in NOD/SCID mice, presumably resulting from residual "leakiness" of NK cells in the animal model. However, when UCB cells are IV transplanted into NOD/SCID IL2R gamma null mice—where no residual NK cells exist, a six-fold increase in hematopoietic engraftment is observed [45, 46], demonstrating the critical role of the initial host immune response in transplanted allogeneic grafts. MSCs may suppress this initial immune response, thereby, enabling greater numbers of viable HSCs to home and engraft into the BM microenvironment [57, 63]. MSCs may also be promoting local expansion of the UCB HSCs, much in the same way that is observed in the bone marrow microenvironment. In fact, there is evidence that co-cultured MSCs with UCB HSCs can promote expansion of HSCs [39, 40, 47]. However, this ex vivo expansion results in significantly less HSC activity, when compared to un-manipulated, native HSCs possibly due to the loss of HSC survival during the ex vivo culture expansion [54–57].

2.1 Umbilical Cord Tissue MSC Banking

Due to the potential clinical applications of MSCs, in both hematopoietic and non-hematopoietic disease indications, MSCs from the umbilical cord are currently being banked, alongside UCB, for future clinical transplantations. The same native MSCs, which are trapped in the umbilical cord during fetal development, can be easily collected and harvested from the Wharton's Jelly at delivery. Otherwise, if not collected, the cells and tissue are discarded as medical waste. This ease of collection has obvious advantages over the collection of ASCs from bone marrow and adipose tissue, for which the donor has to undergo an invasive surgical procedure [55]. This factor, coupled with the great expansion capabilities of umbilical cord derived MSCs enables WJ-MSCs to represent a virtually inexhaustible supply of MSCs for both autologous and allogeneic cellular and regenerative medicine therapies [50, 55, 57, 64].

At the time of delivery, after the collection of the donor's UCB, the entire umbilical cord is clipped, and placed in a sterile collection jar. The separately collected UCB and umbilical cord tissue are placed in a kit and shipped to the respective cord blood bank, whereby the UCB is processed using either a validated manual or automated method to process and cryopreserve the entire collected cord blood cell population (including poly nucleated and enucleated blood cells) or an enriched population of mononuclear cells—which contains the HSC population. For the latter product, semiautomated systems such as Thermogenesis' AXP® and BioSafe's Sepax® system utilize density centrifugation to enrich for the buffy-coat layer (mononuclear blood cell fraction). The mononuclear blood fraction containing the HSCs is cryopreserved in the vapor phase of liquid nitrogen at temperatures below −196 °C in a two-chamber 25-mL cryopreservation bag for later therapeutic use [57].

Currently, there are two main approaches to bank MSCs from the umbilical cord tissue. One is to mechanically chop and mince the umbilical cord tissue into small pieces and cryopreserve the minced tissue for later processing. The advantage of this method for the processing laboratory is that it is relatively simple, quick, and inexpensive to process umbilical cord tissue. The disadvantage is that more processing on the back end would need to be performed—post-cryopreservation—before the cells could be used in a patient, since the MSCs remain within the tissue pieces. The time to process and collect the cells before infusion into a patient will take weeks to months, since the cells from the tissue would need to be cultured and grown in order to attain the final cell product [57].

Another method—referred to as the AuxoCell method—takes advantage of extracting the native MSCs from within the umbilical cord tissue without significantly changing the biological properties of the native cells within the tissue.

This method uses mechanical mincing and/or an enzymatic digestion to "melt" away the tissue, to release the native MSCs. The liberated native MSCs can be easily collected and cryopreserved as a single cell suspension, much like UCB. The advantage of this method is the derivation of native MSCs with high potency that can be directly transplanted into the patient right away—in the same manner as UCB—without any significant manipulation. The disadvantage of this method is that it is relatively more expensive, time-consuming, and complex, than just freezing the minced tissue. The final homogenous single-cell product from the umbilical cord tissue is cryopreserved in a 25-mL cryopreservation bag, similar to the one used for the associated umbilical cord blood unit. Umbilical cord tissue MSCs are then cryopreserved at a controlled rate, and then transferred to the vapor phase of nitrogen for long-term storage, once the units have passed all quality controls and are found to be free of pathogens and contaminants [57].

Although both methods render MSC products, these cell products are quite different. The final transplanted product derived from the cryopreserved minced tissue is biologically different than the native MSCs collected prior to cryopreservation. Since tissue, and not cells, are cryopreserved in the former method, when needed for transplantation, the product would need to be derived by growing cells out of the tissue for a period of several weeks to months in an artificial environment outside the body (e.g, incubator). This manipulation significantly reduces the potency of the derived ex vivo expanded cells relative to their native state. To that end, although theoretically practical to extract native MSCs from frozen minced umbilical cord tissue, in reality, the efficiency in MSC recovery post thaw is approximately 10 % to that of native MSC extraction from fresh (non-cryopreserved) umbilical cord tissue [3]. Taghizadeh RR, Mrowiec ZR, Cetrulo KJ, Cetrulo CL. Unpublished data. 2010.

UCB processing is performed such that the innate, native mononuclear cell population, for instance, is extracted from the whole UCB in a timeframe that would not alter the biological properties of the cells and without any significant ex vivo manipulation (e.g., ex vivo expansion). This same framework can be used to extract native MSCs from the umbilical cord tissue after collection of the UCB, without changing the native biological properties of the derived MSC population. Once the extracted MSCs are collected and cryopreserved, they represent the native MSCs that were originally found in the Wharton's Jelly between day 4 and 12 of embryonic development [56, 61]. Once the native MSCs are placed in in vitro culture flasks or bioreactors, the resultant cell products have biologically changed—in that they no longer represent the same parent population, but are, rather, cell progenies procured under artificial biological conditions (i.e., incubators, plastic-coated flask, bioreactors, medium) [57].

Although, it is the aim of ex vivo culture expansion to maintain the native properties of the derived MSCs, this is not actually the case. MSCs are classically defined as cells that adhere to coated plastic surfaces, express MSC markers such as CD90, CD73, CD105, CD44, CD29, lack expression of hematopoietic markers CD45, CD14, CD34, and have the capacity for multilineage differentiation into osteocytes, chondrocytes, and adipocytes [9]. The challenge with this artificial definition is that it defines MSCs prospectively, rather than defining the properties of the actual native population of MSCs in vivo. The native MSCs in adipose tissue, dental pulp, umbilical cord tissue, or bone marrow, do not adhere to plastic in vivo, although as soon as native MSCs are derived and placed into culture-treated plastic flasks, they selectively adhere to the coated plastic and expand. Culture expansion of MSCs results in loss of MSC potency, as native MSC activity is gradually lost (and not replenished) and found to lead to accelerated aging and senescence in vitro [5, 10, 13, 14, 56]. Culture expanded Wharton's Jelly derived MSCs exhibit lower rates of hematopoietic engraftment compared to un-manipulated, native WJ-MSCs—when co-transplanted at the same UCB and WJ-MSC dose [10, 55–57]. Therefore, for these reasons, methods have been developed to collect native HSCs and MSCs from the UCB and tissue, respectively, as these native cell populations possess the most potent stem cell activity. This way the full therapeutic benefit of each respective stem cell population can be harnessed without the risk of changing the innate biological and/or genetic properties of each stem cell population.

2.1.1 Graft-Versus-Host Disease

Additional properties of MSCs make them useful stem cell candidates for use in various regenerative medicine therapies, beyond UCB hematopoietic engraftment. Recent evidence suggests that WJ-MSCs have the potential to be used to treat GvHD. GvHD, a disease where the immune cells of a transplanted graft initiate an immunological response to the host tissue, causes debilitating illness in patients who have previously received a bone marrow transplant. The complications include skin rashes and abdominal symptoms with diarrhea and liver problems. Bone marrow MSCs have been the most extensively studied for treatment of GvHD. Osiris Therapeutics Inc., has utilized the immunosuppressive ability of MSCs in a multicenter, randomized, and controlled placebo studies for GvHD [18]. In this pivotal phase 3 clinical study, patients with grade II–IV GvHD were randomized to receive two treatments of an off-the-shelf allogeneic BM derived MSC product, Prochymal®, along with corticosteroids. Although 94 % of patients had an initial response to Prochymal®, the trial failed, however, due to not meeting the trials primary clinical endpoint. Prochymal® did not significantly increase complete remission of steroid refractory GvHD lasting 28 days, when compared to placebo controls [11, 35]. Interestingly, in a similar phase 2 clinical trial study, patients with severe GvHD were treated with

BM-MSCs derived using an ex vivo expansion procedure based on the European Group of Blood and Marrow Transplantation. The results of this study demonstrated that nearly half of patients with steroid-refractory, acute GvHD, treated with BM-MSC responded to the treatment, irrespective of HLA-matching to donor MSCs. At 2 years posttreatment, nearly half of the patients with a complete response to the BM-MSC treatment were alive [28]. Although these two specific studies had different clinical endpoints, it is clear that the age of the donor, as well as the ex vivo culture method and length, are important variables that need to be better understood and controlled for future clinical studies for GvHD and other diseases.

Since donor age and ex vivo manipulation are significant variables for BM derived MSCs, a possible source that may dampen the effects of these variables are MSCs derived from the Wharton's Jelly. WJ-MSCs have yet to be tested in the clinic for GvHD, but since these MSCs are derived from the earliest ethically feasible point in human development, they may have reduced donor-to-donor associated variability. Furthermore, since WJ-MSCs have relatively longer telomeres, faster doubling times, greater pluripotency, and take longer to reach senescence, WJ-MSCs may be a more suitable starting MSC source for GvHD treatment [10, 59].

Both hematopoietic and MSCs maintain great therapeutic potential. HSCs, especially those derived from perinatal sources, such as UCB, are currently being investigated in clinical trials in the treatment of numerous hematopoietic and non-hematopoietic diseases. Much effort has been placed in showing the safety and efficacy of MSCs in the clinic, especially those derived from bone marrow and adipose tissue. As the field moves forward, additional clinical studies will investigate the safety and efficacy of MSCs derived from the Wharton's Jelly of the umbilical cord tissue, in not only the setting of accelerating hematopoietic engraftment with UCB but also in regulating tissue repair/renewal, immunosuppression, and mesodermal tissue formation in the treatment of various diseases from GvHD to wound healing to even sports-related injuries.

3 Regenerative Medicine and Tissue Engineering Applications of Umbilical Cord Derived Mesenchymal Stem Cells

Although MSCs have been studied extensively from basic research to the clinic, for uses such as the augmentation of HSC engraftment and the treatment of GvHD, MSCs have been examined most extensively for their utility in tissue engineering and regenerative medicine. This section will extensively review the use of MSCs in regenerative medicine application. Concise reviews of their applicability for wound healing, functional hepatocyte development, lung repair, musculoskeletal tissue engineering applications, to include tendon and cartilage, as well as delivery options currently being pursued in surgical suites will be discussed. Although the presented publications may utilize MSCs from adult sources, they do provide proof-of-concept for use with perinatal sources of MSCs, namely those derived from the Wharton's Jelly of the umbilical cord. Although further studies need to be conducted, it is conceivable that the use of perinatal MSCs from cord tissue, in lieu of adult BM-MSCs, may result in greater regenerative capabilities and, therefore, superior clinical outcomes, mainly due to the inherent relative infancy of perinatal MSCs.

Although MSCs have been derived from diverse tissue sources, the umbilical cord is a readily accessible source for procuring MSCs. For many reasons, umbilical cord MSCs represent a promising agent in the field of regenerative and transplant medicine. Obtained from the Wharton's Jelly region of umbilical cords, these Wharton's Jelly MSCs hold specific potential in regenerative medicine, serving as differentiating cells shown to grow on synthetic scaffolds and compliant cultures. In addition to their advantages of improved accessibility and reduced ethical concerns, WJ-MSCs have also been shown to be non-immunogenic and possess potent immunosuppressive effects. Studies examining immune-related genes have indicated that WJ-MSCs express numerous immune tolerance genes while, concurrently, not expressing the deleterious immune response genes.

In a recent study [7], this expression of immune property genes in WJ-MSCs was examined along with the effect of allogeneic WJ-MSCs on lymphocytes proliferation in vitro and associated changes in blood T-lymphocytes subgroups and plasma IgG and IgM levels. Wharton's Jelly matrix was obtained by caesarian section from full-term births. WJ-MSCs were derived using the "chop and mince" method previously described in this chapter. Briefly, the samples were finely cut and rinsed in sterile phosphate buffered saline solution, and after 14 days of culturing, the Wharton's jelly fragments were further subcultured, and finally removed and prepared for study. The subsequent study confirmed that WJ-MSCs express not only the normal MHC class I genes, but also the noncanonical class I MHC genes (e.g., HLA-E, HLA-F, and HLA-G). Mixed lymphocyte reaction (MLR) experiment illustrated that WJ-MSCs did not induce, but actually inhibited the proliferation of stimulated immune cells and that the inhibitory effect is dose-dependent. Despite having low immunogenicity, the mechanisms related to WJ-MSC immune properties have yet to be fully elucidated but are under vigorous investigation [32]. Furthermore, the effect of WJ-MSCs transplantation on body immune cells has not been completely validated and will require further study.

Current experimental evidence fundamentally demonstrates equivalent function of MSCs, irrespective of tissue source, with similarity in immunomodulatory function, differentiation capacity, and stem cell phenotype (as defined by surface markers). CD29, CD44, CD73, CD90, and CD105 have been observed on MSCs from various tissue sources including placenta and Wharton's Jelly, and another study demonstrated equivalent morphology and growth kinetics of MSCs from placental and bone marrow origin. In addition, similar transcriptomes have been observed in MSCs of placental and bone marrow origin. Finally, regardless of origin, MSCs are immune-privileged and therefore available to be transplanted to allogeneic recipients [6, 9, 10, 63].

Regardless of the source of MSCs, it is likely that quality-controlled and harvested cells from various sources that also meet the accepted cell-surface marker expression definition, transcriptome pattern, and phenotype of MSCs will be utilized for tissue engineering and wound healing applications in the near future. Because of the aforementioned similarity in function of MSCs irrespective of tissue origin, a wide range of potential uses for perinatal MSCs is evident and likely, in the coming years. However, beyond the classic definition of MSCs, the MSC source has important implications on pluripotency and expansion potential. For instance, MSCs derived from the Wharton's Jelly—that formed during the earliest ontogenic time period—result in significant differences in expansion potential compared to (adult) bone marrow MSCs. As previously reported, the number and potency of BM-MSCs specifically decreases with increased age, exhibited by lower in vitro CFU-F (colony forming unit-fibroblast) and proliferative potential, longer population doubling times, lower telomerase activity, and shorter times to senescence [5, 12–14]. WJ-MSCs maintain the same multipotent differentiation potential with relatively higher CFU-F and proliferative potential, higher telomerase activity, shorter population doubling times, and longer times to senescence, without loss of stem cell potency. Thus, WJ-MSCs appear to be more primitive MSCs than those found in bone marrow and represent an earlier stage mesenchymal-like stem cell than those derived from adult fat or bone marrow [10, 59].

3.1 Wound Healing

Despite the previously discussed potential clinical uses of perinatal MSCs, the impact of these stem cells may be felt most significantly in the field of wound healing. Five million new acute and chronic wounds occur each year due to diabetes, venous stasis disease, vascular insufficiency, or trauma. These injuries add significant burden to the USA health system. More than half of all wounds do not heal with current therapy, and require surgical reconstruction or lead to extremity amputation. Interestingly, the instinct for exploiting the healing properties of perinatal tissue originated over a century ago, by application of placentas to burn wounds [36].

For an acute wound to heal, a complicated orchestra of various cell populations is required, each playing a specific role at a designated timeframe in the course of healing. A complex pathway regulates the migration and assembly of new tissue, along with the associated clearance of damaged tissue. This involves cell and extracellular matrix interactions, various paracrine actions, and the secretion of various growth factors and antimicrobial defense mechanisms [36].

MSCs function at various checkpoints in the three stages of wound healing: inflammation, proliferation, and remodeling. The effectiveness of MSCs for improving wound healing has been shown in a number of studies. An equal number of hypotheses have been proposed to explain their efficacy. These possible explanations and the evidence that supports them can be broadly grouped into three categories that correspond to the MSCs effect on the three stages of wound healing: (1) suppression of inflammation and immunomodulation; (2) paracrine effects on cell survival and proliferation, migration, and augmentation of angiogenesis and vasculogenesis; and (3) generation and remodeling of new tissue [40].

3.1.1 Suppression of Inflammation

MSCs dampen the inflammatory response by suppression of interferon-gamma and tumor necrosis factor-alpha (TNF-α), an early initiator of a cascade of pro-inflammatory events in the response to an acute wound. Secondly, MSCs modulate T-cell proliferation, further stifling the overall inflammatory response. Conversely, MSCs promote the expression of the anti-inflammatory cytokines IL-4 (interleukin-4) and IL-10 (interleukin-10). The anti-inflammatory properties of MSCs are particularly interesting because most non-healing wounds do not progress beyond the acute inflammation phase of wound healing and into the proliferation and remodeling phases; rather, due to the microvascular insufficiency of diabetes or venous stasis, these wounds are "arrested" in a chronic pro-inflammatory milieu that perpetuates the non-healing state, and will typically not progress to healing without a therapeutic strategy that mitigates chronic inflammation. MSCs are, therefore, a very attractive modality for chronic wounds. Several excellent reviews summarize the evidence for MSCs valuable anti-inflammatory and immunomodulative properties [32, 36].

3.1.2 Paracrine Effects Amplifying Local Cell Proliferation and Remodeling

Although transplanted MSCs placed into a wound can potentially differentiate and contribute directly to the reconstruction of the wound, such actions require engraftment by both

long-term repopulating stem cells and progenitor cell populations. To date, limited data supporting such a mechanism exists, and it is more likely that MSCs exert their effects on a wound by paracrine signaling of local host cell populations. A wealth of in vitro data demonstrates that: (1) MSCs secrete high levels of known tissue repair factors such as TGF-β (transforming growth factor-beta), bFGF (basic fibroblast growth factor), PDGF (platelet derived growth factor), EGF (epidermal growth factor), and KGF (keratinocyte growth factor); (2) MSCs are potent promoters of angiogenesis; and (3) the function of cell types responsible for wound repair and remodeling, including dermal fibroblasts, keratinocytes, and epithelial cells is improved by MSC co-culture and exposure to MSC culture media [36].

3.1.3 Perinatal MSCs Added to Skin Substitutes

The emerging beneficial effects of MSCs on wound healing have led to the development of "hybrid" therapies utilizing skin substitutes or matrices augmented with MSCs. These products may combine the extracellular matrix and local cell population interactions—known to be essential for re-epithelialization of a wound—with the paracrine amplification added by the presence of MSCs. Such combination therapies are promising for chronic wounds for which current modalities are insufficient.

3.2 Hepatocyte Differentiation and Function

MSCs ability to differentiate into distinct cell lineages represents a highly promising complement to existing clinical organ transplant procedures. Specifically, due to a persistent shortage of liver transplant donors, MSCs obtained from human umbilical cords hold significant potential as a valuable resource for providing the necessary hepatocytes required for successful clinical application. A study by the University of Padova [60] illustrated the plasticity of these cells and demonstrated their ability for differentiation into functioning hepatocytes. Hepatogenic differentiation of Wharton's Jelly derived MSC was concurrently confirmed by another study [1] and further demonstrated that the numerous, critically important immunomodulatory molecules continued to be expressed even after definitive hepatogenic differentiation occurred.

Additionally, in a study by the University of Padua [2], MSCs were seeded on three different supports (untreated plastic, Matrigel, and human acellular matrix) to assess the effects of undifferentiated MSCs in hepatic regeneration. Their findings concluded that not only can MSCs be isolated in a reliable fashion, but the cells also express hepatogenic properties that accelerate acute liver injury resolution.

3.3 Lung Repair

The results of a 2009 study conducted at the University of Melbourne [38] suggest that umbilical cord derived MSCs have anti-fibrotic properties capable of augmenting the clinical treatment of acute respiratory distress syndrome (ARDS). This pathology is characterized by the loss of lung tissue refractory to inflammation and tissue fibrosis as a direct result of damage to the epithelial and endothelial cells and a compromised alveolar–capillary barrier. Patients with ARDS experience hypoxemia from bilateral lung infiltrates, and may be the end result of direct injury from conditions such as inhalation injury, pulmonary contusion, pneumonia, and near drowning. Several treatment strategies exist which include improved surfactant therapy, vasodilators, inhalation techniques, or anti-inflammatory agents. An increased focus on the acceleration of resolution by epithelial restitution and the consequent reduction in fibrosis of ARDS has occurred recently in which MSCs may play a critical future role.

The hypothesis of the study was based on the premise that MSCs derived from the Wharton's Jelly of the umbilical cord could repair lung injury and prevent fibrosis. Mice were treated with bleomycin sulfate to induce lung injury and, subsequently, received various exposures to MSCs, tested against negative controls. MSCs did not survive beyond 14 days in small airway growth medium; MSCs did not differentiate into morphologically typical pneumocytes in small airway growth medium. The cellular extensions of the MSCs retracted and the cells detached from the culture wells, and were not detected in the lungs of healthy mice at 14–28 days following injection. These findings implied that tissue injury is necessary to attract and retain these cells [38].

The repair of injured lung, despite the transient presence of MSCs, is consistent with previous reports involving other organs. MSCs were not observed to differentiate into lung tissue, but they did modify the fibrotic response in the bleomycin-induced model of lung injury. MSC injection did, however, reduce the exudative infiltration in the lung and early fibrosis at 7 days post-injury. This suggests that the early blunting of inflammation may result in the attenuation of the downstream events leading to collagen deposition. Lastly, there was a significant increase in the expression of all cytokines responsible for mediating inflammation and a simultaneous reduction in the expression of those cytokines involved in inflammation and fibrosis following MSC treatment. These results are exciting to date, as there are no safe and effective therapeutic agents to augment lung repair [38].

3.4 Musculoskeletal Applications

3.4.1 Bone Regeneration

Numerous clinical studies have demonstrated that bone marrow, bone marrow concentrates, and ex vivo cell preparations may facilitate bone healing in critically sized segmental defects, nonunions or osteonecrosis. However, at this time the technique of percutaneous injection has not been performed in critical-sized defects [15]. Regardless, due to its biological potency and low associated risks, the use of bone marrow aspirates during multiple other studies have been investigated using a variety of open surgery procedures for bone healing in high-tibial osteotomies. These include the combination with lyophilized bone chips [8], healing of bone defects of variable sizes with devitalized bone allografts [49], and ectopic bone formation together with titanium carriers and recombinant BMP-7 [53].

Several studies have also investigated the use of ex vivo expanded MSCs for the regeneration of bone, in lieu of the single step procedures. Segmental bone defects of three patients were successfully treated with MSCs that were used together with hydroxyapatite (HA) carriers [48]. Significantly higher healing rates were observed than in the control groups without cell-based therapies when bones were treated with ex vivo expanded bone marrow cells together with platelet-rich plasma during distraction osteogenesis approaches that included 20 or 46 cases [19, 20].

3.4.2 Full-Thickness Cartilage Defects

Full-size patella defects in two patients were treated with collagen gel constructs seeded with ex vivo expanded MSCs, covered with a periosteal flap, and sealed with good defect filling, demonstrated markedly improved patient outcome at 1-year follow-up [62]. Using a similar approach, this technology was also used to treat a full-thickness cartilage defect in the medial femoral condyle of one individual, with similar positive clinical and histological outcomes [23]. Although promising, the clinical data on MSC delivery to cartilage defects are still considered as very preliminary.

3.4.3 Arthritis

Any regenerative medicine-based treatment of osteoarthritis (OA) or rheumatoid arthritis (RA) is unlikely to be successful long-term. For the treatment of OA, the intra-articular injection of MSC suspensions conceptually represents the simplest approach. In this manner, cells are distributed throughout the joint space to provide their therapeutic effects. However, data is preliminary and long-term evaluations in controlled, randomized clinical trials are necessary to determine efficacy [53].

Because of their immunosuppressive effects, MSCs from autologous and allogeneic origin have been considered for several autoimmune diseases, including RA [22, 24]. Allogeneic MSC transplantation in four patients with refractory RA was considered safe but revealed only short-term clinical improvement in two patients and none of the patients reached the stage of remission [30]. However, this treatment option for RA has not been pursued further because of disease relapses and the good success with standard therapies complemented with the use of the antagonist in recent years. In lieu of systemic transplantation, fibroblastic cells were used to deliver anti-inflammatory cytokines to diseased joints intra-articularly, following genetic modification, experimentally and clinically. However, it remains to be seen whether such costly and laborious genetic therapies will find broad clinical application, and the safety concerns remains in treatment of diseases that are nonlife threatening.

3.4.4 Tendon Repair/Regeneration

In the field of regenerative orthopedics, significant research data supports the beneficial use of MSCs for treatment of injuries to the meniscus [33], intervertebral disc [37], ligaments and tendon [17, 31], or muscle trauma [43], which has extensively been reviewed [43]. For all of these applications, clinical trials that use MSCs from autologous or allogeneic origin are currently underway [53]. Thus, in combination with these cell-based therapy approaches, the feasibility of the development of these treatments to clinical use is supported. Early clinical trials will clarify whether such approaches will find their way to broader, definitive clinical application. It is conceivable that stem cells might prove ideal to complement or enhance readily available treatment modalities where desired results are often not achieved. It remains to be seen for every specific application whether the addition of cellular entities derived from whole blood, marrow concentrates thereof, or ex vivo processed cell preparations is required and effective to augment optimized healing results [53].

Another great potential for healing enhancement is the facilitation of endogenous tissue stem cells. Mounting evidence continues to demonstrate the paracrine and trophic functions of MSCs that act by stimulating regenerative cell populations in situ. Indirect ascension up the steps of the "therapeutic escalator" may be elicited by administration of MSCs leading to the regeneration of other musculoskeletal tissues, such as cartilage, where no satisfying treatment options are available in the clinic. However, the application of cells that involve either ex vivo expanded or processed cells are considered advanced therapy medicinal products by international and national regulatory agencies. Adherence to these stringent criteria will help assure efficacy and safety of

innovative stem cell interventions, while also protecting study participants.

Utilization of MSCs for the repair of cartilage, and tendon injuries has shown safety and initial efficacy in several phase one clinical trials. Large comparative, randomized clinical trials are still required for adequate comparison of the MSC-based therapies to standard treatment modalities. In other arenas, such as meniscus, intervertebral disc, ligament, or muscle regeneration, MSCs have only been used experimentally with some success [53].

3.5 Musculoskeletal System Cell Delivery Options

3.5.1 Cell Suspensions and Injection Versus Seeded Scaffolds for Musculoskeletal Tissue Engineering

MSCs hold great promise for regenerative therapies in the musculoskeletal system. MSC populations can be readily differentiated into lineages including bone, cartilage, meniscus, ligaments, and tendons by easily reproducible methods [44].

Autologous MSCs have been harvested, processed, and re-administered via numerous protocols for specific musculoskeletal applications—varying from single-step methods such as direct injection of unprocessed or concentrated blood or aspirated bone marrow to multi-step processes such as prefabricated constructs composed of natural or synthetic scaffolds seeded with undifferentiated and differentiated MSC populations. An example is demonstrated in autologous chondrocyte-seeded implants. Given the complexity of each of the therapeutic components involved, along with the different modes of delivery of each alone and in combination, there is currently no fully developed definitive treatment for a given orthopedic application. However, numerous techniques are currently in widespread clinical use [44].

The simplest means of cell delivery to the musculoskeletal system is direct injection of cells. Depending on the application, the suspension can be injected directly into the diseased tissue, where the cells eventually populate the target site and stimulate repair via autocrine or paracrine pathways. Examples of such direct approaches are MSC injection therapies for the treatment of arthritis, tendinitis, or tendon ruptures [16, 42]. In cases where large tissue defects need to be restored, however, cells are usually delivered via a scaffold to the target tissue. Such constructs are seeded with stem subpopulations in vivo prior to implantation and are used to regenerate structural defects in bone or cartilage [4, 34, 41].

An important issue for the success of any stem cell therapy is the quality of the cells administered, with regard to cellular integrity, age, senescence, proliferative ability, capacity for differentiation, and intact paracrine function [25, 52]. In this regard, the use perinatal stem cell sources verses ASC sources may ultimately increase the success rates of these surgical procedures and result in better outcomes for the patient. Furthermore, as ex vivo cell preparations are subjected to additional external influences and stress factors placed on the cellular population, perhaps the AuxoCell method, previously described in this chapter that results in the derivation of native MSCs with high potency that can be directly transplanted into the patient right away without any significant manipulation, will play an increased role in similar surgical procedures in the future.

4 Conclusion

Wharton's Jelly derived MSCs from the umbilical cord tissue is proving to be an advantageous source of primitive, perinatal MSCs, and perhaps will one day be considered the gold standard for MSC sources. Although behind bone marrow and adipose derived MSCs in terms of clinical development, it is anticipated that in the near future MSCs derived from Wharton's Jelly will succeed in some clinical indications and become a useful cellular starting material for regenerative medicine therapies described in this chapter, as well as other regenerative medicine treatments that have yet to be investigated with WJ-MSCs. Certainly, the use of perinatal stem cell populations in cell-based therapies and regenerative medicine is very promising. There is great potential for the treatment and ultimate healing for hematopoietic diseases and cancers, immune-related diseases (e.g., GvHD), as well as auto-immune-related disorders (e.g., Rheumatoid Arthritis, Crohn's Disease, Diabetes, Lupus), musculoskeletal injuries (e.g., tendon, ligament, muscle, bone), neurodegenerative disorders (e.g., Parkinson's Disease, Alzheimer's), cardiovascular-related injuries (e.g., acute myocardial infarctions, stroke) and wound repair. Although, this chapter describes some of the potential therapeutic uses of WJ-MSCs, it does not describe the full spectrum of diseases that could be treated and cured with these potent perinatal stem cells. Certainly, much effort is still needed to bring this promise to clinical fruition for the safe and efficacious treatment of ailing patients.

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Recent Patents on Perinatal Stem Cells

32

Tamara Yawno, Euan M. Wallace, and Rebecca Lim

Abstract

In recent years, emerging interests in stem cell biology and the burgeoning promise of cell therapies has culminated in an increasingly complex patent landscape. The onus is on inventors to patent discoveries in order to protect their intellectual property, which may become commercially valuable. This chapter will discuss the steps involved in patent development and requirements for patent filing. We will also discuss recent patents on the collection, isolation, characterization, and application of stem cells derived from gestational tissues including the placenta, placental membranes (amnion/chorion), amniotic fluid, umbilical cord tissue, and cord blood. The patents described in this chapter have been selected due to their relevance to other chapters in this book.

1 Introduction

There has been an increasing number of patents describing methods for the characterization, isolation, expansion, storage and application of perinatal stem cells. In this chapter we will discuss recent patents surrounding perinatal stem cells. The patents selected have particular relevance to the earlier chapters in this book. These patents will encompass the collection, isolation, characterization and application of multipotent stem cells from gestational tissues including the placenta, fetal membranes (amnion/chorion), amniotic fluid, umbilical cord and cord blood. We will explain the process of applying for a patent, the rights of the inventor(s) upon the granting of a patent and how the potential value of stem cell patents have been exploited to date.

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2 The Patent Process

The rate at which stem cell research has progressed, has led to an exponential number of innovations requiring protection. Intellectual property rights such as trade secrets, trademarks, copyright, and patents can protect some of these leaps in technological advances. Currently, patents are one of the most common forms of intellectual property protection. The patenting of an invention is a long and arduous process, which is often costly and frustrating for inventors (Fig. 32.1). It can take up to a year from the conceptualization of an idea to the time that a provisional patent is filed. And while the provisional patent itself does not offer patent protection, it does allow the inventor(s) a 12-month period with which to consider proceeding onto a standard or innovation patent. The date on which the provisional patent is filed is also referred to as the priority date.

In the next step, the non-provisional patent or patent corporation treaty (PCT) application is filed. Within 3 months of a PCT application, or 9 months from the priority date, the inventor(s) will receive an international search report and an examination report, sometimes referred to as the International

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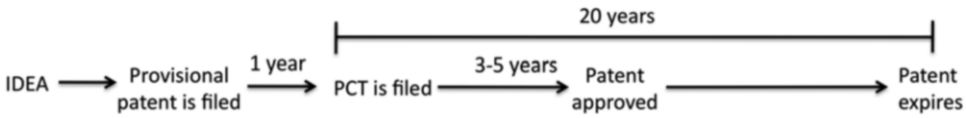


Fig. 32.1 The average timeline for patent filing

Search Opinion or the Written Opinion of the International Searching Authority. This report will assist the inventor(s) in refining a patent strategy since the reports will explain in detail the validity of the patent application and identify similar inventions.

The inventor(s) will then be allowed to amend any claims stated in the PCT within 2 months of receiving the written report or within 16 months of the earliest priority date. There are typically ownership disputes and oppositions, as well as queries on patent claims requiring resolution. This stage can take 3–5 years to resolve and is usually handled by patent attorneys, thus adding to the costs of PCT filing. Once resolved, a standard PCT is then valid for 20 years from the priority date. However the inventor(s) are required to pay ongoing annual maintenance fees. Despite the time and costs involved, this process is important for the protection of the intellectual property and allows the inventor(s) a legal avenue to pursue infringement.

3 Implications of a Patent

Patents are often described as legal monopolies. A patent gives the patent holder exclusive rights over an invented product/process including marketing, hiring, selling or disposing of the products. These are summarized in Box 32.1. The patent holder may, for a limited period of time, prevent anyone else from making, constructing, emulating, using, or selling the patented invention, or any other invention that achieves substantially the same result in substantially the same manner. These exclusionary rights help to encourage competitiveness in the field. Consequently, biotech companies invest heavily in their research and development. For example, Michael West, CEO of BioTime Inc., and his colleagues patented a method for derivation of chondrogenic cell lines from embryonic progenitors, which can be expanded for over 40 passages and retain site-specific purity [1].

Box 32.1: The Rights of a Patent Holder

1. The marketing, hiring, selling, or disposing of the product
2. Offering to make, sell, hire, or dispose of the product
3. The use or import of the product
4. The keeping of the product for any of the above reasons

However, biotech companies do not rely solely on intellectual property developed within company walls nor do they always retain exclusivity of their patent rights. For instance, Advanced Cell Technology has extensively licensed its patent rights to BioTime Inc. and International Stem Cell Corp. Such arrangements are reflective of the commercial value of any given patent. Another level of complexity is added when one considers that as biotech companies grow, they often develop subsidiary companies which they then license their proprietary technology. License holders of patents may also be allowed to sublicense a patent if this is stipulated in the license agreement.

A patent holder may also seek to maximize profit potential by entering into an agreement with a third party allowing that party to exploit their invention in the most commercially viable way. For example, Izadyar and colleagues from the University Medical Center, Utrecht, Netherlands, published their work on the development of a cryopreservation protocol for spermatogonia [2]. While developing this protocol, Izadyar and colleagues entered an agreement with PrimeGen Biotech LLC, which resulted in a patent that was jointly filed [3]. Such arrangements allow biotech companies to invest in academic projects with potential commercial value. Similarly, collaborating academics that are often resource-poor are able to access funds for the project and patent application. The patent is then shared between both parties and allows protection of their joint intellectual property.

Patent law is intended to strike a balance between two conflicting interests: the individual interest of the inventor/investor, which seeks financial recognition, and the promotion of public interest, which also stands to benefit from economic prosperity. Ongoing public debates illustrate the public view that there is currently an imbalance favoring the interests of the inventor/investor which is inherent to patent law, especially where stem cell patents are concerned [4]. Public debate and the inclusion of such concerns in political agendas may lead to restoration of balance in the legal domain.

4 Requirements for the Grant of a Patent

In order for an invention to be patented, it needs to fulfill a number of criteria (Box 32.2). These include novelty, usefulness, an inventive step, full disclosure of the invention, and that the patent relates to a manner of manufacture. In return

Box 32.2: Criteria for an Invention to be Patent-Worthy

1. Is novel
2. Involves an inventive step
3. Is useful
4. Details of the invention are sufficiently well disclosed/described
5. A manner of manufacture, not a product of nature

for the grant of a limited term of commercial exclusivity, the patent holder must disclose the invention in a manner that will enable others to replicate the invention of experimental purposes during the life of the patent and use it freely when the term of exclusivity has expired. This ensures that others can utilize and build on an existing innovation to further develop new and useful technologies [5]. Overall, the patent system attempts to distribute property rights equally between all the inventors who contributed to the overall development process in a manner that encourages exclusivity, efficiency, and wider commercial exploitation without creating barriers to the progress of science [6].

5 Recent Patent Activities

The term 'manner of manufacture' means that a patent must relate to an artificial state of affair. Some argue that genes and DNA are natural parts of the body and should be categorized as products of nature. They are thus unsuitable for patent protection since products of nature can only be *discovered*, not *invented*. It is generally accepted that isolated and purified products are patentable if their new form is substantially different from their natural form [7].

The requirement for novelty is to ensure that a patent is only granted for an invention that is truly new, in the absence of being not previously known in a given field of knowledge. Thus, at the time of the application for a patent, the details of the invention must not have been published [8]. The requirement that an invention involves an inventive step is fundamental to the patent system, as it ensures that an incentive is not awarded for knowledge that was obvious or that would have been available at the time of the patent application [8].

As of May 2013, we found 56,300 patent applications pertaining to perinatal stem cells on the Google Patent database. Of these, 2,230 applications were approved in the past decade (2003–2013). Currently, 645 patents pertain to amniotic fluid stem cells, 1,100 to stem cells from the placenta and fetal membranes, 616 to umbilical cord blood stem cells, and 1,280 to umbilical cord stem cells. The USA remains a world leader in patenting new technologies regardless of whether one considers all patents filed across all disciplines (Fig. 32.2) or patents filed only in the area of stem cell-related technologies (Fig. 32.3). Patent applications can be found in a number of databases and we have listed some of the major databases in Box 32.3.

Box 32.3: A List of Online Patent Databases

World Intellectual Property Organization (WIPO), http://www.wipo.int
Google Patents, http://www.google.com
Scifinder, https://scifinder.cas.org
United States Patent & Trademark Office, http://www.uspto.gov/
European Patent Office, http://worldwide.espacenet.com
Patent Lens, http://www.patentlens.net/
Patent Data Analytics Services, www.freedopatentonline.com
Stem cell patents, http://www.stemcellpatents.com

The patent system is relatively uniform across a number of countries, following many years of efforts to harmonize intellectual property system. This reflects international cooperation in economic and trade development. For the purposes of this chapter, we will review recent patents on stem cells that have been granted to the top five inventor countries—the USA, Germany, UK, Canada, and Australia (Fig. 32.3). We will only review patents that have been granted a publication number. Therefore, inventors/researchers that may have lodged a patent through World Intellectual Property Organization (WIPO) but have not been granted a publication number are not mentioned in this chapter. Each of the following subsections will describe a group of perinatal stem cells, where patents describing the methods of isolation, preparation, and application will be discussed.

6 Patents on Amniotic Fluid Stem Cells

Amniotic fluid stem cells are a multipotent cell source associated with minimal ethical and legal considerations involved in their collection and use. Amniotic fluid stem cells have been differentiated along the adipogenic, osteogenic, myogenic, endothelial, neuronal, and hepatic lineages [9–12]. In preclinical studies, they have been shown to be efficacious in the treatment of bone defects [13], heart disease [14], kidney disease [15], neurodegenerative disease [16], lung disease [17], blood [18], and immune disorders [19].

A patent on amniotic fluid stem cell banking was granted to Martin Haas in 2005 [20]. His patent describes a method of cell banking that allows for easy access to transplantation antigen-compatible amniotic stem cells, which could then be used postnatally for regenerative medicine. Atala and De Coppi were the first to file a patent on methods of isolation, expansion, and differentiation of stem cells from the amniotic

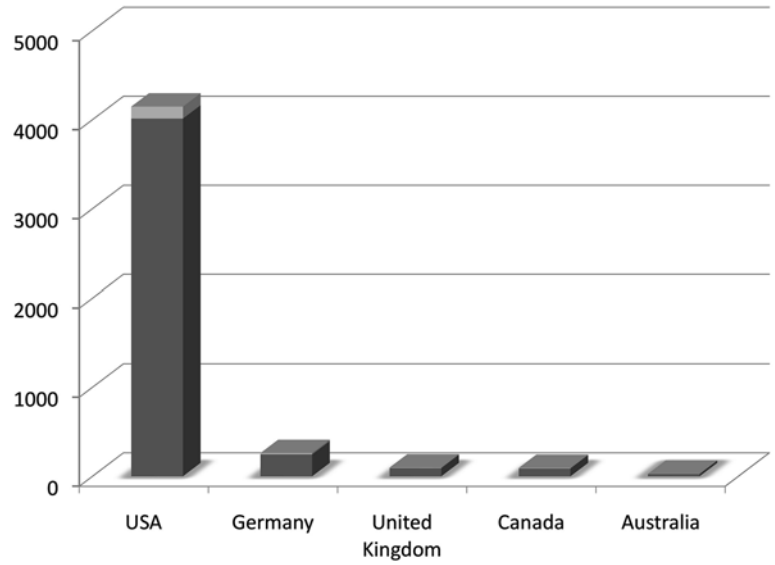


Fig. 32.2 Total number of patents approved (1000s) from the top five most active countries in stem cell patents

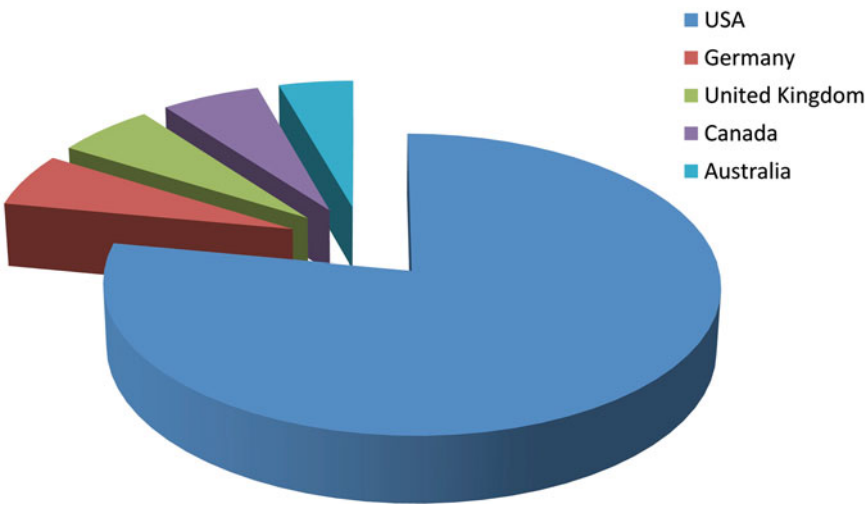


Fig. 32.3 The top five inventor countries

fluid and their therapeutic use [21]. A separate patent by Tsai and Hwang describes a method that contacts the cells isolated with an apoptosis inducing agent under conditions which are apoptotic to non-stem cells and non-apoptotic to stem cells, thereby selecting the stem cells from a heterogeneous population of cells [22]. The selected stem cells may then be used for a variety of applications including transplantation and differentiation [23].

7 Patents on Stem Cells from the Placenta and Fetal Membranes

Gestational tissues, such as the fetal membranes, have had a history of allograft applications in regenerative medicine. One of the earliest descriptions of allografts of gestational tissues was published almost 70 years ago by Sorsby and Symons, who described the grafting of amniotic membranes in caustic burns of the eye [24]. Amniotic membranes remain recognized as a superior biological dressing for wound management [25]. Beyond their mechanical properties, cells derived from the amniotic membranes retain many characteristics that are ordinarily associated with stem cells. These include pluripotency, proliferative ability, and relative lack of immunogenicity. Additionally, stem cells and stem-like cells obtained from gestational tissues maintain a higher proliferative capacity when compared to their adult stem cell counterparts [26].

Also in the past decade, there has been growing interest in the reparative properties and potential use of amniotic membrane stem cells in addressing organ injury, including the treatment of adult neurodegenerative or acute-onset brain disorders [27, 28]. The amniotic membrane plays a key

function during pregnancy and embryonic development. It has anti-inflammatory and immunological properties that contribute to the suppression of the maternal immune response against the fetus. These unique features of the placental membranes have been exploited in a patent that describes the use of methods of transplanting tissues and organs comprising administration of placental stem cells to prevent or inhibit immune-mediated rejection [29].

The cell populations within the amniotic membrane demonstrate pluripotent properties, reflective of their derivation prior to gastrulation. Their immunomodulatory properties, reflective of their biological role during pregnancy, contribute to the basis for their clinical applications [30]. For example, the human amniotic membrane has been used clinically to promote epithelialization in burns and skin ulcers, and as a dressing for skin grafts [31]. In keeping with this wound healing property of the amniotic membrane, John Daniel hold a patent that describes a method for preparing fetal membranes as tissue grafts [32]. His patented technique includes a method for processing a placenta with its associated fetal membranes, cleaning and separating the chorion tissue from the amniotic membrane, and preparing the selected layer into a plurality ready for desired tissue grafts. These tissue grafts include the Schneiderian membrane repair (i.e., sinus lift), guided tissue regeneration, general wound care, and a primary closure membrane.

On a related clinical subject, Herzberg and Gurney patented an application of placental stem cells for the treatment of neuropathic pain, which involves the local or systemic administration of a "therapeutically effective amount of tissue culture plastic adherent placental stem cells" [33]. Furthermore, a patent on the wound healing properties of placental stem cell work has been granted to Smith and colleagues [34]. In their patent, they described the use of conditioned media from amnion derived epithelial cells applied topically across a spectrum of wounds. These included infected chronic wounds, surgical sites to prevent keloid scar formation, burns, fractures, and congenital wounds such as epidermolysis [34]. However, it is important to note that similar patents on the wound healing properties of adipose derived stem cells exist in two separate iterations [35, 36].

In another related medical application, the use of placental stem cells for angiogenesis was recently patented by Abbot and colleagues [37]. Their patent describes the use of stem cells to treat individuals with circulatory system disorders (e.g., diseases or disorders associated with or resulting from, inadequate vascularization or blood flow) by improving angiogenesis. Other patents held by Abbot and colleagues include a method for producing placental stem cells enhanced by RNA molecules to treat individuals having condition caused by, or relating to, an unwanted or harmful immune response [38]. In brief, their patent included methods for increasing the immunomodulatory activity of placental stem cells using oligomeric or polymeric regulatory molecules such as modulatory RNA. The enhanced stem cell could then be used to treat individuals suffering from a disease that involves an inflammatory or immunological component.

7.1 Amnion Epithelial Cells

Miki and colleagues were the first to report a stem cell-like population, isolated from the term amnion, termed human amniotic epithelial cells (hAECs) [39]. hAECs express traditional stem cell markers including Oct 4 and Sox 2, and are capable of multipotential differentiation. Miki and colleagues filed a patent on the therapeutic use of placental derived stem cells and their differentiated counterparts including hepatic, pancreatic, and nervous tissue [40, 41]. Tseng and colleagues filed a patent method on the isolation and ex vivo expansion of amnion cells of animal (nonhuman) origin. Their patented method retains pluripotency and selectively enriches the stem cell population [42]. Subsequently, Kang and colleagues were granted a patent on a unique method for isolating and culturing stem cells derived from human amnion [43]. The method used by Kang and colleagues promises high yields of hAECs, involves the treatment of the amnion with dithiothreitol and a low concentration of trypsin, and then culturing the hAECs in a medium containing a Rho-associated kinase inhibitor [43].

Recent evidence has shown that hAECs are useful in reducing multiple organ injury [44–46]. In particular, the hAECs have been proven to be novel therapies effective in reducing or ameliorating preterm lung and brain injury. Jenkin and colleagues filed a patent on the application of hAECs as a therapy for chronic and acute lung diseases and conditions [47]. When cultured in small airway growth media, hAECs adopted a pulmonary phenotype as evidenced by the expression of lung specific markers and intracellular lamellar bodies degenerative disc disease [48]. Further, when systemically administered into a mouse model of lung fibrosis, hAECs were found to localize to the lung and express the morphology and markers of type II alveolar epithelial cells [49]. Jenkin and colleagues later extended the use of hAECs on the treatment of degenerative disc disease. They were granted a patent on the use of hAECs, mesenchymal stem cells and their derivatives directed towards a disc cartilage type cell for the in situ generation of intervertebral (IVD)-like tissue [50]. The claims of this patent suggest that hAECs have the capacity to differentiate into cells of the chondrogenic lineage [50].

8 Patents on Stem Cells from the Umbilical Cord

The umbilical cord blood contains a rich and diverse mixture of stem and progenitor cells that have the potential to generate a variety of cell types [51, 52]. Cord blood is rich in hematopoietic stem cells, mesenchymal stem cells, regulatory T lymphocytes, monocytes, endothelial progenitor cells, and stromal precursor cells. Umbilical cord blood is being used with increasing frequency as an alternative to bone marrow or peripheral stem cells for transplantation in the treatment of malignant and nonmalignant conditions in children and adults [53]. Transplantation of cord blood delivers hematopoietic stem cells with several advantages. These include their prompt availability, decreased risk of transmissible viral infections, and graft-versus-host disease in both human leukocyte antigen (HLA)-matched and HLA-mismatched stem cell transplants. Compared to the more traditional bone marrow derived hematopoietic stem cells, cord blood can be collected with relative ease and at negligible risk to the mother or newborn [54]. This therapeutic modality is not without its limitations. For instance, an insufficient stem cell dose to reliably treat older children and adult recipients, slow engraftment and potential of genetically abnormal hematopoietic stem cells need to be considered [54].

To address this, an innovative method of collecting cord blood stem cells from an isolated mammalian placenta by placental perfusion is presented in a patent application that was granted to Takebe from Cord Blood Sciences [55]. This patent describes a method involving the use of efficient pulsatile perfusion machines. This patent claims that cord blood collected using this device contains a higher yield of primitive hematopoietic stem cells compared to cord blood from collected using conventional needle and syringe withdrawal or gravity drainage collection.

Umbilical cord blood has been used to treat many disorders or conditions. Their use in transplantation, treatment, and prevention of disease, as well as disease diagnosis is covered by patent to Hariri (Anthrogenesis Corporation) awarded in 2004. This patent also provides a protocol for the use of cord blood or cord blood-derived stem cells from multiple donors without the need for HLA typing [56]. As technologies surrounding stem cell therapies and bioprocessing develop, a more recent patent has been filed, describing a method for expanding stem cells in vitro using a rotating cell culture chamber [57]. The chamber contains a fluid-filled compartment where cord blood stem cells are grown in the presence of the mesenchymal stem cells seeded on a scaffold. Unique to this patent is a dual flow valve within the culture chamber. This valve allows the first flow path to pass under a molecular cutoff membrane that covers a central core, transversing the culture chamber. A second flow path then flows through the culture chamber thus allowing cord blood stem cells to be harvested while in suspension.

Schwartz and colleagues filed a patent on hematopoietic stem cells derived from the umbilical cord, which described a specific method for hematopoietic stem cells expansion in vitro [58]. Following that, a patent was granted to Boyse from Biocyte Corporation on methods for collection and cryopreservation of the neonatal and fetal stem and progenitor cells [59]. This was identified by Konski and Spielthenner as one of the five most dominant patents in the field of hematopoietic stem cells [60]. Currently, Yilmaz and colleagues hold a patent on methods for identification, collection, and enrichment of hematopoietic stem cells [61]. In particular, their patent specified a method for hematopoietic stem cell purification from hematopoietic tissues for clinical use, including the fetal spleen and umbilical cord blood [61].

In addition to being the source of cord blood stem cells, the umbilical cord also contains Wharton's jelly, a primitive tissue of the umbilical cord lying between the amniotic epithelium and umbilical vessels. The main role of the Wharton's jelly is to prevent compression, torsion, and bending of umbilical vessels which provide bidirectional flow of oxygen, glucose, and amino acids to the developing fetus [62]. A method for isolating progenitor cells from the perivascular region of Wharton's jelly was patented in 2005 [63]. The progenitor cells isolated from Wharton's jelly have a remarkably short doubling time. These progenitor cells have a wide range of properties perceived as being valuable for cell therapy including multipotent capacity. Wharton's jelly derived progenitor cells can give rise to a variety of mesenchymal tissues including fat, bone, cartilage, muscle, and endothelium. The method for derivation of progenitors cells from Wharton's jelly that spontaneously differentiate into bone-forming osteoblasts has been patented by the same inventors [64]. As a progression of their earlier work, the inventors were granted another patent that described the recovery of viable umbilical cord perivascular cells from cryogenically frozen umbilical cord tissue, thus addressing the harvest and isolation of a population of progenitor cells extractable from Wharton's jelly [65].

These inventors describe the progenitor cells as being umbilical cord perivascular cells. However, based on claims in the patent, one could argue that the inventors have described a method for isolating *mesenchymal stem cells* from Wharton's jelly. Delineation between the umbilical cord perivascular cells and mesenchymal stem cells is challenging but important when considering patent claims. Notably, there are already a number of patents in existence that describe methods for isolating, enriching, and expanding mesenchymal stem cells from Wharton's jelly. The subtle differences between inventions that allow a patent application to fulfill the criteria of novelty and inventiveness illustrate the complexity and nuances in patenting.

Wharton's jelly also contains the more commonly discussed mesenchymal stem cells. These stem cells travel between the placenta and fetus but predominantly reside in the Wharton's jelly during gestation [66]. Kallis and Matsis patented a method for isolating mesenchymal stem cells from umbilical cord tissue comprising: (a) separation of vascular tissue from Wharton's jelly matrix from a sample of umbilical cord; (b) dissociation of cells from the Wharton's jelly matrix and suspension in plasma; (c) separation of dissociated cells from undissociated cells and matrix; and (d) cryopreservation of the separated dissociated cells, thereby isolating mesenchymal stem cells from umbilical tissue [67].

While the properties of mesenchymal stem cells derived from perinatal tissues may not be unique to their niche, there are arguably benefits to using patented technologies such as that described above. For instance, perinatal mesenchymal stem cells may be isolated and banked for use in treatment of diseases affecting neonates and children. This approach avoids invasive procedures for isolation of mesenchymal stem cells from more traditional sources such as the bone marrow and adipose tissue. Considering the number of clinical trials currently using perinatal stem cells, technological improvements in expansion and banking of umbilical tissue, mesenchymal stem cells are likely to be reflected in more patents in this area.

9 Conclusion and Future Directions

The patents described in this chapter are reflective of a transition. In the past decade, we have moved from understanding the basic biology of perinatal stem cells to developing the necessary technology for clinical application and manufacturing. The patents discussed in this chapter lend themselves to an industry that is primed to meet a predicted medical and commercial demand. Not surprisingly, biotech companies and academics have intensified their efforts in protecting their intellectual property, while increasingly research focus has turned towards understanding clinical utility.

A major caveat remains for many types of perinatal stem cells—the low yield of stem cells, which is often insufficient for clinical application. Many perinatal stem cell patents address this using ex vivo expansion methods that involve growth factor supplementation and/or specially designed cell culture chambers. Another area that dominates perinatal stem cell patents is in clinical application. Given the increasing numbers of clinical trials using perinatal stem cells [68–70], there is little wonder that the bulk of these patents encompass diseases that affect neonates, children, *and adults*. It is tempting to speculate that this busy patent landscape will soon reach saturation. More likely, inventors will find ever more ingenious ways to develop new technologies while concurrently protecting their intellectual property.

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