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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.

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Part I

Amniotic Fluid Stem Cells

Amniotic Fluid Stem Cells for Cardiac Regeneration

1

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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

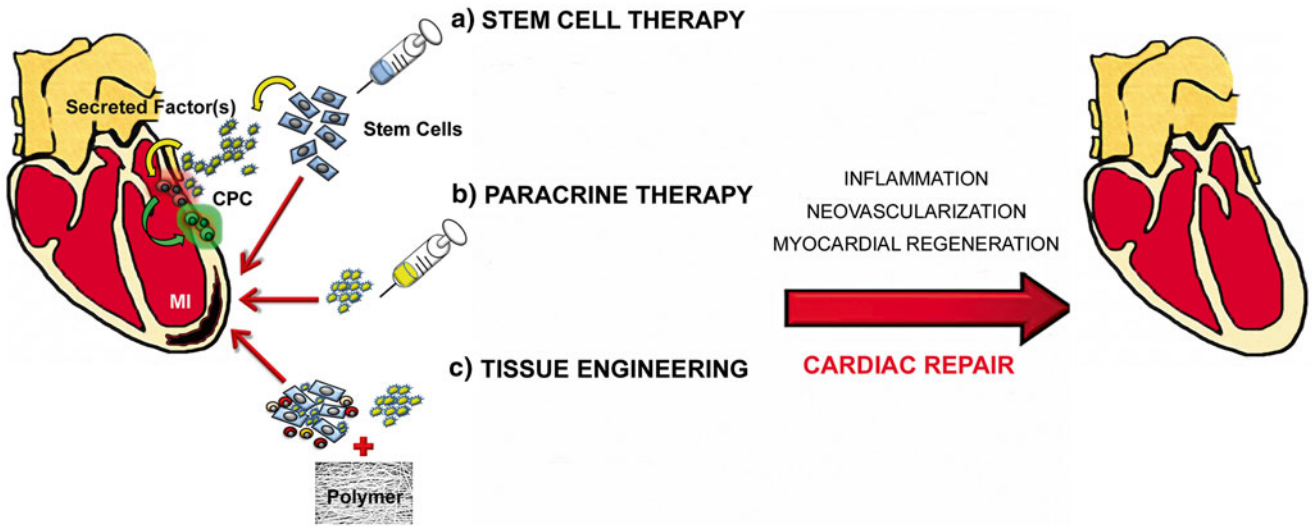

Fig. 1.1 Regenerative strategies for cardiac repair. To provide cardiac repair following ischemic injury such as myocardial infarct (MI), three different aspects should be addressed, that is (1) the modulation of the inflammatory response, (2) supporting neoangiogenesis and (3) providing new functional 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.