Stem Cell Biology and Regenerative Medicine

Lucas G. Chase
Mohan C. Vemuri *Editors*

Mesenchymal Stem Cell Therapy

Humana Press

Stem Cell Biology and Regenerative Medicine

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Preface

Over the past decade, significant efforts have been made to develop stem cell-based therapies for difficult-to-treat diseases. Multipotent mesenchymal stromal cells, also referred to as mesenchymal stem cells (MSCs), appear to hold great promise in regard to a regenerative cell-based therapy for the treatment of these diseases. Currently, more than 200 clinical trials are under way worldwide exploring the use of MSCs for the treatment of a wide range of disorders including bone, cartilage and tendon damage, myocardial infarction, graft-versus-host disease, Crohn's disease, diabetes, multiple sclerosis, critical limb ischemia, and many others (http://www.clinicaltrials.gov/).

MSCs were first identified by Friendenstein and colleagues as an adherent stromal cell population within the bone marrow with the ability to form clonogenic colonies in vitro. In regard to the basic biology associated with MSCs, there has been tremendous progress towards understanding this cell population's phenotype and function from a range of tissue sources. Despite enormous progress and an overall increased understanding of MSCs at the molecular and cellular level, several critical questions remain to be answered in regard to the use of these cells in therapeutic applications. Clinically, both autologous and allogenic approaches for the transplantation of MSCs are being explored. Several of the processing steps needed for the clinical application of MSCs, including isolation from various tissues, scalable in vitro expansion, cell banking, dose preparation, quality control parameters, delivery methods, and numerous others, are being extensively studied. Despite a significant number of ongoing clinical trials, none of the current therapeutic approaches have, at this point, become a standard-of-care treatment. Although exceptionally promising, the clinical translation of MSC-based therapies is still a work in progress.

The extensive number of ongoing clinical trials is expected to provide a clearer path forward for the realization and implementation of MSCs in regenerative medicine. Towards this end, reviews of current clinical trial results and discussions of relevant topics in association with the clinical application of MSCs are compiled in

this book from some of the leading researchers in this exciting and rapidly advancing field. Although not absolutely all inclusive, we hope the chapters in this book can promote and enable a better understanding of the translation of MSCs from bench to bedside and inspire researchers to further explore this promising and quickly evolving field.

Madison, WI, USA — Lucas G. Chase
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Chapter 1
Scaling-up Ex Vivo Expansion of Mesenchymal Stem/Stromal Cells for Cellular Therapies

F. Dos Santos, P.Z. Andrade, C.L. da Silva, and J.M.S. Cabral

Abstract The significantly large cell doses required in clinical trials with mesenchymal stem/stromal cells (MSC) demand for an efficient production of clinical-scale cell numbers. However, traditional cell culture techniques present several limitations making them unsuitable for the production of large numbers of MSC. Moreover, monitoring and control of MSC expansion are critical to provide a safe and reliable cell product for clinical settings. Bioprocess engineering, in particular bioreactors, offers the adequate tools to develop and optimize an efficient, cost-effective, and easily scalable culture system for the large-scale expansion of human MSC for cellular therapy.

Keywords Mesenchymal stem/stromal cells • Expansion • Bioreactors • Scale-up • Cellular therapy

1.1 Introduction

In recent years, the intense research on the multilineage differentiation potential and immunomodulatory properties of human mesenchymal stem/stromal cells (MSC) have indicated that these cells can be used to treat a range of clinical conditions including immunological disorders and degenerative diseases. Consequently, the number of registered clinical trials with MSC has been steadily increasing recently and include a wide variety of conditions namely hemato-oncological

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diseases, bone and cartilage defects, myocardial infarction, autoimmune diseases, and neurological disorders, among others [1].

However, the high cell doses required for MSC clinical applications ($0.4–9 \times 10^6$ cells/kg [2]) represent a major challenge for the field of Stem Cell Bioengineering. In order to meet the approval of regulatory agencies (FDA, EMA), clinical-scale MSC expansion protocols must meet the requirements for Good Manufacturing Practices (GMP) and be a reliable, reproducible, and efficient process able to generate large numbers of a safe and clinically effective cell product.

1.2 Traditional Clinical-Scale Expansion of MSC

Traditionally, the culture of MSC has been performed using standard plastic (i.e., polystyrene) tissue culture flasks. However, these static systems present several limitations: (a) cell productivity for adherent cells is normally limited to the area available in each flask; (b) culture parameters (pH, dissolved O_2) are difficult to monitor and control; and (c) extensive handling and labor hours are required for cell culture processing (i.e., medium renewal, cell passaging). In addition, static culture systems lead to the formation of concentration gradients of nutrients/metabolites/growth factors, temperature, pH, and dissolved O_2 that can affect cell proliferation throughout time in culture.

Moreover, at a clinical scale, the high cell doses applied to patients require considerably long culture times (up to 4 weeks) and a significantly high number of culture flasks. On the other hand, clinical-scale MSC expansion has been traditionally performed using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), from special batches that are highly controlled and approved for the use in clinical trials [2]. Consequently, large volumes of culture medium and FBS are needed throughout the entire cell expansion period.

Additionally, the characteristic tissue culture flask design, with a high headspace ratio *per* flask, together with the limited available cell growth surface area, makes it necessary to have a considerably large number of incubators to house all the required culture flasks for a clinical-scale expansion of MSC. All these factors, in addition to the high maintenance costs of a GMP-compliant facility, result in a large total expansion process cost, which could represent a major economical hurdle for the approval of a systematic use of MSC-based therapies by healthcare systems worldwide.

1.2.1 Alternative Static Culture Systems

With the aim of reducing the required space in the incubators for large-scale expansion of adherent cells, static devices consisting of stacking tissue culture surfaces, such as Cell Factories (Nunc) [3] and CellSTACK (Corning) [4], have been developed (Fig. 1.1). Additionally, these systems can be integrated with culture medium

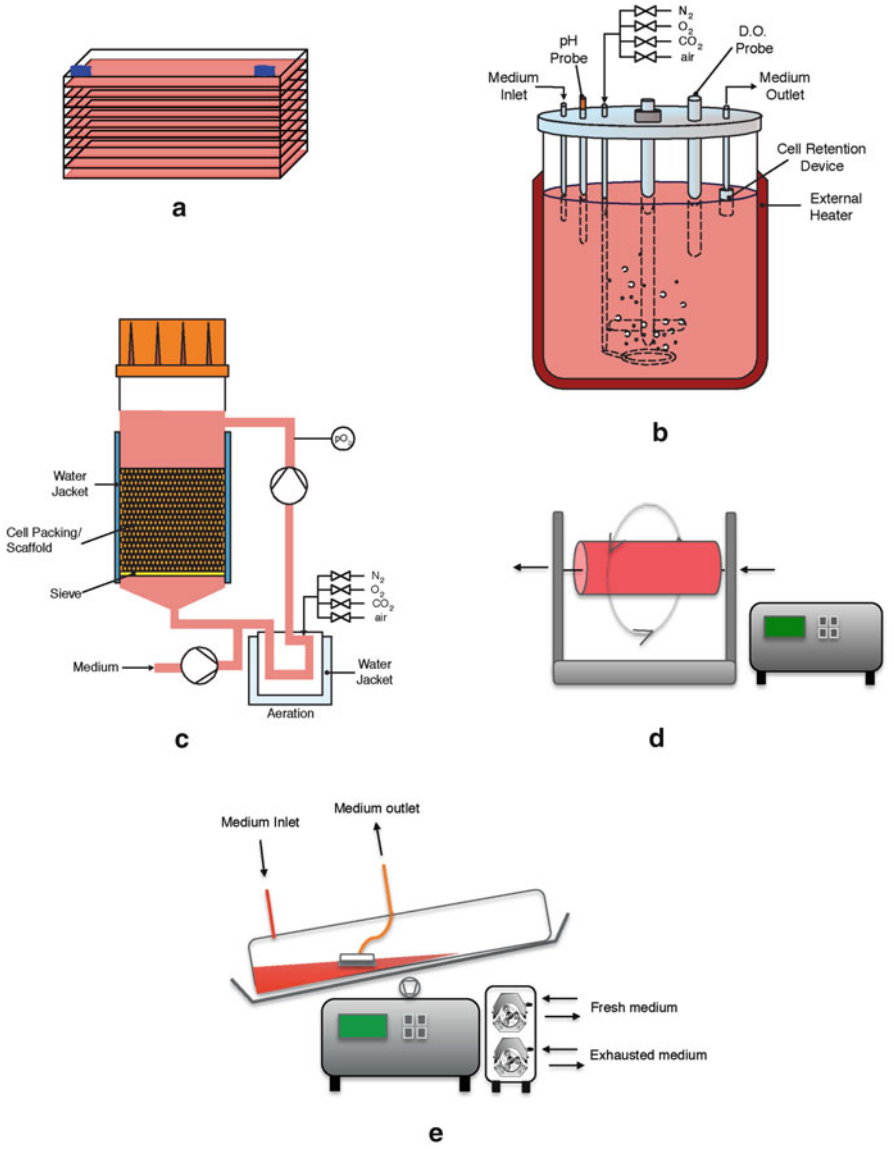

Fig. 1.1 Culture systems for the expansion of human MSC as an alternative to standard tissue culture flasks. (**a**) cell factory. (**b**) stirred tank bioreactor. (**c**) fixed-/packed-bed bioreactor. (**d**) rotating wall vessel bioreactor. (**e**) wave bioreactor

reservoirs in a closed system to reduce culture manipulation needs. Cell culture bags, permeable to gases, offer the alternative for a closed culture system, although are usually more routinely used for non-adherent cells. Nonetheless, a few systems have also been adapted for adherent cell cultures by specific treatments of the inner surfaces (VueLife™ AC Cell Culture Bags, American Fluoroseal Corporation). Petaka3G

(Discovery Scientific) is another cell culture device for adherent mammalian cells with a Gas Transfer Quenching System™ (Fig. 1.1) that enables cell culture without a CO_2 humidified incubator, thus allowing for ex vivo cell in culture transport. Another strategy was followed by Madj and collaborators who were able to successfully expand human MSC for 9 weeks using a dynamically enlarging culture surface, thus avoiding cell proliferation arrest due to cell confluency [5].

1.2.2 Expansion of MSC in Bioreactor Systems

The growing need of clinical-scale numbers of MSC for therapeutic applications requires a large-scale, fully monitored, and controlled bioreactor culture system for MSC production. Moreover, the design and optimization of different bioreactor systems allow for the expansion or differentiation of MSC accordingly to clinical needs. For Tissue Engineering settings, culture on different biomaterial scaffolds and under specific culture conditions can direct MSC differentiation to particular lineages of interest. On the other hand, the use of bioreactor systems for Cellular Therapy has different requirements. The main objective is typically to promote cell proliferation (rather than differentiation), while maintaining intrinsic MSC properties (i.e., immunomodulatory potential, multilineage differentiative potential). It is also necessary to harvest cells after achieving the required expansion levels since most of the MSC used in clinical settings targeting multiple organ systems have been infused intravenously [1].

1.2.3 Expansion of MSC in Stirred Bioreactor Systems

Through efficient mixing, stirred bioreactor systems enable the formation of a homogeneous macro-environment, thus eliminating gradients of nutrient/metabolite and gas concentrations and thereby creating a more favorable microenvironment for cell expansion. These systems can be operated with different feeding modes, such as batch, fed-batch, or continuous mode (using a perfusion system with cell retention). Moreover, advanced bioreactor systems allow for full monitoring and control of several culture parameters such as agitation, pH, and dissolved oxygen concentration (Fig. 1.1), which can be optimized to maximize cell number output. Disposable stirred bioreactor systems have been developed that, in combination with a fully controlled operation, would meet the standards for production of clinical-grade MSC according to GMP for Cellular Therapy settings.

As anchorage-dependent cells, MSC require a support for cellular adhesion, such as microcarriers, for an efficient culture under stirred conditions. Even though a recent report demonstrated that the expansion of human MSC into 3D spheroids enhance MSC anti-inflammatory properties [6], this type of culture requires a close monitoring and control of aggregate size in order to avoid cell necrosis, due to

limitations of nutrient and oxygen transfer to the inner core of spheroids, and to avoid unwanted cell differentiation, since this culture configuration has been shown to enhance multilineage MSC differentiation efficiency [7].

1.2.4 Microcarriers

Since first used by van Wezel in 1967 [8], microcarriers have been developed for culturing anchorage-dependent animal cells in stirred systems, either for cell cultivation targeting cells as a product [9–12] or towards the production of recombinant proteins [13]. Microcarriers present several advantages for cell culture including a large surface area to volume ratio (that allows for high adherent cell density cultures in a reduced medium volume), easy scalability, and simple cell harvesting. Hence, the use of an optimized microcarrier-based culture system can be crucial to reduce cell expansion process costs.

With an average diameter within the range of 100 and 400 μm, microcarriers can be classified by their structure and composition. Microporous (solid) microcarriers (pore size <10 μm) allow the formation of microenvironments that may be important for cell expansion (only attached on the surface) and/or differentiation, while macroporous microcarriers (pore size >20 μm) have a 3D structure that creates an environment that may favor cell expansion, for example, by mimicking an in vivo stem cell niche. Moreover, cells growing inside macroporous microcarriers might potentially be protected from shear stress caused by agitation and aeration that can be prejudicial for cell expansion or induce cell differentiation.

Nonporous microcarriers are also an alternative support for adherent cell culture. These include 2D surfaces, such as NUNC 2D MicroHex™ microcarriers, and 3D microcarriers usually made of polystyrene. In order to improve cell adhesion, these nonporous microcarriers are usually coated with common extracellular matrix proteins. In fact, SoloHill Engineering, Inc., a lead company in microcarrier production, offers a wide variety of animal protein-containing and animal protein-free microcarriers for anchorage-dependent cell culture.

The choice of the type of microcarrier to be used is closely related to the bioreactor design. High-density microcarriers are more suitable for fixed-bed/packed-bed perfusion systems, while stirred bioreactors require low-density microcarriers to generate a homogeneous suspension culture (Fig. 1.1). Another important issue is the protocol to detach cells from microcarriers, which is particularly crucial in Cellular Therapy settings where a single cell suspension has to be obtained for infusion. Some of the core materials used in microcarriers allow for a complete digestion with appropriate enzymes, such as *Cultispher-S*® [10] with trypsin or Cytodex with dextranase [14]. On the contrary, cell harvest from nonporous cross-linked polystyrene microcarriers, more appropriate for a GMP-compliant expansion compared to animal-derived microcarriers, require incubation with an enzyme and a subsequent filtration step to separate cells from microcarriers (i.e., made of polystyrene, dextran). However, a prolonged exposure to a proteolytic enzyme may

result in cell damage, particularly in terms of membrane receptors, and affect final characteristics of the cellular product. Consequently, other alternatives are currently being studied, such as microcarriers coated with thermosensitive polymers that allow cell detachment with a small temperature variation [15].

The phenomenon of cell bead-to-bead transfer described by several groups [16, 17] reveals another important advantage of using a microcarrier-based culture system. The ability to continuously increase the available surface area for cell expansion by simply adding fresh microcarriers, thus avoiding several cycles of potentially damaging enzymatic cell detachment and further seeding, is an advantage of a microcarrier-based culture system as compared to the standard tissue culture flask-based cell expansion.

1.2.5 Spinner Flask

Most studies of MSC cultivation in stirred systems have been performed in spinner flasks using microcarriers. Normally built in glass or plastic with a magnetic stirrer shaft, spinner flasks are available in different sizes, which allows for scalability from a few milliliters up to several liters (Fig. 1.1). Hence, spinner flasks are an ideal agitated system for laboratory-scale studies of different culture parameters, such as microcarrier type, agitation and feeding schemes, and cellular expansion kinetics.

The initial step of cell adhesion is a crucial step in microcarrier-based culture systems under stirred conditions, and it is dependent on cell source, microcarrier type, seeding method chosen, and culture medium used. For instance, Frauenschuh et al. observed that after 6 h of incubation, 80% of porcine bone marrow (BM) MSC adhered to *Cytodex™-1* microcarriers [18], while Schop et al. obtained a seeding efficiency of only 57% with human BM MSC using the same microcarriers under serum-containing conditions [17]. Moreover, in the latter study, *Cytodex™-1* microcarriers not only had the best performance in a wide comparison of seeding efficiency for different microcarriers, but also initial cell adhesion was enhanced in lower serum content medium [17]. Microcarrier coating with adhesion-related proteins, such as fibronectin (a major component of FBS), is another strategy to improve cell seeding. Eibes et al. drastically reduced the lag phase of BM MSC expanded in spinner flasks by coating *Cultispher-S®* microcarriers with FBS, thus obtaining a 100% cell seeding efficiency and promoting immediate MSC proliferation from day 0 [16]. In contrast, the absence of serum seriously impairs the cell attachment step to microcarriers [9]. Therefore, the optimization of the initial cell seeding, either using a more efficient coating of microcarriers or agitation scheme, is of major importance to maximize MSC expansion in stirred systems.

The first MSC expansion studies in spinner flasks were performed with nonhuman MSC (Table 1.1). Although MSC from different species were used, similar cell expansion kinetics was observed and, in all these studies, *Cultispher-S®* and *Cytodex™-1* microcarriers were shown to be the more efficient for MSC seeding

Table 1.1 Summary of the ex vivo expansion of nonhuman MSC in spinner flasks

Cells	Culture system	Microcarriers	Feeding scheme	Expansion	References
Rat BM MSC	Stirred plates	*Cultispher-S*® (cross-linked gelatin) *Cytodex™-1* (positively charged dextran) *Cytopore™-2* (cross-linked cellulose)	Medium renewal every 3 days after day 5	8.3-fold no expansion no expansion	[21]
Porcine BM MSC	Spinner flask 40 mL scale	*Cytodex™-1* (positively charged dextran)	50% renewal every 2 days	~9×10^5 cells/mL	[18]
Rabbit BM MSC	Spinner-flask	NA		6.2×10^5 cells/mL	[45]
Rat ear MSC	Spinner flask 100 mL scale	*Cultispher-S*® (crosslinked gelatin)	50% renewal on day 3	9×10^5 cells/mL	[20]
Goat BM MSC	Spinner flask 50 mL scale	*Cytodex™-1* (positively charged dextran)	30% renewal + microcarriers every 3 days	4 PD	[19]

PD population doublings

and proliferation. In fact, Frauenschuh et al. [18] and Schop et al. [19] used *Cytodex™-1* microcarriers to expand porcine and rat BM MSC, respectively, while *Cultispher-S®* microcarriers proved to be efficient in the expansion of rat ear MSC [20]. In terms of feeding regime, each study adopted different medium renewal periods and volumes. Although culture metabolic analysis was not performed for all the studies, Schop et al. demonstrated that frequent feeding was important to prevent cell growth arrest by nutrient limitation or excessive metabolite accumulation. In addition to medium renewal, this team added fresh microcarriers to the culture with the objective of increasing the available surface area and, consequently, to prevent or minimize the formation of cell and bead aggregates [19]. As an alternative to spinner flasks, Yang et al. successfully expanded rat BM MSC in a spin microcarrier culture using stirred plates [21]. However, this type of system is not able to achieve the same level of culture homogeneity as compared to spinner flasks, thus not avoiding the formation of concentration gradients that may affect cell expansion. Even though MSC expansion kinetics usually varies between species [22], these studies provided important information (such as cell seeding on microcarriers and more efficient feeding regimens) for the subsequent studies of human MSC expansion in spinner flasks.

Few reports on human MSC expansion in spinner flasks exist in the literature (Table 1.2). Both BM-derived MSC and adipose-derived stromal/stem cells (ASC) have been used. Based on their previous work, Schop et al. were able to expand human BM MSC up to a cell density of approximately 1.75×10^5 cells/mL using *Cytodex™-1* microcarriers and a feeding strategy of 50% medium renewal together with 30% addition of medium containing empty microcarriers every 3 days [17]. Likewise, Eibes et al. used *Cultispher-S®* microcarriers pre-coated with FBS to improve initial cell adhesion and successfully reached a cell density of 4.2×10^5 cells/mL (an 8.5-fold increase in total cell number), with a 25% daily medium renewal [16]. Foreseeing an urgent need for the expansion of human MSC for clinical applications in the absence of animal-derived serum, dos Santos et al. were able to adapt a microcarrier-based culture system to xeno-free conditions for the successful expansion of both human BM MSC and adipose-derived stem/stromal cells (ASC) up to 2.0×10^5 cells/mL and 1.4×10^5 cells/mL, respectively [9]. On the other hand, Zhu et al. demonstrated the feasibility to expand human ASC without microcarriers in spinner flasks, by using a framework that sustained collagen/chitosan scaffolds suspended in the spinner flask and achieving a 26-fold increase in total cell number [23].

The easy scalability of a microcarrier-based stirred culture system to large-scale fully controlled bioreactors makes it a very promising approach for the clinical-scale expansion of human MSC. In fact, MSC expansion results obtained thus far with spinner flasks demonstrate that a 1–2 L scale would be sufficient to reach a clinically relevant cell dose for a patient [9]. Moreover, fully controlled stirred bioreactor systems will allow for a more complete optimization of culture parameters (such as dissolved oxygen tension, aeration rate, and pH) when compared to laboratory scale basic spinner flasks [24].

Table 1.2 Summary of the ex vivo expansion of human MSC in spinner flasks

Cells	Culture system	Microcarriers	Feeding scheme	Expansion	References
hBM MSC	Spinner flask, 50 mL scale	*Cultispher-S*® (cross-linked gelatin) pre-coated with FBS	25% daily renewal	4.2×10^5 cells/mL 8.5-fold	[16]
hBM MSC	Spinner flask, 100 mL scale	*Cytodex™-1* (positively charged dextran)	50% renewal + 30% addition of microcarriers	~1.75×10^5 cells/mL 4.8 PD	[17]
hBM MSC hASC	Spinner flask, 80 mL scale	Plastic pre-coated with CELLStart™ daily renewal	Xeno-free medium, 25% renewal upon day 3	2.0×10^5 cells/mL 18-fold 1.4×10^5 cells/mL 14-fold	[9]
hASC	Spinner flask	Collagen/chitosan scaffolds Suspended by needles		2.2×10^6 cells/mL 26-fold	[23]

1.2.6 Rotating Wall Vessel

The use of rotating wall vessels (RWV) (Fig. 1.1) for MSC culture has been more focused on Tissue Engineering studies targeting MSC differentiation, rather than MSC expansion. Firstly developed by the National Aeronautics and Space Agency (NASA) with the objective of studying the effect of microgravity on cell/tissue culture, it enables the creation of a low shear microenvironment suitable for the expansion of shear-sensitive cell types. For instance, Sheyn et al. studied the effect of microgravity on MSC osteogenic potential [25], while Frith's group cultured MSC as spheroids and observed an enhancement of osteogenic and adipogenic differentiation [26]. The few studies focusing on MSC expansion in RWV consisted of coculture systems (with hematopoietic stem cells) both from BM [27] and umbilical cord blood (UCB) [28] and resulted in limited levels of MSC expansion.

1.2.7 WAVE Bioreactor™

The WAVE Bioreactor™ (GE Healthcare) system is a simple culture system composed by gas-permeable bags on a rocking platform to create a low shear wave fluid dynamics, with high oxygen transfer and a large range of working volumes (0.1 to 500 L) (Fig. 1.1). The WAVE Bioreactor™ system also provides a simple and reliable perfusion method using a disposable Cellbag™ bioreactor with an integral perfusion filter. In addition, the disposable bags represent an important feature for a GMP-compliant expansion protocol. Although there are no reported applications for MSC expansion, the wave bioreactor system was already shown to efficiently expand tumor-infiltrating lymphocytes in suspension [29] and embryonic feline lung fibroblasts on *Cytodex™-1* microcarriers [30].

1.3 Flow Perfusion Bioreactor Systems

In a perfusion bioreactor, the continuous renewal of medium and its distribution throughout the bioreactor core (cell packing or scaffold) allows for efficient mass transfer rates [31]. Most perfusion bioreactor systems consist of a media reservoir, a pump, and a perfusion cartridge/chamber interconnected by a tubing circuit, allowing medium perfusion directly through the scaffold (which usually fill the whole space of the perfusion chamber) (Fig. 1.1). Moreover, the different possible configurations (column bioreactors, parallel plates, hollow fiber bioreactors, among others) widen the range of materials that can be used for cell cultivation (such as microcarriers or biomaterial scaffolds).

Nonmechanically agitated flow perfusion bioreactors were first used for the expansion of BM and UCB mononuclear cells (MNC) [32–34] in an attempt to mimic the intricate microenvironment of the BM. This system allowed cell-to-cell and cell-to-matrix interactions, which do not take place in suspension culture systems. In fact, perfusion chambers, due to their low-flow nature, better mimic the BM microenvironment by allowing a low-shear environment that promotes the concurrent development of stroma, stem cells, progenitors, precursors, and mature cells from an MNC fraction of BM [33]. In addition, a tubular perfusion bioreactor can be adapted to mimic specific in vivo niches, such as the BM, which can be important for expansion/differentiation studies of MSC [35].

In the literature, the majority of studies whereby MSC were cultured in perfusion systems were focused on the production of tissue engineered constructs. The ability to control important culture parameters (i.e., shear stress and biomaterial scaffolds) that are critical for the creation of an efficient cell differentiation-inducing microenvironment makes perfusion systems a powerful tool for Tissue Engineering. Examples where this system has been modulated in order to promote MSC differentiation include bone grafts [36, 37] and cartilage [38]. In particular, it has been suggested that shear stress higher than 0.1–0.15 dyn/cm^2 is sufficient to significantly enhance MSC differentiation towards the osteogenic lineage [39], namely by increasing the amount of mineralization and/or the upregulation of osteogenic genes such as osteopontin and osteocalcin [40].

However, once a correct balance between low shear stress (achieved by low flow rates and/or high cross-section area of medium flow) and suitable mass/oxygen transport conditions is found and the correct physical and chemical stimuli of the scaffold has been determined, perfusion bioreactor systems can be a powerful tool for the expansion of MSC for Cellular Therapy.

1.4 Culture and Cell Characterization Tools

The development of large-scale culture systems for the expansion of MSC should take in consideration the integration of culture and cell product analysis tools. A continuous on-line measurement of nutrients (i.e., glucose and glutamine) and metabolites (i.e., lactate and ammonia) can allow for not only adapting the feeding regimen, but can also be used as an indirect method to periodically determine cell number without manual cell sampling. Throughout time in culture, bacteriological and mycoplasma testing should be rigorously performed to guarantee cell product safety. In addition, standardized controls should be implemented to attest the phenotype and functionality (i.e., differentiation potential, immunomodulation, hematopoiesis support, and clonogenicity), as well as safety (i.e., karyotyping assays, transcriptomics, and proteomics) of MSC upon ex vivo culture before for the release of a GMP clinical grade MSC-based product [41].

1.5 Conclusions and Future Challenges

In the near future, a more widespread, cost-effective, and regular use of MSC for Cellular Therapy must be sustained by an efficient, reliable, and reproducible MSC production system. Large-scale bioreactor technology offers significant advantages in terms of cell productivity, culture homogeneity, monitoring, and control. Moreover, in order to be GMP-compliant, the ex vivo expansion of MSC will require clinical-grade media systems, as well as effective standards and methodologies for preclinical safety and efficacy evaluation, product characterization, and process validation and control [42].

The influence of earlier developments in microbial and mammalian cell culture for production of molecular medicines on the development of novel culture systems to be used in the field of Regenerative Medicine is remarkable [43]. The trend towards disposable bioreactors for the biopharmaceutical protein sector (available up to 1000 L) has become very strong, as the cost of cleaning, the risks associated with sterilization, and the amount of effort required for validating the safety of a reusable process are extremely high [44]. Following that trend, there is already disposable stirred-bioreactor technology available (highly controlled and closely-monitored) which is GMP-compliant available for application in Cellular Therapy and Tissue Engineering settings. The integration of such large-scale disposable bioreactors in completely closed culture systems, requiring minimal intervention, together with the development of efficient downstream operations for cell purification, will represent a major advance towards the production of clinical-grade MSC numbers for therapeutic applications.

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Chapter 2
Mesenchymal Stromal Cell Mechanisms of Immunomodulation and Homing

J. Barminko, A. Gray, T. Maguire, R. Schloss, and M.L. Yarmush

Abstract The identification of therapeutic immunomodulatory mesenchymal stromal cells (MSC) with specific homing capabilities has simultaneously contributed to the potential development of powerful cellular immune therapies, with applications for a variety of inflammatory associated diseases. MSC have the ability to directly abrogate T cell, macrophage, dendritic cell (DC), neutrophil, and B cell pro-inflammatory functions. Specifically, T cell, macrophage, and DC MSC-mediated immunosuppression results in the adoption of phenotypes indicative of type II anti-inflammatory functional cells. These findings collectively suggest that MSC directly combat inflammation by controlling endogenous immune mechanisms. In this chapter, the molecular/cellular mechanisms governing these phenomena are discussed for each MSC-immune cell interaction. Furthermore, MSC homing mechanisms are discussed, highlighting our current understanding of the modes and limitations of MSC direct implantation modalities.

Keywords Inflammation • Mesenchymal stromal cell

2.1 Introduction

Mesenchymal stromal/stem cells (MSC) have become a promising therapy for various inflammatory disease applications. MSC are being explored as a treatment for myocardial infarction [1], graft-versus-host disease (GVHD), colitis [2], liver failure [3], kidney failure [4], Crohn's disease [5], central nervous system (CNS) trauma [6], and several autoimmune diseases [7–9]. Despite the fact that the precise MSC therapeutic mechanisms are unclear, to date there are many clinical trials

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ongoing to evaluate their safety and efficacy [10]. In many of these applications MSC differentiation was thought to be the primary mechanism of action. However, a considerable amount of data suggest improved outcomes after transplantation even in the absence of apparent MSC long-term engraftment [11]. The contemporary paradigm being popularized is that MSC promote therapeutic benefits via secretion of soluble factors and cues which control immune cell functions and provide trophic support. This idea is supported by in vitro coculture studies as well as in vivo transplantation studies. As such, numerous researchers have begun to regard MSC, not only as traditional differentiating stem cells, but also as cellular drug delivery vehicles [11, 12]. Therefore, it has become evident that by evaluating the molecular factors that these cells may contribute to the local inflammatory milieu, we will ultimately be able to assess the effectiveness of their therapeutic applications. While the evaluation of MSC as a potential therapy is moving forward, the mechanisms of action are still uncertain. Ostensibly, MSC provide immune support in these systems via different mechanisms, depending on the specific disease state. Therefore, depending on the application, different MSC-secreted products will be vital. To effectively characterize these, one must understand the specific cellular mediator(s) which accompany the respective pathologies. MSC effects could then be evaluated based on the MSC's ability to modulate particular immune cell(s) functions. As a result, therapies could be tailored to maximize these interactions. Just as important to the success of MSC as a therapy is the efficiency of MSC targeting as well as persistence at the site of injury. MSC abundance and persistence in vivo will likely be crucial for therapy evaluation and clinical translation. However, specific MSC mechanisms, homing potential and persistence in vivo are currently controversial. Here we discuss the current knowledge pertaining to MSC mechanisms of immunomodulation and review the effects of MSC on several immune system networks. In addition, the chapter will conclude with a discussion on MSC mechanisms of homing.

2.2 MSC Modulation of T Cells

Thymus-derived (T) cells recognize antigens and are critical for acquired immunity. These cells originate in the bone marrow and mature within the thymus into one of several subtypes with diverse functions as either direct effector cells or immunomodulating cells. These functions include maintenance of self-tolerance, lysis of infected cells, activation of other lymphocytes, and interaction with cells of the innate immune system. Some of the T cell subsets that have been investigated in the context of MSC-mediated immunomodulation are described in Table 2.1.

The first evidence that MSC can regulate immunosuppression in vivo came from models of GVHD [13]. These studies demonstrated that MSC could reduce allograft rejection, which is partly mediated by T lymphocytes [14, 15]. Shortly after, MSC T cell immunosuppression was demonstrated in vitro [16]. Subsequently, MSC became a candidate therapy for several autoimmune-related syndromes, where

Table 2.1 T cell subsets

T cell subtype		Major functions	Identifying proteins	Important secreted proteins	Effect of excessive or disrupted activity	References
Helper T cells	T_h1	Cell-mediated immunity. Enhancement of inflammation by promoting cytotoxic T cell proliferation and macrophage eradication of intracellular microbes	CD4	TNF-α, TNF-β, IFN-γ	Autoimmunity	[146, 147]
	T_h2	Antibody-mediated (humoral) immunity: induction of B cell production of IgE2 and promotion of eradication of parasites; antagonize effects of T_h1	CD4	IL-4, IL-5, IL-13	Allergies, hypersensitivity	[146, 147]
	T_h17	Induction of neutrophil mobilization in response to injury and infection to eradicate pathogens	CD4	IL-17, IL-23	Autoimmunity	[43, 148–150]
Cytotoxic T cells (CTL)		Destruction of cancerous or infected cells expressing MHC class I and foreign antigens	CD8	Perforin, granzymes	Autoimmunity	[56, 151, 152]
Regulatory T cells	T_{reg}, including T_h1 and T_h3	Maintenance of self-tolerance and immune homeostasis via immunosuppression and inhibition of helper T cells	CD4, CD25, Foxp3	I1-10, TGF-β	Autoimmunity	[153, 154]

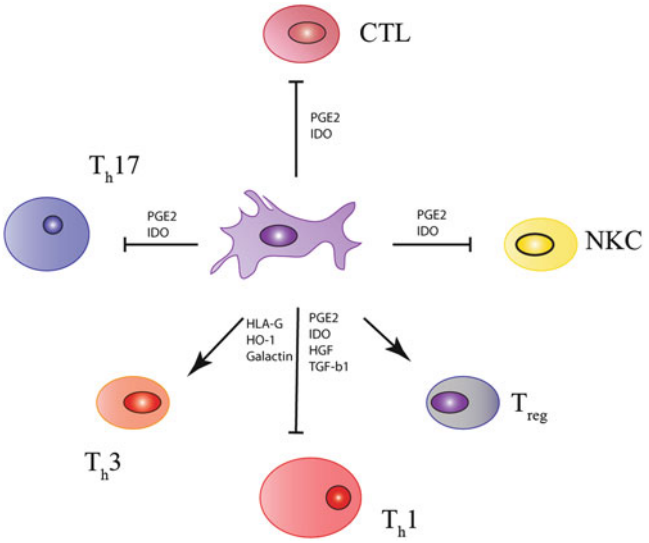

Fig. 2.1 The control of T lymphocyte differentiation and effector function by MSC. MSC modulate several aspects of T cell behaviors, primarily preventing the differentiation and expansion of pro-inflammatory mediators, T_h1 and T_h17. Simultaneously, MSC promote T cell phenotypes which possess immunomodulatory behaviors, T_h3 and T_{reg}. Additionally, MSC demonstrate the ability to prevent cytotoxic T and natural killer cell (NKC) functions. Overall, MSC dictate T cell functions which suppress the adaptive immune response potentiating their application in T cell associated disorders

MSC effects on destructive T cell behaviors could be harnessed. MSC transplantation in animal models of experimental autoimmune encephalomyelitis (EAE) has resulted in a marked decrease in myelin degeneration [7]. MSC reduce T cell expansion in vivo and provide neuroprotection as seen by preserved axons and reduced CNS inflammation [9]. Also, MSC have been found to promote differentiation of naïve T cell into T_h2, providing protection against demyelination and axon loss [17–19]. MSC have been explored as a potential therapy for rheumatoid arthritis (RA), controlling T cell-mediated degradation of collagen [8]. In all of these disease states, MSC control T cell-mediated autoimmunity. We now discuss the proposed mechanisms which drive the observed phenomena (summarized in Fig. 2.1).

It has been shown that MSC do not promote T cell apoptosis, but rather induce T cell anergy [7, 16]. Gonzalez et al. reported MSC suppression of activated $CD4^+$ and $CD8^+$ T cell proliferation and simultaneous promotion of T-regulatory responses as measured by enhanced interleukin (IL)-10 secretion and an increase in Foxp3-expressing $CD4^+CD25^+$ T cells [20]. These regulatory T cells (T_{reg}) were found to suppress collagen-specific T cell responses in RA models. This same group observed an identical phenomena in an experimental model of colitis [21]. Others have similarly observed MSC promotion of T_{reg}, but also T_h3 phenotypes [22]. To date there have been several reports of the ability of MSC to reduce T_h1 activities and simultaneously promote T_{reg} phenotypes [23, 24]. Najar et al. reported that this capability is

dependent on cell–cell contact as well as specific MSC to T cell ratios, with low ratios resulting in enhanced rather than reduced proliferation [25]. However, the percentage of T_{reg} in the population with a low MSC to T cell ratio was still elevated. Therefore, it will be important to consider the number of MSC required to achieve a desired effect. This will be regulated not only by the actual number of injected MSC, but also by successful cell homing and persistence. Beyth et al. similarly found that MSC facilitate T cell unresponsiveness in a cell contact and ratio-dependent manner, which is dictated by the microenvironment [26]. It was also determined that MSC reduced interferon (IFN)-γ secretion in PHA activated T cell cultures. However, this could be partially restored when lipopolysaccharide (LPS) or CD40 was used as stimulators. MSC modulation of T cell immunity may also involve other immune cells. Beyth et al. demonstrated the MSC effect on T cells to be dependent on monocytes in a dose-dependent manner [26]. This suggests that multiple cells may be involved in the overall mechanism of MSC-mediated modulation of T cell functions.

While the effects of MSC on T cell subsets is well established from in vitro and in vivo studies, the precise mechanism driving these responses is not well understood. There is evidence that Toll-like receptor (TLR)-2 stimulation augments MSC immunosuppressive behaviors by increasing secretion of indoleamine 2, 3-dioxgyenase 1 (IDO1) and thus elevating the levels of kynurenines in the environment [27]. IDO1 is the rate limiting enzyme in kynurenine-dependent catabolism of tryptophan, which will halt T cell proliferation. It was shown that this was dependent on autocrine secretion of IFN-β, which was dependent on protein kinase R (PKR), but independent of IFN-γ [28]. DelaRosa et al. also identified IDO to be essential to MSC T cell immunosuppression amongst several candidate mediators including IL-10, hepatocyte growth factor (HGF), prostaglandin E2 (PGE2), transforming growth factor-beta 1 (TGF-β1), and nitric oxide (NO) [29]. IFN-γ was found to be an inducer of MSC IDO1 secretion, but alternative modes of IDO1 activation were not excluded. Ryan et al. identified a similar phenomenon; however, HGF, TGF-β1, and PGE2 were found to partially mediate immunosuppression [30, 31]. In fact, PGE2 was recently identified to play a significant role in effecting T lymphocyte subset functions [32, 33]. Therefore it is likely that not one mediator and molecular pathway is solely responsible for MSC immunosuppression but rather a synergistic effect results in maximal immunosuppression. Interestingly, the IDO1 mechanism of immunosuppression has been found to be specific to human MSC. Rat and murine MSC do not exhibit IDO1-mediated immunosuppression [34]. Instead, NO secretion in rodent models has been shown to mediate MSC T cell immunosuppression [28, 35, 36]. Ren et al. showed that MSC immunosuppression is driven by IFN-γ and tumor necrosis factor (TNF)-α mediated stimulation of NO, which locally promotes T cell anergy. MSC secretion of chemotactic factors attracts T cells and NO is secreted locally to promote anergy. In addition, several investigators have highlighted human leukocyte antigen G (HLA-G) as a mediator of MSC T cell immunosuppression [37]. MSC HLA-G secretion was found to be IL-10 dependent and maximum when in direct contact with the T cells [38]. HLA-G blocking reversed MSC immunosuppressive effects. Others have observed that MSC secretion of

galectin 1 and 3 mediates their immunosuppressive behaviors [39, 40]. siRNA knockdown of these proteins completely abolished MSC T cell immunosuppression [39, 40]. Most recently the stress protein Heme oxygenase (HO)-1 has been found to promote the MSC-mediated adoption of the T_{reg} phenotype [22]. However, when implemented in an inflammatory T cell reaction, HO-1 effects were trumped by other mediators. At the transcriptional level, signal transducer and activator of transcription 3 (STAT3) has been shown to play a crucial role in MSC effects on antigen presenting cells (APC); when STAT3 was blocked, attenuation of pro-inflammatory T cell secretion ceased [41].

It is clear that there is conflicting data supporting the exact mediators of MSC T cell immunosuppression. These discrepancies may be attributed to varying MSC isolation and culture techniques. One must also keep in mind that the mechanisms used to stimulate and isolate T cells will result in variable MSC responses and could therefore lead to differing observed mechanisms. Lastly, it may be important to distinguish the functions used to assess specific MSC factor effects. For example, Aggarwal et al. utilized T cell IFN-γ secretion as the functional outcome to prove that PGE2 is responsible for MSC immunosuppression [42]. However, Ren et al. evaluated proliferation as the T cell output parameter and identified that IDO was the primary MSC secreted factor responsible for immunosuppression [35]. In fact, it may very well be that both factors contribute to the overall response. One factor may be responsible for promoting T cell annergy (IDO) and another for promoting T_{reg} (PGE2) phenotypes. It is important to note these distinctions in the literature to fully appreciate the mechanisms MSC exploit to carry out immunosuppression.

2.2.1 MSC Inhibit T_h17 Naïve T Cell Differentiation

The recently identified $CD4^+$ T_h17 subset secretes IL-17 and has been implicated in several models of autoimmunity as an integral component of disease progression [43]. These cells are essential for effective microbial elimination through secretion of several cytokines which facilitate microbial clearance [44]. However, with respect to their role in autoimmunity, they contribute to a persistent inflammatory response and recently MSC have been shown to control T_h17 inflammatory functions. Ghannam et al. investigated the role IFN-γ and TNF-α have on enhancing MSC CD54 expression, thus permitting T_h17 adhesion to MSC via the CCR6-CCL20 interaction [45]. It was observed that $CD4^+$ T cells could not differentiate into T_h17 cells when cultured in direct cell contact with MSC. There was decreased secretion of IL-17, IL-22, IFN-γ, and TNF-α, hallmark T_h17 secretion patterns, and this effect was partially mediated by PGE2. Also, there was enhanced IL-10 secretion as well as epigenetic alteration leading to T_{reg} expression of Foxp3. All these phenotypes were enhanced when MSC were pre-incubated with IFN-γ and TNF-α. Duffy et al. observed a similar phenomenon. When indomethacin (PGE2 blocker) and selective COX-2 inhibiter were added to the coculture, MSC inhibition was reversed [46]. It was then shown that PGE2 binding of the prostaglandin

E receptor 4 (EP4) was responsible for mediating the modulatory effects of MSC in preventing T_h17 differentiation. Recent studies by Tatara et al. indicate that MSC prevent naïve T cell T_h17, but not T_{reg}, differentiation and that the inhibition was partly attributed to MSC PGE2 and IDO secretion [47]. These in vitro findings have been found to be consistent with in vivo observations. Park et al. identified reduced T_h17 and elevated T_{reg} populations in an experimental model of autoimmune arthritis after MSC transplantation [48]. Rafi et al. demonstrated the same in vivo result, but in a model of EAE. They found that IL-17 and TNF-α levels were reduced as was $CD4^+$ T cell infiltration into the spinal cord [49]. While these data are very encouraging, other reports suggest that MSC promote an opposing phenomenon, where they enhance T_h17 proliferation and function [50]. It is important to note that this study incorporated MSC at a 1:10 ratio. It has been observed that at lower ratios MSC promote inflammatory responses [25]. These types of findings convey the importance of understanding the cell doses needed to elicit desired MSC effector functions. Overall, it appears that MSC exert their control over T cell function both by preventing inflammatory phenotypes and simultaneously promoting the differentiation of anti-inflammatory T cell subtypes.

2.2.2 MSC Modulation of Natural Killer Cells and Cytotoxic T Cells

Natural killer (NK) cells are granular cytotoxic lymphocyte effector cells belonging to the innate immune system. The most well-known function of NK cells is the lysis of foreign or infected cells via release of cytotoxic granules or death receptor activating molecules [51]. This is accomplished by the complex interaction of stimulatory and inhibitory signals on target cells with NK receptors [52]. The MSC NK cell interaction is a very interesting one. Spaggiari et al. demonstrated that coculture of autologous or allogeneic MSC with IL-2 activated NK cells resulted in MSC lysis [53]. NK cell cytotoxic activity has been attributed to receptors NKp30, NKp44, and NKG2D, whose ligands, ULBPs, PVR, and Nectin-2, are expressed on MSC. Interestingly, when MSC were pre-activated with IFN-γ, NK cells no longer exhibited cytolytic activity [53]. More recently MSC have been found to prevent IL-2-induced NK cell proliferation as well effector functions [54]. In addition, MSC reduce cytotoxic activity and cytokine production as well as surface expression of the activating NK receptors NKp30, NKp44, and NKG2D. It was determined that MSC secretion of IDO1 and PGE2 was responsible for regulating this effector function. Others have suggested that HLA-G5 may also promote MSC effects on NK cell effector functions [38]. Seemingly, MSC would have to be present while NK cells are stimulated. Rasmusson et al. observed that MSC could not prevent NK cell functions when implemented post-NK cell activation; however, these studies were performed in a mixed lymphocyte reaction (MLR) [55]. Sotiropoulou et al. also observed similar MSC NK cell interactions. They claimed that certain MSC effects on NK cells are dependent on cell number, where low ratios of MSC to NK

cells can modulate NK cell function most effectively [56]. They reported that PGE2 and TGF-β1 both have roles in mediating MSC effects on NK cell cytotoxic function.

Cytotoxic T cells (CTL) are a subset of $CD8^+$ lymphocytes which are primarily responsible for inducing somatic and tumor cell lysis. They have been implicated in the progression of autoimmune diseases as well as other tissue pathologies [57, 58]. MSC effects on CTL functions are dependent on the time of implementation. MLR assays have been employed to assess MSC effects on CTL temporally. MLRs are initiated with allogenic T cell cocultures leading to proliferation and after approximately 48 h, the formations of CTL with cytotoxic capabilities. If MSC are added in the beginning of the MLR, they reduce CTL lysis by 70 %, in the absence of cell–cell contact [55]. However, they did not affect cell lysis when added 3 days into the reaction. In contrast to NK cells, direct CTL lysis of MSC has not been observed [55, 59]. Others have observed that MSC failed to modulate CTL proliferation and IFN-γ secretion once CTLs were exposed to the pathogenic viruses CMV or EBV [60]. It appears that MSC modulation of NK cells and CTL will depend upon their state of activation, which will make the timing and persistence of MSC administration critical for controlling immune cytolytic behaviors.

2.3 MSC Modulation of B Cells

B cells play an essential role in adaptive immunity. They are directly responsible for the humoral immune response via the secretion of antibodies against pathogenic or foreign antigens. A subset of B lineage cells differentiates into memory B cells, which can mediate a rapid response upon secondary exposure to that same antigen. Aberrant antibody production by B cells has been implicated in several autoimmune diseases, including systemic lupus erythematosus (SLE), and MSC have been found to modulate these responses. Stimulated B cells were arrested in the G0/G1 phase of the cell cycle when cocultured with MSC [61]. Reduction in IgM, IgG, and IgA production indicated a decrease in B cell differentiation. Furthermore, B cell responses to chemotactic ligands, specifically SDF-1 and BCA-1, were reduced as well as their expression of several membrane expressed chemotactic receptors [62]. Tabera et al. also observed B cell arrest in the G0/G1 phase of the cell cycle when cocultured with MSC, independent of cell–cell contact [63]. Furthermore, dendritic cell (DC) promotion of B cell differentiation, as seen by an increase in $CD38^{++}CD138^{++}$, as well as increased immunoglobulin secretion, was inhibited in the presence of MSC. Asari et al. reported similar findings with MSC and LPS-stimulated B cell contact-independent cell cultures [64]. Schena et al. suggest that this inhibitory effect was augmented in the presence of IFN-γ; however, IDO1 was not the mediator of this response as it was with T cells [65]. They claim that cell–cell contact enhances MSC function and that the interaction between programmed death 1 (PD-1) receptor and the PD-1 ligand mediates the inhibitory effects of MSC (Fig. 2.2). This has been observed previously by Augello et al. [66]. Interestingly,

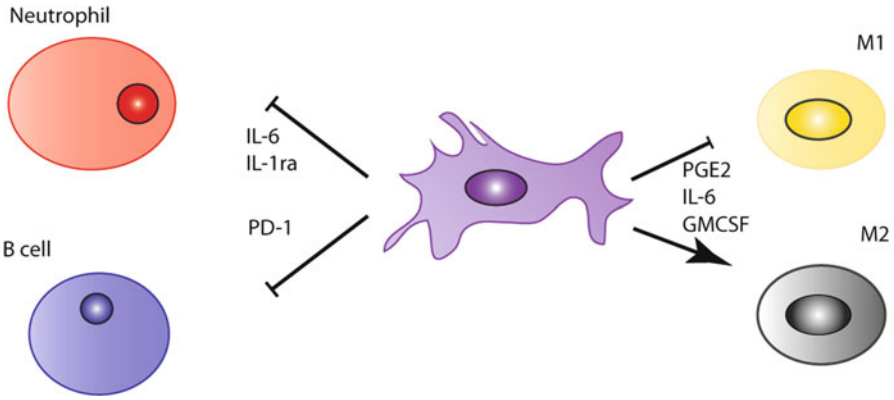

Fig. 2.2 Modulation of macrophage, B and neutrophil cell pro-inflammatory functions. MSC direct macrophages toward an M2phenotype and simultaneously suppress M1 functions. MSC inhibit B cell differentiation via the PD-1 surface receptor, which in turn reduces immunoglobulin and chemokine production. MSC also provide effector function on neutrophils via HLA-g and IL-6, preventing pro-inflammatory secretion and migration

TLR-9 activation of B cells did not induce an MSC inhibitory response, but B cell receptor (BCR)-dependent activation was inhibited by MSC [65]. Furthermore, there is no conclusive in vivo data establishing an MSC ability to affect B cell functions. Schena et al. observed that MSC, in a model of lupus, reduce nephron glomerulosis [65]. However, neither reduced immunoglobulin levels nor other changes in B cell phenotypes were detected. Others have observed no effect whatsoever on B cells post MSC transplantations. Youd et al. reported that MSC do not have therapeutic potential in lupus models driven by type II inflammation-associated disorders [67]. However, this study used one transplanted dose of MSC and did not titrate or evaluate multiple injections. Also, this group waited until disease onset to implant MSC [67]. While the in vitro data on the MSC effect on B cells is suggestive, more studies need to be done to reveal the potential of MSC in treating B cell-mediated disorders.

2.4 MSC Modulation of Macrophages: Promotion of the M2 Phenotype

Macrophages are phagocytic cells of the myeloid lineage, differentiated from monocytes and present in essentially all tissues. They play major roles in adaptive and innate immunity and are able to perform pathogen clearance in the absence of phagocytic labels for pathogen ingestion/destruction (opsonization) and act as APC. Considering their abundance throughout the body, the macrophage is an essential player in tissue damage as well as the overall immune response.

Macrophage behaviors have been implicated in pathology after organ trauma [68], allograft organ rejection [69] and atherosclerosis [70]. Over the past several years, the complexity of the macrophage response has been documented as well as the role of phenotypic plasticity in macrophage responses. The major implication of these observations has been the distinction of classically (M1) and alternatively (M2) activated macrophages [71]. M1 macrophages represent the pro-inflammatory arm of the macrophage response while M2 is the anti-inflammatory arm. Intriguingly, MSC secrete several of the factors found to promote M2 phenotypes either constitutively or in the presence of certain soluble cues (Fig. 2.2). Furthermore, considering the tremendous amount of data supporting the ability of MSC to modulate immune responses, it is no surprise that MSC have been found to promote M2 macrophage phenotypes in the presence of stimuli which normally lead to M1 phenotypes. Kim and Hematti were the first to observe that macrophages cultured in the presence of MSC adopted phenotypes indicative of M2 macrophages ($CD206^{high}$, $IL\text{-}10^{high}$, $IL\text{-}12^{low}$) after 48 h of culture [72]. These studies were performed in the absence of cell–cell contact, suggesting that soluble factors were responsible for the phenomenon. Gonzales et al. cocultured colitis-derived macrophages with MSC and found that the pro-inflammatory secretion of TNF-α and IL-12 was diminished [21]. Anti-inflammatory IL-10 secretion was found to be elevated and, when PGE2 blocking antibodies were introduced, inflammatory functions were partially reverted. Reports by Cutler et al. suggested that MSC can modulate monocyte functions, which ultimately resulted in the suppression of T cell proliferation [73]. They suggested that this response was dictated by MSC secretion of PGE2. Similarly, Maggini et al. observed that thioglycolate-treated peritoneal macrophages cultured with MSC adopted a regulatory phenotype [74]. These macrophages exhibited reduced secretion of pro-inflammatory mediators and enhanced secretion of anti-inflammatory mediators [74]. Furthermore, LPS-dependent upregulation of major histocompatibility complex (MHC) class II and co-stimulatory CD86, factors which are responsible for macrophage antigen presentation, were mitigated [74]. They claimed that MSC secretion of PGE2 was responsible for these changes. Zhang et al. also observed that macrophages assumed M2 phenotypes in the presence of MSC. Macrophages expressed mannose receptors (CD206) and secreted IL-10, hallmarks of M2 macrophage phenotypes. This was observed with a concomitant reduction in M1 secretion of TNF-α as well as the ability to stimulate T_h17 expansion [75]. It was suggested that MSC drive macrophage phenotype through the synergistic interaction between granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-6, which when blocked, reduced macrophage expression of CD206 [75]. The studies of Barminko et al. support these findings as well. They observed that THP-1 pro-inflammatory secretion of IL-1β, TNF-α, IP-10, and MIP1-α was reduced in the presence of MSC. These macrophages exhibited elevated CD206 expression as well as IL-10 secretion [76]. Collectively, these observations strongly suggest that MSC can dictate macrophage plasticity toward regulatory M2 behavior. MSC promote similar phenomena in vivo. In an animal model of sepsis, Nemeth et al. found that MSC reprogrammed macrophages to secrete IL-10 and this was dependant on MSC secretion of PGE2 [77].

Gonzalez et al. reported that MSC reduced T_h1 driven histopathology as well stimulated systemic levels of IL-10 in an experimental model of colitis [21]. They claimed that MSC act directly on activated macrophages to partially facilitate these benefits. In skin injury models, MSC transplantation accelerated wound healing by increasing the number of macrophages infiltrating the wound site [78]. Zhang et al. observed that subcutaneous administration of MSC increased M2 macrophages and enhanced wound healing [75]. Ohtaki et al. explored the effects of MSC on inflammation in an animal model of stroke and found that microglia exhibited M2 phenotypes. The implication of these findings is that if appropriately implemented in vivo, MSC could be utilized as a means of driving endogenous macrophage expression of M2 phenotypes. This could potentially provide an approach to enhance resolution of chronic inflammation.

2.5 MSC Modulation of Neutrophils

Neutrophils are phagocytic granulocytes and are among the first cells to arrive at sites of inflammation. They are recruited and activated by chemoattractants such as IL-8, CXCL1, CXCL2, CXCL5, CCL3, and CCL4, produced by tissue-resident and circulating macrophages that have been activated in response to microbial components or tissue damage-associated molecular patterns (DAMPs) [79]. The main targets of neutrophil activity are pathogenic microorganisms. Upon invasion into the inflamed tissue, neutrophils that encounter microbes phagocytize them and fuse the phagosome with intracellular granules containing acidic hydrolases and bactericidal proteins [80]. If no microbe is encountered within a short time activated neutrophils will release these and other granules containing proteolytic enzymes into the extracellular space. This combined with the abrupt release of reactive oxidative species (respiratory burst) can cause further damage to the tissue [81]. MSC have been reported to affect the neutrophil contribution to inflammation by influencing their recruitment and invasion into tissues in several models of inflammatory conditions, including sepsis [77], acute lung injury [82, 83], diabetes [84], and tetrachloride-induced cirrhosis [85]. The mechanism of this effect may be indirect (Fig. 2.2). In a model of sepsis, Nemeth et al. reported that septic mice treated intravenously with MSC had higher numbers of circulating neutrophils, lower levels of myeloperoxidase (a granulocytic enzyme) in the liver and spleen, and higher secretion of IL-10 from macrophages [77]. They hypothesized that the decreased invasion of neutrophils was due to this direct effect of MSC on macrophages, since IL-10 has been reported to inhibit neutrophil migration from the vasculature [86–88]. Ortiz et al. reported that certain subpopulations of MSC produce IL-1 receptor antagonist (IL-1ra), which may block the production of pro-inflammatory cytokines from macrophages and therefore the subsequent expression of adhesion molecules and chemokines by endothelial cells, resulting in decreased neutrophil recruitment [82]. This proposed mechanism was supported by the observation of a reduced number of neutrophils in the bleomycin-injured lungs of mice treated with MSC.

There is also evidence of more direct relationships between MSC and neutrophils. Raffaghello et al. reported that MSC have an anti-apoptotic effect on neutrophils, which is independent of cell–cell contact. Instead, MSC secretion of IL-6 is apparently the key soluble factor responsible for this effect [89]. The neutrophil production of reactive oxidative species was also inhibited, while phagocytosis was unimpaired. Interestingly, activation of MSC through TLR3 and TLR4 may enhance this anti-apoptotic affect as well as enhance the respiratory burst function of neutrophils [90]. No effect on neutrophil adhesion molecules or migration was observed, again supporting the notion that the effect of MSC on neutrophil tissue invasion is indirect in nature [64].

2.6 MSC Modulation of Dendritic Cells

DC are phagocytic APC which link the innate immune system to the adaptive immune system. After differentiation from myeloid progenitor cells in the bone marrow, DC distribute to the blood and many peripheral tissues [91]. DC in non-lymphoid tissues are considered immature and in a state of surveillance characterized by low expression of MHC class II, very low expression of co-stimulatory molecules, and little secretion of IL-12 [92]. Encounter with a bacterial, viral, or parasitic component activates DC, allowing them to phagocytize the antigen, process it, and present it on their cell surface [93]. During this maturation process, surface expression of MHC class II and co-stimulatory molecules are upregulated, as is IL-12 secretion. These activated DC then migrate to lymphoid tissues, where they present their antigen complexes to T and B lymphocytes, thereby initiating the adaptive immune response.

MSC have been shown to affect each aspect of DC participation in inflammation (differentiation, maturation, and function; Fig. 2.3) in numerous in vitro coculture systems [94–100]. MSC are consistently reported to inhibit DC differentiation, decrease the expression of MHC class II and co-stimulatory molecules, decrease the secretion of IL-12 and inhibit the capacity to stimulate T cell proliferation for both $CD34^+$-derived and monocyte-derived DC. Several mechanisms for these effects have been proposed. Ramasamy et al. reported that the differentiation of DC from peripheral blood monocytes was inhibited due to a human MSC-derived arrest of cell cycle in G_0 [97]. The reduced expression of MHC class II and co-stimulatory molecules and impaired stimulation of T cell proliferation observed by Djouad et al. were attributed to the secretion of high levels of IL-6 by murine MSC [94]. IL-6 has previously been suggested to be an important regulator of DC differentiation [101]. Action of MSC-derived PGE2 has also been implicated as having a central role in these effects on DC [98, 100].

In addition, MSC have been shown to exert effects on mature DC (maDC). Zhang et al. demonstrated that MSC increase maDC proliferation, which display high endocytic capacity, low immunogenicity, and strong immunoregulatory function [102]. Likewise, Wang et al. observed a reduction in maDC expression of maturation

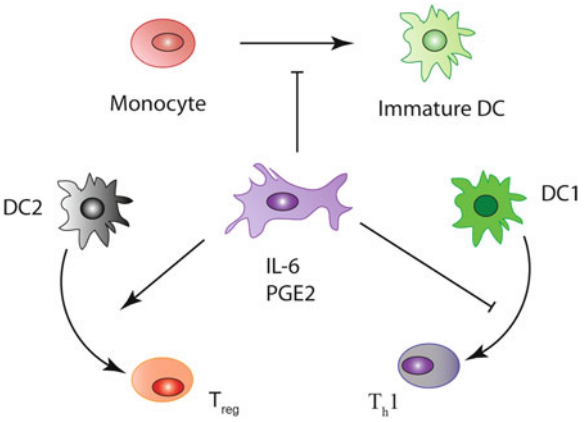

Fig. 2.3 MSC impede DC maturation and augment DC anti-inflammatory functions. Dendritic cells are potent APC, possessing a great deal of control over the adaptive immune response. MSC prevent monocyte commitment to DC differentiation and subsequent maturation. Furthermore, MSC favor DC2 phenotypes within mature maDC populations, while simultaneously inhibiting DC1 phenotypes. The consequence of these observations is that MSC treated DC will favor type II T cell immune functions in vivo

marker CD83 as well as an increase in endocytic activity [103]. These DC orchestrated a shift from pro-inflammatory T_h1 to anti-inflammatory T_h2, suggesting that MSC can promote DC immunoregulatory phenotypes. Interestingly, DC exhibit type I and II phenotypes as do most cells of the immune system. These cells have been referred to as DC1 (pro-inflammatory) and DC2 (regulatory) [104]. Studies by Aggarwal et al. indicated that MSC enhance DC2 functions, while subduing DC1, within a maDC population [42]. The data suggest that MSC can direct DC to adopt regulatory phenotypes. Considering the tremendous control of DC subpopulations over the immune system, MSC success both in treating autoimmune disorders and overcoming allogeneic organ transplantation, may be partially attributed to their effects on DC.

2.7 Differential MSC Activation

The immunomodulatory potential of MSC has been described by many investigators using numerous in vitro systems and in vivo models of inflammatory diseases/conditions. This is not, however, a constitutive function of MSC since activation by external factors is required to attain MSC immunomodulatory activity [105]. Further, the outcome of MSC activation is dependent upon the types of stimulating factors as well as the order and timing of MSC exposure [106]. Many reports related to this have recently emerged regarding the function of TLR in MSC. TLR are a class of pattern recognition receptors (PRR) which recognize bacterial, viral,

fungal, and protozoal pathogen-associated molecular patterns (PAMPs), and therefore are very important in innate immunity [107]. There is also ample evidence that they can be activated by endogenous danger signals (DAMPs) [108]. Stimulation of these receptors results in activation of MyD88-dependent (NF-κB) and -independent (IRF) pathways, resulting in the production of pro-inflammatory cytokines and type 1 interferons [109, 110].

Human MSC have been reported to express TLR1, 2, 3, 4, 5, and 6 mRNA and TLR2, 3, 4, 7, and 9 protein [111]. The effect of TLR3 and TLR4 activation on MSC functions, including migration, differentiation, and immunomodulation, has been the particular focus of many recent studies, sometimes describing contradictory results [27, 90, 112–116]. Liotta et al. observed that activation of MSC TLR3 and TLR4 resulted in NF-κB activity, the secretion of inflammatory cytokines and chemokines (IL-6, IL-8, CXCL10), and the inhibition of the suppression of T cell proliferation [112]. This was attributed to a downregulation of Jagged-1 in MSC after TLR3 and TLR4 ligation, which resulted in impairment of MSC signaling to T cell Notch receptors. Contrary to these findings, Opitz et al. reported that engagement of TLR3 and TLR4 enhanced the MSC-mediated suppression of T cell proliferation by inducing an IFN-β autocrine signaling loop that led to the MSC production of IDO1 [27]. The findings of Waterman et al. fall in the middle of these two contradictory reports. MSC were able to suppress T cell activation after TLR3 priming, but were unable to have this effect after priming of MSC TLR4 [116]. There is further evidence of the anti-immunosuppressive effect of TLR4 activation in vivo. Wang et al. investigated whether MSC from TLR4 knock-out mice could have a therapeutic effect after myocardial ischemia/reperfusion injury [115], a disease model in which MSC have been shown to impart therapeutic benefit [117, 118]. They reported that TLR4-deficient MSC were better able to impart cardioprotection due in part to increased production of angiogenic factors and increased activation of the STAT3 pathway.

Despite some contradictory reports, there is evidence that MSC can have differential states of activation with different immunomodulatory outcomes based on which molecules they are exposed to. Due to this apparent plasticity in MSC phenotype, it has been suggested the MSC be considered as adopting either MSC1 or MSC2 phenotype, following the paradigm used in the monocyte literature [116]. Differential MSC activation may prove to be important when considering their therapeutic use. Further investigation of MSC activation and related underlying mechanisms of immunomodulation may also prove to be a valuable therapeutic tool in that MSC can be preprogrammed/pre-activated to the particular phenotype that will be the most beneficial for the specific disease/condition under consideration.

2.8 MSC Homing

Just as understanding MSC mechanisms of action are important in designing an effective therapy regimen, ensuring that MSC will target the proper tissue is equally as important. MSC have been heralded for their ability to specifically home to areas

of tissue damage. The ability to noninvasively transplant MSC and then have them specifically home to areas of tissue injury is an intriguing and controversial concept. Much can be gleaned from leukocyte and hematopoietic stem cell (HSC) homing, which is a multistage process of (1) chemotaxis, (2) tethering and rolling, (3) firm adhesion, and (4) diapedesis. Most MSC targeting studies attempt to evaluate potential mechanisms of homing in the context of what is known about leukocyte extravasation. We begin our discussion with molecular cues that initiate MSC migration to areas of tissue trauma.

2.8.1 Chemotaxis

Post injury, chemokines activate local endothelial cells to increase expression of cell surface P-selectin, E-selectin, and vascular cell adhesion protein 1 (VCAM-1). These chemokines are also released into the systemic circulation and selectively activate specific leukocyte subsets. Chemokines potentially secreted post-trauma are CXCL12 (SDF-1), CCL2, CCL3 CCL4, CXCL8 (IL-8), CXCL1, and CXCL-10. MSC express receptors for several of these chemoattractant proteins [119–122]. The stromal cell-derived factor 1 (SDF-1)/CXCR4 axis has been described to induce MSC mobilization [123, 124]. Others have found that SDF-1 acts synergistically with other factors, such as HGF, to potentiate MSC targeting [125]. In vitro transmigration assays identified that MCP-1, MIP-1α, IL-8 as well as ischemic brain tissue extract enhance MSC migration [126]. Platelet-derived growth factor (PDGF) [127] and vascular endothelial growth factor A (VEGF-A) [128] have displayed similar functions in vitro. These factors have also been implicated in directing MSC into the injury site locally, once they have adhered to the endothelial lumen. However, in vivo studies directly linking these factors to extravasation efficiency have not been reported.

2.8.2 Tethering and Firm Adhesion

Once a leukocyte migrates to its destination, adhesion molecules on endothelial cells bind leukocyte receptors to facilitate tethering. Tethering decelerates the leukocyte flow and permits strong adherence to the luminal wall. Several protein interactions have been identified to govern this phenomenon and include endothelial P-, L-, and E-selectin binding to carbohydrates on leukocyte transmembrane glycoproteins [129]. Leukocyte firm adhesion is mediated by β1 integrins, particularly α4β1 (VLA-4) and α5β1 (VLA-5) [130]. Ruster et al. indicated that MSC adhere to endothelial cells via P-selectin and VCAM-1/VLA-4, similar to HSC and peripheral blood mononuclear cell (PBMC) [131]. Firmness of adhesion was increased upon TNF-α endothelial stimulation, which likely increased cell surface expression of

integrin adhering proteins. There is conflicting evidence that MSC do not utilize P- or any other selectins as an endothelial tethering mechanism [122]. However, in that study MSC expression of VLA-4 was detected and found to bind VCAM-1. Likewise, Steingen et al. showed that antibody blocking of either VCAM-1 or VLA-4 significantly diminished MSC-endothelial cell adhesion [132]. In vivo, pre-blocking MSC with an antibody against integrin β1 before transplantation significantly reduced MSC homing to myocardial infarct sites [133]. Semon et al. published a thorough analysis of MSC integrin expression. They indicated that integrin subunits β1, β2, and α3 were expressed on over 80 % of the MSC population [134]. While the authors highlight the many discrepancies regarding integrin expression on MSC, integrin β1 has unequivocally been detected on these cells. While the data supporting MSC use of classical selectin tethering mechanisms are debatable, the VCAM-1/VLA-4 axis has consistently been found to play a major role in homing. Some have suggested that since MSC are larger than HSC, both passive and active homing mechanisms may be involved in successful MSC targeting [122, 135]. Therefore, MSC may not need to exhibit classical tethering mechanisms to attach to the endothelium. Furthermore, endothelium from different tissues utilize distinct subsets of these integrins to facilitate adhesion [132, 134], suggesting that MSC homing efficiency will depend on the specific nature of the targeted tissue. Others have suggested that clotting factors such as fibronectin could potentially bind integrin subunits on MSC [136].

2.8.3 Diapedesis

The final step in MSC extravasation is trans-endothelial migration into the targeted tissue. Unlike rolling and adhesion, the mechanisms driving diapedisis are not well understood. There is evidence for para- and trans-cellular routes of tissue entry [137]. However, considering the size of an MSC, transcellular entry would be an unlikely route as MSC would need to transverse the basement membranes of these tissues. Son et al. demonstrated MSC matrix metalloproteinase 2 (MMP-2) and membrane type 1 matrix metalloprotease (MT1-MMP) mediated transmigration across a Matrigel basement membrane [125]. Similarly, Becker et al. also described the MMP-2 mediated transmigrations of MSC [138]. Others have described the role of the MMP1/protease-activated receptors (PAR1) axis in MSC migration, as blocking of this interaction resulted in reduced migration in a glioma model [139]. These findings suggest that MSC would degrade the endothelial layer to enter the tissue.

The disparity in the mechanisms proposed to dictate MSC homing capabilities may be attributed to several factors. MSC expression of homing mediators begin to decline over passage number and is dependent upon culture conditions [120]. Therefore, depending on the isolation techniques and cultures conditions, MSC from different laboratories will display varying homing potential and mechanisms. To complicate the issue further, MSC isolated from different tissues may also express these homing mediators differently [140]. Some investigators have suggested

that MSC homing mechanisms are trumped by their tremendous size [122], as MSC will get trapped in nonspecific locations and will therefore display reduced homing efficiencies which are chemotaxis-independent [135]. This may explain why localized delivery of MSC enhances engraftment efficiencies [141–143] since intravenously injected MSC have been observed to be systemically delivered to unintended tissues, mainly the lung and liver [141, 144]. Furthermore, MSC have not been found to persist at a tissue site long term and are sometimes indetectable as early as 1 week post transplantation [145]. To date MSC homing potential and the mechanisms which govern homing control continue to be debated.

2.9 Conclusion

It is clear that MSC have a tremendous effect on the immune system. MSC seem to exert their effects by controlling white blood cell differentiation into regulatory phenotypes as well as attenuating pro-inflammatory functions. Also, MSC appear to affect differentiated immune cell functions; however, the degree of regulation appears to be dependent upon the particular cell type and the activation state of MSC. The specific mediators which control these unique cell–cell interactions vary, and it is likely that several mediators synergistically contribute to the overall effects. In addition, the homing mechanisms governing MSC targeting are poorly understood and are also extremely controversial. It appears that MSC targeting is not as efficient as once thought. Furthermore, since persistence may not be long term, MSC therapeutic potential may be maximized with continuous implantations. Considering the complexity of MSC immunomodulation and the factors or cell–cell interactions necessary for effector functions to be activated, maximal immune regulation may require constant MSC surveillance. Therefore, approaches to prolong MSC persistence will be crucial in translating their immunotherapeutic potential.

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Chapter 3
Mesenchymal Stem Cell Exosomes: The Future MSC-Based Therapy?

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Abstract The ease of isolation from adult tissues, large ex vivo expansion capacity, and apparent therapeutic efficacy in a wide range of disease indications have made mesenchymal stem cells (MSCs) the stem cell of choice for regenerative medicine. Clinical and animal studies have demonstrated that secreted trophic factors, and not stem cell differentiation, likely mediated much of the therapeutic efficacy of MSCs. This paradigm shift in the therapeutic mechanism of MSCs has started to transform MSC therapy from a cell- to biologic-based therapy. Our group has identified the exosome, a secreted membrane vesicle, as an active therapeutic factor in MSC secretion. An exosome is thought to mediate cell to cell communication. It carries a large and varied protein cargo that could regulate a wide array of biochemical and cellular processes. These include enhancing glycolysis which increases not only cellular ATP production but also glycolytic intermediates for anabolic activities, inducing adenosine-mediated activation of survival kinases (e.g., ERK and AKT via

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CD73) and reducing complement activation through CD59. As these processes are fundamental, non-tissue specific processes in ameliorating tissue injury and promoting tissue repair, MSC exosomes could potentially underpin the therapeutic efficacy of MSC in diverse disease indications. This could transform present MSC-based therapies into MSC exosome-based therapies.

Keywords Mesenchymal stem cells • Exosome • Proteome • Glycolysis • Ecto-5′ nucleotidase • Complement-mediated cell lysis • Therapy

3.1 Background

Mesenchymal stem cells (MSCs) were first described in 1968 as a population of multipotent fibroblast-like cells that reside in the bone marrow and have the potential to differentiate into osteocytes, chondrocytes, adipocytes, and myoblasts [1]. Since then MSCs have been isolated from adipose tissue [2, 3], liver [4], muscle [5], amniotic fluid [6], placenta [7, 8], umbilical cord blood [2], dental pulp [9, 10], and other sources [4, 11]. Their differentiation potential has also expanded into an amazing array of cell types that include nearly every major cell types in the adult body [12]. To better facilitate the study and comparison of MSCs from different tissue sources, the International Society for Cellular Therapy has issued a position statement for a minimal criterion to define multipotent MSCs [13]. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, they must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules. Third, MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts in vitro.

Among the different kinds of stem cells identified to date, MSCs are presently the stem cell of choice for regenerative medicine. The main allure of MSCs lies in their reported potential to exert protective and reparative effects on an amazingly wide spectrum of tissue injury. This is further bolstered by their ease of isolation from ethically palatable adult tissue sources (e.g., bone marrow and adipose tissue), a large ex vivo expansion capacity, as well as demonstrated multipotency and immunosuppressive activity (reviewed in [12, 14, 15]). MSCs are currently the most evaluated stem cells. It was estimated in 2010 that there were 101 clinical trials using MSCs to treat a variety of disease conditions [16]. MSCs have been and are currently being evaluated for their efficacy in treating a myriad of diseases such as cardiovascular diseases (e.g., acute myocardial infarction, end-stage ischemic heart disease, and prevention of vascular restenosis), osteogenesis imperfecta (OI) or brittle bone disease, amyotrophic lateral sclerosis (ALS), lysosomal storage diseases (e.g., Hurler syndrome), steroid refractory Graft versus Host Disease (GVHD), periodontitis, and bone fractures [17].

Many studies on the use of MSCs as therapeutics were predicated on the hypothesis that transplanted MSCs home and engraft in injured tissues, and then differentiated into cells to replace damaged cells. Although there have been many reports that MSCs could migrate and engraft at sites of injury where they then differentiate to replace damaged tissues and restore tissue function after transplantation in animal

models, it has been estimated that <1% of transplanted cells actually reached the target tissue with most of the cells being trapped in the liver, spleen, and lung [18]. Furthermore, evidence for reported differentiation of transplanted MSCs at the site of injury was often limited to the presence of new phenotypic features or markers that could be acquired by fusion with endogenous host cells [19–21]. More importantly, it has been increasingly observed that the therapeutic efficacy of MSC therapy is not dependent on the engraftment of MSCs at the site of injury or differentiation capability of the transplanted MSC [22–26], essentially eliminating the need for MSCs to be in the vicinity of their target tissue or differentiate to exert a therapeutic effect.

To reconcile this discrepancy between the therapeutic efficacy of MSC and the lack of MSC engraftment or differentiation at the site of injury, it was proposed that MSCs exert their therapeutic effects through secreted trophic mediators. MSCs are known to secrete a broad spectrum of growth factors and cytokines [27]. This diversity could potentially provide a basis for the therapeutic efficacy of MSCs in a wide range of disease indications and injuries [28–35]. Increasingly, MSCs are being used for their trophic secretions to reduce injury and repair tissues rather than as stem cells to differentiate and regenerate injured tissues. Of the 101 MSC clinical trials in 2010, 65 were rationalized on trophic secretion while 36 were based on differentiation potential [16].

This paradigm shift in the therapeutic mechanism of MSC from one based on cell engraftment, differentiation, and replacement to one based on secretion and paracrine signaling could potentially engender the development of biologic- instead of cell-based therapeutics. From clinical and manufacturing perspectives, biologics offer several advantages. In contrast to cells, biologics are more amenable to development as an "off-the-shelf" therapeutic in a rigorously regulated and monitored manufacturing process. This will translate into better qualified and safer products that could be delivered to patients in a timely manner. In cell-based therapy, the need to preserve cell viability adds a layer of complexity to its manufacture, storage, transport, and delivery/transplantation. The use of relatively large viable cells as therapeutics also carries its own unique safety risks and challenges. First, their large size increases the risk of occlusion in the distal microvasculature as demonstrated by the intra-arterial administration of MSCs in mice which resulted in pulmonary embolism and death in 25–40% of the animals [36]. Second, the viability of transplanted cells would result in the persistence or amplification of biological potency of the agent even after the need has been resolved. This may lead to an increased risk of tumor formation and immunological reactions. Such persistence or amplification could be more ominous if treatment had to be terminated as a result of adverse outcomes. Finally, the differentiation potential of MSCs could generate inappropriate and potentially deleterious cell types. For example, cardiac sympathetic nerve sprouting was thought to contribute to the proarrhythmic effects of MSC therapy [37–39], while a high frequency (51.2%) of ossifications and/or calcifications was observed in cryo-infarcted hearts after MSC transplantation [40]. The benefits of a biologic-based therapy vis-a-vis the risks of a cell-based therapy have prompted a close examination of MSC secretions. MSCs have been reported to secrete a wide diversity of factors and these active therapeutic factors were initially presumed to be the small soluble chemokines, cytokines, or growth factors that are abundantly secreted by MSCs (as summarized in Table 3.1). These secreted factors

Table 3.1 Therapeutic role of MSC secretions in various tissue injury models and the proposed soluble factors mediating these effects

Author	Year	MSC source	Disease model	Soluble factors involved	Therapeutic effects	Remarks	References
Kinnaird et al.	2004	BM[a]	Unilateral hindlimb ischemia	VEGF, bFGF, PIGF, MCP-1	Angiogenic (enhance collateral remodeling)	Injected MSC were not seen incorporated into mature collaterals	[19]
Hung et al.	2007	BM	Hypoxia-induced apoptosis in human aortic endothelial cells	IL-6, VEGF, MCP-1	Angiogenic, anti-apoptotic	Therapeutic effect cannot be explained by these three factors alone, other paracrine factors involved	[18]
Togel et al.	2007	BM	Ischemia/reperfusion acute renal failure	VEGF, HGF, IGF-1	Anti-apoptotic	Little engraftment and differentiation of transplanted MSCs	[22]
Chen et al.	2008	BM	Excisional wound healing	More VEGF-α, IGF-1, EGF, KGF, Ang-1, SDF-1, MIP-1α, MIP-1β and EPO than dermal fibroblasts	Chemoattractive, angiogenic	Conditioned medium recruited macrophages and endothelial lineage cells into wound to enhance healing	[17]
Li et al.	2008	BM	Isoproterenol-induced global heart failure	ADM	Anti-fibrogenic	Transplanted MSCs improved heart function and decreased collagen volume fraction in myocardium	[20]
van Poll et al.	2008	BM	D-galactosamine-induced fulminant hepatic failure	OSM, AR-1, TGF- β, HGF, TNF-α, EGF, IL-6, SCF, HB-EGF, TIMP-3	Anti-apoptotic, anti-inflammatory, promitotic	Conditioned medium inhibited cell death and stimulated regeneration in vivo and in vitro	[23]
Lin et al.	2011	UC[b]	Middle cerebral artery occlusion and reperfusion (ischemic stroke)	BDNF, bFGF, PDGF-AA, Ang-2, CXCL-16, NAP-2, VEGFR-3	Angiogenic, anti-apoptotic (neuroprotective)	Transplanted MSCs scattered in the infarct cortex 36 days after transplantation	[21]

[a]BM—Bone marrow
[b]UC—Umbilical cord

could potentially provide a basis for the therapeutic efficacy of MSCs in wide range of diseases and injuries [28–35]. However, recent studies have discovered that the paracrine factors secreted by MSCs involve small secreted lipid vesicles such as microvesicles [41, 42] and exosomes [43]. These lipid vesicles could potentially carry a cargo that is sufficiently large and diverse to underpin the therapeutic efficacy of MSCs observed in a plethora of diseases.

3.2 Microvesicles and Exosomes

Microvesicles and exosomes are two of the several classes of secreted membrane vesicles that include microvesicles, ectosomes, membrane particles, exosome-like vesicles, or apoptotic bodies [44]. Microvesicles are highly variable in size with a diameter of 100–1,000 nm and originate primarily from the plasma membrane. They are generally not as well characterized as exosomes. The latter vesicles are much better defined secreted membrane vesicles that originate in the endosomes. They are smaller with a much narrower diameter range of 40–100 nm, a flotation density in sucrose of 1.1–1.18 g/mL; and membranes enriched in lipid rafts of cholesterol, sphingomyelin, and ceramide [45, 46]. The presence of exposed phosphatidylserine on exosome membrane was reported to be present for some exosomes [47, 48] and absent for others [49, 50]. The exosome cargo contains both proteins and RNAs. Most exosomes have an evolutionary conserved set of proteins molecules including tetraspanins (CD81, CD63, CD9), Alix, Tsg101 but they also have unique tissue/cell type-specific proteins that reflect their cellular source [51].

When membrane vesicles were first found to be shed by maturating sheep reticulocytes by Johnstone and colleagues in 1983, they were thought to be "garbage bags" for disposal of unwanted transferrin receptors [52]. These membrane vesicles were subsequently described as "exosomes" [53]. Pulse-chase and electron microscopy studies determined that these membrane vesicles were released during the fusion of multivesicular late endosomes with the plasma membrane [54, 55]. In 1996, B-lymphocytes were also observed to secrete exosomes and unlike those from reticulocytes, these exosomes were found to have important biological functions. They could stimulate T cell proliferation [56] and suppress tumor growth [57]. More recently, exosomes were found to contain mRNA [58] and miRNA [58–60] that could be transferred into recipient cells to modulate protein synthesis. Together these studies suggest that the function of exosomes extends beyond the disposal of unwanted proteins and may mediate intercellular communication through protein–protein interactions and exchange of proteins and genetic materials.

As exosomes were first observed to be secreted by in vitro cultures of different cell types such as B cells [56], dendritic cells [57], mast cells [61], T cells [62], platelets [63], Schwann cells [64], tumor cells [65], mesenchymal stem cell [43], human embryonic kidney cell [66], various cancer cell lines [67], and sperm [68], they were initially suspected to be culture artifacts. However, the subsequent discovery of exosomes in physiological fluids including bronchial lavage fluid [69],

human urine [70, 71], and human blood [71] helped establish exosomes as physiological cellular products. The large diversity in exosome-secreting cell types and the presence of exosomes in different physiological fluids indicate that secretion of exosomes is a general cellular function.

Although exosomes from different cell sources have been shown to carry a similar set of proteins, they also carry proteins that reflect their cellular origin and the physiological state of the cells from which they originate [72]. For example, exosomes released from maturing reticulocytes are rich in transferrin receptors that the reticulocytes have to dispose of while those from lymphocytes and dendritic cells have few transferrin receptors [56, 73, 74]. Similarly, exosomes from epididymis are rich in proteins that are essential for the maturation of male gametes [68] and urinary exosomes secreted by kidney tubules carry aquaporin, a kidney-specific protein [70]. The cargo of exosomes has also been found to correlate with the physiological state of its cellular source. For example, tumor-derived exosomes have been shown to contain either tumor antigens [65, 75–77] or tumor-specific microRNAs [59]. Together, these observations are consistent with the hypothesis that exosomes facilitate intercellular communication through protein–protein interactions and exchange of proteins and genetic materials [78]. The list of proteins and RNAs reported to be present in exosomes could be accessed at Exocarta, a freely accessible web-based compendium of exosome proteins and RNAs set up by Richard Simpson and his colleagues (http://exocarta.ludwig.edu.au) [79].

The most defining feature that distinguishes exosomes from other secreted membrane vesicles is their biogenesis through the endosomal pathway where the endosome membrane invaginates to form intraluminal vesicles (ILVs) such that the entire complex becomes a multivesicular body (MVB). The fate of the ILVs depends on whether the MVB fuses with lysosome or plasma membrane. Fusion with lysosomes lead to the degradation of the ILVs while fusion with the plasma membrane releases the ILVs into the extracellular space as exosomes [80]. While ESCRT (endosomal sorting complex required for transport) has been shown to be important in recognizing and sequestering of proteins in the endosomal membrane and subsequent budding of the endosomal membrane [81], it is clearly only one element in the complex process of recognizing and targeting proteins to ILVs or exosomes. Recent reports suggested that some proteins are targeted to exosomes in an ESCRT-independent manner. For example, higher-order oligomerization alone was sufficient to target some plasma membrane proteins such as CD43 and HIV Gag to exosomes in Jurkat cells, while others, e.g., proteolipid protein (PLP) are targeted to exosomes in a ceramide-dependent but ESCRT independent process [82, 83].

3.2.1 Exosome Functions

For many years after its discovery, exosomes were perceived to be a unique cellular product of reticulocytes, a highly specialized cell type and had little function

beyond the disposal of obsolete membrane proteins such as transferrin receptors during reticulocyte maturation [84–86]. However, this perception of exosomes was transformed as increasing numbers of cell types have been observed to secrete exosomes during normal physiological activities, thus suggesting that exosomes are a highly ubiquitous cellular vehicle for modulating or mediating cellular processes. The cell types that were first observed to secrete exosomes were mainly of hematopoietic origin such as reticulocytes [53, 87, 88], B- and T-lymphocytes [56, 89], dendritic cells [57], mast cells [90], and platelets [50]. Exosomes secreted by B-lymphocytes and dendritic cells have been shown to stimulate T cells [56, 57, 89] and therefore play a role in mounting host immune responses. Furthermore, exosomes from dendritic cells could be pulsed with tumor peptides to prime cytotoxic T cells in vivo and suppress growth of established tumors in mice [91]. Subsequently, non-hematopoietic cells were also found to secrete exosomes to facilitate some of their cellular activities. For example, neurons were reported to secrete exosomes during synaptic activities for neurotransmission [92, 93], while oligodendrocytes secreted exosomes to coordinate myelin membrane biogenesis [94]. Cardiomyocyte progenitor cells promote cardiac regenerative activity through secretion of exosomes to stimulate migration of endothelial cells [95]. The fusion of egg and sperm was postulated to involve exosomes secreted by the egg [96]. Exosomes are also increasingly implicated in disease pathogenesis and host responses. Exosomes from non-immune cells such as mesenchymal stem cells have also been shown to have the capacity to influence biological processes such as reducing myocardial ischemia/reperfusion injury [43] or acute tubular injury [97].

The functions of exosome are not always benign or beneficial to cells or tissues. Diseased cells have been shown to secrete exosomes that could transfer some of the disease phenotype to recipient cells. Exosomes secreted from cultured epithelial and neuroglial cell lines infected with scrapie were found to carry the infectious PrPSc [64]. It was subsequently proposed that such exosomes mediate the intercellular spreading of infectious prions protein (PrP) which is responsible for the transmissible spongiform encephalopathies [84, 86]. Virally infected cells such as HIV-infected Jurkat and primary T-cells or Epstein-Barr Virus (EBV)-infected B cells are reported to secrete exosomes that contain virally encoded materials (e.g., HIV *Nef* [85], EBV glycoprotein gp350 [98], EBV latent membrane protein 1 [98], or EBV-encoded miRNA [91]). Such exosomes could potentially transmit viral products to uninfected cells and cause deleterious effects. HIV *Nef*-containing exosomes have been shown to induce cell death in uninfected bystander T cells [85]. Like virally infected cells, bacterially infected cells such as *Mycobacterium tuberculosis*-, *Mycobacterium bovis*-, or *Toxoplamsa gondii*-infected macrophages also secrete exosomes that contain pathogen-derived antigens [99].

Exosomes have also been implicated in the pathogenesis of cancer or development of a metastatic phenotype. Uptake of exosomes derived from highly metastatic melanoma cells was observed to transform poorly metastatic tumor cells into highly metastatic cells [100], while exosomes from human brain tumor cells carry oncogenic

receptor EGFRvIII that could be transferred to other cells [13]. Tumor exosomes have also been reported to enhance the generation of immunosuppressive myeloid cells and potentially enable tumor evasion of the immune system [101]. In 2008, Taylor et al. showed that miRNA profile of circulating exosomes in ovarian cancer patients' blood is highly similar to the originating tumor cells [59]. This observation together with Jan Lotvall's 2007 report that exosome is a vehicle for intercellular exchange of mRNAs and miRNAs [102] provide a hypothetical mechanism for the dissemination of the cancer phenotype.

Complex neurodegenerative diseases such as Parkinson and Alzheimer diseases have also recently implicated exosomes in the formation of disease-associated protein aggregates, namely aggregation of alpha-synuclein in Parkinson's disease and amyloid beta protein (Aβ) in Alzheimer disease. Neurons have been shown to secrete exosomes containing α-synuclein [14, 19] or Aβ [103], but it remains to be determined if these exosomes contribute to the nucleation or physical dissemination of the protein aggregates that characterize Parkinson and Alzheimer diseases.

As a consequence of the close association between the secretion and modulation of exosomes with changes in physiological and pathological states of the secreting cells, exosomes are good sentinels of cellular health and pathology and have become an attractive source of biomarkers for diseases (reviewed [104]).

3.2.2 MSCs Secrete Therapeutic Exosomes

In 2010, our group described the purification of exosomes from human ESC-derived mesenchymal stem cells and their efficacy in reducing myocardial ischemia-reperfusion injury [43]. Prior to this, we demonstrated that intravenous administration of a single bolus of culture medium conditioned by human embryonic stem cell-derived MSCs (hESC-MSCs) reduced relative infarct size in a pig and mouse model of ischemia/reperfusion injury [105]. By molecular weight fractionation of the conditioned medium, we further demonstrated that the active component had a presumptive size of 50–200 nm in size [43]. Using size exclusion high performance liquid chromatography, we purified a population of homogenously sized particles that have the biophysical parameters of exosomes, namely a hydrodynamic radius of 55–65 nm and a flotation density in sucrose of 1.10–1.18 g/mL. They also have common exosome-associated proteins such as the tetraspanin proteins, CD9 and CD81, Alix, Tsg101. They also contained RNA which consists primarily of short RNAs of less than 300 nt. Some of these RNAs were microRNAs that are predominantly in the pre-microRNA form [106]. These purified particles reduced infarct size to the same extent as CM but at one-tenth of the protein dosage used in CM. We also discovered that despite a loss of adipogenic differentiation potential, the production of cardioprotective exosomes by myc-immortalized hESC-MSCs was not compromised [107]. The proteome of these purified exosomes was profiled by mass spectrometry and antibody array, and found to contain 857 unique gene products (http://www.exocarta.org). These proteins are distributed

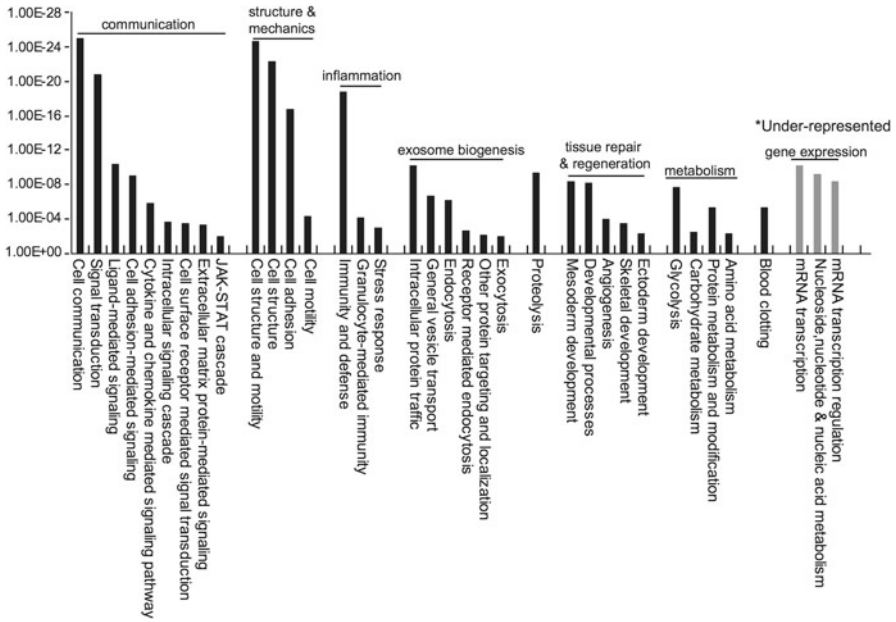

Fig. 3.1 Functional distribution of MSC exosome proteins. 857 proteins or unique gene products were found in MSC exosome (www.exocarta.org). The observed frequency of unique gene products in the exosome proteome for each biological process was compared with the reference frequency of genes in the NCBI database for that biological process. The 857 unique gene products could be clustered into 32 biological processes that were overrepresented ($p < 0.001$) and 3 that were underrepresented ($p < 0.001$)

over a wide array of biochemical and cellular processes such as communication, structure and mechanics, inflammation, exosome biogenesis, tissue repair and regeneration, and metabolism (Fig. 3.1).

3.3 Biochemical Potential of MSC Exosomes

Our demonstration that MSC-secreted exosomes are cardioprotective and carry a diverse cargo suggests that exosomes have the potential to be the secreted trophic factors mediating the therapeutic efficacy of MSCs against a plethora of diseases. A fundamental requisite for such a factor would be its capacity to be as efficacious as MSCs against a complex multifactorial tissue injury such as myocardial ischemia injury, and an equally complex and heterogenous range of diseases and injury. MSC exosomes with their diverse array of proteins distributed over a wide range of biochemical and cellular processes could potentially fulfill this requisite. Below is an analysis of three biochemical activities found in MSC exosomes to illustrate how exosomes could contribute to the therapeutic paracrine effects of MSCs in mediating tissue repair and reducing tissue injury in a wide range of diseases.

3.3.1 Exosomes Enhances Cellular ATP Production Through Glycolysis

One prominent feature of the MSC exosome proteome was the presence of all five enzymes in the ATP generating stage of glycolysis (Fig. 3.2a): glyceraldehyde 3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase (PGK), phosphoglucomutase (PGM), enolase (ENO) and pyruvate kinase m_2 isoform (PKm_2) (www.exocarta.org). Of these, GAPDH, PGK, and PKm_2, that generate either ATP or NADH, were further confirmed to be present by immunoblotting (Fig. 3.2b). Their enzymatic activities were determined as 1.1, 3.59 and 5.5 μU per μg protein respectively (Fig. 3.2c). Whereas 1 unit (U) of enzyme activity is defined as the activity required for the production of 1 μmol of product per minute.

MSC exosomes also contained PFKFB3, which converts fructose 6-phosphate to fructose 2, 6-bisphosphate. PFKFB3 is one of four PFKFB isoforms. The four isoform are encoded by four different genes, PFKFKB1, 2, 3, and 4. PFKFBs are responsible for maintaining the cellular level of fructose-2, 6-bisphosphate, a powerful allosteric activator of phosphofructokinase [108] which catalyses the commitment to glycolysis. These proteins are thought to be responsible for the high glycolytic rate or "Warburg effect" in cancer cells [109]. The kinase activity of PFKFB3 is upregulated by phosphorylation by protein kinases such as cAMP-dependent protein kinase and protein kinase C. Mass spectrometry analysis and immunoblotting revealed the presence of phosphorylated PFKFB3 in the exosome (www.exocarta.org) (Fig. 3.2b). Exposure of oligomycin-treated H9C2 cells to MSC exosomes, which can be internalized by H9C2 cells [106], increased intracellular ATP level by 75.5 + 28.8% or 55.8 + 16.5% in 15 or 30 min of exposure to exosomes, respectively. Since oligomycin inhibits mitochondrial ATPase [110], the increased ATP level is likely to be derived from increased glycolysis. This ability to increase glycolysis may underpin part of the therapeutic efficacy of MSC exosomes.

Rapidly proliferating cells such as cancer cells and stem cells are known to maintain a high rate of aerobic glycolysis despite its inefficient ATP production in comparison to that by oxidative phosphorylation (reviewed [111]). However, the importance of increased glycolysis lies not in its ATP production, but rather the glycolytic intermediates which are essential in anabolic reactions to generate biomass for growth and repair. By extrapolation, exosome through increasing aerobic glycolysis in injured cells would increase anabolic activities to promote tissue repair and minimize tissue damage.

3.3.2 Exosome Phosphorylates ERK and AKT via CD73 (Ecto-5′-Ectonucleotidase, NT5E)

Two of the most important signaling cascades that regulate proliferation and apoptosis are Ras/Raf/MEK/ERK (MAPK) and PTEN/PI3K/AKT/mTOR [112].

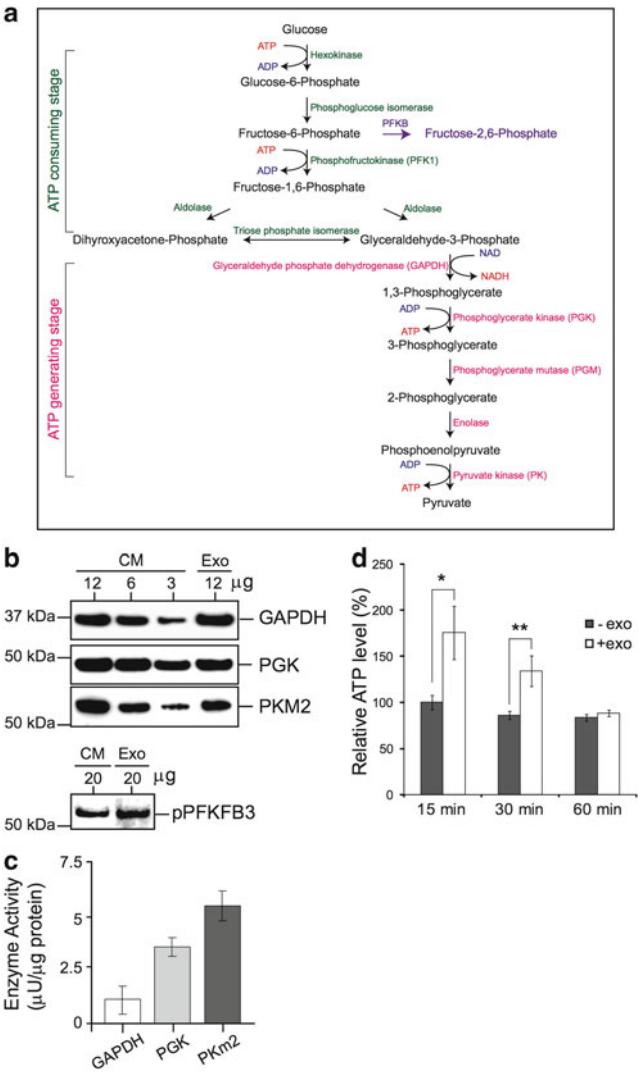

Fig. 3.2 Exosomes enhances glycolysis. (**a**) Schematic diagram of biochemical reactions in glycolysis. (**b**) Presence of geraldehyde phosphate dehydrogenase (GAPDH), phosphoclycerate kinase (PGK), pyruvate kinase m_2 isoform (PKm_2), and pPFKFB3 in MSC conditioned medium (CM) and exosomes (Exo). CM and Exo were analyzed by standard immunoblotting assays using antibodies from Santa Cruz Biotechnology, Santa Cruz, CA, except mouse anti-PK which was from Abcam Inc., Cambridge, MA. (**c**) Enzymatic activities of GAPDH, PGK and PKm_2 in MSC exosomes. PKm_2 activity was measured using the PK assay kit (Biovision), GAPDH and PGK activity was measured using KDalert GAPDH assay kit (Ambion Inc., Austin, TX) and ApoSENSOR ADP/ATP ratio assay kit (Biovision). Each activity was measured in triplicate using two independent exosome preparations. Each bar represents mean ± SEM. One unit (U) enzyme activity is defined as the activity to generate 1 µmol product per minute at 37 °C. (**d**) Effect of exosome on ATP production in oligomycin-treated cells. H9C2 cardiomyocytes were washed twice with Tyrode's buffer and then incubated in Tyrode's buffer containing 20 µmol of a mitochondrial inhibitor, oligomycin, 6 mmol glucose, and with or without 0.1 µg/mL exosomes for 15, 30, and 60 min. Cellular ATP concentration was measured using ATPlite 1 step luminescence ATP detection assay system and normalized to that of sample without exosomes at 15 min. Each bar represents mean ± SEM of three independent assays with five replicates for each sample. $*p = 0.0173$, $**p = 0.0090$

Activation of these pathways are important in tissue repair and amelioration of tissue injury (e.g., myocardial ischemia reperfusion injury [113], sepsis [114], epithelial wound [115]). In myocardial ischemia reperfusion injury, a key activator of ERK and AKT phosphorylation is adenosine and it was effective in reducing infarct size when used as an adjunctive to reperfusion in a clinical trial [116]. Adenosine has been shown to exert a nonredundant role in attenuating inflammation and tissue damage and mediates diverse cardioprotective, neuroprotective, vasodilatory, and angiogenic responses (reviewed [117]). In animals, adenosine is produced by the degradation of extracellular ATP and ADP that are released during tissue trauma such as shear stress induced hemolysis of red blood cells, working skeletal muscle, perfused heart or isolated heart muscle cells under hypoxic conditions (reviewed [118]), and apoptotic cells [119]. Extracellular ATP and ADP in the body are rapidly degraded into AMP by enzymes in the plasma or ecto-enzymes on the surface of red blood cells and the vascular lining with half-lives in the blood estimated to be <1 s [119] and 3.2 min [120], respectively. ATP can be degraded to either ADP by ecto-ATPase or AMP by a pyrophosphatase. Extracellular AMP is dephosphorylated to adenosine primarily by CD73, an ecto 5′ nucleotidase [121]. Therefore, injured or stressed tissues such as reperfused hypoxic cardiac tissues could activate adenosine-mediated activation of survival signaling pathway through the release of ATP or ADP.

CD73 was found to be present in the MSC exosomes by mass spectrometry analysis (www.exocarta.org) and confirmed by immunoblotting (Fig. 3.3a). The enzyme activity in exosomes was determined to be 22.04 μU/μg protein. Exposure of serum-starved H9C2 cardiomyocytes to exosomes and AMP led to phosphorylation of ERK1/2 and AKT (Fig. 3.3b, c). This phosphorylation was abolished in the presence of theophylline, a nonselective adenosine receptor antagonist that antagonized A1, A2A, A2B, and A3 receptors [122]. The presence of biologically active CD73 on exosomes that can elicit pro-survival signaling by phosphorylating ERK and AKT provides another mechanistic basis for the MSC paracrine effect in enhancing tissue repair and reducing tissue injury.

3.3.3 Exosome Inhibits Complement Activation

The complement system is a tightly regulated proteolytic cascade that clears invading microorganisms, circulating immune complexes, dead cells, apoptotic bodies, and cellular debris. It represents one of the first lines of defense in innate immunity and facilitates communication between innate and adaptive immunity (reviewed [123]). This system can elicit a number of proinflammatory responses including the recruitment of leukocytes, degranulation of phagocytic cells, mast cells and basophils, smooth muscle contraction, and increased vascular permeability. These responses could be further escalated by the production of toxic oxygen, arachidonic acid metabolites, and cytokines [123]. The complement system is complex and involves at least 30 serum proteins. Deficiencies in the complement system results

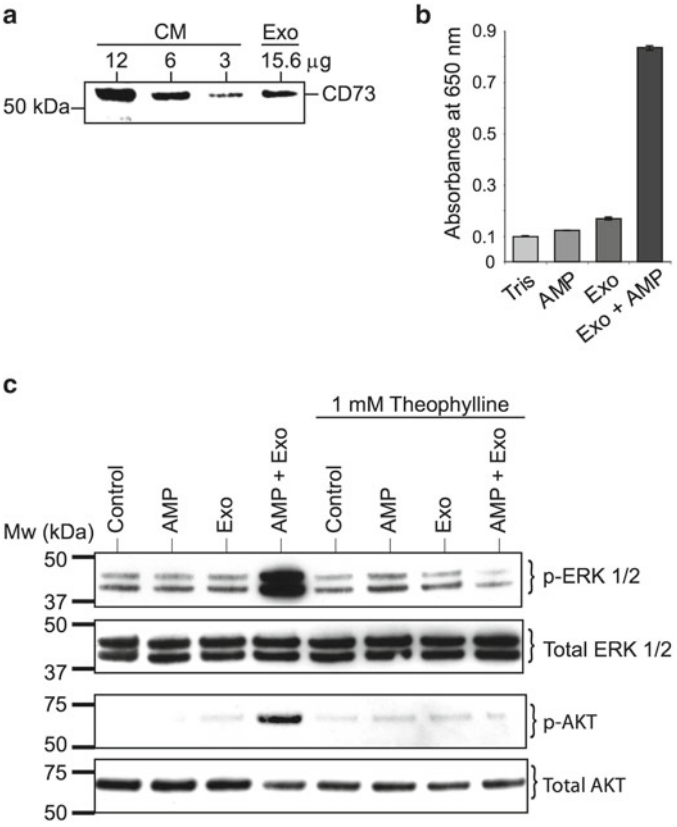

Fig. 3.3 Activation of ERK and AKT pro-survival signaling by exosome CD73. (**a**) Western blot analysis of MSC conditioned medium (CM) and exosomes for CD73 using a specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). (**b**) CD73 activity in exosomes (Exo) was measured by incubating 2.5 μg of exosomes in 100 μL Tris buffer pH 7.4 containing 50 μM AMP (Sigma-Aldrich, St. Louis, MO) and measuring the amount of phosphate ions released from the hydrolysis of AMP by Colorlock Gold kit (Innova Biosciences, Cambridge, UK). Each bar represents mean ± SD of duplicate sample. (C) H9C2 cells were serum starved overnight and then incubated with medium with or without 1 mM theophylline for 1 h. The cells were then exposed for 5 min to medium that had been pre-incubated for 30 min with 50 μM AMP, 0.1 μg/mL exosome or AMP and exosome. The cells were then harvested and lysed. 10 μg of total proteins were immunoblotted using 1:2000 dilution of rabbit anti-pERK 1/2 (Cell Signaling, 9101S), 1:2000 dilution of rabbit anti ERK1/2 (Santa Cruz, sc-94), 1:500 dilution rabbit anti-pAKT (Cell Signaling, 9271S), or 1:500 dilution of rabbit anti AKT (Cell Signaling, 9272S)

in diseases such as autoimmunity (e.g., lupus), recurring infections, glomerulonephritis, angioedema, and hemolysis (reviewed [124]). Conversely, excessive or aberrant activation also contributes to the pathogenesis of diseases such as inflammatory diseases (e.g., Crohn's disease), autoimmune diseases (e.g., multiple sclerosis), neurodegenerative disease (e.g., age-related macular degeneration), and others (e.g., stroke, myocardial infarction, trauma, burn).

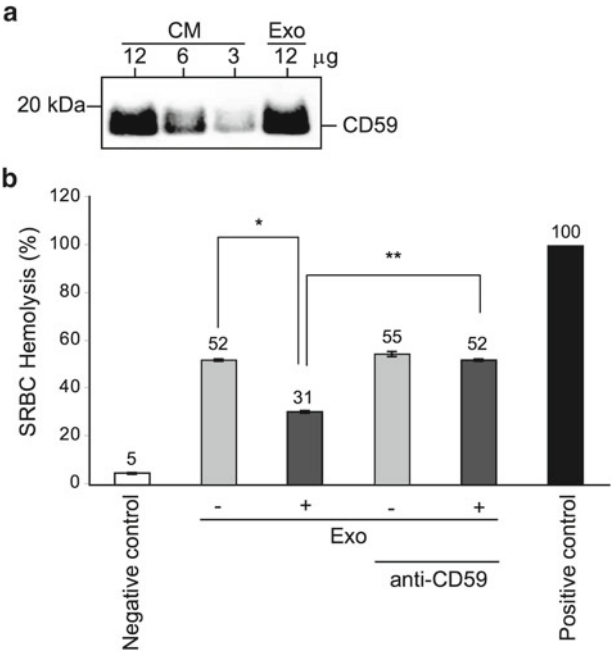

Fig. 3.4 Inhibition of membrane attack complex (MAC) formation. (**a**) Western blot analysis of MSC conditioned medium (CM) and exosome (Exo) using a CD59-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). (**b**) Sheep red blood cells (SRBCs) (Innovative Research, Southfield, MI) were washed and then resuspended in PBS with C5b6 and C7 in the presence or absence of exosomes. The mixture was incubated at 37 °C for 15 min before C8 and C9 were added with or without a blocking CD59 antibody (AbD Serotec, Oxford, UK) for additional 30 min incubation. The cells were centrifuged and the amount of hemoglobin released by the lysed SRBC in the supernatant was measured by absorbance at 415 nm. The positive control was SRBCs lysed with Triton X-100. The negative control is the sample without addition of complement components. The absorbance value of positive control was normalized to 100%. Each bar represents mean ± SEM of three independent experiments with triplicate for each sample. *p = 2.8E-06, **p = 3.51E-08

Activation of the complement system initiates a biochemical cascade that generates several key products: C3b, which binds to the surface of pathogens and enhances phagocytosis of these pathogens; C5a, which recruits inflammatory cells by chemotaxis; and C5b, which initiates formation of the membrane attack complex (MAC) consisting of C5b, C6, C7, C8, and polymeric C9. MAC deposited on the target cell forms a transmembrane channel that causes cell lysis. MAC-mediated lysis can be inhibited by CD59 [125] (protectin) a widely expressed glycosylphosphatidyl inositol (GPI)-anchored membrane protein that binds to C8 and C9 in the assembling MAC and interferes with membrane insertion and pore formation.

Incidentally CD59 is also present on MSC exosomes (www.exocarta.org) (Fig. 3.4a), and this provides a possible mechanism for the well-documented MSC efficacy in treating immune or inflammatory diseases (e.g., GVDH) [126, 127].

Complement-mediated lysis of sheep red blood cells is inhibited by MSC exosomes in a CD59-dependent manner (Fig. 3.4b). The mechanism by which CD59 on exosome membranes could inhibit MAC formation on the membrane of sheep red blood cells has not been elucidated. However, recent studies [128, 129] have shown that soluble recombinant CD59 could also inhibit MAC formation albeit less efficiently than GPI-linked CD59, suggesting that CD59 need not be on the same membrane where MAC is being assembled to exert an inhibitory effect.

3.4 Conclusion

A therapeutic component in MSC secretion is a lipid vesicle that has a complex protein and RNA cargo. A preliminary analysis of the protein cargo in MSC exosomes revealed that the biochemical and cellular processes driven by MSC exosomes are rather generic processes. They also share a common feature in that they are highly relevant to tissue injury and cellular repair (e.g., glycolysis, survival signaling, and MAC formation). These processes enable injured cells to better ameliorate tissue injury and enhance tissue repair by promoting anabolic activities through increased anabolic activities, decreasing cell death by activating the adenosine-mediated survival signaling pathway, and reducing complement-activated cell lysis. The rather generic nature of these exosome-driven processes suggested that the therapeutic activity of MSC exosomes is targeted at fundamental processes in cellular trauma and repair. This may provide a rationale for the therapeutic efficacy of MSCs in diverse disease indications. It also suggests that MSC exosomes could provide adjunctive therapy to alleviate tissue injury and enhance cell repair in many pathological conditions.

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Chapter 4
The Biology of Mesenchymal Stem Cells in Health and Disease and Its Relevance to MSC-Based Cell Delivery Therapies

Catherine M. Browne, Tung-Liang Chung, and Kerry Atkinson

Abstract Mesenchymal stromal cells (MSCs) are dynamic cells that orchestrate tissue morphogenesis during development, support haematopoiesis throughout life, and are key mediators of tissue repair and disease resolution. In certain disease states, however, MSCs mediate tissue disrepair and disease exacerbation. Herein we describe the salient features of MSCs, and highlight the need for a deep understanding of the molecular mechanisms that underpin the biological function of these cells, in order to develop safe and effective MSC-based therapies for acute and chronic disorders that are currently untreatable.

Keywords Mesenchymal stromal cells • Multipotency • Cellular therapies

4.1 Introduction

The three basic characteristics of stem cells are that they are self-renewing, multipotent and clonogenic [1]. Clonogenic cells are genetically identical. These cells can form colonies with potential to give rise to various differentiated cell types of the origin in which the stem cell exists [1, 2].

During the development of an organism, there are many different types of stem cells that feature in various developmental stages. At one extreme, totipotent stem cells, of which the zygote is the archetype, can give rise to all cell types in the body, plus the extra-embryonic structures (placenta, yolk sac). At the other extreme,

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multipotent stem cells or progenitor cells exhibit limited potency within their native niche, as a result of having undergone commitment to a select few lineages [2].

The pioneering work of Owen and Friedenstein over the last two decades has laid the foundation of mesenchymal stromal cells (MSC) research [3]. MSCs are plastic-adherent cells defined by the expression of CD73 and CD105 and lack of CD45 expression with CFU-F (colony forming unit fibroblast) ability [4]. Following on Friedenstein's original observation, bone marrow-derived stromal cells have been reported as the common progenitors of mesenchymal tissues. Thus, the mesodermal germ layer is the origin of MSC, which can give rise to connective tissues. There are different names for MSCs such as osteogenic stem cells (Friedenstein) and marrow stromal stem cells (Owen), as these cells generate stromal cells in long-term cultures [5]. Based on the properties of self-renewal and differentiation, Caplan suggested that bone marrow-derived stromal cells be considered as mesenchymal stem cells (MSCs) [6]. However, a position paper from the International Society of Cell Therapy (ISCT) promoted the term multipotent MSCs, since evidence of true mesenchymal stem cells has been lacking until recently, and the vast majority of MSCs in *ex vivo* expansion cultures are transit amplifying cells. Some groups recently have reported that multipotential stromal cells can also differentiate into lineages unrelated to the mesodermal germ line (known as *trans*-differentiation) [7, 8], but this remains controversial.

In this chapter, we discuss the functional features of MSCs, describe their mechanisms of action and suggest how these findings can be translated to the clinical setting.

4.2 Multipotent Mesenchymal Stromal Cells (MSCs)

Although the majority of the literature uses the term mesenchymal stem cells [9], it has been thought to be inadequate by many researchers as it has been shown that human MSCs lose their multipotent properties when these cells are cultured indefinitely *in vitro*. However, recently three studies have shown that true mesenchymal stem cells do exist in the foetal human lung and in the bony compartment of the bone marrow [10–12]. The retention or loss of the stem cell phenotype by MSCs above passage 10 remains to be investigated. However, one report suggested that bone marrow-derived MSCs at or above passage 25 failed to show signs of senescence or malignant transformation following engraftment into immune-deficient mice [10].

Another difficulty in defining MSCs has been that a period of *ex vivo* expansion is required in order to gain adequate numbers of therapeutically useful cells. The cultured cells may be phenotypically different from their *in vivo* progenitors [13]. Thus, the general accepted definition of MSCs is of an *in vitro* expanded cell population, usually isolated by plastic adherence and defined using some markers that are characteristic of, but not unique to, MSCs. According to the guideline established by the ISCT, "multipotent MSCs" are the cells with the

following properties: (1) adherence to plastic surface in culture; (2) co-expression of mesenchymal markers such as CD105, CD73, and CD90 and loss of haematopoietic antigens; and (3) capability to differentiate *in vitro* toward the osteoblast, adipocyte and chondroblast lineages [4, 14].

4.2.1 Distribution and Isolation of MSC In Vivo

MSCs reside in specialised niches within various tissues. Using marking techniques, their distribution can be identified. For example, the labelling of MSCs by quantum dots *in vitro* can help to locate transplanted cells *in vivo* and can define their tissue distribution [15, 16]. An additional approach is the systematic isolation of MSCs from different tissues and their subsequent characterization. For example, through the establishment of long-term culture and functional characterization of MSC populations, *in vivo* distribution of post-natal murine MSCs was analysed from different tissues and organs [17]. It has been shown that MSC distribution is wider than previously expected. MSCs can be found in virtually all post-natal organs and tissues. In particular, there is a reservoir of progenitor cells along blood vessel walls that may be related to the origin of MSCs [18]. Recent research suggests that in bone marrow and some other tissues, MSCs might occupy a perivascular zone. In those areas, MSCs would support blood vessels and contribute to tissue and immune system homeostasis [19].

At present, most studies of MSCs use cells isolated from bone marrow. *In vivo*, MSCs are present as a rare population, representing 0.0001% of nucleated bone marrow cells. MSCs appear to play an important role in the haematopoietic stem cell (HSC) niche and in the regulation of haematopoiesis [11]. However, only a small number of stem cells with multipotential self-renewing capability exist. Recently, these cells have been identified as sub-endothelial cells that are $CD146^{+}$ [12]. The therapeutic application of MSCs requires *ex vivo* expansion to obtain a large number of cells. It is worth noting that while cells isolated from various tissues share many characteristics, they exhibit some differences in their gene expression profile and differentiation potential [20]. *In vivo,* the total number of MSCs decreases with an individual's age. For example, a dramatic decrease in MSCs per nucleated marrow cell could be observed when grouped by decade, with a tenfold decrease from birth to a teenager and another tenfold decrease from a teenager to elderly [21]. It has been shown that a direct correlation exits between advanced age and decreased osteogenic potential. This fact may contribute to diseases in the ageing population, such as osteoporosis [22]. In addition, MSCs with lower proliferation potential isolated from older donors may be responsible for the reduced healing capacity found in older patients [23].

MSCs can be derived from many different organs and tissues such as placenta, adipose tissue, blood vessels (as perivascular cells) [3, 7, 24–27], amnion [28], amniotic fluid [29], fat [30], lung [31] and liver [32]. Most of these sources are relatively difficult to access as a tissue source for the isolation of MSCs. In particular,

the collection of bone marrow not only remains an invasive procedure with significant discomfort for the donor, but also results in a relatively low yield of MSCs [33]. In contrast, placenta is readily and widely available. As there is no significant difference between MSCs grown from bone marrow and placenta [34], the use of placenta as a source of human MSCs for clinical trials might be to bone marrow.

4.2.2 Culture and Expansion of MSCs In Vitro

It is widely accepted that stem cells *in vivo* can regenerate and expend indefinitely throughout an individual's life. However, they may show limited proliferation and differentiation in an *ex vivo* setting [35]. This is the case for MSCs. The capacity of MSC to expand *ex vivo* is highly variable, even from two samples from the same donor [35]. All these observations have posed a challenge for comparing data from different groups. In addition, many factors including culture parameters such as nutritional level, cell confluence, oxygen level, number of passages and plastic surface quality can influence MSC behaviour [36]. For example, Vacanti *et al.* examined passage number and its effect on MSC characteristics [36]. They compared early (<5 passages) to late (>15 passages) MSCs and showed that late MSCs had characteristics associated with cell ageing as depicted by actin accumulation and reduced substrate adherence [37]. Furthermore, early MSCs remained pluripotent, while late MSCs had limited differentiation capacity [37]. To address this concern, researchers have tried to identify the MSC niche *in vivo* in hope of mimicking this environment in an *ex vivo* setting to allow for maintenance of the multipotent state of MSCs.

MSCs have also been reported to undergo malignant transformation after *ex vivo* expansion [38]. Malignant transformation or an altered karyotype are a concern, particularly after long-term *ex vivo* expansion. However, it has been shown that carefully controlling the culture conditions of MSCs can reduce the chance of malignant transformation [39]. Of note, aneuploidy recently reported in culture-expanded human MSC populations was not necessarily associated with transformation; instead, these aneuploid MSCs became senescent and their growth was arrested [40].

4.2.3 Surface Markers for MSC

There is no specific and unique single marker for *ex vivo* cultured MSCs. The consensus is that human MSCs do not express the haematopoietic markers CD45, CD34 and CD14 or the co-stimulatory molecules CD80, CD86 and CD40, whereas they do express variable levels of CD105 (also known as endoglin), CD73, CD44, CD90 (Thy-1), CD71 (transferrin receptor), the ganglioside GD2 and CD271 (low-

affinity nerve growth factor receptor) [4, 7, 41]. Furthermore, Simmons *et al.* have also suggested that multipotent MSCs can be preferentially enriched by using the markers Stro-1 and VCAM-1 [42, 43]. In addition, Battula *et al*. [44] recently reported a panel of monoclonal antibodies with strong selectivity for MSCs, including the monoclonal antibodies W8B2 against human mesenchymal stem cell antigen-1 (MSCA-1) and CD56. As shown by CFU-F assays, MSCA-1$^+$ and CD56$^+$ MSCs had the capacity to differentiate into mesodermal lineages. However, there remains diversity within all these populations, even at the clonal level. Thus, there is still no universally accepted phenotypic definition of a MSC. At present, MSCs are expanded *ex vivo* before any clinical application, and it should be noted that the properties attributed to these cells are those of *ex vivo* expanded cells. The use of a definitively phenotyped MSC population remains an unmet goal in the MSC research field.

It is therefore important that researchers continue to gather new and additional information regarding MSC characteristics, using an acknowledged standard to evaluate the behaviour of MSCs. These data will be helpful for comparing information from different groups and in the translational application of MSCs. In particular, detailed descriptions of cell markers and phenotypes may help us to identify the most appropriate tissue source for a specific therapy at a specific *ex vivo* stage and for a certain individual.

4.2.4 Multipotential Capacity of MSCs

Since their multi-lineage potential was identified a decade ago, MSCs have generated significant biomedical interest [7, 45]. MSCs are capable of differentiating into cells of the mesodermal lineage including chondrocytes, adipocytes and osteocytes, making MSCs suitable for a wide range of potential therapeutic applications [3, 7]. Furthermore, they can differentiate into endothelial cells, form capillaries *in vitro* and secrete growth factors such as vascular endothelial growth factor (VEGF) to support angiogenesis [7]. In line with these observations, undifferentiated MSCs express many lineage-specific genes other than those of the mesenchymal lineage [46]. Due to their differentiation capacity, reproducible isolation, high expansion potential and capacity for genetic modification, MSCs are a good candidate for the repair and regeneration of a large variety of tissues.

Recently, Sakaguchi *et al*. [47] isolated MSCs from bone marrow, synovium, periosteum, skeletal muscle and adipose tissue in a comparative study to evaluate their colony-forming capacity and differentiation in defined conditions. Their study suggested that even if cultured in exactly the same conditions, MSCs sourced from different tissues varied in their abilities to undergo terminal osteocyte, adipocyte and chondrocyte differentiation. Furthermore, manipulation and other technical artefacts may impact the phenotype of MSCs.

4.2.5 *Immunosuppressive and Immunomodulation Capacity of MSCs*

Both *in vitro* and *in vivo,* culture-expanded MSCs are immunosuppressive and anti-inflammatory and thus are attractive candidates in treating human disease such as inflammatory autoimmune conditions. Also, these cells are MHC II negative and lack co-stimulatory molecules such as CD40, CD80 and CD86. Allogeneic transplantation of MSCs can be performed without immunosuppression in adult outbred immune competent adult animals and humans [48–50]. The mechanism for this remains unclear at present, but appears to be an active process involving the suppression of T-cell function [51–53]. It has been reported that MSCs can inhibit the proliferation and cytokine production by T cells, B cells, NK cells and dendritic cells via multiple mechanisms in a dose-dependent manner. Mixtures of cytokines and cell-to-cell contact molecules are involved in mediating these effects *in vitro* and *in vivo* [54, 55]. For the therapeutic application of MSCs, major histocompatibility complex (MHC) matching between MSC donor and recipient is not required, thus making them available in a timely manner for patients in a variety of acute and chronic clinical settings. However, it has been reported that induced functional HLA-DR appears in MSCs after exposure to expansion media containing mitogenic growth factors such as FGF-2 and PDGF, molecules used to enhance consistency of bioprocessing of the cellular product [56]. It will be important to determine whether, after transplantation, these MSCs can act as nonprofessional antigen-presenting cells.

In addition, MSCs can modulate immune cells associating with immune-related disorders, especially autoimmune diseases [57, 58]. It has been suggested that MSCs express a wide range of receptors and manufacture and release a number of cytokines and chemokines. Additional investigations into the mechanisms underlying the immunomodulatory effects of MSCs may increase our understanding of the immune system. Bartholomew and colleagues demonstrated for the first time that injection of allogeneic MSCs prolonged skin graft survival in baboons [59]. These *in vivo* and *in vitro* studies have provided support for the immunomodulatory role of MSCs. Interestingly, MSCs show both immune enhancing and suppressing capabilities. For example, they can enhance immune function by serving as antigen presenting cells (APCs) through an autocrine interferon-γ (IFN-γ)-dependent pathway. However, they can also directly inhibit antigen presentation and promote immune suppression when the level of IFN-γ increases above a given threshold [60]. This suggests that the small window of immune activity of MSCs may provide protection against foreign antigens while limiting damage caused by an exacerbated inflammatory response. In addition, MSC-induced immune suppression by IFN-γ is associated with an up-regulation of B7-H1, a co-stimulatory surface molecule on stem cells. This suggests that cell-to-cell contact is important for immune function of MSCs [61]. Additionally, secretion of soluble factors is important to the immune-regulatory role of MSCs [62, 63]. These MSC-secreted soluble factors can arrest B-cells in the G0/G1 phase, inhibit B-cell differentiation

and impair B-cell chemotaxis [62]. MSCs modulate monocyte function in a contact-independent manner through secretion of IL-1[beta]. This event induces the expression of TGF[beta]1 expression by MSCs and leads to the inhibition of alloreactive T-cells and the down-regulation of expression by MSCs of CD25, CD38 and CD69 cell surface markers. MSCs also modulate the function dendritic cells and NK cells using a similar mechanism [53, 64–67].

4.2.6 Migration of MSCs

When MSCs are injected intravenously in the normal healthy animal, the bone marrow has traditionally been thought of as the preferred (default) organ to which MSCs home after passage through the lungs [68–70]. However, when inflammation is present this does not appear to be the case as intravenously injected MSCs appear to preferentially home to the site of inflammation [71, 72]. The molecular mechanisms responsible for homing to injured site are not fully understood.

The tissue in which MSCs will exert functional effects is normally the homing site. For example, leucocytes migrate to peripheral sites, such as secondary lymphoid tissues or sites of inflammation. The molecular mechanisms involve a complex process required to cope with shear forces generated in the blood stream. This represents a likely paradigm for MSC homing. While the exact mechanisms of MSC homing remain elusive, some studies have shed light on factors that may govern MSC trafficking. For example, when rats were placed in a hypoxic chamber over a 3-week period, a 15-fold increase in the pool of circulating MSCs was observed [73]. This increase was specific to MSCs, while the number of haematopoietic precursors remained unchanged. It has been suggested that a matrix metalloproteinase (MMP)-dependent pathway may be responsible for such hypoxia-induced cell trafficking [74]. In addition, stromal cell-derived factor-1(SDF-1, also known as CXCL12) plays a crucial role in MSC migration by its selective expression at sites of injury [75, 76]. Furthermore, Ceradini *et al.* showed that attraction of CXC chemokine receptor-4 (CXCR-4)-positive progenitor cells to injured tissue is facilitated by hypoxia-inducible factor-1 (HIF-1) which enhances overexpression of SDF-1 in a gradient proportional to tissue hypoxia [77]. A decline in HIF-1 expression has been shown to be age-dependent. This may relate to the impaired ability of MSC homing and tissue repair found in the elderly [78]. Of note, A CXCR4-SDF-1 dependent homing mechanism has also been reported to be related to the migration of MSCs toward sites of malignant growth [79, 80].

Increased inflammatory chemokines secreted at the site of inflammation likely cause MSCs to preferentially migrate to these sites. MSCs express the receptors for several chemokines released after tissue damage [81]. It should also be noted that MSCs are large cells with a size of about 20–100 μm diameter in cell suspension and 10–20 μm in tissues. This likely causes the cells to be caught up in capillary beds, especially those of the lungs after intravenous injection.

4.2.7 Tissue Engraftment: Therapeutic Potential of MSCs

For a variety of unmet medical needs, MSCs have potential as a beneficial biological therapeutic agent [82]. MSCs have been studied for tissue regeneration, and increasing evidence supports their use for treating both genetic and acquired human diseases relating to loss of specialised tissues. However, it is still under debate in terms of the capacity of MSCs to engraft *in vivo*, differentiate into mature long-term surviving cells, and restore damaged cell functions. They nonetheless represent one of the most promising applications for regenerative medicine. Many studies indicate that systemically injected MSCs exert an efficient therapeutic potential by MSC-secreted soluble mediators [83] as well as a constitutive immunosuppressive capacity. [84]. MSCs express both chemokine receptors and adhesion molecules enabling their homing function to injured sites *in vivo* in response to likely specific chemokine gradients [85].

A growing body of evidence suggests that MSCs may impart a therapeutic benefit in various disorders that result from cell injury or cell loss. Preclinical studies have shown that MSCs improve myocardial function after myocardial infarction [86–89], liver damage [90], lung damage [72], cerebral function (after cerebral infarction), liver and joint damage [91–93], repair of non-healing bone fractures and resolution of corticosteroid-refractory graft-versus-host disease [49]. In particular, several pre-clinical investigations have reported that MSCs attenuate maladaptive left ventricular (LV) remodelling and preserve and/or promote recovery of pump performance after myocardial infarction. The restorative mechanism of MSC transplantation in the latter setting is still unclear. It has been suggested that these effects have been variously attributed to *de novo* cardiomyogenesis and neoangiogenesis. On the other hand, some evidence indicates that the therapeutic effects of MSC transplantation are due mainly to indirect stimulation (or paracrine effects) of neovascularisation and protection from ischemia-induced cell death [94, 95].

4.2.8 Advantages of Using MSC in Clinical Application

Perhaps, as a cell therapy, the most important aspect of MSCs is their potential for mass production and cryopreservation as an allogeneic MSC cell bank. MSCs could be the first cell type to become an “off-the-shelf” therapeutic cellular product for human disease. Because there seems to be no need to match MHC from the donor to the intended recipient, a single production run of MSCs can service many different patients in a number of clinical applications. That is, they can be mass-produced, cryopreserved and shipped to medical facilities for immediate use in both acute and chronic disease settings. Thus, MSCs have the potential to become novel cellular therapeutic agents in the twenty-first century.

4.3 The Role of Mesenchymal Cells in the Perturbation of Tissue Homeostasis

4.3.1 Cellular Origins of Mesenchymal Cells Involved in Tissue Perturbation

Mesenchymal cells, including MSCs, are implicated in the pathogenesis of a variety of inflammatory, fibrosing and metastatic diseases. Mesenchymal cells may be recruited from tissue-resident or circulating MSCs and fibrocytes [96–104], tissue macrophages, myofibroblasts, as a result of epithelial-to-mesenchymal transition (EMT), endothelial-to-mesenchymal transition (EndMT) [105–109], the accumulation of mesenchymal cells that have failed to undergo mesenchymal-to-epithelial transition (MET), or from cancer stem cells [103, 110, 111]. In this section, we describe the salient features of these cells as mediators of tissue morphogenesis during development, wound repair throughout life, and of tissue pathogenesis in disease.

4.3.2 Circulating and Tissue-Resident MSCs in Health and Disease

MSCs, alternatively defined as multipotent MSCs, are a heterogeneous group of non-haematopoietic progenitor cells that were originally classified as colony-forming unit-fibroblasts (CFU-F). MSCs are plastic-adherent, exhibit a spindle-shaped morphology and can differentiate into osteoblasts, chondrocytes, adipocytes, myocytes and connective tissue. Also, although somewhat controversial, MSCs have been reported to be able to differentiate into neuron-like cells, hepatocytes and pancreatic-like cells [84, 112, 113]. MSCs contribute to tissue homeostasis and repair by virtue of their potent pro-angiogenic, anti-proliferative and anti-inflammatory properties [114]. However, these same attributes underpin tissue pathogenesis in certain disease processes, most notably growth and metastasis of solid and haematological cancers [84, 109–111] and leukaemia relapse following MSC co-transplantation with haematopoietic stem cells to prevent graft-versus-host disease [111]. Prolonged MSC proliferation *in vitro* carries the risk of accumulation of cytogenetic abnormalities and subsequent differentiation into tumour cells following engraftment *in vivo* [84]. In these conditions, MSCs may be a potential source of mesenchymal tumour cells (MTCs), which are described below.

4.3.3 Fibrocytes

Fibrocytes were first described more than 150 years ago as a circulating, bone marrow-derived cell with the ability to adopt a mesenchymal phenotype [99–101, 115].

Fibrocytes arise from $CD14^+$ $CD34^+$ $CD45^+$ peripheral blood mononuclear cells and account for between 0.1% and 0.5% of circulating leukocytes [98, 100]. Following their expansion *in vitro*, fibrocytes express markers of haematopoietic stem cells (CD34, CD105), leukocytes (CD45, LSP-1), monocytes (CD11a, CD11b, CD13, CD32, CD64), Fcγ receptors (CD16, CD32a, CD32b, CD32c), cell surface molecules involved in antigen presentation (MHC Class I, II, CD40, CD54, CD80 and CD86) and integrins (CD18, CD29, CD49a, CD49b, CD49e, CD61). Fibrocytes also express several receptors (CCR1, CCR3, CCR4, CCR5, CCR7, CCR9, CXCR1, CXCR3, CXCR4), secrete a range of extracellular matrix (ECM) proteins (Collagens I, III, IV and V, fibronectin, vimentin, tenascin, α-smooth muscle actin (αSMA), matrix metalloproteinase-9 (MMP-9)) and glycosaminoglycans (perlecan, versican, hyaluronan, decorin and biglycan). *Ex vivo* expanded fibrocytes do not express detectable dendritic cell markers, nor B-cell or T-cell markers [97, 103] Collectively, these molecules reflect the multi-faceted functions of fibrocytes, notably cytokine production, immune cell trafficking, ECM production, αSMA production, lipid metabolism, antigen presentation, angiogenesis, MMP production and chitinase production [103].

Perhaps best known for their roles in tissue morphogenesis and remodelling during development and wound healing throughout life, fibrocytes, *ex vivo*-expanded mesenchymal stem cells, and terminally differentiated, activated tissue fibrocytes, known as myofibroblasts, have each been associated with diverse forms of tissue perturbation in both experimental and clinical settings. In humans, these cells are implicated in a range of pathogenic processes namely (1) fibrosing diseases including interstitial pulmonary fibrosis, idiopathic pulmonary fibrosis, scleroderma, nephrogenic systemic fibrosis, cardiac fibrosis, atherosclerosis, liver fibrosis, reactive fibrosis in chronic pancreatitis and cystitis, radiation-induced fibrosis and severe asthma [97, 100, 102, 104, 107, 108, 116], (2) the conversion of parasite-infected macrophages to a regulatory role, enabling persistence of intracellular infection [117], (3) the formation of tumours of mesenchymal origin, (4) cancer cell invasion [106, 109, 118–122] and (5) the pathogenesis of ophthalmopathy associated with Graves' disease [123, 124]. Some of the mechanisms by which these outcomes are achieved include: (a) TGFβ1-, ET1-, semaphorin 7A- and β1 integrin-mediated unrestrained differentiation of circulating monocyte progenitors to fibrocytes. TGFβ signalling by tissue fibrocytes induces their terminal differentiation to activated fibroblasts and myofibroblasts, and these in turn deposit collagen within the local milieu, express αSMA and attain contractile force and motility [97, 100, 103, 116]; (b) the recruitment of fibrocytes to the site of tissue injury aided by tissue secretion of chemokine signals; (c) fibrocyte production of pro-inflammatory cytokines, ECM proteins and angiogenic factors; and (d) the expression by fibrocytes of MHC Class II molecules, and their presentation of antigens.

4.3.4 Myofibroblasts

Myofibroblasts are activated fibroblasts with features of both mesenchymal and smooth muscle cells. These cells may be derived from bone marrow-derived and

circulating MSCs, tissue-resident mesenchymal cells, through EMT or EndMT, or from circulating fibrocytes [105, 113]. Myofibroblasts are a rich source of growth factors, including hepatocyte growth factor (HGF), TGFβ and interleukin-6 (IL-6) [113]. These cells mediate the repair of damaged epithelium and scar formation and, in their activated state, co-operate with epithelial and endothelial cells to secrete matrix metalloproteinases that digest injured tissue and facilitate the synthesis of a provisional ECM [105]. Cessation of the inflammatory reaction leads to resolution of the initial EMT/EndMT phenotype and the elimination of myofibroblasts via apoptosis [119].

Dysregulation of EMT/EndMT may lead to the persistence of myofibroblasts and the exacerbation of the inflammatory response, or tumour formation, growth and metastasis. In disease, myofibroblasts are otherwise known as tumour-associated (myo)fibroblasts (TAF), carcinoma-associated fibroblasts (CAF), fibrocytes or pericytes. As mediators of tumorigenesis, myofibroblasts form fibrovascular stromal networks, a diverse arrangement of septa within tumours, and microvasculature that contain αSMA- and desmin-expressing cells, and their presence is associated with heightened tumour aggressiveness and a poor prognosis [113]. Mouse xenograft models have also shown that intravenous infusion of an admixture of human MSCs with ovarian adenocarcinoma cells resulted in the transition of human MSCs to myofibroblasts, evidenced by their expression of tenascin-C (Tn-c), thrombospondin-1 (TSP1), fibroblast-specific protein (FSP), fibroblast activating protein (FAP), αSMA, desmin and tubulin [113], as well as expansion of the tumour. Together, these observations highlight the tropism of MSCs for tumours and their potential to be transformed into a pro-inflammatory and tumour-supporting phenotype.

4.3.5 Epithelial-to-Mesenchymal Transition (EMT) in Health and Disease

EMT is an indispensable process that is conserved among all metazoan organisms [122]. This process was first demonstrated *in vitro* in three-dimensional collagen gel cultures of epithelial cells, isolated from the embryonic and adult anterior lens [125]. EMT drives the conversion of polarised, immotile epithelial cells to cells that are apolar, exhibit significant reorganisation of the actin cytoskeleton, and are highly migratory. Concomitant with this transition is the loss of expression of markers of epithelial cells, notably E-cadherin (CDH1), loss of tight junctions and cell polarity proteins, and the induced expression of mesenchymal markers including vimentin, fibronectin, CD44, ECM metalloproteinases and N-cadherin (CDH2) [126].

During early embryogenesis mesenchymal cells arise from the primitive epithelium. EMT is associated with normal tissue morphogenesis, organogenesis, tissue remodelling and wound healing. Three distinct types of EMT have been described, based on the primary stimulus and the outcome(s) (Table 4.1). Inappropriate

Table 4.1 Epithelial-to-mesenchymal transition

	Stimulus for initiation	Event(S)	Outcome(S)	Diseases/disorders	References
Type 1 EMT	Embryogenesis	Gastrulation • EMT resulting in production of multipotent MSCs • MET resulting in formation of new epithelial structures Neurulation Myogenesis Palatogenesis Cardiac valve formation Nephrogenesis (via MET)	Formation of endoderm, mesoderm ectoderm	*Developmental defects*: Cleft palate Heart valve Cranio-facial Peripheral nervous system	[5, 7]
Type 2 EMT	Noxious stimuli	Coagulation, clot formation; enhanced matrix metalloproteinase production; recruitment of immune cells and platelets to denuded wounds; production of provisional extracellular matrix; activation of angio-genesis; activated myofibroblasts mediate wound healing and closure, and *de novo* epithelial synthesis via MET	Wound healing Tissue homeostasis Resolution of EMT phenotype (inflammation; scar formation); elimination of myofibroblasts by apoptosis	None	[5]
Type 3 EMT	Persistent infection Autoimmune reactions Allergic responses Chemical insults Radiation Tissue injury Genetic predisposition	EMT	Invasion Migration Metastasis	*Fibrosis* (asthma; idiopathic pulmonary fibrosis; pancreatitis; cystitis; liver cirrhosis, systemic sclerosis, progressive kidney disease, cardiovascular fibrosis; thyroid-associated ophthalmopathy) *Cancer* (oesophageal, ovarian, breast, papillary thyroid, cervical, colorectal, glioblastoma multiforme, pulmonary adeno-carcinoma)	[7, 8]

activation of EMT contributes to the pathogenesis of a variety of human diseases, with inflammatory and fibrotic components, as described below.

4.3.6 Type 1 EMT

The earliest known EMT event that takes place in most metazoan embryos is the formation of multipotent mesenchymal cells. These in turn give rise to endoderm, mesoderm and ectoderm from the invaginating primitive streak in the process known as gastrulation [119]. Subsequently, EMT facilitates neural crest development, secondary palate formation, cardiac valve formation, myogenesis, nephrogenesis and male Müllerian duct regression [121, 122]. Type 1 EMT and its reverse process, MET, are indispensable for the differentiation and migration of specialised cell types, resulting in tissue morphogenesis and organogenesis [126, 127]. Type 1 EMT is not governed by inflammatory reactions nor is it associated with tissue fibrosis, inflammation nor aberrant migration of cancer cells. Small, non-protein-coding microRNAs (miR) have recently been identified as regulators of EMT. In metazoa, these regulatory molecules serve to inhibit both the abundance and translation of their target mRNAs [128]. The induction of EMT by ectopic expression of protein tyrosine phosphatase Pez (PTP-Pez) in Madin Darby canine kidney (MDCK) epithelial cells resulted in the loss of E-cadherin expression, induction of the mesenchymal genes, fibronectin, ZEB1 and SIP1, loss of cohesion, induction of cell motility and a change in cell morphology. A comparison of miR expression levels in MDCK and MDCK-Pez cells by microarray analysis revealed that all five members of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141) and miR-205 were down-regulated in cells that had undergone EMT, and that ectopic expression of miR-200 genes alone was sufficient to inhibit TGFβ-mediated EMT [128].

4.3.7 Type 2 EMT

Type 2 EMT is governed by inflammatory reactions and is essential for restoring tissue homeostasis by wound repair and tissue remodelling in response to noxious insults [119]. Normal epithelia that arose during gastrulation experience an insult or injury, which triggers endothelial and epithelial cells to produce factors that mediate coagulation and clot formation. These events, coupled with enhanced matrix metalloproteinase production, promote the recruitment of immune cells and platelets to the lesion, leading to the production of a provisional ECM and activation of angiogenesis. The healing process is enhanced by TGFβ-stimulated induction of EMT in myofibroblasts. The resulting cells differentiate, become activated and migrate into the lesion to initiate wound repair, closure and re-epithelialisation [119].

4.3.8 Type 3 EMT

Type 3, or pathological EMT occurs in response to profound tissue disturbances that are associated with many disorders, most notably mammary cancer progression and metastasis [119]. Type 3 EMT is distinguished from Types 1 and 2 EMT by virtue of its occurrence in oncogenically transformed cells that harbour genetic and epigenetic abnormalities, and subvert the molecular events that are required for EMT, to induce metastatic dissemination. At the gene expression level, Type 3 EMT may be induced by the direct binding of SNAIL, ZEB1 and ZEB2, E47 and KLF8 factors to the CDH1 gene promoter, resulting in the repression of E-cadherin expression. Alternatively, Type 3 EMB may be induced by indirect silencing of E-cadherin transcription by Twist, Goosecoid, E2.2 and FOXC2 transcription factors [127]. Suppression of miR-205 by ZEB transcription factors, activation of TGFβ, canonical and non-canonical Wnt-β-catenin signalling, and chromatin remodelling by histone deacetylases HDAC1 and HDAC2, collectively reinforce Type 3 EMT to facilitate tissue pathogenesis [121, 122, 126, 127, 129–131].

4.3.9 Endothelial-to-Mesenchymal Transition (EndMT)

The endothelium is a single cell layer of mostly squamous epithelium that lines the blood and lymphatic vasculature. During development, EndMT-derived cells delaminate from an organised cell layer, lose cell–cell cohesiveness, down-regulate their expression of the endothelial marker CD31 (PECAM-1) and acquire the migratory and invasive phenotype of mesenchymal cells [106]. EndMT is a critical event in heart development; however, it has also been associated with cancer, angiogenesis, cardiac fibrosis, pulmonary hypertension, atherosclerosis, wound healing and acute and chronic kidney injury. In each case, EndMT-derived cells function as fibroblasts in damaged tissue; however, the molecular mechanisms by which these cells mediate tissue pathogenesis remain to be investigated [106].

4.3.10 Mesenchymal-to-Epithelial Transition (MET)

During development, MET is essential for kidney organogenesis [132], cardiogenesis [133], hepatogenesis [134] and somitogenesis [135]. MET is also implicated in the establishment of metastases of colorectal cancer [136] and of invasive breast cancer cells with a mesenchymal phenotype [128]. This highlights the vulnerability of mesenchymal cells to undergo transition to an epithelial phenotype, which enables them to integrate into and proliferate within remote organs. The loss of expression of miR-200 family members is thought to be a pivotal event in breast cancer metastasis [128].

4.3.11 Mesenchymal Tumour Cells (MTCs)

MTCs likely arise from cell-autonomous or environmentally induced changes in the phenotype of the precursor cell. MSCs that are expanded *ex vivo* for several generations, for example, may accumulate cytogenetic abnormalities and epigenetic changes as a consequence of adaptation to their artificial environment. One reported outcome of this treatment is the incidence of fibrosarcoma following transplantation of *ex vivo*-expanded MSCs into experimental animals [111].

MTCs and MSCs are members of the same histogenetic lineage, exhibit similar morphological, immunophenotypic, gene expression and stemness features, are involved in similar biological programs and share an extraordinary capacity to generate new blood vessels. MTCs are, however, unique in their ability to support neoplastic growth of the epithelial compartment and mediate tissue pathogenesis in a cell-autonomous manner [114].

Human mammary carcinoma results from the complete replacement of the epithelial parenchyma by MTCs, and the recruitment of reactive stromal cells is a prerequisite for tumour growth and survival [114]. The spontaneous establishment of MTCs in HER-2/neu transgenic mice also demonstrated their ability to generate mesenchymal tumours [114]. Although stromal cell activation in tumours recapitulates many of the reparative processes that occur in wound healing, including activation of the angiogenic switch, it is unclear whether the neo-vascularisation of stromal tumours is a consequence of the recruitment of MSCs to the site, *trans*-differentiation of MSCs to endothelial cells, or MSC-to-MTC transition.

Compelling evidence from experimental models suggests that cancer stem cells or cancer initiating cells that are found in solid tumours of the breast, colon, brain and prostate result from EMT [137], whereas mesenchymal tumours of the kidney [109] may arise from aberrant EMT, inhibition of MET, mesenchymal-to-MTC transition, or other cause(s). In conclusion it would appear that neoplastic transformation, invasion and metastasis by mesenchymal cells occur by several alternate processes. These are governed by the genotype, epigenetic profile and molecular phenotype of the cell, the composition of the niche, autocrine and paracrine effectors, and the nature of the insult. Further investigation will also reveal whether benign mesenchymal tumours are regulated differently from their metastatic counterparts.

4.4 Perspective

4.4.1 Which Patients Are at High Risk of Adverse Events Following MSC Therapy and Why?

MSCs hold great promise as cellular mediators of tissue repair in fibrosing, inflammatory and metastatic disease, due to their tropism for diseased and damaged tissues, their reputation for safety in MHC-mismatched recipients following allogeneic

transplantation, and their immunosuppressive and migratory properties. These attributes are tempered by the potential of MSCs to undergo indirect transition in the damaged tissue into activated fibrocytes, myofibroblasts, activated fibroblasts, epithelial cells (via MET), or direct transition to cancer stem cells. These processes may result from cell-intrinsic and/or cell-extrinsic mechanisms and may lead to the exacerbation rather than resolution of the disease or injury. For these reasons, a clear understanding of the molecular pathways that facilitate cross-talk between MSCs and their injured or diseased niche, together with a full knowledge of the patient's medical history and disease susceptibilities, should assist in determining the suitability of MSC-based cellular therapy as a treatment strategy.

4.5 Conclusions

MSCs are essential for tissue morphogenesis during development, haematopoiesis throughout life, and the orchestration of tissue repair following acute and chronic injuries. Their involvement in such diverse biological activities are in large part attributable to their ability to undergo *trans*-differentiation to and from epithelial and endothelial cells via EMT and EndMT, respectively, their ability to function as stromal cells, and their ability to express a vast number of trophic and ECM factors. Unlike other cells, MSCs do not provoke rejection following engraftment into MHC-mismatched recipients. Furthermore, they are amenable to amplification on a large scale, using bio-reactors, and they survive cryopreservation. These features underpin their usefulness in cell-based therapies in MHC-mismatched recipients suffering a range of tissue disorders.

Published data suggest that MSC homeostasis is a delicately balanced state that is easily perturbed by environmental and/or cell-intrinsic mechanisms, and may lead to malignant transformation via Type III EMT, or exacerbation of tissue damage in certain autoimmune diseases. A thorough understanding of MSC biology in health and disease, the underlying molecular mechanisms that lead to these states, and the validation of potential MSC-based cellular and/or molecular therapies in clinically relevant animal models are pre-requisites for the development of safe and effective treatments for a variety of acute and chronic diseases.

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Chapter 5
Pulmonary Clinical Applications for Mesenchymal Stem Cells

D. Chambers and P. Hopkins

Abstract Lung disease remains a significant health and economic burden to societies throughout the world and is projected to increase in prevalence. Recent medical advances have enhanced clinical care but further research is needed in the areas of inhibition of lung fibrosis, altering airway inflammation and manipulating the pulmonary vascular endothelium. Stem cells have the potential to address these deficiencies by their remarkable ability to differentiate into various tissue lines and regulate internal repair systems. These unique regenerative abilities provide a novel approach to management for those suffering from pulmonary disease.

Keywords Mesenchymal stem cells • Lung disease • Immunosuppression

5.1 Introduction

While therapies based on embryonic or induced pluripotent stem cell delivery to treat disease are many years away, adult stem cell treatment is much closer to the clinic. Among adult stem cells, mesenchymal stem cells (MSC) hold particular promise for the treatment of lung diseases. In this chapter we will review the potential clinical applications of MSC for lung disease by highlighting relevant preclinical and early human studies before touching on the implications of the discovery of a lung-resident population of MSC.

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5.1.1 The Burden of Pulmonary Disease

Respiratory disorders are a leading cause of death worldwide with further increases in mortality expected in the future. In Western countries, respiratory disease ranks second only to cardiovascular disease in terms of mortality, incidence, prevalence and socioeconomic cost. The aetiology of lung disease can be generally summarised into environmental, occupational, genetic, lifestyle and other causes. Nonetheless, for a significant number of disorders no aetiology is discernable and these are termed idiopathic. The societal burden is substantial and future management strategies need to focus on prevention and effective therapies introduced early in the disease process. In terms of non-malignant lung disease, the most pressing avenues for research are centred on altering airway inflammation, inhibiting lung fibrosis, manipulating the pulmonary vascular endothelium and developing novel therapeutic options for patients with hereditary and congenital disease processes.

5.1.2 Pulmonary Disease: Where Is the Clinical Need?

Chronic obstructive pulmonary disease (COPD) is a common respiratory disease characterised by irreversible airways obstruction culminating in progressive decline in lung function. Currently, COPD is the fifth leading cause of death worldwide with estimates of prevalence in the USA of 5% and 6.3% in Asia [1, 2]. Data from the UK General Practice Research Database estimate about 883,200 patients in the UK have a diagnosis of COPD. In England, in 2002/2003, COPD was recorded as the reason for hospital admission in 109,243 cases and accounted for 1,094,922 bed-days, with a median duration of stay of 6 days [3]. Mortality from COPD is expected to continue to increase in the coming decades while deaths from heart and cerebrovascular disease are expected to decrease. Reducing the burden of COPD requires better evaluation and diagnosis but also improved management strategies aimed at preserving lung function. Pathophysiologically COPD is a multicomponent disease with inflammation central to its pathogenesis along with parenchymal destruction and airway remodelling. Emphysema is almost invariably induced by cigarette smoking, and it is widely accepted that the disease is caused by excessive proteolytic enzyme activity by proteases and a chronic inflammatory process characterised by a cellular influx consisting of macrophages, neutrophils and T lymphocytes.

Medical therapy with inhalers and pulmonary rehabilitation has no significant impact on lung function trajectory in patients with COPD. Lung volume reduction either through novel bronchoscopic techniques or the conventional surgical approach targets only those patients with upper lobe dominant emphysema disease—at most just 25% of patients. Lung transplantation is an option for a select group of patients, but lack of donor organ availability, advancing age of potential recipients and the high economic cost of transplantation limit more widespread application. A theoretical

protective effect of MSC transplantation on pulmonary emphysema may be partly mediated by modulation of T cell function and inhibiting the apoptosis of lung cells by influencing the vascular endothelial growth factor signalling pathway [4].

Bronchial asthma is the most common chronic respiratory illness typified by reversible airflow obstruction and with a varied prevalence in Western countries of 10–12% [5]. Unlike COPD, asthma is an important cause of morbidity and mortality in children. Whilst mortality has fallen dramatically in the last 20 years, asthma rates have increased continuously during recent decades. There are several ways to estimate the burden of disease with one approach being the disability adjusted life year (DALY) score as adopted by the World Health Organization. This disability score describes the number of years of healthy life lost due to disability or premature death. In 2003, asthma was the eleventh-leading contributor to the overall burden of disease in Australia, accounting for 2.4% of the total number of DALYs. In that year, 63,100 years of healthy life were lost due to asthma—59,054 of these due to years lived with disability and 4,045 due to premature death. Morbidity from asthma remains substantial despite medical advances with preventative therapy over the years.

Idiopathic pulmonary fibrosis (IPF) is a relatively common chronic, fibrosing lung disease of unknown cause that is characterised by severe, refractory and progressive breathlessness. The course of disease is relentless with an average survival of 3.6 years from diagnosis and age at onset 61 years. Epidemiological studies suggest an annual incidence of 16.3 cases per 100,000 population and prevalence of 42.7 cases per 100,000 [6]. Although respiratory failure is the most common cause of death, other modes include congestive cardiac failure, lung infection, pulmonary embolism and bronchogenic carcinoma. No recommended or approved therapy exists currently for IPF with the exception of anti-inflammatory and anti-fibrotic agent pirfenidone which is licensed in Europe and Japan for those with mild to moderate disease. However, the Food and Drug Administration in the USA is demanding a further phase 3 trial of pirfenidone given inconsistencies in some previous studies. Central to the pathogenesis of IPF is abnormal epithelial repair and epithelial-mesenchymal transition. Endothelin receptor antagonists (ERA) are another potential treatment line although bosentan, a dual ERA, had no influence on disease progression in a large multicentre randomised controlled trial. MSC have theoretical benefits in the IPF patient in switching injured epithelia down the pathway of repair rather than fibrosis.

Acute lung injury (severe variant known as Adult Respiratory Distress Syndrome or ARDS) is defined as acute onset of severe hypoxia and pulmonary infiltrates within 12–72 h of a precipitating event. Sepsis is the leading cause, followed by pneumonia, aspiration of gastric contents, massive blood transfusion, multiple trauma and other tissue injury. ARDS is a significant issue for intensive care units with an estimated 18 to 34 cases per 100,000 population each year [7]. In published clinical trials, prolonged corticosteroid treatment at an initial dose of 1 mg/kg/day of methylprednisolone [8] significantly improves patient centred outcome variables. Nonetheless, despite recent improvements in critical care, the mortality rate still remains at about 50%. With the pathogenesis of acute lung injury/ARDS involving lung endothelial injury,

alveolar epithelial injury and the accumulation of a protein rich cellular debris in the alveolar space, one possible candidate for therapy is the MSC.

Idiopathic pulmonary arterial hypertension (PAH) is a syndrome characterised by a progressive increase in pulmonary vascular resistance leading to right ventricular overload and eventually to right ventricular failure and premature death. The annual incidence of PAH is estimated at 7.1 cases per million population and prevalence at 52 cases per million population [9]. The increase in pulmonary vascular resistance is related to a number of progressive changes in the pulmonary arterioles, including vasoconstriction, obstruction through proliferation of smooth muscle, fibroblasts and endothelial cells, inflammation and in situ thrombosis. The main histological features include medial hypertrophy, intimal thickening and plexiform lesions. The plexifom lesion represents a focal proliferation of endothelial and smooth muscle cells and is pathognomonic of PAH. Medical therapies for PAH centre on selective pulmonary vascular bed vasodilatation, anticoagulation and long-term antifibrotic and remodelling agents. Despite these advances, the condition remains invariably progressive with markedly reduced life expectancy. Future directions of therapy may focus on the delivery of MSC to alter endothelial-mesenchymal transition and directly promote vascular remodelling.

Two noteworthy congenital and genetic respiratory conditions respectively are neonatal bronchopulmonary dysplasia (BPD) and cystic fibrosis. BPD is a chronic lung disease that develops in infants born prematurely, particularly if they require treatment with oxygen and positive pressure ventilation. It has a complex pathogenesis incorporating contributions of hyperoxia, hypoxia, shear stress from mechanical ventilation, vascular maldevelopment, inflammation, malnutrition and genetics. The clinical picture of BPD has evolved with advances in medical care including surfactant replacement, antibiotic management and protective modes of mechanical ventilation. A significant number of infants with BPD are now surviving to adulthood, manifesting with a range of chronic lung diseases including emphysema [10]. Prevention of alveolar growth arrest with cell-based therapies remains an attractive and durable long-term therapeutic goal. Finally, cystic fibrosis is the most common autosomal recessive inherited condition with an incidence of approximately 1 in 2,400 births. The condition is typically caused by mutations in the gene coding for a protein called the cystic fibrosis transmembrane conductance regulator (CFTR). The defect in CFTR results in poor sodium-chloride ion flow regulation across cell membranes and the accumulation of thick tenacious mucus in the lung and digestive tract. Cystic fibrosis is potentially a model disease for stem cell treatment as the continued lung inflammation and infection may promote engraftment of circulating progenitor cells, corrected for the chloride channel defect [11].

5.2 The Lung: An Attractive Target for MSC Therapy

Due to their immunosuppressive properties, capacity to remodel extracellular matrix and perhaps also their ability to differentiate into lung epithelia, MSC have been proposed as a potential cellular therapeutic agent for lung diseases. One of the

difficulties with MSC therapy for other organs may also be an inherent advantage when pulmonary biotherapy is considered. One of the major barriers to non-pulmonary cellular therapy is the pulmonary first pass effect. Following intravenous infusion, due to the filtering function of the pulmonary vasculature, only a small proportion of cells pass through into the systemic circulation. This effect is particularly pertinent to MSC-based therapy due to the large physical size of the mesenchymal stromal cells. The first pass effect has impeded the development of regenerative therapy approaches such as MSC therapy for heart disease [12] and has led to the development of strategies to deliver MSC directly to the affected organ. For instance, in the case of the heart this has involved using direct intracoronary and myocardial stem cell injection, but local stem cell delivery strategies increase the potential risks and side effects of therapy (e.g., bleeding and tissue injury following direct tissue injection or occlusion and embolisation following direct arterial administration). Therefore, the ability to deliver cellular therapy to the lung via a simple intravenous approach is a major advantage and gives the potential for large-scale engraftment. Even more attractively, engrafting cells appear to target areas of injured lung [13, 14]. Direct intra-tracheal, intrapulmonary [15] and intrapleural [16] inoculation represent additional relatively non-invasive routes of administration which are specific to the lung.

Another advantage of MSC therapy is the ability to potentially transplant cells across the human leukocyte antigen (HLA) barrier. While it is clear that MSC have reduced immunogenicity when compared with many cell types [17] since they express only low levels of class I HLA, and no class II HLA or co-stimulatory molecules [18, 19], there is a substantial body of data which questions the degree of immunologic privilege awarded MSC [20]. For instance, MSC are immunogenic in that they can induce memory T-cell responses [21] and, as MSC express the activating NK cell-receptor ligands NKG2D and UL16 [22], they are susceptible to lysis by NK cells [23]. The practical implication is that preclinical work in HLA-matched and/or immunosuppressed animals needs to be cautiously interpreted in the planning of human phase I studies which are likely to involve HLA mismatching.

Despite this caveat, MSC represent an attractive and novel therapeutic agent for inflammatory and fibrotic lung diseases where the clinical need for treatment advances is strong. Although MSC are multipotent and are able to differentiate down lung epithelial lineages [24, 25], it is unlikely that the degree of parenchymal cell engraftment required to achieve a therapeutic effect will ever be achieved. It is much more likely that a therapeutic role for MSC will be created by exploiting their ability to remodel extracellular matrix [26, 27], or their ability to suppress the immune response through contact-dependent and soluble mediators [28–30].

The demonstrated immunosuppressive ability of MSC has translated to clinical trials currently being undertaken in graft-versus-host disease (GVHD) following allogeneic haematopoietic stem cell (HSC) transplantation, Crohn's disease, multiple sclerosis, lupus, COPD, insulin-dependent diabetes mellitus and in the renal transplant setting. The tissue repair capability of MSC is being investigated in clinical trials for cardiac repair, bone disorders (osteogenesis imperfecta), bone fracture, meniscectomy and liver repair (cirrhosis), as well as for enhancing engraftment after HSC transplantation. Studies have also been carried out using MSC to treat

various metabolic disorders, ischaemic stoke and neurological disorders. We will review the preclinical studies which have identified a potential niche for MSC therapy in the treatment of human lung disease, the current early phase human trials and finally the possible role of lung-resident MSC in the pathogenesis of lung disease.

5.2.1 Preclinical Studies: Acute Lung Injury

As outlined above, acute lung injury is a common complication in patients admitted to the intensive care unit, and still carries a substantial risk of mortality and residual morbidity despite decades of research. Unfortunately, care remains largely supportive. Due to their immunomodulatory effects, effects on epithelial repair and potential to reduce alveolar oedema, MSC have been considered as a potential treatment option [31]. Preclinical studies largely employing the endotoxin-induced model of acute lung injury have been encouraging [15, 32]. The therapeutic effect of MSC in this setting appears to be mediated largely by paracrine rather than contact-dependent effects, perhaps through the secretion of the keratinocyte growth factor (KGF) [32].

5.2.2 Preclinical Studies: Idiopathic Pulmonary Fibrosis

As highlighted above, IPF is a relatively common chronic, fibrosing lung disease of unknown cause. IPF affects older individuals (typically older than 50) and is characterised by severe, refractory and progressive breathlessness. On clinical examination, patients usually have fine bibasal crackles on auscultation of the chest and digital clubbing. Open lung biopsy, if performed, reveals geographic and temporally heterogeneous fibrosis with areas of active fibrosis (fibroblastic foci) and areas of normal lung. The course is usually relentless, with an average survival from diagnosis of only 3.6 years. To date, there is no approved or recommended therapy for the treatment of IPF, other than lung transplantation in highly selected individuals.

The therapeutic potential for MSC in IPF was first recognised when it was noted that lung fibrosis was diminished in a study designed to assess the effect of bleomycin-induced pulmonary fibrosis on pulmonary engraftment of MSC [27]. Since that study, multiple preclinical studies, summarised in Table 5.1, have demonstrated the therapeutic efficacy of MSC in the bleomycin model with MSC leading to reduced lung connective tissue (hydroxyproline and collagen) content and fibrosis scores.

Although there appears to be a consistent effect of MSC if delivered soon after the administration of bleomycin, the therapeutic effect diminishes considerably if treatment is delayed until 7 days after administration [27, 33]. This effect highlights an inherent deficiency of the bleomycin model. Bleomycin induces an initial inflammatory response which is followed by a fibrotic response, whereas IPF is now known to be a fibrotic disease from the outset with minimal or no preceding fibrosis.

Table 5.1 Preclinical studies of MSC in the treatment of lung fibrosis

Author	Model	Intervention	Outcome
Ortiz 2003 [27]	Mouse bleomycin	5×10^5 BM-MSC @0, 7 days via jugular vein	↓ Hydroxyproline—not significant with day 7 infusion
Cui 2007 [33]	Rat bleomycin	BM-MSC @ 1, 7 days via tail vein	↓ Hydroxyproline and lung fibrotic score—more pronounced with day 1 infusion
Zhao 2008 [45]	Rat bleomycin	5×10^6 BM-MSC @ 12 h via tail vein	↓ Hydroxyproline and pro-fibrotic cytokines
Moodley 2009 [13]	Mouse bleomycin	1×10^6 umbilical cord-derived MSC @ 1 day	↓ Hydroxyproline, collagen and pro-fibrotic cytokines
Bitencourt 2011 [46]	Mouse belomycin	Autologous MSC engraftment encouraged by hyaluronidase	↓ Collagen content and fibrotic score

BM-MSC bone marrow-derived mesenchymal stromal cells

Agents which have a predominantly anti-inflammatory, rather than anti-fibrotic effect may therefore appear effective in preclinical studies but be ineffective in humans. Successful later delivery of potential therapeutics is more likely, therefore, to reliably predict efficacy in human IPF. This is particularly important to recognise since the timing of MSC delivery appears to determine the fate of the engrafting cell, with later delivery favouring the differentiation of MSC into cells which are pro-fibrotic [14]. Taken together, however, and in the absence of suitable large animal models of IPF, the small animal studies performed to date have provided sufficient evidence for potential efficacy in human IPF for phase I trials to be planned.

5.2.3 Preclinical Studies: Asthma

While most patients with asthma enjoy excellent disease control due to the efficacy of currently available inhaled corticosteroid +/− long-acting β2-agonist therapy, a minority of patients are less responsive and have persistent asthmatic symptoms (cough, wheeze and breathlessness) and airflow obstruction. This group typically has largely irreversible airway remodelling with persistent airflow obstruction despite maximal use of bronchodilator therapy. MSC have been trialled in preclinical studies to determine their ability to reverse the airway remodelling characteristic of chronic asthma, with early reports of success [34]. Our group is currently exploring the use of murine MSC in a murine model of allergic asthma due to house dust mite (K. Atkinson, personal communication), and if successful preclinical data are obtained we will take it into a phase I clinical trial in people with severe treatment-refractory asthma.

5.2.4 Preclinical Studies: Other Applications

MSC have also been studied in the preclinical setting in other lung diseases where a strong clinical need for improved therapeutics exists. Post-transplant obliterative bronchiolitis is the major cause of long-term mortality and morbidity after lung transplantation and is refractory to treatment. In a heterotopic tracheal transplant model, Grove and colleagues have recently demonstrated the therapeutic potential of intravenously delivered MSC to attenuate airway obliteration through the production of IL-10 and modulation of TGFβ expression [35]. MSC have also been shown to be of benefit in preclinical studies of PAH [36, 37], particularly when they are transgenically treated to induce hyper-expression of heme oxygenase-1 [36].

5.2.5 Human Studies of MSC Therapy in Lung Disease

The largest study of MSC therapy in human lung disease began recruitment in 2008 and is listed as closed to recruitment but ongoing (http://www.clinicaltrial.gov/ct2/show/NCT00683722, accessed 1 June 2011). The primary aim of this phase II clinical trial was to establish the safety and efficacy of multiple administrations of allogeneic MSC (Prochymal™, Osiris Therapeutics Inc., osiristx.com) in patients with moderate and severe COPD. Human adult MSC were derived from the bone marrow of normal healthy adult volunteer donors. A total of 62 patients, between the ages of 47 and 80 years, with a diagnosis of moderate ($n=23$) or severe ($n=39$) COPD have been enrolled and are being followed for 2 years in this placebo-controlled study. The primary outcome measure is safety, with secondary outcome measures listed as pulmonary function tests, exercise capability and quality of life. Interim 6-month results were made available on 23 June 2009 (http://investor.osiristx.com/releasedetail.cfm?releaseid=391580, accessed 17 March 2010) but have not been formally published. All patients in the trial completed the planned course of four infusions without any evidence of infusional toxicity. Adverse event rates were comparable for patients receiving Prochymal™ and placebo, but the pulmonary function efficacy endpoint was not met [38].

Our group has initiated two human phase I trials of MSC therapy for lung disease. In the first study (http://clinicaltrials.gov/ct2/show/NCT01175655), the primary objective is to establish the safety of infusions of bone marrow-derived MSC from related or unrelated HLA-identical or HLA-mismatched donors in the management of bronchiolitis obliterans syndrome (BOS) after lung transplantation. The secondary objectives are to document changes in lung function, 6 min walk distance (6MWD) and survival following MSC infusion. Patients ($n=10$) with single, bilateral or heart-lung allografts and deteriorating chronic allograft dysfunction manifesting as either BOS grades 2 and 3, or grade 1 [39] with an additional risk factor for subsequent death, will receive open label treatment with 2×10^6 MSC/kg bodyweight twice weekly for 2 weeks.

In the second study, a phase I, open-label, investigator-driven, non-randomised dose-escalation evaluation of the safety and feasibility of MSC treatment for subjects

diagnosed with IPF, we will be assessing the feasibility and safety of delivering allogeneic placenta-derived MSC to patients ($n=8$) with IPF. A total of eight subjects will be studied, four will receive 1×10^6 cells/kg and the next four will receive 2×10^6 cells/kg. The primary endpoint is to provide evidence of safe delivery of MSC in doses as per protocol. The secondary endpoints are the effectiveness at 1, 3 and 6 months post MSC infusion, compared to baseline, as assessed by lung function, exercise capacity (6MWD) and gas exchange as assessed by resting PaO_2 and pulse oximetry during exercise testing. Enrolled patients will have moderate disease as assessed by honeycombing > 5% in 0–3 of 6 lung zones; forced vital capacity (FVC) > 50% of predicted and a diffusing capacity for carbon monoxide (DLCO) > 25% of predicted capacity.

The only other human study listed on www.clinicaltrial.gov as currently recruiting involves the intra-tracheal administration of umbilical cord blood-derived MSC to infants with BPD (http://clinicaltrials.gov/ct2/show/NCT01297205, accessed 1 June 2011).

5.2.6 Lung-Resident MSC: Their Role in Lung Physiology and Disease

In 2007 a population of lung-resident MSC were identified in the bronchoalveolar lavage (BAL) fluid of lung transplant recipients. Astonishingly, this cellular population was donor-derived, as discerned from their ability to reflect the sex of the lung donor, even many years after sex mismatched lung transplantation [40]. This startling discovery was consistent with an emerging body of literature suggesting that MSC occupy niches in many non-haematopoietic organs, not simply bone marrow. It is likely that these so-called "tissue resident" MSC have a different function to that of bone marrow-derived MSC, but this area of human biology is in its infancy. The apparently long-lived nature of lung-resident MSC confirms their ability to self-renew or their "stemness".

Lung-resident MSC, like bone marrow-derived MSC, are multipotent in that they are able to differentiate into adipocytes, chondrocytes and osteocytes. Their phenotype is similarly $CD73^+CD90^+CD105^+$ and $CD14^-CD34^-CD45^-$, and they are able to inhibit T cell proliferation via a contact-independent mechanism, potentially by the secretion of PGE_2 [29]. Although there are multiple similarities to the better characterised bone marrow-derived MSC, lung-resident MSC have a subtly but distinctly different gene expression profile. This is consistent with the concept that tissue resident populations of MSC have organ-specific functions. It is currently not clear what the function of this curious population of lung cells is nor in which pulmonary niche they usually reside.

Since lung-resident MSC were first identified in BAL fluid [40], and as this procedure involves sampling of the intra-alveolar pulmonary compartment, it must be that these MSC either reside within, or are able to migrate into, the alveolar space. In either case their niche must be either intra- or peri-alveolar. Recent evidence from

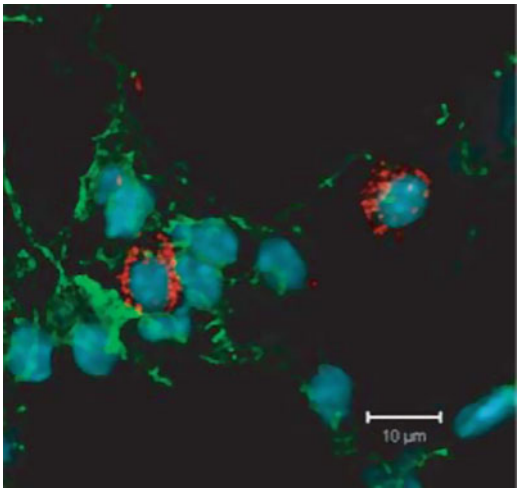

Fig. 5.1 A putative pulmonary niche for lung-resident mesenchymal stromal cells. Lung-resident mesenchymal stromal cells (MSC, red fluorescent PKH-26 staining) reside in the alveolar region in close proximity to alveolar type 2 cells (green fluorescent cytokeratin staining) either in the corners of the alveoli (*arrow*) or attached to the alveolar septa (*solid arrow*). Blue is nuclear DAPI staining. Like bone marrow-derived MSC, lung-resident cells are multipotent and immunomodulatory [41]. Reprinted with permission from the American Thoracic Society. Copyright of American Thoracic Society

the same group at Ann Arbor, Michigan, using a chimeric pulmonary model suggests that MSC reside in the alveolar region either attached to the alveolar septa or in the corners of the alveoli in close relationship to type 2 alveolar cells [41]. As far as their function is concerned, one can only speculate; however, it seems likely that lung-resident MSC will provide regenerative support to the surrounding epithelium, analogous to the support Sca-1 positive cells provide in the mouse [42] and much like the support their bone marrow cousins provide to adjacent lineages. Further clarification of the role of lung-resident MSC in human lung biology will depend heavily on the identification of suitable and specific markers. One such marker may be forkhead box F1 (FOXF1) [13, 43].

5.3 Conclusions

Lung disease is a major and growing cause of morbidity and mortality. While a number of lung diseases, most notably asthma, are now able to be relatively safely and effectively treated due to huge improvements in the available pharmacologics, substantial therapeutic gaps remain. It is likely that adult stem cells such as MSC may fill some of these gaps; however, in order for this promise to be achieved safely, and in order to avoid a repeat of the problematic headlong introduction of gene therapies to large scale clinical trials [44], a much deeper understanding of basic MSC biology will be required.

Of particular interest in the future will be the role of tissue resident MSC in lung physiology and disease. In this way, while initially stem cell technology was seen as potentially therapeutic because of engraftment potential, it is more likely that therapeutic aims will be achieved through the potent paracrine and contact-dependent effects of adult stem cell populations on adjacent somatic and inflammatory cells.

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Chapter 6
Stem Cell Therapy for Bone Disorders

Elizabeth Rosado Balmayor and Martijn van Griensven

Abstract Stem cells of various origins have shown enormous potential to enhance bone repair and regeneration. From fracture healing to bone loss, including well-known medical conditions like osteoporosis, stem cells are being applied more and more frequently. Furthermore, they are being proposed for the treatment of genetic bone disorders with satisfactory outcomes. However, key questions remain on the type of cell to use, and the isolation and expansion protocols to select. Moreover, the in vitro modification of the cells to induce a specific phenotype, enabling regeneration of new bone is being matter of extensive research. In that respect, a possibility also exists for the enhancement of vascularization during bone regeneration events by using coculture systems or endothelial-based cell therapies. It is our intention in this chapter, to bring to the reader an update on the use of stem cells to treat bone disorders. In light of that, important concepts and definitions will be presented, as well as some examples of relevant findings. Finally, clinical trials on this topic will be discussed.

Keywords Bone disorders • Fracture healing • Mesenchymal stem cells • Clinical trials

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6.1 Cell Selection: Mesenchymal Stem Cells or Osteoprogenitor Cells?

6.1.1 Genesis: Bone Development at the Cellular Level

During bone development, most of the skeleton is created either by endochondral ossification process (the formation of a cartilage template that mineralizes) (Fig. 6.1a) or intramembranous ossification (direct differentiation of mesenchymal stem cells into bone forming cells (osteoblasts)) (Fig. 6.1b) [1]. Most of the bones in the body are formed by endochondral ossification. Thereby, an initial cartilaginous tissue creates a support for osteoblast cells to colonize and subsequently secrete a bony matrix that mineralizes. The formation of intramembranous bone occurs from mesenchymal condensation that differentiates into pre- and mature osteoblasts to create bones of the skull, clavicle, and sternum [2]. At the cellular level, chondrocytes become hypertrophic, mineralize their matrix, and signal the migration of chondroclasts (cells that destroy and resorb cartilage) and blood vessels during endochondral ossification. Blood vessels facilitate the influx of hematopoietic cells which interact with the stroma, and form the future bone marrow. Cells in the perichondrium, connective tissue which surrounds the cartilage of developing bone, are signaled to become osteoblasts and to secrete bone matrix proteins resulting in the formation of a bone surrounding structure [1, 3]. The cellular process of intramembranous ossification can be described as follows: MSCs proliferate but stay close together. Thereby, a dense aggregation of cells is formed. This aggregation is a so-called primary bone nodule. The MSCs in this nodule start to differentiate into osteoprogenitor cells and finally osteoblasts. The latter produce collagen type I that serves as a matrix for mineralization. The cells in the middle further differentiate to osteocytes. Thus, bone tissue is formed with lining osteoblasts and central osteocytes in a mineralized matrix.

These mechanisms of bone development, when complete, will transit to a bone remodeling process during the adult age of an individual (Fig. 6.1c). During bone remodeling, the main events are bone maintenance and adaptation to their mechanical environment. These two processes allow maintaining a healthy bone structure and are performed by the so-called "bone cells." Three main cell types populate bone tissue. *Osteoblasts*, which are the cells that secrete bone matrix proteins, are originated from local osteoprogenitor cells and are present in the bone surfaces. The produced proteins can be divided in two main groups: the collagenous and the noncollagenous proteins. The collagenous proteins like collagen type I represent 90% of the bone matrix proteins [4]. The noncollagenous proteins, representing about 10% of the matrix, are a heterogenous group containing among others different glycoproteins. Maybe the most popular and well characterized of these proteins is osteocalcin. Its amino acid sequence is very well preserved across the vertebrate phylogenetic tree, suggesting that it plays an important role but this is as yet unelucidated. Osteocalcin is produced only by osteoblasts and high serum levels of this protein have been reported in diseases which are associated with increased bone

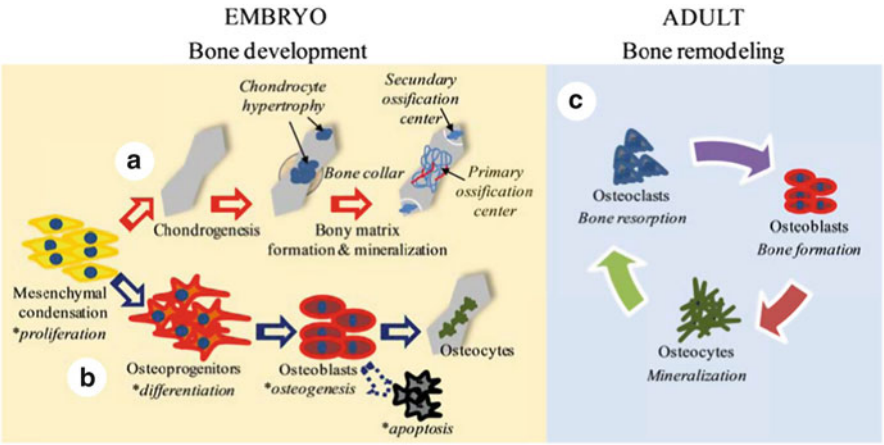

Fig. 6.1 Endochondral ossification (**a**) and intramembranous ossification (**b**) process during bone development. Both processes begin with a mesenchymal condensation phase. In the endochondral ossification, an initial cartilagenous tissue is formed. Subsequently, chondrocyte hypertrophy, cartilage matrix mineralization, osteoclast activity, and vascularization result in the formation of the primary, and then secondary ossification centers. In the intramembranous ossification, undifferentiated mesenchymal cells differentiate into osteoprogenitor cells that will progress to mature osteoblasts. These cells deposit and mineralize bone matrix. Osteoblasts either die by apoptosis or are embedded in the matrix, becoming osteocytes. After the mechanisms of bone development are complete, a bone remodeling process takes place to maintain normal healthy bone (**c**)

turnover such as Paget's disease, renal osteodystrophy, and primary hyperparathyroidism. This has led to interest in the measurement of osteocalcin as a biochemical marker of bone formation. Overall, the bone matrix proteins will serve as the scaffold for bone formation and development.

Another cell type present in bone is the *osteoclast*. Osteoclasts are of eminent importance for bone remodeling. These multinucleated, hematopoietic-derived cells are responsible for bone resorption. They attach to bone surfaces and secrete hydrolytic enzymes that resorb bone. Interestingly, the osteoclast formation is partially regulated by the activity of the osteoblast cells. As a result of the action of bone cells at the surface of the bone, bone is turned over in a process known as bone remodeling. Bone formation and bone resorption are coordinated as part of the turnover mechanism by which old bone is replaced by new bone. In a healthy adult, bone formation only occurs when bone resorption has already occurred [5]. Thus, an intricate balance between the activities of these two types of bone cells, osteoblast and osteoclasts, determines an individual total bone mass and the maintenance of normal healthy bone.

After conclusion of the bone remodeling cycle, most osteoblasts undergo apoptosis. Some cells will remain at the bone surface to form lining cells that may serve as osteoprogenitors or to prepare bone for osteoclast attachment [2, 6]. A few osteoblasts, however, become buried or trapped in their own calcified matrix, thus generating the *osteocytes*. They constitute the most prevalent cell in bone. Although the

metabolic activity of an osteoblast is decreased when becoming an osteocyte, these cells still produce small amounts of matrix proteins that can subsequently calcify. Osteocytes influence bone remodeling by recruiting osteoclasts to sites where bone remodeling is required. Their apoptosis is one of the critical signals for the induction of this process to occur [2]. This may be the main function of these cells.

6.1.2 Bone Repair and Regeneration

When bone repair or regeneration (healing) is needed, after for instance bone fractures, both endochondral and intramembranous ossification may occur. The healing process generally involves coordinated responses of the bone marrow, bone cortex, periosteum and the surrounding soft tissues, including regulation of cellular proliferation, migration and differentiation [7, 8]. The process combines, indeed, elements of endochondral and intramembranous ossification recapitulating many of the developmental steps. This process begins with the formation of a hematoma due to the damaging of blood vessels at the fracture site, accompanied by an inflammatory response. Many signaling molecules, like fibroblast growth factors (FGF), bone morphogenetic proteins, platelet-derived growth factor and vascular endothelial growth factor (VEGF) are then involved in the regulation of new bone formation. They are strongly associated with the inflammation process resulting from a bone fracture or injury [7]. Through the release of cytokines, hypoxia and vascular disruption, cells are recruited to the fracture site. Fibroblastoid periosteal bone lining cells differentiate into osteoblasts and begin the process of callus formation (which subsequently undergoes chondrogenesis). After the callus forms, cell proliferation decreases, chondrocytes undergo hypertrophy and begin to calcify the matrix. The calcified cartilage is targeted by in-growing blood vessels in a process that is highly similar to endochondral ossification. Chondroclasts resorb the calcified cartilage and osteoblastic progenitors begin the process of new bone formation, in which the mechanical continuity of the cortex is regained by subsequent remodeling. The fracture gap is eventually closed at first by immature bone (woven bone). Subsequently, this primitive bone will transform to more structural bone (lamellar bone) by the process of remodeling.

Fracture repair is, in general, a very straightforward process. Bone expresses excellent ability for healing naturally. Thus, even relatively large bone defects may be bridged by natural mechanisms of bone repair including callus formation, woven bone to final lamellar bone formation. However, risk factors for failure in fracture healing and repair include aging, nutritional status, diabetes, and smoking among others [2]. Extensive fractures are very difficult to heal and result in 5–10% of the cases in bone nonunions. Additionally, in some clinical situations, the natural bone repair may be too slow or inadequate although the fracture gap is not too large. Therefore, several different treatments have been proposed for bone regeneration. Relevant examples, presenting satisfactory results, can be found by using biomaterials [9, 10], bone grafting [11, 12] and stem cell transplantation [13] for bone regeneration.

When considering the use of cells to improve or regenerate bone, the natural source of cells following a fracture should be considered. Repair cells are from the inner osteogenic layer of the periosteum, osteoprogenitor cells, cells from the endosteum lining the inner cortex and the undifferentiated mesenchymal stem cells of the bone marrow and of the surrounding muscle and connective tissue based on their ability to differentiate as needed [14]. Which source of cells serves as the primary repair agent is determined by the environment provided for repair.

Much of the current investigations and proposed treatments are based on the use of MSCs, although a direct application of osteoblast cells could have a stronger benefit. An open question arises, therefore, concerning the appropriate cell selection. Why select MSCs over osteoblasts when the latter could perform their function without the need of stimulus and pre-differentiation? During development, as was already mentioned in this chapter, osteoblasts are derived from mesodermal sclerotome condensations that form MSCs. MSCs are multipotent cells with the capacity to differentiate into other cell types including chondrocytes, myocytes, adipocytes, and connective tissue fibroblasts. In fact, MSCs differentiate and form bone during normal development. They persist into adulthood in different niches and provide a supply of osteoblasts for normal adult bone remodeling. The majority of MSCs allocated for the osteoblast lineage likely reside in bone marrow. Moreover, the availability of MSCs prevails over the less differentiated state (compare to osteoblasts to form new bone). MSCs are abundantly present in bone marrow and adipose tissue. These are two easily approachable tissues and contain sufficient amounts of MSCs. Osteoblasts are the most active anabolic bone cells as described before. However, they are solely aligned at the bone edges. Thus, to harvest sufficient amounts of osteoblasts, large volumes of bone tissue needs to be excided. This would mean filling a gap by creating another gap. Therefore, although MSCs need differentiation cues, and osteoblasts are already differentiated, MSCs are the preferred option for bone regeneration therapies.

6.1.3 Vascularization: The Ultimate Need for Healthy Bone Formation

Vascularization is a crucial process during bone growth and development, where a close connection between blood vessels and bone cells would ultimately maintain skeletal integrity. The microvascular structure ensures the transport of oxygen, nutrients, soluble factors, and numerous cell types through the whole tissue. It also guaranties the removal of waste products. In fact, during bone development, both endochondral and intramembranous ossification have as common feature the prerequisite of vascularization [15–17]. For example, an invasion of capillaries occurs prior to intramembranous bone formation. These capillaries will transport the MSCs that ultimately differentiate into osteoblasts depositing bone matrix [18]. In the case of endochondral ossification, the hypertrophic chondrocytes in their path to mineralize their cartilaginous matrix secret angiogenic growth factors that promote the

invasion of blood vessels. These blood vessels are of eminent importance for the subsequent transport of highly specialized cells that will replace the mineralized cartilaginous matrix with bone and bone marrow [16, 18].

The lack of vascularization during fracture healing remains one of the main obstacles to overcome. During bone regeneration, similar to the process of bone development, a microvasculature and microcirculation is critical for the homeostasis and regeneration of bone. Without this structure, and therefore deprived of blood circulation, the tissue would simply degenerate and die [19]. Most of the current proposed therapies to repair bone defects, including the use of bone grafts, implants, and scaffolding materials, face significant limitations due to insufficient integration with the surrounding host tissue. The main reason behind that is the lack of an active blood vessel network. In that sense, several strategies have been proposed to improve vascularization. The combination of polymeric scaffolds, growth factors, and stem cells is claimed to promote angiogenesis and osteogenesis. For instance, the polymeric scaffold is characterized by a high porosity and porous interconnectivity to promote vascularization. Larger pores present on the scaffold after implantation result in higher oxygen tension, promoting the differentiation of MSCs into osteoblasts. Moreover, the integration of the implant with the surrounding tissue is highly favored, where cells and the needed oxygen and nutrients, soluble factors, and ECM proteins can be freely transported and are available as needed during the healing process.

To this approach, if growth factors like basic FGF and VEGF which are desired to promote blood vessel formation are added, a better cellular repair process is expected. VEGF is, for instance, the main angiogenic growth factor involved in bone healing. This growth factor stimulates very important cell populations like chondrocytes, osteoblasts, and osteoclasts toward angiogenesis [20]. The therapeutic approaches in this case could be the localized and sustained delivery of these growth factors at the site of injury [21, 22]. In this case, a scaffolding material could be used as matrix for the growth factor delivery. Thus, the combination of a porous matrix with highly porous interconnectivity, loaded with angiogenic growth factors, could bring a very promising solution to improve vascularization.

Cell therapy is gaining popularity in the field of bone engineering and regeneration. Whether directly administered to the lesion site or pre-seeded onto a scaffold, stem cells have shown significant osteogenic potential. Several approaches exist in the application of stem cells to promote vascularization during bone regeneration. A majority of these approaches include the utilization of endothelial or endothelial progenitor cells. Coculture systems that combine MSCs or osteoprogenitors cells and endothelial cells are popular approaches. Endothelial progenitor cells are resident in the bone marrow and home to ischemic sites to initiate vasculogenesis. They have been shown to increase blood vessel formation when administered to an osteogenesis site [13]. In fact, histological findings have indicated that osteoblasts and osteoprogenitors are constantly located adjacent to endothelial cells in blood vessels at the site of new bone formation [23].

An ultimate example of a promising approach can be described by the combination of all the above mentioned factors. For instance, a porous, VEGF loaded,

3D-biomaterial scaffold that can be used pre-seeded with MSCs or osteoblasts and endothelial cells prior to implantation, creating a pre-vascular structure to be implanted in the site of the defect. This type of construct could provide a live bone graft substitute that can be successfully integrated with the surrounding bone tissue. This combination has been shown to accelerate the establishment of a vasculature in the implanted construct [24, 25].

6.2 Cell Isolation and In Vitro Culture for Clinical Treatments

Human adult MSCs have been proven clinically relevant in cell-based therapies, including myocardial infarction, multiple sclerosis, amyotrophic lateral sclerosis, graft versus host disease, osteogenesis imperfecta, and Crohn's fistula [26–30]. Methods for the effective isolation of adult human MSCs from specific tissues have been developed. Adipose tissue and bone marrow are an abundant source of MSCs, and these cells are a well-accepted source for bone regeneration. Adult bone marrow mesenchymal stem cells (BMSCs) differentiate in vitro to different cell types and form new tissue in vivo, including bone [31, 32]. They have been claimed as the gold standard for engineering skeletal tissues. BMSCs are isolated from the marrow aspirate based on their ability to adhere and grow on tissue culture plastics [33]. Their collection is easy and they are characterized by a relatively high frequency (10^4–10^5 bone marrow mononuclear cells [34]). During the clinical practice, bone marrow of a patient is usually collected by aspiration from the iliac crest or sternum through a special needle. Small volumes are preferred to prevent hemodilution and/or contamination from other cells types [35]. Due to their density (1.073 g/dL), BMSCs can be isolated by the use of gradient centrifugation at 600×g for 30 min. After resuspension in a growth medium, cells can be either be directly used or plated at a density of 10^5–10^6 BMSCs per cm^2 for in vitro expansion [34]. BMSCs have also been satisfactorily isolated by enzymatic digestion by the use of solutions of collagenase type II or equivalent proteases, but this procedure is more laborious and time consuming. This is especially true for clinical applications, where it is very often needed to readminister the isolated cells to the patient within a short period of time. Attention need to be given to the fact that the quantity of cells initially isolated varies between different patients and aspirate preparations, and reportedly declines with a patient's age [36]. Nevertheless, the bone marrow aspirates for the BMSCs isolation is recognized as a straightforward procedure and is being extensively applied to several clinical treatments. In the case that in vitro culturing of these cells is preferred prior to clinical use, studies have shown that specific growth factors supplemented to cell culture medium can help maintain the differentiation potential of these cells during culture and expansion [1]. The need to utilize the right cell phenotype for engineering of human tissues has been widely recognized. For engineering and regeneration of bone, the properties of choice include high biosynthetic activity, expression of osteogenic markers, and phenotypic stability [1].

Another accessible and abundant source of MSCs is adipose tissue. Isolation protocols of adipose mesenchymal stem cells (AMSCs) include aspiration of the fat tissue (lipoaspirate or liposuction) with subsequent enzymatic digestion by the use of collagenase type II. After centrifugation, the pellet containing the vascular-stromal fraction can be washed with PBS and contaminating erythrocytes can be removed by lysis. The cell suspension can be passed through cell strainers to remove small tissue debris and cellular aggregates. The final suspension of AMSCs can then be prepared in growth or culture media for further expansion.

After isolation, both BMSCs and AMSCs can be labeled with antibodies against well-known MSC surface markers to confirm their stemness. According to the International Society for Cellular Therapy guidelines, the minimal criteria that define MSCs are adherence to plastic under standard (serum-containing) culture conditions, multipotent differentiation potential, and specific surface antigen (Ag) expression (positive for CD105, CD73, CD90 and negative for CD45, CD34, CD14 or CD11b, CD79a, or CD19) [37]. Moreover, they can be characterized in vitro for their ability to generate colony forming (CFU-F) units.

The isolation and expansion of MSCs for clinical application need to follow clinical good manufacturing practice (cGMP) to ensure safety, reproducibility, and efficient use. All steps and assays, including cell source selection, isolation protocol, and culture method, must be well defined. In addition, special care is needed for the materials and reagent use. Even when MSCs have been isolated and cultured from nearly all tissues, to date the most accepted and preferred source for clinical application remains the bone marrow. Some other tissues, such as trabecular bone [38], cord blood [39], amniotic membrane [40], and adipose tissue, could have clinical use potential in the future.

6.2.1 Ex Vivo Modification of Isolated Cells Prior to Clinical Use

Diverse molecules, cytokines, and genes can be used in vitro for the modification or "instruction" of the cells before therapeutic transplantation. It is documented, for instance, that in vitro stimulation of MSCs with bone morphogenetic protein-2 (BMP-2) induces osteogenesis. Subsequently, these cells can be administered in vivo to achieve bone regeneration. In this case, the growth factor can be directly added to the culture medium as a supplement. A fair number of investigations have reported, however, benefits of loading this growth factor into biomaterials (e.g., nano- or microparticles, fibers, or 3D scaffolds) for the controlled or sustained release of this molecule to the cells. The cells are then seeded onto the growth factor loaded materials or culture them in contact with those. We have developed biodegradable starch-polycaprolactone microparticles loaded with BMP-2 for bone regeneration [41]. In this study, AMSCs were cultivated in contact with these particles and their osteogenic differentiation was followed up to 35 days in culture (Fig. 6.2). The developed BMP-2 loaded microparticles were able to induce osteogenesis in AMSCs in vitro. After 7 days of culturing the cells in contact with the

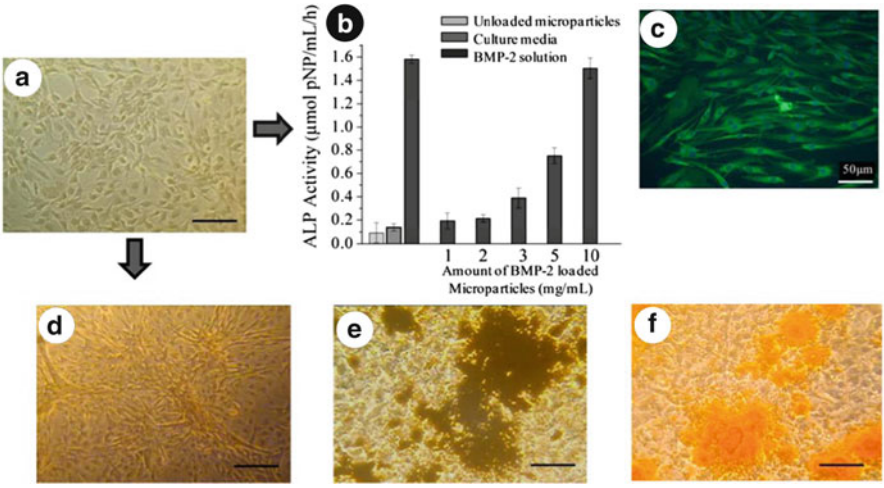

Fig. 6.2 In vitro osteogenic differentiation of human AMSCs cultivated in contact with BMP-2 loaded microparticles. Human AMSCs in culture (**a**). ALP activity (**b**) and osteocalcin expression (**c**) after 7 days of AMSCs culture in direct contact with the BMP-2 loaded microparticles. Control samples for ALP include unloaded microparticles, culture medium, and BMP-2 supplemented medium (100 ng/mL). Samples include BMP-2 loaded microparticles incubated directly with the cells (1–10 mg/mL). Photomicrographs illustrate mineralization and calcium deposition in the AMSCs upon incubation with BMP-2-loaded microparticles stained with von Kossa and alizarin red. Nodule formation after 3 days of culture (**d**) and mineral (**e**) and calcium deposits (**f**) after 14 days of culture. Bar = 50 μm. (b), (e), and (f) Reproduced from figures in [39] with permission

loaded particles, increased levels of alkaline phosphatase (ALP), and high expression of osteocalcin were observed (Fig. 6.2b and c). ALP levels were found to be higher when higher amounts of loaded particles were added to the culture medium. Von Kossa staining demonstrated, after 3 days of culture, that the AMSCs have started the formation of nodules and mineralization (Fig. 6.2d). Longer times of culture (up to 35 days) demonstrated more mineralization and calcium deposits (Fig. 6.2e and f). This indicated a full differentiation of AMSCs to osteoblasts. These data suggested that the use of microparticles reduced the amount of BMP-2 needed to induce in vitro osteogenic differentiation of AMSCs (in comparison to the BMP-2 added as supplement to culture medium). In a separate set of experiments, these BMP-2 loaded particles were precultured for 7 days with the AMSCs, and then the construct (cells and BMP-2 loaded microparticles) was administered to a 2 mm drill hole model in the femur of healthy rats. The evaluation of a new bone formation was performed by micro-CT and histology after 4 weeks of administration. As a result, the combination of AMSCs and BMP-2 loaded microparticles was found to accelerate significantly bone regeneration. Indeed, after 2 weeks of administration, the group with AMSCs plus BMP-2 particles showed new bone formation in the micro-CT scans in contrast to the control group (unpublished results).

Another example of the combination of MSCs and BMP-2 for bone regeneration can be found in the study from Keibl et al. [42]. The authors investigated bone

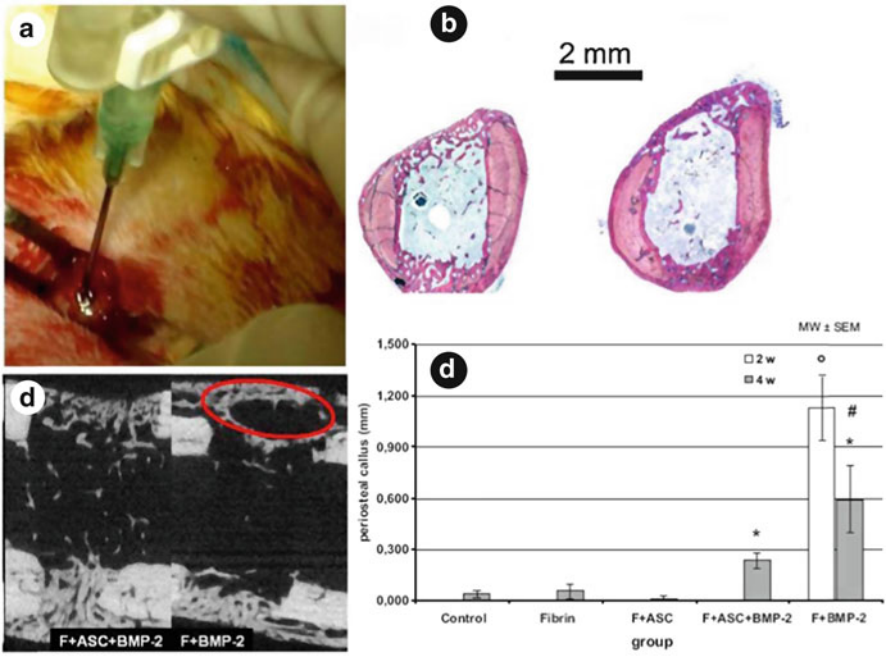

Fig. 6.3 Drill hole model in a rat femur for bone regeneration. A 2 mm drill hole filled with AMSCs and BMP-2 embedded in fibrin glue (**a**). Histological images of the drill hole after 4 weeks. Massive callus formation is visible in the group where only BMP-2 was administered (right image), whereas less or no callus formation can be seen in group were AMSCs and BMP-2 were administered together (left image) (**b**). Micro-CT longitudinal sections after 4 weeks. Callus formation is visible in the BMP-2 group, whereas less or no callus formation can be seen in group were the BMP-2 was administered together with the AMSCs (**c**). Comparison of the micro-CT data after 2 weeks and 4 weeks (mean value ± SEM) periosteal callus formation (mm). $*p < 0.05$ for 2 weeks versus 4 weeks; $\#p < 0.05$ for BMP-2 group versus control, fibrin group and AMSCs group at 4 weeks; $^{o}p < 0.05$ for BMP-2 group versus all other groups at 2 weeks (**d**). Reproduced from figures in [40] with permission

healing upon administration of BMP-2 embedded with AMSCs in a fibrin matrix to a small noncritical size defect in rats (Fig. 6.3). The most significant finding was that the combination of AMSCs and BMP-2 significantly reduced callus formation compared to BMP-2 alone (Fig. 6.3c and d). Interestingly, when BMP-2 was administered alone the callus formation was significantly increased (Fig. 6.3c). On the other hand, when the AMSCs were administered alone no bone regeneration was observed. This study points out that MSCs and growth factors are two major components for regeneration influencing each other.

A different approach described in the literature is the induction of therapeutic molecule secretion (e.g., proteins and growth factors) by the cells itself. Thus, MSCs act as smart systems delivering the needed therapeutic molecules at the injured site of the body. Hsain-Chung Shen et al. have described the transduction of muscle-derived MSCs with an efficiency of approximately 80% by using a retroviral vector

expressing human bone morphogenetic protein-4 (BMP-4) [43]. The transduced cells secreted high levels of BMP-4 and differentiated toward the osteogenic lineage. Von Kossa staining indicated that mineralized bone was formed after 7 days of implantation of the BMP-4-expressing cells into immunocompetent mice. Meinel et al. have followed the same approach but using human BMSCs and silk fibroin biomaterials. BMSCs were transduced with adenovirus containing a human BMP-2 gene at clinically reasonable viral concentrations and cultured for 4 weeks [44]. Transduced cells strongly expressed osteopontin and secreted a matrix that underwent mineralization on silk fibroin scaffolds. Remarkably, the authors found that the expression of osteogenic marker proteins and alkaline phosphatase was significantly higher in the transduced MSC group than in the exogenous protein BMP-2 group used as control.

However, it seems difficult to move the above-mentioned results to clinical practice. Mainly due to safety issues, the future of the in vitro modification of MSCs via cytokines or genes with subsequent transplantation into patients remains unclear. Undoubtedly, accomplishing reproducibility and safety of the used protocols as well as the improvement of nonviral transfection methodologies will help move these technologies forward.

6.3 Current Treatments: An Update on Clinical Trials

MSCs prepared for cell therapy applications may require extensive in vitro expansion. Long-term cultures of MSCs may result in alterations such as spontaneous transformation or loss of responsiveness to differentiation signals [45, 46]. Extended ex vivo expansion and manipulation bears the risk of contamination with pathogens [47], and since the explanted cells have to be transported and cultivated mainly in external facilities using good manufacturing practices, production and banking are very expensive. Therefore, the higher operation expenses of cell therapeutic GMP facilities and potentially hazardous graft contamination are major drawbacks of ex vivo cellular therapies.

In the clinical field for bone regeneration, only a few clinical studies exist, including one in long bone, one in short bone, and one in the maxillofacial field. The first clinical report was provided by Quarto et al. [48] describing the treatment of three long bone defects (4 cm bone defect in the tibia, 4 cm in the ulna, and 7 cm in the humerus) with MSCs. The cells used in this study were expanded ex vivo and loaded onto a hydroxy-apatite collagen scaffold. The scaffold was reconstructed according to the shape of the defects. As the scaffold itself is not weight bearing, external fixators were used. The external fixators could be removed after 6.5 months for the tibia, 6 months for the ulna, and 13 months for the humerus. All three patients showed consolidation of the fracture with good callus formation and they regained functionality. Importantly, no adverse reactions were observed [48]. However, it is unclear whether these results are due to the presence of the MSCs per se. If the MSCs did make a difference, it is unknown whether the cells

differentiated into bone cells or if they provided growth factors that attracted and differentiated progenitors from the periosteum. A similar hydroxyl apatite scaffold was used to treat an injured thumb. The cells used for this approach were derived from the periosteum. They can be considered as progenitors or stem cells [49]. The clinical outcome was satisfactory, but not better than standard treatment. Although the patient did well after implantation and hand function recovered significantly, quantitative histomorphometric analysis of a biopsy revealed that eventually only 5% of the implant volume was bone. The outcome of this clinical trial was not superior to that of conventional reconstructive approaches [50]. Periosteal cell-seeded polymer fleeces or mesenchymal stem cells and platelet-rich plasma, immobilized in beta-tricalcium phosphate scaffolds, induced bone formation in sinus lift operations [51–53]. Despite anecdotal reports of successful implantation of engineered bone tissues, the small number of patients makes it difficult to assess the efficacy of these constructs and a comparison with conventional methods has not been performed yet.

The second published clinical study [51] described the augmentation procedure of the posterior maxilla in 27 patients, using a matrix derived from mandibular periosteum cells on a polymer fleece (Ethisorb; Ethicon, http://www.ethicon.com). In 12 patients, only radiographic and clinical assessments were performed. Limited conclusions can be drawn from the radiographic findings, as discussed above. The other 15 patients were treated according to a two-step method. First, reconstruction of the host area was performed. After a healing period of 3 months, in advance of dental implant placement, a biopsy was taken. In 8 of these 15 patients, an unsuccessful outcome was observed and a replacement resorption with connective tissue was found. In the case of a positive biopsy (seven patients), the investigators failed to mention if the observed bone formation was induced by the implanted cells (osteoinduction) or by osteoblasts from the preexisting bone surface (osteoconduction).

Interestingly, a considerable number of clinical trials are being performed or have been terminated on the application of MSCs to treat bone genetic disorders (www.clinicaltrials.gov). Conditions like osteopetrosis (in which the bones become overly dense) and osteogenesis imperfecta (in which the bones are so fragile that they break easily) are some of the most studied diseases [54–58]. Two clinical trials are currently recruiting participants for the application of hematopoietic stem cells for the treatment of osteopetrosis patients [57, 58]. These groups claim that the transplantation of stem cells will help these patients by generating functioning osteoclasts. Clearly, this is claimed to assist in the resolution of the abnormal bone architecture. One of these studies is being performed by the Tehran University of Medical Sciences from Iran and the other one by the Masonic Cancer Center from the University of Minnesota, USA. Several clinical trials have been completed at the St. Jude Children's Research Hospital to treat osteogenesis imperfecta [54]. At this institution, Dr. Horwitz and his team performed the world's first bone marrow transplantation for osteogenesis imperfecta by replacing the bone marrow of the ill patient with donor marrow. As a result, the patient's body began to produce new, healthy cells. In the hospital's first clinical trial for osteogenesis imperfecta, physicians transplanted whole bone marrow. The donor

cells were found to engraft and help the bone to grow more normally. However, with time the growth rate slowed down and benefits from the transplant decreased. In a second study, patients received infusions of BMSCs, aiming that the cells would differentiate into bone or connective tissue. Again, however, the benefits were disappointing. Using a technique called retroviral integration site analysis, the type of bone marrow cell that could mature into bone producing cells were identified. This institution has conducted more than four clinical trials on the treatments of osteogenesis imperfecta with remarkable results. Unfortunately, none of them is standard clinical care to date.

6.4 Conclusion

There is a clear need for improving bone regeneration of patients that have suffered from massive trauma including long bone fractures. Most likely, the combination of MSCs, growth factors, and scaffolds will be the most effective. It is important to include the use of, for instance, cells associated with angiogenesis to the cellular treatments and/or the use of angiogenic growth factors to ensure good vascularization. Thus, a good integration of the construct to the defect site and functional new bone formation is anticipated.

MSCs from diverse sources have been evaluated for their potential to differentiate into bone cells. Recently, an additional point of interest is their well-documented immunomodulatory properties. Thus, the clinical application of MSCs for bone regeneration is clearly supported by their ability to promote bone tissue repair/regeneration and to prevent inflammation. Up to today, a fair number of in vitro and in vivo studies have undoubtedly proven the benefits of using MSCs for bone therapies. A strong need persists, however, for more clinical studies to be performed. The available clinical experience presents a significant variation amongst the cases in terms of, for instance, administered cell population, time and location of the application, application route, etc. This variation complicates the comparison of results from one study to another. Thereby, the standardization of MSCs for clinical use is impaired. Moreover, previous data cannot be used as a base for further studies.

Before a solid and successful step forward can be taken to use MSCs to treat patients for bone disorders, the potential risks need to be eliminated. In light of that, several aspects need to be considered. For instance, their proliferation and differentiation in vitro need to be accomplished under well-controlled, reproducible, and standardized conditions. In addition, a good understanding on the mechanism and/or factors behind MSCs migration, integration to the application site, and therapeutic action is highly needed. Finally, a complete certainty about their lack of capability to induce tumor formation will be required. A thorough evaluation of the risks factors to the patient needs to be adequately evaluated in every case, before setting up the trials. Helpful for the future on the field, is minimizing the variation on different conditions for clinical trials treating, for instance, the same pathology. With this, we should be able to use previous results and move forward with the development of new therapies based on the use of these cells.

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Chapter 7
Mesenchymal Stem Cell Therapies for Bone and Tendon Conditions

Mark Young and Michael R. Doran

Abstract Bone, tendon, and cartilage are highly specialized musculoskeletal connective tissues that are subject to injury and degeneration. These tissues have relatively poor healing capabilities, and coupled with their variable response to established medical treatments, produce significant morbidity. Mesenchymal stem cells (MSCs) are capable of regenerating skeletal tissues and therefore offer great promise in the treatment of connective tissue pathologies. Adult MSCs are multipotent cells that possess the properties of proliferation and differentiation into all connective tissues. Furthermore, they can be gene modified to secrete growth factors and utilized in connective tissue engineering. Potential MSC-based therapies for bone and tendon conditions are reviewed in this chapter.

Keywords Osteogenesis • Osteointegration • Osteoconduction • Osteoinduction • Tenocyte • Tendinopathy • Tendon reconstruction

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7.1 Introduction

7.1.1 MSCs and Connective Tissue

Recent studies suggest that all human tissues and organs contain mesenchymal stem cells (MSCs). These MSCs appear to function as the mediators that facilitate the growth, maintenance, and repair of connective tissues during normal aging or following injury [1]. Whilst tissue-specific MSC populations share common features, there is now overwhelming evidence that these populations can equally be considered unique [2]. Derivation of these original tissue-specific stromal cell populations and their maintenance throughout life are beyond the scope of this discussion, and are reviewed elsewhere in this text. There is increasing evidence that MSCs reside in tissue-specific perivascular niches, and that they help generate a microenvironment that both modulates immune function and facilitates tissue regeneration through paracrine mechanisms [3, 4].

The rare MSC population, by definition, has the potential for self-renewal as well as the ability to differentiate into progenitor cells of the mesenchymal lineage. These latter terminally differentiated connective tissue cells have a finite lifespan and perform specialized musculoskeletal functions, including the production of collagen-rich matrix in connective tissues such as bone, tendon, and cartilage.

During childhood growth, the number of terminally differentiated connective tissue cells increases. Maximal muscle mass and peak bone mineral density are achieved in the third decade of life, after which cell numbers are largely maintained through a process of replacement from local progenitors and MSC pools. This cellular homeostasis in the collagen-rich tissues occurs, providing that a degree of mechanical stimulation (e.g., exercise) is applied in conjunction with complex paracrine and autocrine signaling. In later life, age-related and degenerative connective tissue pathologies develop, which are generally associated with reduced numbers of appropriately differentiated connective tissue cells.

7.1.2 MSC Ex Vivo Therapies

Given that MSCs contribute to connective tissue homeostasis through the indirect provision of immune modulation, the generation of trophic factors as well as through the renewal of tissue-specific progenitor cells, there is solid motivation to utilize MSCs in the form of an ex vivo cell therapy to promote connective tissue repair. Indeed there are a few published controlled clinical trials confirming that the provision of ex vivo expanded MSCs do promote the regeneration of human connective tissues. Most reports are promising but are largely confined to small case series, providing level III or IV evidence. Promising early data have spawned a number of recent trials that are evaluating MSCs sourced from a number of donor tissues to treat a variety of indications. The outcome of these trials will likely be varied, with

success dependent on the alignment of the in vivo potential of the selected donor MSC population with the specific indication. Our understanding of in vivo potential and the mechanism by which MSC populations might contribute to tissue repair and regeneration continues to evolve, and these trials will make substantial contributions to this knowledge.

Many early reports in the literature indicated that the probable mechanism by which MSCs contributed to tissue repair was through direct tissue engraftment and the provision of tissue-specific differentiated cells, which integrated seamlessly with the repaired tissue. However, recent studies suggest that the main mechanism of action is more likely through a trophic effect. MSCs secrete of a variety of soluble autocrine and paracrine growth factors, which promote cell survival and enhance the proliferation of endogenous connective tissue cells [5, 6]. These growth factors stimulate mitosis in tissue progenitors, induce angiogenesis, and reduce apoptosis [6–8]. This effect is well illustrated in ex vivo MSC therapies involving children with the genetic disease osteogenesis imperfecta (OI). This disease is associated with the production of abnormal collagen resulting in a lower fracture threshold. In studies where children suffering from OI were infused with allogeneic donor MSCs, there was enhanced total bone mass, growth velocity, and reduced fracture rates. Whilst these improvements were considered significant, the detectable number of donor cells in bone and skin tissues was less than 2%, suggesting that the partial reversal of the pathology is in response to a trophic effect, and not direct engraftment and repopulation of the tissues with donor cells.

Understanding the precise mechanism by which ex vivo expanded MSCs may elicit tissue repair is vital as it will influence critical clinical considerations such as:

1. Route of administration—local injection (percutaneous, intra-arterial, or intra-articular), surgical implantation, or systemic administration.
2. Implantation with or without tissue engineered scaffold.
3. Autologous or allogeneic MSC sources.
4. Source of MSC harvest site.
5. Requirement for expansion.
6. Number of cells administered.
7. Requirement for preimplantation differentiation.

Searches of the current clinical human trial databases reveal that MSCs are being evaluated for the regeneration of bone, tendon, and cartilage, and these studies will address some of the considerations listed above. The following section outlines some of the basis on which particular MSC populations might be selected for specific clinical applications.

The use of allogeneic MSCs permits more efficient harvesting and expansion obviating the requirement for a small host biopsy. Allogeneic cells have the advantage of always being available, and thus can be used in emergency situations such as in the treatment of acute injuries. However, allogeneic MSCs have a potential risk of virus or prion transmission. Allogeneic MSCs do not incite host immune rejection and a number of mechanisms have been shown to contribute to this

effect. MSCs do not express MHC class II antigens and appear to prevent T cell responses by disrupting NK, CD8+, CD4+, and dendritic cell function. They also produce an anti-inflammatory environment, by secreting cytokines such as interleukin 10 and prostaglandins [9]. Once differentiated, the evidence regarding persisting allogeneic properties and an immuneprivileged status is inconclusive. Even though MSCs do not express MHC class II antigens on their cell surface, these antigens can be detected intracellularly by Western blotting, and their cell surface expression is upregulated following exposure to IFN-gamma or tumor necrosis factor. In one in vitro study, human (h)MSCs did not elicit alloreactive lymphocyte proliferative responses following differentiation [10]. Consistent with this result, Toma et al. showed that a limited number of hMSCs were shown to persist and differentiate into cardiomyocytes after engraftment in murine hearts, indicating that these allogeneic cells were not rejected [10]. In contrast to Toma's study, Huang et al. compared the implantation of allogeneic and syngeneic MSCs in rat myocardia, and found that allogeneic (but not syngeneic) cells were eliminated by 5 weeks, and functional benefits were lost by 5 months [11]. In another study, Tsuchida showed that allogeneic MSCs were detected in rat femur repair, when a short dose of immunosuppressant was administered with implantation [12]. The results of these animal-based studies, coupled with the previous OI example, may suggest that long-term engraftment of allogeneic MSCs and their differentiated progeny is not be a realistic expectation. This does not mean that allogeneic MSCs are not useful, but suggests rather that the mechanism by which they might contribute to tissue regeneration will more likely be through the provision of trophic factors.

If the desired outcome is MSC engraftment and subsequent direct contribution to the cellular component of the regenerated tissue, then an autologous transplant may be necessary. However, as MSCs can be obtained from a number of tissue sources, tissue source selection must be considered carefully. MSCs isolated from the iliac crest bone marrow and adipose tissue are perhaps the best studied [13, 14]. Other sites include periosteum, umbilical cord, placenta, lung, liver, and fetal tissues [13]. Whilst in vitro differentiation assays might suggest multilineage potential, in vivo assays suggest that cell fate may be largely restricted to that of the so-called "tissue of origin" [2]. Further research is required to determine the relative differentiation potential of MSC populations harvested from different sites, and whether specific populations can repopulate any of the mesenchymal tissues. These limitations may be overcome through a process of differentiation prior to in vivo implantation. The process of preimplantation differentiation may be achieved through the use of induction medium that guides the differentiation process through biochemical cues or through genetic engineering. Genetic engineering may allow us to overcome pathologies associated with specific genetic deficiencies as well as to enhance the quality of the regenerated tissues. However, as with all genetic manipulation strategies there is the added risk of immune reaction, insertional mutagenesis, and uncontrolled transgene expression [15].

In the following section, the role of MSCs in the development of specific connective tissues is reviewed, as well as the current and predicted future use of MSCs in the regeneration and repair of pathologies associated with these tissues.

7.2 Bone Applications

7.2.1 The Role of MSCs in Bone Formation

Skeletal bone formation is initiated with the process of cellular condensation, where dispersed MSCs migrate and proliferate, as they become bound together by the expression of adhesion molecules [13, 16]. Bone tissue develops by one of the two processes: endochondral ossification—when the bone forms in a cartilage template, or intramembranous ossification—when MSCs differentiate directly into osteoblasts. The majority of adult bone is formed by endochondral ossification, whereby the MSCs differentiate into chondral progenitors and chondrocytes, which secrete both alkaline phosphatase for matrix mineralization and vascular endothelial growth factor (VEGF). The resulting migration of blood vessels facilitates the influx of hematopoietic cells, which later form the bone marrow. These blood vessels also transport osteoprogenitors, which form cancellous (trabecular) bone. Only 20% of the total bone mass is trabecular, but this has a very large surface area which is important for the metabolic functions of the bone marrow. The long bones have joints at each end with a zone of proliferating chondrocytes called the epiphyseal plate. Once the epiphyseal plate ossifies, axial bone growth ceases, and peak height and limb length is attained. Bone width can vary throughout life. The perichondrium is a rich source of MSCs, which start differentiating into osteoprogenitors, and thus the periosteum is formed. These osteoprogenitors ossify directly without a chondral template, and produce compact or cortical bone, which contributes to approximately 80% of the total bone mass. The periosteum and the metaphysis (the region between the epiphyseal plate and the diaphyseal shaft) remain a relatively rich source of MSCs, possibly permitting improved healing from bone injury at these sites. Periosteal preservation is an important factor in the surgical treatment of fractures.

Intramembranous ossification and bone formation occur primarily within the formation of the flat bones such as the skull, scapula, and mandible. This process is not well characterized, but there is direct differentiation of MSCs into osteoprogenitors and osteoblasts [13].

New bone contains irregular disorganized fibers and is described as woven, before remodeling into more organized lamellar bone, which can be cortical or cancellous. This remodeling occurs due to the balance between bone formation by osteoprogenitors (osteoblasts), and bone resorption by osteoclasts (which are derived from hematopoietic rather than from MSC lineages) [17]. Osteoclasts are especially active after bone fracture and are found in high concentration on the endosteal surfaces.

7.3 Current Therapies for Bone Repair

Bone repair occurs either by direct (primary) healing without callus, or by indirect (secondary) healing. Direct healing usually occurs with rigid surgical fixation, and MSCs differentiate directly into osteoprogenitors forming mature lamellar bone.

However, most fractures repair by indirect healing which involves callus formation. After a fracture occurs, there is bleeding from local vessels and a hematoma is formed. The bone injury initiates the release of bone morphogenic proteins (BMPs) and inflammatory mediators, such as insulin-like growth factor 1 (IGF-1) and transforming growth factor beta (TGF-β) [18]. These growth factors attract MSCs to the hematoma from the local bone marrow, periosteum, and muscle, as well as systemically from MSC reservoirs [18]. It has been shown that increasing age, illness, and infection are factors associated with lower numbers of systemic MSCs in bone marrow reservoirs and an increased likelihood of disordered bone healing [1, 18–20]. The hematoma acts as a scaffold for the MSCs and after about 2 weeks, the initial soft callus is primarily fibrous or cartilaginous in nature [21]. The MSCs then differentiate into osteoprogenitors and a collagen-rich matrix is laid down, which is later mineralized. The fracture stabilizes and the woven bone enters a phase of remodeling into lamellar bone, which can take between 1 and 4 years and occurs without scar formation [22].

It is estimated that between 5 and 20% of all fractures fail to unite [15]. The definition of fracture nonunion varies slightly, but is usually defined as pain, fracture micromotion and radiological evidence of persisting fracture nonunion 6 months post-injury. There are many causes of fracture nonunion, which are related to the fracture, the host, or the surgery. The local fracture-related causes include loss of blood supply, periosteal or muscle damage, instability, local irradiation, or infection [15]. Host factors include malnutrition, infection, smoking, systemic disease (e.g., diabetes mellitus), and increasing age [18]. Surgical factors include inaccurate surgical reduction and persisting motion.

Fracture nonunions are further classified as atrophic, oligotrophic, and hypertrophic. In the case of hypertrophic nonunion, there is too much fracture motion, which stimulates the production of excessive fracture callus rich in MSCs; and the treatment is correct fixation and immobilization [23]. Oligotrophic fractures produce minimal callus and are often displaced or have inaccurate internal fixation. Atrophic nonunions have minimal or no callous and are often associated with fibrous or cartilaginous bridging. Another clinical condition that produces failure of union (but is not classified as a true nonunion) is a critical or segmental bone defect, which occurs when the displacement or bone loss cannot be bridged by the normal healing process [24].

Bone grafting is required to facilitate the healing of atrophic (and some oligotrophic) nonunions and segmental defects. The four critical requirements of a bone graft in order to produce new bone formation (osteogenesis) are: an osteoconductive matrix (scaffold); osteoinductive growth factors; osteogenic cells; and a blood supply [25, 26]. Osteoconduction is the property of a matrix, which supports the attachment of bone-forming cells for subsequent bone formation [27]. Osteoinduction is a process that supports the mitogenesis of undifferentiated MSCs, leading to the formation of the osteoprogenitor cells which form new bone. The most utilized and efficacious osteoinductive agents are BMPs 2 and 7 (level 1 evidence) [28]. The fourth requirement is a good blood supply, which is required to deliver oxygen and nutrients and remove waste products.

Traditional approaches for bone grafting include autologous bone grafts, allogeneic bone grafts (freeze dried cadaveric bone graft or demineralized bone matrices), and calcium phosphate synthetic scaffolds [25]. An autologous cortico-cancellous bone graft is usually obtained from the patient's iliac crest, as this provides three of the four critical elements of bone repair. However, there is morbidity associated with the harvest site, including pain, infection, and nerve damage, and there is usually insufficient iliac crest bone for large defects [25]. When a vascularized bone graft is required, the fibular shaft is usually the harvest site, but this is a technically difficult and destructive procedure. Therefore, there is a requirement for non-autologous grafting materials. Allogeneic bone grafts are a popular alternative, and may be fresh-frozen, freeze-dried, or demineralized bone matrix (DBM). The freezing process makes the allograft acellular, meaning that only osteoconduction and some osteoinduction are provided, but the osteogenic properties are inferior to those of autologous grafts, and incorporation is relatively slow [25, 26]. Immune responses to foreign proteins can occur, and there is the potential for transmission of viral or prion infection [29, 30]. This has led to the use of homologous DBM, which is produced by acidic extraction of bone allograft. DBM has osteoconductive properties, but has limited osteoinduction and no osteogenic cells, and therefore some manufacturers recommend the addition of BMPs at the time of surgery. Synthetic scaffolds are osteoconductive only, and may be relatively brittle with little tensile strength. However, once osteogenesis is achieved, and biomechanical support is obtained, the scaffold can then integrate with native bone, a process referred to as osteointegration [13].

7.4 Tissue Engineered Bone Bioscaffolds

With the recent advances in the understanding of the osteogenic properties of MSCs, there has been a great deal of research focusing on tissue-engineered synthetic scaffolds. The scaffold needs to be highly porous and permeable for cell attachment and migration, and to support tissue ingrowth, nutrition, and angiogenesis [25, 31]. The scaffold also needs to be biocompatible and degrade into nontoxic and non-allogeneic products. Synthetic materials such as hydroxyapatite, tri-calcium phosphate, and calcium sulfate are available as polymers, ceramics, or composites. Polymers such as type 1 collagen sponges do not provide enough biomechanical support for cells [13, 32]. Synthetic polymers and copolymers of polylactic acid (PLA) and polyglycolic acid (PLGA) are popular, due to their mechanical properties and controlled degradation, but they may form acidic by-products [1]. Ceramics such as corals have good biocompatibility, but the high dissolution rate has meant that use is limited to grafts requiring minimal load bearing (e.g., digital phalanges). Similarly, synthetic calcium-based ceramics, such as calcium hydroxyapatite (+/− tricalcium phosphate), are osteoconductive but may be fragile [13, 33]. When ceramics are combined with polymers, the biomechanical properties can be improved and further surface-modified to permit cellular attachment, migration, and incorporation of growth factors [13].

In vitro bone engineering is currently being evaluated to provide and assess scaffolds in situations of segmental or critical bone loss. Parameters such as seeding density, scaffold property, and culture medium composition have been studied in various bioreactor systems [13, 34, 35]. Evaluations of stirred and rotated mass transport systems have not provided the uniform cellular growth required, as there is relatively poor nutrient diffusion into the interior [13]. Therefore, these bioreactors are not suitable for larger constructs. Perfusion bioreactors have been shown to permit the flow of culture medium throughout the construct, which provides oxygen, nutrient, and metabolite removal and results in a more uniform cellular distribution [36, 37].

In order to osteointegrate with native bone, scaffolds need to become vascularized to facilitate gas and nutrient exchange, and remove waste products. At distances up to 300 μm, molecular diffusion can provide for these requirements. However, vascularization needs to be considered with larger scaffolds, otherwise oxygen diffusion is impaired and acellular regions develop [13, 37, 38]. Tissue-engineered methods to improve vascularization include in vivo pre-vascularization, use of angiogenic factors, and pre-vascularized engineered scaffolds [13]. When pre-vascularization is required, the scaffold can be implanted in a rich vascular environment (e.g., intramuscular or the intraperitoneal space) so that new blood vessels form at random. The vascularized tissue is then excised and implanted at the desired site, but this can also damage the neovessels making anastomosis with the native vessels technically difficult [24, 39]. Other in vivo techniques involve transplanting MSC-seeded scaffolds around a medium-sized vessel, or creating an arteriovenous shunt. Angiogenic factors such as VEGF, platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) can also be incorporated into scaffolds, but these factors have short tissue half-lives meaning that slow release preparations are required [13, 40]. Vascularized bone scaffolds have been engineered in bioreactors by culturing MSCs with osteogenic and angiogenic factors. The adult MSCs have been shown to differentiate into vascular endothelial cells and to form new blood vessels [13].

Osteogenic culture medium can be useful for promoting differentiation and confirming osteogenic potential, and this is achieved by culturing purified MSCs in the presence of dexamethasone, ascorbic acid, and glycerophosphate [17, 41]. The MSCs assume a cuboidal osteoblastic shape, associated with transient induction of alkaline phosphatase activity [17, 42]. The cells express BMP messenger RNAs and matrix is laid down, which is then mineralized [17, 41, 43]. The secreted BMPs are osteoinductive and have been shown to induce further differentiation of other local MSCs along osteoprogenitor lines.

Gene therapies can manipulate the proteins being generated by the host cells. Animal studies have shown that MSCs can be genetically modified to express BMPs or other growth factors; and the MSCs can then be returned to the patient [22, 44]. This has a potential advantage over current surgical techniques, which are expensive and require an intraoperative, supra-physiological, short acting bolus of growth factors. Gene therapy delivery of growth factors can be sustained for short or long periods, which is determined by the size of the defect [25, 45, 46]. The local production

of increased growth factors has been shown to accelerate fracture healing in animal studies [25]. The requirements for successful genetic modification are a cDNA which encodes the desired protein, a vector that mediates the entry of genetic material into the cell, and target cells with the ability to transcribe and translate the genetic information into proteins (e.g., osteoinductive growth factors) [25]. These vectors are classified as viral or nonviral. Due to the concerns regarding the use of viral vectors such as adenoviruses or retroviruses, nonviral vectors are also being investigated. These include the use of liposomes, electroporation (e.g., nucleofectin), or transfection reagents (e.g., FuGENE 6) [24, 25].

Preclinical studies have yielded some promising results with genetically modified BMP-producing MSCs, but immune reaction has been problematic and immunosuppression has been required in some animal studies [25, 47, 48].

Genetic engineering of MSCs may eliminate the requirement for large numbers of cells, which can only be manufactured over several weeks of expansion and culture. However, optimization of the vector, cDNA, MSC, and carrier is still required, before genetically modified MSCs can be routinely used in human bone repair.

See Table 7.1 for a summary of the properties of scaffolds.

7.5 Clinical Studies of Therapeutic Use of MSCs

7.5.1 Fracture Nonunion

Delayed or nonunion of fractures is an important clinical problem. Current treatments include surgical fixation, bone grafting, immobilization, bone stimulation, and treatment of contributing comorbidities. The first successful report of the use of cellular therapies was in 1978, when autologous iliac crest bone marrow aspirates were added to calf tibia xenografts to treat 15 various pseudarthroses (nonunions) [49]. In 1986, Connelly published a successful case report when he injected bone marrow cells directly in and around a tibial nonunion [50, 51]. In 1991, Connelly et al. reported success in treating 20 cases of tibial nonunion with bone marrow aspirate cells, and concluded that this technique has "numerous advantages compared to standard open grafting technique" [24, 52]. Hernigou et al. later refined the surgical technique by concentrating the bone marrow aspirate [53]. It was shown that the iliac crest aspirates of 60 patients with tibial nonunion contained 612 ± 134 progenitors per milliliter, but after centrifugation and separation of the buffy coat, the original 300 mL was concentrated to about 50 mL with $2{,}579 \pm 1{,}121$ progenitors per milliliter. Hernigou recommended that a total volume of 20 mL be injected into and around the fracture, so that there was no risk of compartment syndrome. Hernigou also retrospectively reported that 53 out of the 60 cases of established tibial nonunion progressed to clinical union with this minimally invasive technique (level III evidence) [54]. The seven cases with persisting nonunion all received lower numbers of progenitors.

Table 7.1 Summary of properties of bone scaffolds

	Osteoconductive	Osteoinductive	Osteogenic cells	Vascularized	Comment
Autograft	+	+	+	–[a]	Significant donor site morbidity Lack of available tissue
Allograft (freeze-dried bone)	+	±	–	–	Potential risk of infection transmission or immune reaction
Allograft (demineralized bone)	+	±	–	–	Theoretical risk of infection transmission or immune reaction Expensive
Synthetic	+	–	–	–	Only osteoconductive
Osteoinducers (e.g., BMPS)	–	+	–	–	Level I evidence of efficacy [28] Expensive Can be added to scaffolds
MSCs (percutaneous injection)	–	+	+	–	Level III evidence
Engineered MSC bioscaffolds	+	+	+	–[b]	Can be prepared at the time of surgery or inside bioreactors

[a]Vascular fibular graft is occasionally used but is a technically difficult harvesting procedure
[b]Prevascularized grafts can be prepared in vivo or in vitro bioreactors

Maneerit et al. conducted a prospective randomized trial on 30 cases of established or expected tibial nonunion, whereby subjects received either a percutaneous injection of bone marrow cells or open bone grafting [55]. The authors concluded that the union rates in both techniques were similar.

With improved isolation and expansion techniques, it is now possible to administer smaller volumes containing higher concentrations of purified MSCs. These cells may undergo genetic modification or osteogenic culture, to encourage osseous differentiation. Currently, phase 1 and 2 clinical trials are being undertaken, with synthetic scaffolds and demineralized bone matrix, to assess the safety and efficacy of both autologous and allogeneic MSCs in treating bony nonunion.

7.6 Critical Bone Defects

Critical bone defects typically require bone scaffold, and arise as a result of a trauma, tumors, genetic conditions, and orthopedic interventions, e.g., arthrodesis, osteotomy, spinal fusion, arthroplasty, or lengthening procedures. Controlled animals trials have demonstrated that when MSCs are seeded on hydroxyapatite or demineralized bone matrix, the healing is accelerated compared to carrier alone [24, 56, 57].

Quarto et al. published a small case series of three patients with varied critical defects, who underwent successful treatments with expanded autologous bone marrow-derived MSCs, seeded on macroporous hydroxyapatite scaffolds ex vivo [57]. All patients were immobilized in an external fixator, and osteointegration was achieved in all three cases by the second postoperative month. There are also a number of similar case reports of autologous MSCs mixed with platelet-rich plasma (PRP), being successfully administered to treat craniofacial defects and distraction osteogenesis [24, 58, 59].

Phase 1 and 2 clinical trials have been completed assessing the use of MSCs in treating critical bone defects, but results are yet to be published. Currently a phase 3 trial (www.mesoblast.com) is being planned to assess the efficacy of allogeneic MSCs in promoting bony union in anterior cervical spinal fusion.

7.7 Osteonecrosis

Osteonecrosis (ON) usually occurs in the femoral or humeral heads, and is caused by vascular insufficiency from whatever cause. The necrotic subchondral bone may collapse before revascularization and osteogenesis occurs, leading to pain and disability. Often the final result is osteoarthritis that can only be treated by joint replacement surgery (arthroplasty). In principle, MSCs could improve outcomes by their ability to secrete cytokines and growth factors, resulting in angiogenesis and osteogenesis [24]. In 2002, Hernigou and Beaujean reported a noncontrolled study of femoral head ON [60]. Concentrated bone marrow aspirates were injected using

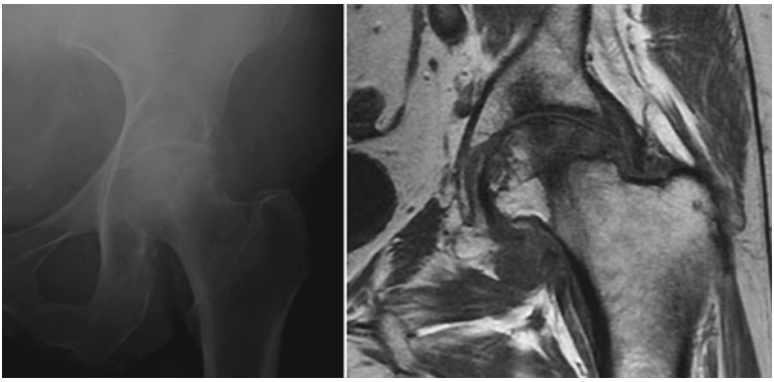

Fig. 7.1 Hip X-ray shows early loss of joint space with suspicion of early femoral head collapse. A T1 MRI of the same hip confirms ON with the typical femoral sub-chondral collapse due to an avascular process

a trocar needle into 145 femoral heads with stage 1 or 2 ON (pre-collapse). Hip replacements were required in seven cases, as the subjects progressed to femoral head collapse. Of the 44 cases presenting in stage 3 or 4 (with femoral head collapse), 25 hip replacements were performed (level 4 evidence).

A double-blind controlled study by Gangji in 2004 compared ten cases of femoral head ON treated by core decompression and concentrated bone marrow aspirate, to a control group of eight cases treated by core decompression only [61]. At 24 months, five of the controls and one of the treatment group had progressed to collapse ($P=0.016$). These encouraging results have been supported by further studies with bone marrow aspirate alone, or mixing aspirate on a demineralized bone matrix scaffold [50, 62, 63]. Current trials include the use of expanded autologous MSCs administered into the femoral head arteries (NCT 00813267) (Fig. 7.1).

7.8 Osteoporosis

Osteoporosis is a disease characterized by a reduction in the quality and quantity of bone, which results in increased fracture susceptibility [64, 65]. It is particularly common in aged individuals and postmenopausal women, who demonstrate lower bone mineral density and higher bone marrow fat [66, 67]. Recent advances of drug treatment for osteoporosis include the use of bisphosphonates such as zoledronic acid and the use of RANK ligand antagonists such as denosumab.

As adipocytes and osteocytes are derived from MSCs, the question is raised as to whether one cell line forms at the expense of the other. Osteoblastic differentiation of MSCs can be encouraged by mechanical stimulation and growth factors, such as BMPs and TGF-βs [66, 68]. Glucocorticosteroids and PPAR-γ agonists, such as the thiazolidinedione (TZD) drugs for diabetes mellitus, reduce bone mass and increase

marrow fat [66]. Both of these adipogenic drugs are associated with an increased fracture risk [66, 69]. It is possible that PPAR-γ antagonists could be developed to promote osteogenic differentiation of MSCs and increase bone mass without producing insulin resistance.

MSCs have been shown to improve bone mass when used as a cell-based therapy. In a controlled trial, autologous rabbit MSCs were injected into osteoporotic bone marrow cavities, and demonstrated improved bone apposition and trabecular thickness [64].

This recent knowledge of MSCs does have potential clinical applications in the treatment and prevention of osteoporosis—either by manipulation of differentiation, gene therapy production of growth factors, or MSC implantation. However, currently there are no registered clinical trials investigating the role of MSCs in the treatment of osteoporosis.

7.9 Osteogenesis Imperfecta

OI is a heterogeneous group of inherited disorders characterized by the production of abnormal type I collagen by osteoblasts [70, 71]. The clinical phenotypes are variable, but include osteopenia, multiple fractures, severe bony deformity, and short stature. The condition may range from a subclinical state to osteoporosis and premature death. Current treatments involve pharmacological agents, such as bisphosphonates and fracture management. In theory, allogeneic MSC transplantation could alleviate the effects of this genetic disorder, and the cells could be gene modified ex vivo, to secrete osteoblastic growth factors such as IGF-1. Animal studies demonstrate that transplanted marrow stromal cells can migrate and incorporate into bone. Horwitz et al. performed allogeneic sibling bone marrow transplants followed by MSC infusions, from the same respective sibling in three children with OI [72]. Three months after engraftment, less than 2% of stromal marrow cells were of donor origin, but there were significant increases in bone mineral content and growth velocity, with a resulting decrease in fractures. Further studies with longer follow-ups and slightly larger case series have shown similar results [72]. Le Blanc et al. performed an intrauterine transfusion of male allogeneic MSCs into a female fetus with severe OI at 32 weeks gestation [73]. At 9 months of age, bone histology showed regularly arranged trabeculae. By 2 years of age, the child had experienced only three fractures, with normal psychomotor development. Cells of male origin were still detected in bone and demonstrate that HLA mismatched MSC can provide a continual source of osteoblastic progenitors, without rejection.

7.10 Tendon Applications

7.10.1 Tendon Pathophysiology

Tendons are a specialized connective tissue, which link muscle to bone and are integral to the function of the musculoskeletal system. Tendon injuries contribute significantly to morbidity in the active young and in the elderly. It is estimated that

in the USA annually, there are more than 32 million traumatic and repetitive motion injuries to tendons and ligaments, and 50,000 rotator cuff tendon repairs. Collagens are responsible for over 80% of tendon dry mass (the large majority of collagen is type I), with elastins contributing to about 2%. Type I collagen is arranged in a hierarchical structure and gives tendons their high tensile strength [74]. The reparative type III collagen (small minority) is thinner, but rapidly forms crosslinks and stabilizes acute tendon injury [75–77]. Tendons are relatively hypocellular, but arranged along the long axis of the collagen are tenoblasts and tenocytes. The tenoblasts are immature cells and are the precursors to the terminally differentiated tenocytes, which lay down the extracellular matrix (ECM) including the collagens. Tendons predominantly utilize anaerobic energy systems, and their oxygen consumption is 15% of skeletal muscle [78, 79]. This allows tendons to maintain tension for long periods of time, whilst avoiding necrosis and ischemia; however, this results in slower healing after acute or overuse injury [78].

Tendons demonstrate a nonlinear stress strain curve, which is dependent on the type of collagen, and intra- and inter-molecular bonds [80]. At higher tendon strain (>4%), microscopic failure starts to develop, and the tendon does not return to the original length. Greater stress (>8%) can cause macroscopic failure and rupture occurs.

Following acute tear or laceration in a healthy tendon, five overlapping healing phases have been identified [81]. After the acute post-injury phase there is an inflammatory phase with invasion of red and white blood cells (especially neutrophils), forming a hematoma, which acts as an early scaffold. There is release of vasoactive, differentiation, proliferation, and chemotactic factors, resulting in angiogenesis and tenocyte hyperplasia. The proliferative and reparative phases follow, and are characterized by early repair with synthesis of type III collagen which can constitute up to 30% of total collagen. After 6 weeks the remodeling stage commences, which may last up to 10 weeks post-injury, with cellular maturation and type I collagen deposition. The resulting tendon is fibrotic, thickened, and less resistant to tensile stress, when compared to pre-injury structure. This process can take 1–2 years if there is preexisting degeneration present, and is occasionally refractory to all treatment [82]. Tendons will repair with scar tissue, if there is stable apposition of both free ends; however, immobilization is difficult for some joints and for tendons with an active muscle contraction. Therefore, acute tendon rupture is often surgically repaired and then immobilized for 6–10 weeks. If the rupture is chronic then unopposed muscle contraction results in a shortened atrophic muscle, the tendon defect cannot be bridged, and scaffold reconstruction is undertaken. Sometimes degenerate tendon tears result in a weakened primary repair, and scaffold augmentation of the repair is required. The current scaffolds of choice are autologous tendon grafts (e.g., patella, hamstring, or palmaris longus), tendon allografts, or artificial acellular synthetic engineered scaffolds. These reconstructive procedures have recognized complications including donor site morbidity in autografts, potential immune rejection or infection transmission in allografts, and possible delayed implant failure in synthetic grafts [83–85]. Tendon allograft or autograft scaffolds require prolonged postoperative rehabilitation and activity restriction for between 6

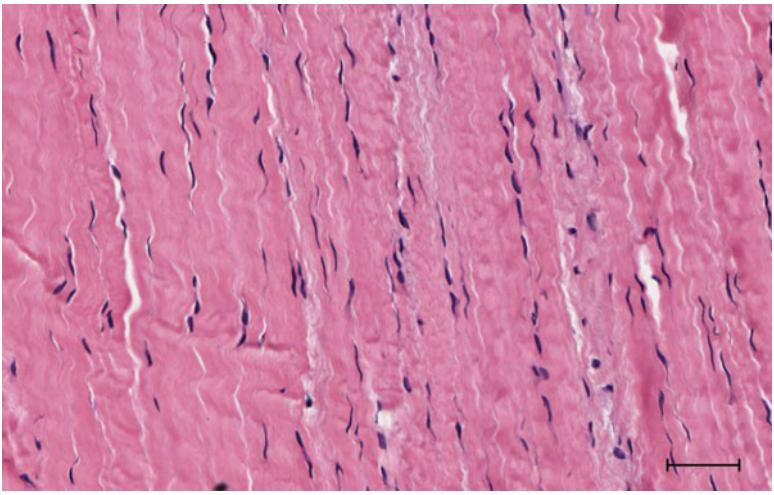

Fig. 7.2 Histology of healthy tendon demonstrates the relative paucity of cells (Courtesy of University of Western Australia)

and 18 months. During this period, the scaffold integrates with native tendon, and type I collagen is replaced with type III (reparative) collagen, before new type I collagen is laid down. Hence, there is a clear clinical need for a cell-based tissue-engineered scaffold for both tendon and ligament injury.

Tendons also develop overuse and degenerative pathology with microscopic collagen breakdown, which leads to pain and altered function, and contributes to degenerative tearing at lower strain thresholds. This degeneration is referred to as tendinopathy, which is an all-encompassing term that includes both tendinosis and tendinitis [86]. The histological changes include increased ground substance, increased type III collagen, variation in tenocyte morphology, and reduction in the number of healthy tenocytes [87]. Common tendinopathies are found in the rotator cuff of the shoulder, gluteal tendons of the hip ("greater trochanteric bursitis"), lateral epicondyle of the elbow ("tennis elbow"), and the Achilles tendon. Initial treatment is always conservative and is usually prolonged. There is good evidence that exercise rehabilitation is beneficial, but limited evidence of efficacy for any of the injectable or other nonoperative treatment [88, 89]. If tendinopathy is refractory to conservative treatments, then surgical tendon debridement is sometimes undertaken; but this is expensive and disabling, and the success is only modest [88]. The resulting tendon is fibrotic, thickened, and histologically has scar tissue fibroblasts with lower numbers of healthy tenocytes, associated with an increase in non-collagenous material [75, 87]. Therefore, tendinopathy may become a chronic and disabling condition with few effective treatments. Cellular therapies offer great potential in the more chronic tendinopathies, as they lead to regeneration of new tendon, rather than repair with scar tissue. It is important to note that improved ultrasound techniques mean that cell-based treatments can be accurately implanted by precision intratendinous injection (Figs. 7.2 and 7.3).

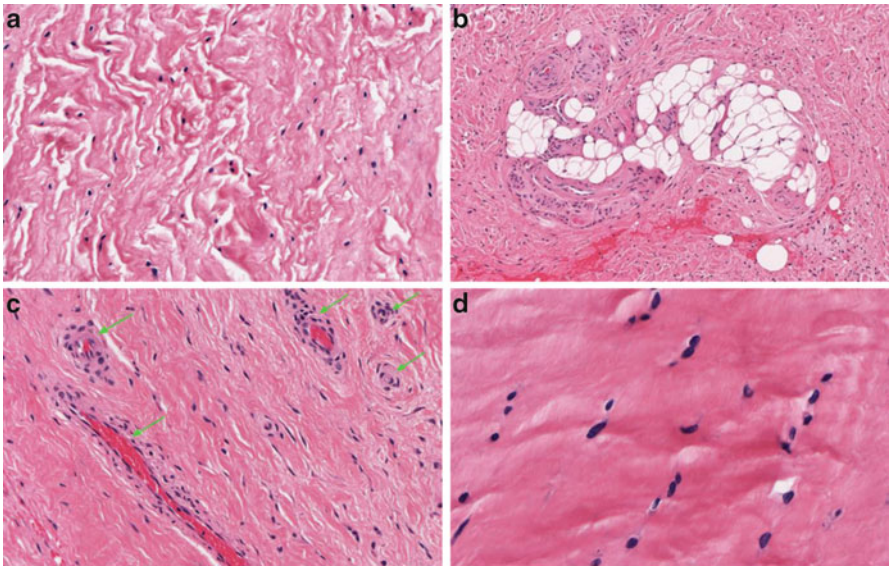

Fig. 7.3 Histopathology of rotator cuff tendinopathy reveals: fiber disruption (**a**), adipose tissue deposition (**b**), vascular hyperplasia (**c**), and rounding of nuclei (**d**) (Courtesy of University of Western Australia)

7.11 Gene Transcription Factors

Introduction of transcription factors into stem cells leads to reprogramming and phenotype transition [90]. Gene modification may lead to therapeutic approaches to treat cellular injury, such as degenerative tendinopathy, when healthy tenocyte numbers are usually reduced. However, no specific master transcription factor has been isolated for the tendon lineage. Scleraxis (Scx) has been the most studied potential marker of neotendon formation [91]. It is a basic helix-loop-helix transcription factor expressed in the syndetomal compartment of developing embryonic somites [92]. Other candidate genes involved in tendon formation may be Six-1, Six-2, Eya-1, Eya-2, THBS4, and TNMD, which are expressed during limb formation in developing tendons and ligaments [74, 93].

Hoffman et al. showed that MSC differentiation into tendon-like cells was mediated by intracellular signaling factor Smad-8 expression, and simultaneous stimulation with bone morphogenic protein 2 (BMP2) [94]. The authors concluded that Smad-8 inhibited the normal osteogenesis pathway induced by BMP2.

7.12 Growth Factors

Growth factors are proteins that regulate cellular processes including the growth, proliferation, and differentiation of cells. No tendon- or ligament-specific paracrine growth factors have been discovered yet. A number of connective tissue

growth factors have been shown to facilitate the differentiation of MSCs into tenoprogenitors, as well as cellular migration and collagen synthesis [91]. These include fibroblast growth factor (FGF)-2, transforming growth factor (TGF)-β, insulin-like growth factor (IGF)-1, VEGF, PDGF, and members of the BMP superfamily—including growth and differentiation factors (GDFs) [81, 95–98]. Wolfman et al. administered intramuscular injections of GDFs 5, 6, and 7 into rats, and induced ectopic neotendon-ligament tissue formation [99]. Aspenberg and Forslund reported positive results after local injection of GDF 5 and 6 into rat Achilles defects [100].

Blood platelets are known be a rich source of growth factors, which can induce tendon cell proliferation and angiogenesis in vitro [101]. Current clinical treatments for tendinopathy often include the intratendinous injection of autologous PRP. However, there is inconsistent evidence regarding the efficacy of PRP in vivo. There are only two published double-blinded randomized controlled trials, and one showed no statistically significant benefit over placebo, whilst the other showed a benefit of PRP over corticosteroid [102, 103]. Tendon growth factors have a restricted biological half-life; and repeated intratendinous injections to facilitate healing are impractical and cause tendon damage. Possible solutions include slow release preparations, or gene-enhanced cellular therapies, which temporarily produce tenogenic growth factors. Thomopoulos et al. showed that platelet-derived growth factor (PDFG-BB) linked to a sustained release delivery system was able to improve tendon range of motion and excursion in a dog tendon repair model; but the tensile properties were unchanged [104]. Gene therapy can transiently or permanently engineer DNA to produce growth factors. This can be performed in 2 ways: in vivo gene transfer where the gene is transferred directly to the recipient, or ex vivo gene transfer of the gene to a stem cell in tissue culture, which is then implanted. Rickert et al. injected adenovirus-GDF5-infected particles into rat Achilles tendons, permitting transitory transgene expression [105]. This resulted in thicker, stronger tendons at eight weeks; however, on histological analysis, there were increased chondral cell lines and more type II collagen was produced, compared to controls. Hou et al. implanted MSCs infected with adenovirus carrying human TGF-β cDNA into rabbit Achilles tendon defects, and demonstrated improved recovery and biomechanical properties [106]. Similarly, IGF-1 gene-enhanced MSCs improved histological scores in horse flexor tendons [107]. These experiments are promising but most current gene delivery methods require viral vectors, with potential risks including immune reaction, insertional mutagenesis, and uncontrolled transgene expression [9]. There are no current human clinical trials with genetically modified stem cells in tendon-ligament conditions.

7.13 Tissue Engineered Tendon Bioscaffolds

When designing the ideal tendon bioscaffold for cell seeding, important factors such as biocompatibility, biodegradation rates, mechanical properties, internal space for cell infiltration (porosity), and nutrient transmission all need to be considered

[91]. Scaffold materials are classified as natural or synthetic. Natural scaffolds tested to date include collagens, small intestine submucosa, chitosan, renal capsule matrix, and silk fibers [108]. Synthetic scaffolds have been derived from poly-L-lactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA) [109]. Modern scaffold design also needs to consider the important biological roles of the ECM, including the proliferation and differentiation of the tendon stem/progenitor cells [91]. The elasticity of the matrix has been shown to determine cell fate [110]. Softer substrates promote MSC differentiation into neuronal like cells, moderate elasticity favors myogenic differentiation, and a rigid scaffold is osteogenic.

As most of the mechanical properties of natural tendon are from type I collagen, the majority of studies have involved cell-seeded collagen gels. Contraction of the gel is related to cell seeding density, which provides mechanical stability, and is generally followed by cellular alignment and reorganization of the matrix [111]. The biomechanical properties of cellular scaffolds are further improved if preliminary cell seeding is performed in vitro, before implantation in vivo. Collagen gels have been enhanced by collagen hybridization with PLA, and cross-linking collagen with di-catechol nordihydroguaiaretic acid (NDGA) [111, 112]. At present, no tenocyte-collagen scaffold constructs have been able to achieve similar mechanical properties to native tendon [111].

MSC-enhanced collagen gel scaffolds for the rabbit model have been shown to produce ectopic calcification (due to osteogenesis) in 28% of cases—irrespective of the cell seeding density [113]. In follow-up studies, the authors found that the alkaline phosphatase activity (a sign of preosteoblastic phenotype) was elevated around the sutures, but only when the cells were in a 3D construct, and not when the MSCs were in a monolayer [114]. The authors later concluded that the osteoblastic proliferation was due to in vitro factors, independent of cell seeding density. However, Butler et al. advocated lower seeding density, with end posts rather than sutures, and augmentation of the gel with type I collagen sponge; this produced bioscaffolds with improved repair stiffness and improved force to failure [115]. No ectopic calcification was produced.

Ouyang et al. showed that PLGA scaffolds seeded with allogeneic MSCs repaired 1 cm defects in rabbit Achilles tendons, with improved tensile stiffness and modulus, compared to an acellular scaffold. However, the grafted tendon only had 62% of the tensile stiffness compared to control repaired tendons at 12 weeks [116].

The local administration of VEGF improves revascularization, but not the mechanics of the scaffold [117, 118]. TGF-β1 promotes improved strength in Achilles tendon regeneration by regulating collagen I and III synthesis, cross-link formation, and matrix remodeling [106]. Wei et al. transfected bone marrow (bm)MSCs with an adenoviral vector expressing TGFβ1/VEGF165, which were implanted into a rabbit tendon ACL model [119]. The treated grafts demonstrated accelerated remodeling, angiogenesis, and mechanical properties compared to controls.

Stem cell scaffold design requires a multidisciplinary strategy combining cell technology, engineered scaffolds, and mechanical stimulation [115]. Currently, there are no registered human stem cell trials using scaffolds to repair tendons or ligaments recorded on the database registries.

7.14 Mechanical Stimulation

Tendon studies have demonstrated a role for mechanical loading in tissue homeostasis and healing. In uninjured musculoskeletal tissues, increased loading leads to an improvement in biomechanical properties, but the role of loading on injured or healing tissues is less clear. Thomopoulos et al. showed that complete removal of load, by proximal tendon transection, resulted in tendon-to-bone repairs with less range of motion and lower biomechanical properties, compared to repairs in which the muscle-tendon-bone unit was left intact [120]. In the clinical treatment of tendinopathy, exercise rehabilitation is the most effective nonoperative evidence-based intervention [88]. However, there is no consensus regarding the duration, frequency, amplitude and type of exercise, but some evidence suggests eccentric loading is the most efficacious [121]. Mechanical loading of tendons has been shown to produce a trophic cellular response with cellular proliferation, differentiation of tendon stems cells, and resulting increased deposition of ECM [122–124]. Loading also promotes secretion of cellular proteins, including TGF-β and IGF-I [125, 126]. The type of loading and the axis of application alter the cellular response. Compression loading has been shown to lead to the formation of more cartilaginous tissue, and shear stress produces increased matrix metalloproteinases (MMP-1 and 3) in rabbit tendon fibroblasts, which cause matrix disruption and resultant collagen degradation [127, 128]. Repetitive in vitro stretching, at higher construct strains, has been shown to cause production of PGE_2 and BMP2, leading to differentiation into non-tendon lineages [129, 130]. Zhang and Wang demonstrated that in vitro uniaxial loading of rabbit patella and Achilles tendons, at 0.5 Hz for 12 h, upregulated type 1 collagen synthesis at 4% strain, but increased adipogenesis and osteogenesis at 8% strain [131]. This has been postulated as a reason why degenerate tendinopathy is associated with calcification [130].

Cyclic uniaxial mechanical stretching of bioscaffolds increases ECM production and the alignment of collagen fibrils along the stress axis in a number of cell lines, including cultured tendon fibroblasts, isolated tendon fascicles, dermal fibroblasts, and MSCs. Stretching increases scleraxis upregulation, and ultimate failure to stress by a factor of six times [91, 132]. Chen et al. found that poorer outcomes resulted, when stress was applied in the first 3 days after cell seeding in silk fibroin matrices [133]. The authors concluded that prerequisites include established cell-to-cell contact and sufficient ECM before load is applied.

The optimal mechanical stimulation for in vitro tendon scaffolds and in vivo application of stem cells in tendinopathy is yet to be established.

7.15 Mesenchymal Stem Cells for Tendinopathy

MSCs are cells that have the ability to either proliferate, or differentiate into progenitors of mesenchymal tissues such as bone, cartilage, fat, tendon, and muscle [99]. MSCs are also characterized by (but not unique) cell surface markers, adhesion

molecules, growth factors (and their receptors), and ECM molecules [134]. MSCs can be isolated from a variety of tissues including bone marrow, adipose tissue, periosteum, muscle, tendon, and articular cartilage [91]. Sakaguchi et al. demonstrated that the differences in the properties of MSCs depended on their cell source [135]. For example, adipose-derived MSCs favored adipogenesis, whereas bone marrow-derived MSCs favored osteogenesis. Bi et al. expanded murine tendon stem cells in vitro, and demonstrated that they preferentially formed neotendon when reinjected into the mice [123].

It has been assumed that MSCs could regenerate tissue due to their "stemness"; however, there is increasing evidence that the mechanism of action may not be due to direct engraftment or differentiation [6]. MSCs secrete a variety of soluble autocrine and paracrine growth factors, which promote cell survival and enhance the proliferation of endogenous connective tissue cells. These growth factors stimulate mitosis in tissue progenitors, induce angiogenesis, and reduce apoptosis [6–8].

In preclinical animal studies, two different MSC experimental tendon models are commonly used. These are the tendon laceration/defect model or the collagenase-induced tendinopathy model. In a controlled study, Chong et al. showed that allogeneic MSCs with a fibrin carrier, implanted into lacerated and sutured rabbit Achilles tendons improved the histological and biomechanical parameters in the early stages of tendon healing [136].

In tendinopathy, the microscopic collagen degeneration can be partly reproduced experimentally by the administration of collagenase, either in vivo or in vitro. In an in vivo study, Lacitignola et al. demonstrated that both autologous bone marrow-derived MSCs (95.5×10^6 cells) and bone marrow mononuclear cells (bmMNCs) (122.3×10^6 cells) could be injected intra-lesionally into equine collagenase-treated tendons, and both produced effective tendon regeneration [137]. In a similar study, Crovace et al. showed that there was type I collagen in the tendons of the actively treated horses and type III collagen in the control tendons [138]. No calcification or ectopic tissue was detected on serial ultrasounds or at autopsy. Schnabel et al. injected autologous bmMSCs and IGF-I gene-enhanced bmMSCs into collagenase-produced equine flexor digitorum superficialis (FDS) tendinopathy [107]. At autopsy, the horses treated with both bmMSCs and adenovirus IGF-MSCs had improved histological scores compared to controls. In a small case series, Guest et al. injected green fluorescent protein-labeled autologous and allogeneic equine MSCs into collagenase-treated FDS tendons. At postmortem, there was evidence of engraftment of both MSCs, without rejection at 34 days [139].

Pacini et al. successfully treated 9 out of 11 horses with FDS tendinopathy, with targeted intra-lesional injection of undifferentiated MSCs [140]. Serial ultrasounds showed improved tendon morphology and no tendon calcification. Allogeneic equine adipose dermal MSCs have also been shown to successfully treat 14 out of 16 horses with FDS tendinopathy [141]. No complications were reported. In a controlled trial, Smith et al. showed that by injecting 1×10^7 autologous bmMSCs intratendinously, the resulting tendon was significantly improved in terms of cross-sectional area, cellularity, crimp pattern, and DNA content

compared to controls [142]. MSCs are commonly used as a therapeutic intervention in the equine thoroughbred industry to treat equine FDS tendinopathy. Currently, over 1,800 horses have received autologous bmMSCs for tendinopathy (www.vetcell.com). The recurrence rate of this injury is 56% with conventional treatment, but with MSC treatments is reportedly 27% [143, 144]. There have been no reported cases of ectopic tissue production detected on serial ultrasounds. Twelve horses have now undergone postmortem (17 tendons), which has revealed good healing with minimal inflammatory cells, with crimped organized collagen fibers, and no ectopic or neoplastic tissues [144]. Currently, the author of this chapter is undertaking a trial in the use of allogeneic MSCs in the treatment of human chronic (refractory) Achilles tendinopathy.

7.16 Conclusions

7.16.1 Bone Applications

Even though bone has one of the best healing potentials of any of the collagen-producing connective tissues, a wide range of heterogeneous pathologies of bone continue to produce significant morbidity. Bone healing is a relatively slow and inconsistent process and is dependent on both intrinsic and extrinsic factors. Over the coming decade, MSC therapies will likely revolutionize the treatment of these conditions. MSCs have the potential to accelerate bone healing, and regenerate new bone in circumstances where healing will not occur. In preclinical studies, MSCs have the ability to be pre-differentiated and gene-modified to secrete growth factors, and can be seeded onto tissue engineered 3D scaffolds (in vivo and in vitro). The therapeutic plasticity of MSCs means that implantation can be via open operation, percutaneous injection, or systemic routes. MSCs can be harvested from a number of sites, and their immunoprivileged status means that allogeneic cells can be used without the need for immune suppressive medication after MSC administration.

MSC use in bone therapies is the most widely researched lineage of any of the connective tissues. Phase I and II trials have been completed in a number of bone conditions. A phase III trial is proposed for allogeneic MSCs in multilevel cervical fusion. This technology holds great promise and is likely to become a clinically important therapy in the near future.

7.16.2 Tendon Applications

Tendon disorders are a common cause of morbidity and a significant health burden on society. Tendons are relatively acellular and have limited blood supply, resulting in a poor capacity to self-heal. Current clinical treatments are only moderately effective and include prolonged exercise regimes, injections, and surgery.

The resulting tissue repair contains mechanically inferior scar tissue and is prone to reinjury, resulting in lifestyle changes such as activity modification or cessation.

Preclinical studies suggest that MSC therapies will prove to be an important therapeutic intervention in the treatment of tendon disorders. MSCs can be administered by precision intratendinous injection under ultrasound guidance, or on bioengineered scaffolds. Much is yet to be discovered about the roles of gene modification, mechanical stimulation, the preferred scaffold, and the need for predifferentiation. Regardless, MSC therapies hold great potential in the future treatment of tendon disorders.

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Chapter 8
Mesenchymal Stromal Cells and the Repair of Cartilage Tissue

Michael R. Doran and Mark Young

Abstract Articular cartilage has a limited intrinsic repair capacity, and thus defects are more likely to further degrade rather than undergo spontaneous self-repair. Whilst a number of surgical techniques have been developed to repair cartilage defects, their efficacy is generally poor and total joint replacement remains the gold standard, albeit last resort, treatment option. Cell-based therapies hold the greatest promise, as they appear uniquely capable of generating de novo cartilage tissue. Two approved therapies (ACI and MACI) are based on the premise that the transplantation of ex vivo expanded autologous chondrocyte populations, harvested from a non-load bearing region of the same joint, could be utilized to effectively regenerate cartilage tissue in the primary defect site. These therapeutic strategies are partially limited by our inability to harvest and expand adequate numbers of autologous chondrocytes that retain the appropriate phenotype. By contrast, the harvest and expansion of large numbers of mesenchymal stem/stromal cells (MSC) derived from tissues such as bone marrow and adipose is comparatively straightforward and has become routine in laboratories worldwide. Additionally, our understanding of the biochemical and biophysical signals required to drive the chondrogenic differentiation of MSC is rapidly increasing. It is conceivable that in the near future MSC expansion and differentiation technologies will offer a means to generate sufficient cell numbers, of an appropriate phenotype, for use in cartilage defect repair. In this

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chapter we review the relative potential of MSC and their likely contribution to cartilage regeneration.

Keywords Cartilage • Mesenchymal stem cell • Tissue engineering • Chondrocytes • Autologous chondrocyte implantation (ACI) • Matrix-induced autologous chondrocyte implantation (MACI)

8.1 Introduction

Cartilage tissue can be categorized as elastic cartilage, fibrous cartilage, or hyaline cartilage based on its structural role and composition [1]. Elastic cartilage can be found in the outer ear, larynx, and epiglottis. The primary structural protein is elastin, and this tissue is designed for repeated elastic distortion rather than compressive distortion. Fibrous cartilage (fibrocartilage) is found in the menisci, intervertebral discs, tendons, ligaments, and the temporomandibular joint [2]. This tissue has incredible tensile strength and is characterized by its high collagen I content [3]. Hyaline cartilage covers the surface of articular joints, and is often referred to as articular cartilage. Greater than 95% of this tissue volume is composed of the extracellular matrix that confers its function as a shock absorber and as a smooth surface for joint articulation [4]. The matrix is rich in collagen II, proteoglycans (aggrecan), and multiadhesive glycoproteins which make up approximately 15%, 10%, and 5% respectively of the tissue's mass. Much of this chapter will focus on the biology and repair of hyaline articular cartilage with mesenchymal stem/stromal cells (MSC).

Hyaline articular cartilage is a specialized tissue that enables decades of smooth joint articulation and cyclic loading. This tissue is unique, relative to non-cartilaginous tissues, in that it has a low cell density, high matrix content, and is avascular. Whilst these features are particularly well suited for cyclic loading, they limit the intrinsic repair capacity of the tissue. In fact once damaged, articular cartilage is more likely to degrade further, than spontaneously repair. Radiographic evidence of articular cartilage damage is evident in the majority of individuals aged over 65 [5]. The disease most commonly responsible for such damage is osteoarthritis (OA), which is crudely defined as a degenerative joint disease [6]. The significance of OA is often underestimated, in spite of the fact that in some western countries OA is a major cause of pain and disability [7]. The combined pressure of an aging population, and the obesity epidemic, is driving a rapid increase in the frequency of total joint replacement procedures in the western world. For example, between 1996 and 2000 there was a 30% increase in knee and hip replacements procedures in Australia [7].

The risk factors for the development of OA include: increasing age, internal joint derangement (ligamentous or meniscal cartilage damage), joint malalignment, obesity, and chondral cartilage injury. Once OA is established, the most popularly prescribed treatments are lifestyle modifications, exercise rehabilitation, and weight loss. These treatments cannot reverse the underlying process, but rather serve to reduce pain and improve function. Total joint replacement (arthroplasty) is frequently required, but this procedure is treated as a last resort measure, as it is an irreversible

step and does not fully restore function. Prosthetic implants are prone to accelerated wear and revision arthroplasty is a technically complicated and destructive procedure. Hence, the goal of many interventional treatments in younger people is not only symptom control, but the long-term prevention of OA. Examples include meniscal repair, ligamentous reconstruction, joint realignment (osteotomy), and articular cartilage debridement or repair.

Despite the fact that the first highly publicized engineered tissue in 1995 (so-called "earmouse"), produced by Dr. Charles Vacanti, was cartilaginous in nature, there remains no highly efficacious tissue engineering procedure for cartilage repair. However, there has been the development of one type/class of clinically approved tissue engineering therapy, and considerable excitement regarding the potential of bone marrow-derived MSC to further advance such therapies. In the following sections we will discuss the clinical strategies used to treat cartilage defects, as well as tissue engineering strategies currently used and under development, with a particular focus on the potential role of MSC.

8.2 The Clinical Management of Articular Cartilage Defects

Articular cartilage defects may arise from either acute trauma or repetitive microtrauma. Treatments aim to control symptoms and restore function, and in the active young, prevent long-term OA. Current therapies can be categorized as debridement, marrow stimulation techniques, whole-tissue transplantation, and chondrocyte tissue engineering therapies. Each of these therapies will be reviewed in brief below.

8.2.1 Chondral Debridement (Chondroplasty)

Chondral debridement and lavage of most joints can be performed arthroscopically. Debridement involves the physical removal, via cutting or abrasion, of cartilage pieces or flaps that might be catching and hindering joint motion. Lavage functions to essentially wash away debris and loose pieces of tissue that may be retained within the joint fluid [8]. The simple excision of damaged tissue has been shown to relieve symptoms for up to 5 years [9]. Whilst this procedure has been shown to be effective in pain management of acute localized cartilage defects, there is a lack of consensus as to if it is effective in the management of established OA [10].

8.2.2 Marrow Stimulation Techniques

Microfracture and Pridie Drilling can all be categorized as marrow stimulation techniques. The underlying objective is to penetrate through the subchondral bone plate,

from within the cartilage defect site, into the adjacent marrow such that there is bleeding from the bone marrow into the defect site. A blood clot will then fill the defect site, with mesenchymal stem cells from the bone marrow trapped inside. It is proposed that this MSC-laden clot will ultimately function as repair tissue and fill the cartilage defect.

Marrow stimulation techniques are appealing, as in theory they are relatively simple low-cost procedures. However, in practice the execution of the procedure requires significant technical rigor to ensure success. The proper preparation of the calcified cartilage at the base of the defect and the shaping of the defect walls appear to be critical considerations that ensure that the blood clot is both able to effectively adhere to the underlying tissue and that it is contained within the defect site [11, 12]. The subsequent adherence to rehabilitation protocols may be an even more significant factor influencing positive outcomes from procedures such as microfracture [13]. There is a tendency for patients to overestimate their functional recovery, and to damage the fragile repair tissue through inappropriate activity. When proper surgical repair and rehabilitation methods are followed, microfracture appears to generate acceptable clinical outcomes [14]. Magnetic resonance imaging of defect sites has been used in previous studies, revealing that 54% of patients had good fill of the defect site with repair tissue, 29% had moderate fill, and 17% had poor fill [15]. Favorable imaging results correlated with clinical outcomes. It is important to note that the patient cohort used in this and related trials had full thickness traumatic cartilage injuries, and were not suffering from OA [15, 16]. Further, patient age was found to be an independent predictor of clinical success [17].

While the marrow stimulation techniques likely do not represent a clinical solution for older patients suffering from OA, there are some significant insights that can be made from these studies that are relevant to the future use of MSC in cartilage repair. First, unlike other cartilage repair techniques, marrow stimulation procedures do not involve the provision of mature chondrocytes into the lesion [17]. This implies that chondrocytes in the repair tissue either migrated from adjacent tissue, or were derived de novo within the defect site. It is generally assumed that the chondrocytes are derived de novo from MSC that populated the clot from the bone marrow. Second, the repair tissue matrix is ultimately fibrocartilage rich in collagens type-I and type-III, unlike hyaline cartilage, which is rich in collagen type-II [18, 19]. These observations imply that bone marrow-derived MSC can play a role in cartilage repair, but suggest that generation of true stable hyaline chondrocyte populations and regeneration of the appropriate zonal cartilage matrix may not occur. Indeed, our discussion later in this chapter identifies this as a potential limitation in the application of bone marrow-derived MSC to articular cartilage defect repair.

8.2.3 Whole-Tissue Transplantation

In whole-tissue transplantation the defect area is excised and the tissue is replaced with mature tissue from either an autologous donor site, or with allogeneic tissue.

The significant advantage gained by using such a procedure, relative to procedures such as microfracture, is that the defect site is immediately filled with mature organized hyaline cartilage tissue. Whilst this procedure has been widely adopted, there are some significant limitations.

In mosaicplasty, cylindrical osteochondral plugs are generally harvested from the peripheries of the femoral condyles, at the level of the patellofemoral joint, and inserted as a mosaic to fill the defect site [10]. Whilst donor site morbidity is a legitimate concern [20], perhaps more concerning is the fact that lateral integration of the plugs within the defect site rarely occurs [21]. Animal studies confirm that there is a persistence of full thickness gaps between the implanted plugs [22]. Poor integration likely reflects a loss in viable chondrocytes at the edge of the plug. Studies which specifically investigated this phenomenon found that within 2 hours of harvest there was a 400 μm zone of cell death around the periphery of the plug [23], and that this margin could be measurably reduced, but not eliminated, using specific harvesting techniques. By contrast, there is generally solid osseous integration with the subchondral bone [24]. Regardless of the poor integration and potential for donor site morbidity, the follow-up of nearly 1,000 patients suggests that good to excellent results can be achieved using this methodology [20]. From a tissue engineering perspective, these studies highlight the probable need for repair strategies to include an interface with the subchondral bone to promote stability and tissue integration.

8.2.4 Chondrocyte Tissue Engineering Therapies

In 1987 Peterson et al. performed a procedure they termed "autologous chondrocyte implantation (ACI)" to repair cartilage defects [25]. This was a pivotal moment as it represented the first use of cell engineering in orthopedic surgery [10]. In ACI, cartilage tissue is harvested arthroscopically in a preliminary operation [25]. Chondrocytes recovered from the biopsied tissue are then expanded in culture for 4–6 weeks. In a second surgery, the expanded chondrocyte population is injected into the cartilage defect beneath a lid formed from periosteum.

The actual quality of the ACI repair tissue remains under debate. Encouraging results have been reported from a number of studies [26–29]. In one of the studies, of the 23 patients followed up at 39 months, good or excellent results were recorded in 70% of the cases [26]. Biopsies from these patients revealed that 11 of the 15 tested had hyaline-like cartilage tissue. As the number of ACI recipients is increasing, it will be possible to evaluate large cohorts and begin to accurately screen outcomes. From 1996 to 2003, 294 adverse events were reported for the 7,500 procedures facilitated by Genzyme Tissue Repair [30]. Of this group, 273 required revision surgeries. Thus, whilst the overall rate of adverse events is very low, such events generally require subsequent surgeries to remedy.

Studies which contrast ACI against other therapeutic options indicate varied relative efficacy. Some studies indicate that ACI is superior to microfracture [31], whilst others suggest that it is not [32, 33]. Similarly, some studies indicate that ACI

is superior to mosaicplasty [34], whilst others suggest that it is not [21]. The ACI procedure has continued to evolve and the next generation procedure uses a collagen membrane to function as a lid and scaffold rather than a piece of periosteum. The membrane is composed of porcine-derived collagen types I and III [35], and the modified procedure is termed matrix-induced chondrocyte implantation (MACI). Cells are seeded onto the MACI membrane that also functions as a scaffold. This feature makes surgical handling and correct placement of the cells easier. Critically, the use of the MACI membrane eliminates the need to harvest a periosteal membrane, which itself is associated with complications in 10–25% of cases [36, 37]. Whilst this evolution makes the surgical procedure technically easier [35], it is not yet clear if clinical outcomes differ.

8.3 MSCs Versus Chondrocytes?

Chondrocytes have been logically utilized in cartilage repair procedures such as ACI and MACI. Unfortunately, the harvest and expansion of chondrocytes is not without its complications. First, the harvesting of donor cartilage tissue for chondrocyte isolation can result in donor-site morbidity. Second, in cases where there is a degenerative pathology involved, it may not be possible to recover a healthy and functioning chondrocyte population for use in subsequent tissue repair. Third, chondrocytes generally undergo a process of dedifferentiation during ex vivo expansion [38–40], meaning that the expanded cells will have lost the characteristics that made them ideal in the first instance. By contrast, bone marrow-derived MSC harvest does not require preliminary surgery, isolation and expansion of MSC is reasonably easy, and differentiation into chondrocyte-like cells is possible. Critically some [41, 42], but not all [43] studies indicate that MSC derived from OA patients behave similarly to those derived from healthy patients.

8.4 MSC from Which Tissue?

MSC can be found and have been isolated from almost all postnatal organs and tissues [44, 45]. These populations are defined by the International Society for Cellular Therapy as having (1) the ability to be selected by plastic adherence in culture, (2) the expression of cell surface antigens CD105, CD73, and CD90 in greater than 95% of the culture population, and lacking the expression of markers including CD34, CD45, CD14 or CD11b, CD79α or CD19, and HLA-DR in greater than 95% of the culture population, (3) the ability to differentiate into osteoblasts, adipocytes, and chondrocytes in vitro [46]. Despite its appearance, this definition is not especially rigorous and allows for considerable differences between populations that meet these same criteria. Whilst "MSC" populations derived from different tissues may share these defining characteristics, they have significant functional differences

in their capacity to differentiate into chondrocytes and to produce cartilage tissue. Most studies indicate, for example, that bone marrow-derived MSC have greater in vitro chondrogenic potential than adipose-derived MSC [47–49]. Some studies suggest that MSC derived from the synovium have a greater chondrogenic potential than bone marrow-derived MSC [50]. These results are consistent with the concept that MSC might preferentially differentiate into a tissue resembling their tissue of origin [1, 51]. Alternatively, these observations might simply indicate a contamination of MSC cultures with tissue-specific progenitors.

The quanta of data suggesting that bone marrow-derived MSC have a propensity to differentiate into chondrocytes, and the relative ease with which bone marrow can be aspirated, makes these cells a logical focus for much of the cartilage tissue engineering field. Given the previous discussion on tissue of origin and differentiation, it is not surprising that these cells have a tendency to undergo hypertrophy and take on osteogenic characteristics [52]. In fact this remains one of the greatest challenges in the field, and is something we address in the subsequent section of this chapter.

8.5 MSC Differentiation into Chondrocytes

The process of cartilage formation has been rigorously studied in both chick embryo and mouse models. These studies provide some insight into the cell–cell interactions and temporal cytokine cascades that result in cartilage tissue formation. This process proceeds in defined stages, and commences with MSC recruitment, proliferation, and condensation [53]. MSC condense, forming a cartilaginous anlagen which functions as a template for the skeletal long-bones. This initial increase in cell adhesion is facilitated by an upregulation of cell adhesion molecules, specifically including neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM) [54]. Transforming growth factor-β (TGFβ) is one of the earliest signal molecules involved in directing MSC condensation, and activates N-CAM. This corresponds to increased Sox 9 expression, a key chondrogenic transcription factor, that drives collagen II and other cartilage-specific matrix gene expression [55]. The three-dimensional (3D) organization of the tissue is thought to be guided by fibroblast growth factor (FGF), hedgehog, bone morphogenic protein (BMP), and Wnt signaling [56]. BMPs were originally discerned as directing endochondral ossification, or the hypertrophy of cartilaginous tissue and its conversion into bone tissue [57]. However, it is now appreciated that BMPs are also required for the formation of precartilaginous condensations, the differentiation of precursors into chondrocytes [58] as well as playing a role in the later stages of chondrogenic maturation, as well as terminal differentiation and hypertrophy.

Once a template is established, the balanced signaling of FGFs and BMPs guide both chondrocyte proliferation and differentiation [59]. BMP-2 and BMP-6 are found exclusively in hypertrophic chondrocytes, whilst BMP-7 is expressed by proliferating chondrocytes. Within the lower proliferative and prehypertrophic zones, chondrocyte proliferation is controlled through a negative feedback loop

involving signaling by parathyroid hormone-related protein (PTHrP) and Indian Hedgehod (Ihh) [53].

In 1998, Johnstone et al. demonstrated that bone marrow-derived MSC could be differentiated into chondrocytes in vitro through their aggregation into a micromass culture in the presence of TGFβ-1 [60]. Since this time other members of the TGFβ super-family have been assessed for their ability to guide chondrogenesis in vitro. Some studies suggest that both TGFβ-2 and -3 stimulate differentiated MSC to produce significantly more proteoglycans and collagen II than TGFβ-1 [61]. Further, BMP-2, BMP-4, and BMP-6 have been shown to promote collagen II production [62], but as mentioned earlier they are also associated with hypertrophy [59]. There is also evidence that MSC-derived chondrocytes respond to specific cell–matrix interactions, with natural cartilage matrix components such as collagen II [63] and hyaluronan supporting enhanced MSC differentiation both in vitro and in vivo [64].

Generation of a stable chondrocyte phenotype from MSC has proven nontrivial, and the propensity for these tissues to undergo endochondral ossification remains problematic. Studies in which in vitro chondrogenically differentiated MSC are implanted subcutaneously in either nude or NOD/SCID mice often result in the formation of unstable or hypertrophic bone tissue [65–67]. This inability to maintain a stable chondrogenic phenotype is the primary factor limiting the use of MSC in cartilage regeneration. One of the most promising solutions to this problem is the use of PTHrP, which appears to prevent hypertrophy both in vitro and in vivo [68, 69].

8.6 In Vitro MSC Cartilage Tissue Engineering

Prior to translation into in vivo animal models, most tissue engineering strategies are first investigated and rigorously tested in vitro. Whilst the in vitro environment generally fails to function as a true in vivo mimic, it enables relatively inexpensive multifactorial analysis within a controlled environment. Such experimentation has been used to identify promising cartilage scaffolding material, MSC differentiation protocols, and other factors likely to contribute to successful tissue regeneration. In our laboratory we are focusing on enhancing MSC chondrogenesis, whilst preventing hypertrophy as well as retaining the valuable matrix molecules secreted by the MSC-derived chondrocyte population in the de novo tissue.

The classic MSC-to-chondrocyte differentiation platform is the aggregation of MSC into a pellet in the presence of TGFβ containing chondrogenic induction medium [60]. Typically these aggregates contain 200,000–500,000 MSC and are approximately 1–2 mm in diameter. These dimensions are large in terms of diffusion length scales, and significant gradients develop within the aggregate tissue resulting in the heterogeneous deposition of cartilage-like matrix [61, 70–72]. We reasoned that we could improve on this classic and important MSC differentiation platform by simply reducing the aggregate diameter, thereby enhancing mass transport within the de novo tissue construct. However, if the diameter of the aggregate is reduced, this will eliminate the natural gradients that produce regions of hypoxia within the

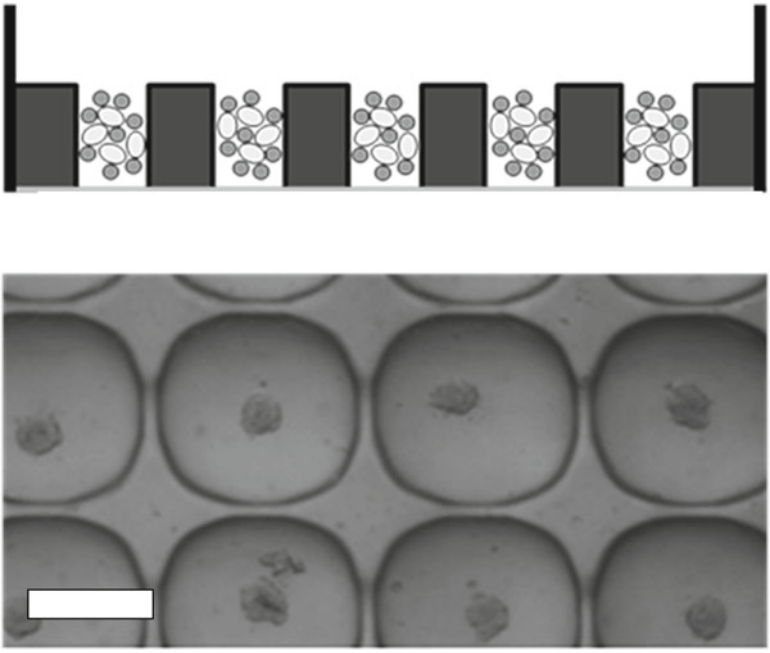

Fig. 8.1 Microwell surface for producing micropellets of cartilage. The top caption shows how aggregates are formed in microwells at the bottom of a modified tissue culture plate. The bottom caption is an image of MSC aggregates in the microwells. We produce microwell inserts where each microwell is 320×320×120 μm, with ~600 microwells/cm^2. The microwell inserts are fabricated from polydimethylsiloxane (PDMS) and surface modified to minimize cell attachment to the surface [90]. Scale bar is 200 μm

tissue construct. There is significant experimental evidence suggesting that hypoxia is an important microenvironmental feature that directly impacts on the expression of the primary chondrogenic transcription factor SOX9, and the subsequent expression of key cartilage matrix genes such as collagen II and aggrecan [73]. Thus we hypothesized that we could enhance outcomes by reducing the aggregate diameter as long as the hypoxic environment was maintained. To test this hypothesis we contrasted classical macroscopic pellet cultures containing 200,000 cells/pellet and micropellets containing ~176 cells/micropellet in both a 20% and a 2% oxygen atmosphere [74]. Figure 8.1 demonstrates conceptually how we produce micropellets in a well plate using a polydimethylsiloxane (PDMS) microwell insert that has ~600 microwells/cm^2 (each 320×320×120 μm). The bottom caption is an actual image of MSC micropellets produced using this microwell surface.

Using the micropellet strategy we were able to significantly enhance chondrogenic gene expression and matrix production [74]. More importantly, our histology results demonstrate that this method results in a more homogeneous tissue product. Much of our current research is focused on exploiting this platform to generate defined chondrocyte and osteoblast populations from MSC for use in the generation of osteochondral tissue constructs.

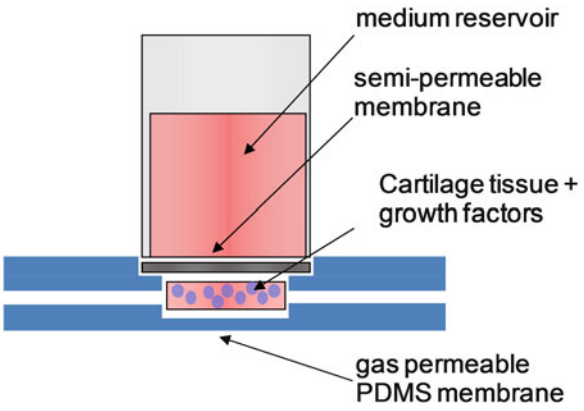

Fig. 8.2 Membrane bioreactor for the retention of cartilage matrix molecules in the developing tissue. The 10 kDa molecular weight cut-off of the semipermeable membrane prevents large molecules such as TFGβ, GAGs, and collagen from being diluted into the bulk medium. This concept may reduce culture costs and increase tissue quality (77).

Cartilage tissue is greater than 90% matrix (by volume content) [75, 76], and this matrix is key to providing functional attributes such as the capacity to withstand repetitive cyclical loading and smooth joint articulation. However, when MSC-derived cartilage constructs are cultured in vitro, 50–80% of this valuable matrix material can be lost to the bulk medium rather than being integrated into the de novo tissue [74, 77]. To overcome this loss, and to enhance the quality of the de novo cartilage tissue, our group has fabricated a membrane bioreactor that is designed to retain these valuable large matrix molecules within the developing tissue. Figure 8.2 shows how we use a semipermeable membrane with a 10 kDa molecular weight-cut-off to isolate the developing tissue within 1% of the culture volume, and from 99% of the bulk medium. Our studies indicate that this strategy is effective in increasing the cartilage matrix content by approximately 50% [77]. Further in vitro studies will enable us to identify opportunities to enhance both the quality and homogeneity of engineered cartilage constructs.

8.7 MSC in Animal Model Cartilage Repair

There are many reports of cartilage tissue repair in animal models using MSC populations. Small animal models are often used for the study of subcutaneous tissue development, and represent excellent models for inexpensive investigations prior to large animal studies. This is especially true of immunodeficient animals, which enable the in vivo study of tissues formed from human MSC populations. Joint repair in small animals, using cells derived from syngeneic animals, may be of dubious value as the joint loading in these animals is not similar to humans, and both the cells and the actual joint's capacity for spontaneous repair are significantly greater than that observed in humans. Regardless, the study of joint repair in rabbit models (for example) appears promising, with multiple studies reporting the successful

regeneration of hyaline-like cartilage using MSC in conjunction with various scaffolds [78–80]. Similarly, promising cartilage repair has been demonstrated in a large animal model (ovine) using MSC in conjunction with TGFβ-3 and a chitosan scaffold [81]. In one study, porcine MSC seeded within a collagen gel were placed into a fabricated osteochondral defect to test the repair capacity of differentiated and undifferentiated MSC populations. MSC populations not previously induced to differentiate into chondrocytes, with TGFβ, appeared to facilitate more robust cartilage repair than those that were [82]. This counterintuitive observation may indicate, as discussed in our bone repair chapter, that the factors secreted by the transplanted MSC population may make a greater contribution to tissue repair than the actual cells themselves. In such cases it is thought that MSC function in a paracrine manner, with their secretions recruiting and upregulating the endogenous repair capacity of the host cells. Whilst the precise mechanism of action is not clear in this study, there are other studies involving inflammatory joint disease which indicate that paracrine factors secreted by MSC do dampen autoimmune disease and block cartilage damage [83]. A critical observation is that the specific localization of the MSC in the target tissue does not appear to be essential, thus indicating that the mechanism of action is via cellular secretions, not through direct contribution to de novo tissue synthesis. Thus MSC may play a role in future cartilage repair strategies, where their contribution is to enhance the regenerative capacity of cells within remaining intact tissues, or of co-transplanted chondrocyte populations, through their secretion of trophic factors.

8.8 MSC in the Clinical Repair of Cartilage

Most clinical studies, and all approved cell therapies, utilize adult chondrocytes in cartilage repair applications. The ease of both harvest and ex vivo expansion of bone marrow-derived MSC makes them an appealing alternative to autologous adult chondrocytes. In addition to the technical challenge of generating a stable chondrocyte population from MSC, there is the regulatory challenge associated with the incorporation of a differentiation protocol. The introduction of growth factors and/or dexamethasone to modify the phenotype of the cell population are significant deviations from protocols used in numerous completed and on-going MSC phase I safety trials. Thus to more efficiently build on the existing body of work, only trials, the first rounds of clinical investigation into the potential use of MSC in human cartilage repair are utilizing undifferentiated MSC populations.

A few case studies, involving one to three patients, indicate clinical improvement following the treatment with MSC-based cartilage repair therapy [84–86]. A more comprehensive study, which contrasted MSC-based treatment relative to a cell-free control, reported no clinical benefit through the inclusion of MSC in their clinical repair protocol, but did note that the MSC repair tissue appeared to be superior when evaluated by arthroscopic and histological techniques [87]. A more recent study utilized a variation of ACI, to contrast the performance of chondrocytes or

MSC in cartilage defect repair [88]. Defects were capped with a lid formed from periosteum rather than the collagen MACI membrane. The study results, which involve data from 72 patients, indicate that MSC are as effective as chondrocyte in facilitating cartilage tissue repair. In recent months a case report describing a similar procedure, but this time involving the use of the collagen MACI, has been published featuring outcomes from two patients [89]. The results presented by the authors indicate significant and robust cartilage tissue repair using MSC in combination with the MACI membrane.

8.9 Conclusions

MSC have been found in most postnatal organs and tissues [44, 45], with bone marrow-derived MSC appearing to being readily capable of being differentiated into chondrocyte-like cells [47–49]. This potential, coupled with the relative ease of both bone marrow-derived MSC harvest and expansion, has motivated the development of an array of MSC-to-chondrocyte differentiation cytokine cocktails, scaffolds, and animal models in the hopes of developing effective articular cartilage repair strategies. These technologies have evolved sufficiently that modest human clinical trials have been completed, and their promising results have motivated further investigation. These studies have been simplistic in the sense that cells alone have been placed or anchored into cartilage defects, rather than the transplant of functional 3D tissues. The near future will see the execution of more thorough investigations using similar strategies, but likely involving more patients, as well potentially chondrogenically induced MSC. Simultaneously, it is likely that bioreactor and MSC differentiation technologies will continue to evolve such that functional cartilage tissue can be produced in vitro, and subsequently transplanted in vivo. It is envisioned that these two strategies for cartilage regeneration will supersede total joint replacement as the gold standard for the treatment for joint repair.

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Chapter 9
Mesenchymal Stem Cells and Haematopoietic Stem Cell Culture

Matthew M. Cook

Abstract Haematopoietic stem cell (HSC) transplantation is an established cell-based therapy for a number of haematological malignancies and immunodeficiency diseases. However, the limited number of HSC from umbilical cord blood (UCB) limits the efficacy of transplants from this source. This limitation could be overcome by expanding the HSC population prior to transplantation. Although such processes have shown little success to date, it is thought that self-renewal of HSC in vitro may be possible through replication of the environmental cues found in the bone marrow (BM) stem cell niche. It is thought that non-haematopoietic cell types residing in the putative HSC niche could provide these cues. Mesenchymal stem/stromal cells (MSC) are one such cell type found in the BM niche that provide these cues. Thus, this review will explore how MSC have been used in the ex vivo expansion of HSC.

Keywords Haematopoietic stem cells (HSC) • Ex vivo expansion • Mesenchymal stem/stromal cells (MSC) • Osteoblasts • Umbilical cord blood • Bioreactors

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9.1 Introduction

Haematopoietic stem cells (HSC) are the source of all blood and immune system cell types and continuously replenish the haematopoietic and immune systems throughout the life. HSC transplantation (HSCT) is a potentially curative therapy for patients following treatment for leukaemias, lymphoproliferative diseases, some solid tumours (including ovarian cancer and neuroblastoma) and some non-malignant disorders such as immunodeficiencies, autoimmune disorders, haemoglobinopathies and inherited diseases of metabolism [1–5]. The sources of allogeneic HSC for transplantation include bone marrow (BM), mobilised peripheral blood (mPB) or umbilical cord blood (UCB).

HSC have been used in the clinical setting for over 50 years and are the only stem cell in routine clinical use [6]. Patients undergoing HSCT get a combination of chemotherapy or chemotherapy and radiotherapy in a period known as the pre-transplant conditioning phase. The predominant aim of this phase is to destroy all malignant cells and usually lasts between 1 and 2 weeks. In the case of allogeneic HSCT, conditioning also ablates the recipient's immune system and thus decreases the chances of donor HSC rejection by host leukocytes not destroyed by the pre-transplant chemo-radiotherapy [7]. Most conditioning regimens markedly reduce host haematopoiesis and cause a rapid onset of pancytopaenia. Therefore a HSCT is required to replenish the haematopoietic system and thus prevents events due to marrow failure such as leukopaenia, anaemia and thrombocytopaenia and their consequent complications of infection and haemorrhage. The ideal donor graft for a HSCT contains a mixture of cells including committed progenitor cells that provide rapid short-term recovery of neutrophils and platelets and HSC that provide durable long-term engraftment [8].

This review will outline the shortcomings of the therapeutic use of HSC and explore some of the efforts to overcome these limitations and the potential that these hold. Specifically, it will focus on mesenchymal stem/stromal cells (MSC) and their osteoblast (Ob) progeny, which have been used in HSC expansion systems.

9.2 HSCT Limitations

The high-dose chemotherapy/total body irradiation conditioning regimens used prior to HSCT leave the patient in an immune-compromised state. This can result in pancytopaenia, including neutropenia, lymphopenia and thrombocytopenia, and render the patient susceptible to bacterial, fungal and viral infections, as well as haemorrhage [1]. The duration of these blood cell deficits can be extended if donor HSC engraftment is delayed. Delay may result from either a lack of sufficient donor HSC or, in the case of allogeneic HSCT, by graft rejection by the recipient's residual immune system. Furthermore, depending on the type of conditioning, damage to non-haematopoietic organs may also develop including renal, hepatic and gastrointestinal complications [9].

Once engraftment has been achieved, the next major complication of allogeneic HSCT is graft-versus-host disease (GVHD) which occurs when the donor graft and recipient are insufficiently tissue-matched. This occurs even when the donor and recipient are matched for the major human leukocyte antigens (HLA) but mismatched for non-HLA (or minor) histocompatibility antigens and may occur after either HLA-matched sibling transplants or matched unrelated donor transplants. GVHD is initiated by donor T-cells recognising non-shared histocompatibility antigens in the recipient as foreign and subsequently mounting a potentially fatal immune response against the host (reviewed in [10]).

UCB-derived HSC are an alternative HSC source when a sufficiently HLA-matched relative cannot be found as it has been shown that UCB-HSC elicit less frequent and severe GVHD even with less rigorous HLA-matching between donor and recipient [11–17]. However, the application of UCB is limited by the HSC dose available for transplantation. This is particularly important in adult recipients where multiple UCB units are often needed to permit a successful transplant (reviewed in [18]). The low number of HSC within UCB units causes a slow rate of blood cell recovery after the transplant, especially in adult recipients. This leads to longer periods of potentially life-threatening pancytopaenia compared to transplantation with BM or mPB [19]. UCB transplantation, however, still represents a viable alternative source of allogeneic donor HSC for patients lacking a matched related or unrelated living donor. The realisation that the limitations associated with UCB could theoretically be overcome by expanding the HSC population prior to transplantation to the patient has motivated the development of numerous expansion processes and clinical trials in the area. These ex vivo expansion techniques are directed towards both true HSC self-renewal for enhanced engraftment and production of clinical doses of committed blood progenitors and immune cells with the aim of reducing the early period of pancytopaenia occurring immediately post-transplant. Historically, two main approaches have been taken towards achieving these aims. These are HSC expansion (1) using in vitro culture systems supplemented with various combinations of haematopoietic growth factors, and (2) using a specific feeder cell monolayer to provide a supportive microenvironment. This review will focus on feeder cell-based haematopoietic expansion systems.

9.3 HSC Expansion Using MSC

Cells of the haematopoietic niche provide a multitude of signals that play a pivotal role in the regulation of HSC. They do this by providing specific colony-stimulating factors, interleukins, transmembrane proteins and cell adhesion molecules. Specific signalling molecules that have been shown to influence haematopoiesis include angiopoietin 1 (Ang1) [20], thrombopoietin (TPO) [21], stromal cell-derived factor-1 (SDF-1 or CXCL12) [22], stem cell factor (SCF; also known as Kit ligand, KL) [23, 24], osteopontin (OPN) [25], Wnts [26] and calcium ions [27]. There is also emerging evidence that the notch signalling pathway is a potentially key

component of the way niche support cells regulate HSC (reviewed in [28]). Therefore, it is logical that these cells would provide a similar environment in vitro and support the expansion of HSC [29]. Accordingly, isolated niche cells such as MSC, and their osteoblast (Ob) progeny, have been used as support/feeder layers in ex vivo HSC cultures.

The first report of co-cultures was by Dexter and colleagues in 1977 using murine cells and thus became commonly known as Dexter cultures [30, 31]. The culture method consisted of seeding whole BM (containing both stromal and haematopoietic cells) into flasks with the supplementation of 20–25 % horse serum [30, 31]. The method built upon Dexter's previous studies using thymic cells as a feeder layer [32, 33]. Although the predominant cell type produced in these cultures was granulocytes, these cells could be maintained for several months. This system was subsequently translated into the human setting (using human BM) with similar results 3 years later [34, 35]. In the following years the stromal cells themselves were investigated for their potential to supply specific haematopoietic growth factors and ability to regulate haematopoietic proliferation [36–41]. This was followed by the development of the cobblestone area forming cell (CAFC) [42] and long-term culture initiating cell (LTC-IC) [43] assays for the in vitro enumeration of HSC-like colonies on stromal monolayers.

The most frequently studied cell type used as a feeder layer for HSC expansion is the MSC. MSC may additionally be beneficial due to their immunomodulatory characteristics [44–46]. The most common source of MSC is from BM; however, they may also be effective in supporting HSC expansion when sourced from other tissues including human placenta [47], umbilical cord [48–50] and adipose tissue [51]. Recent papers have shown that many of the specific cell–cell interactions between HSC and stromal cells are critical for HSC regulation, both in vivo [52] and in vitro [53–57]. Indeed, a majority of studies have shown that cell–cell contact between HSC and MSC is essential for their ex vivo expansion [53–57]. There is also evidence that the most primitive HSC directly interact with stromal cells [54, 58]. Although MSC may provide growth factors themselves, a drawback of this technique, at least in the human setting, is that the co-cultures still require additional supplementation with growth factor cocktails [59–61].

As previously mentioned, one type of MSC progeny, namely the Ob lineage, has now been identified as key regulators of the HSC niche through the provision of signalling networks that direct cell fate. Surprisingly, the use of Ob as feeder layers for HSC growth is a relatively under-utilised concept. The first report of Ob to support HSC growth in vitro was in 1994 [62] and has only recently been revisited [63–65]. These studies show that HSC can be maintained by co-culture with Ob. However, due to the lack of definition of cells of the Ob lineage, it is likely that these supporting Ob monolayers represent a heterogeneous population of cells. This, inadvertently, may be a logical approach for ex vivo HSC culture as cells of different Ob maturation have been shown to be key components of the niche [20, 25, 66–69].

Clinical trials using co-culture expanded HSC are few in number. The proprietary Replicell technology developed by Aastrom Biosciences Inc. was shown to be feasible but not definitively effective in enhancing myeloid, erythroid and platelet

engraftment in the clinical setting [70, 71]. This system used stromal co-cultures while also providing a continuous supply of culture medium containing foetal calf serum and horse serum, along with cytokine supplementation [72]. Perhaps the most comprehensive co-culture clinical trial has recently been initiated by the commercial company Mesoblast PTL using a BM-derived mesenchymal progenitor cell (MPC) product to expand UCB (mesoblast.com). As with clinical trials using cytokine-expanded HSC, the study transfused one unmanipulated UCB unit along with one 14-day expanded unit. Expansion using this method enhanced neutrophil recovery by 14 days and the grafts were shown to elicit less GVHD compared to that of published reports using unmanipulated UCB transplants [73, 74]. While long-term follow-up results are yet to be reported, it will be interesting to see whether the therapeutic value of the expanded unit is purely for short-term myeloid support.

9.4 Perfusion HSC Co-culture

The media components that HSC are exposed to during culture can significantly alter their expansion and that of their progeny [75–78]. These components may either be cellular by-products such as lactate or endogenous factors produced by the cultured cells. In particular, differentiated cells have been shown to secrete negative regulators that can limit HSC proliferation in culture [75, 77, 79]. Furthermore, most HSC cultures are initiated with a small cell population ranging from 10^3 to 10^4 cells/ml. Over the culture period this population may expand to 5×10^5–10^6 cells/ml. In these simple static batch cultures, the many log-changes in cell number result in the medium composition changing with time and, importantly, a considerable difference in the cell micro-environment. The temporal instability of these factors make knowing the specific culture conditions of the microenvironment at any given time-point difficult. Thus, numerous strategies have been proposed to control these factors and preferentially drive the production of more primitive cells. This section will briefly discuss how some of these approaches have been applied to HSC expansion cultures.

Intermittent or continuous media exchanges have been used to tightly control the physiochemical aspects of the culture microenvironment. These allow control over the oxygen concentration, the concentration of available metabolites, the pH, the availability of exogenously supplied growth factors and the development of cell-derived signalling networks. Thus, many bioreactors have been developed that allow the constant perfusion of fresh medium for the cultured cells and removal of any waste products [70, 80–84]. The Aastrom Biosciences Inc. Replicell technology is an example of a perfusion system that uses radial diffusion to supply fresh supplements for its cultures [70–72, 85]. Other strategies such as stirred bioreactors and rotating wall bioreactors have been designed to prevent the local build-up of negative regulators (reviewed in [86]). Another approach, using a similar rationale to that of perfusion cultures, involves the physical removal of any maturing progeny from the HSC cultures. This approach has been used in both a stromal co-culture

system [61] and a growth-factor liquid expansion system [78]. In the co-culture system, mononuclear UCB cells were overlaid onto a pre-established MSC monolayer. The UCB cells were cultured for 7 days, after which the non-adherent fraction (likely to contain maturing haematopoietic cells) was transferred to a secondary liquid culture, while the original stromal co-culture (likely to contain more primitive haematopoietic cells) was supplemented with fresh media. This was repeated on day 10 of culture and both fractions were then expanded for an additional 3 days. The cultures were harvested at day 13 of culture and the adherent fraction and suspension fractions were combined and analysed. This culture method produced a 10- to 20-fold expansion of total nucleated cells, a two- to five-fold expansion of primitive progenitors [by colony-forming cell (CFC) assays] and a 16- to 37-fold expansion of $CD34^+$ cells [61]. Similarly, Madlambayan and colleagues (2005) used a 7-day liquid HSC expansion culture to show that magnetic removal of mature haematopoietic cells at day 4, along with a complete medium exchange, led to increased $CD34^+$ cells and primitive progenitors (by CFC and LTC-IC assays). Furthermore, this paper described >3.3-fold increase in mice with severe combined immune deficiency (SCID)-repopulation ability using this culture system [78]. Thus, these studies demonstrate how removal of mature haematopoietic cells from bulk cultures may be a useful technique in HSC expansion systems.

9.5 HSC 3D Co-culture

To further replicate the in vivo niche, it has been proposed that three-dimensional (3D) cultures may be beneficial to HSC growth and proliferation [86, 87]. The evidence that 3D culture results in more in vivo like behaviour of cells has been shown in numerous in vitro cell models [88–93]. This is logical since 3D culture mitigates the negative influence of artificial surfaces and promotes cell–cell interaction. The importance of 3D culture is now becoming apparent in the HSC field as it has been demonstrated that the critical niche support cells (osteoprogenitors or MSC) maintain their supportive nestin-expression when cultured in 3D spheres, but that this expression is lost when cultured in 2D on tissue culture plastic [89]. Previously, 3D HSC cultures have been attempted using materials including carbon, polyethylene terephthalate, ceramic foams and a tantalum-coated porous biomaterial [94–98]. Notably, carbon matrix and polyethylene terephthalate were able to increase UCB $CD34^+$ cell number whilst retaining SCID-repopulation ability [95, 96]. Furthermore, co-cultures of HSC and stromal cells in 3D using non-woven plastic porous carriers [99, 100] and polyethylene terephthalate woven mesh [58] have been reported. These studies show an increase in cell number and colony-forming ability. Additionally, the 3D co-culture study using polyethylene terephthalate woven mesh was able to show that the cultured haematopoietic cells retained their SCID-repopulation ability [58]. While these studies show promising results, a major limitation to 3D scaffold-based cell expansion systems is the need for mechanical or enzymatic dissociation methods that may cause cell damage. Thus, scaffold-free 3D

HSC cultures may be a logical solution to this problem. To date only a single study has reported scaffold-free HSC culture [101]. In this study, the authors established MSC spheroids by culturing them on a non-adherent surface (agarose coated 96-well plates). While this study was primarily focused on the migration of a $CD34^+$ HSC population into the MSC spheroids, the authors did report that spheroids were able to support the growth of $CD34^+$ HSC populations and showed that myeloid CFCs were maintained in 3D cultures [101]. Progression to 3D HSC culture is likely to be a critical step in artificially recreating a HSC supportive environment.

9.6 Conclusion

The extensive amount of HSC research has led to them being the best-understood stem cell population. While there are still extensive efforts to further this understanding, their potential in a clinical setting was first noted over 50 years ago and is currently the only stem cell therapy in routine clinical use. Despite the extensive and ever-evolving experience surrounding the transplantation of HSC, there are still numerous obstacles that prevent the application of this therapy from achieving its true potential. Specifically, a major limitation is the number of HSC that can be obtained from some donor tissues, namely UCB. The theory that this limitation could be overcome by using cells from the HSC niche to expand HSC populations prior to transplantation has motivated numerous laboratories to develop ex vivo expansion processes. These processes are directed towards both the expansion of HSC for true in vitro self-renewal and for the generation of mature blood cells with the aim of decreasing periods of post-conditioning pancytopaenia and obtaining durable engraftment and reconstitution. Although recent clinical trials using existing HSC expansion techniques show some promising results, extensive long-term engraftment of HSC from expanded donor material has yet to be shown. Furthermore, patients in these trials still suffered from greater than 2 weeks of neutropaenia. Thus, numerous ongoing efforts, including those from our own laboratory, are currently directed towards the generation of robust co-culture platforms to advance and improve existing HSC ex vivo expansion techniques.

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Chapter 10
Mesenchymal Stem Cells for Treatment and Prevention of Graft-Versus-Host Disease and Graft Failure After Hematopoietic Stem Cell Transplantation and Future Challenges

Olle Ringdén

Abstract Graft-versus-host disease (GVHD) is a cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). The rationale to use mesenchymal stem cells (MSCs) to prevent and treat GVHD and graft failure is due to the ability of MSCs to suppress alloantigen-induced T-cells in vitro. This effect was seen despite HLA-incompatibility between MSC and stimulatory or alloreactive cells in vitro, enabling the potential use of third party MSCs in the clinic. Because MSCs home to target organs of tissue toxicity and have low immunogenicity, they may be important in regenerative medicine, for instance in the treatment of acute GVHD. Clinically, MSCs were found to completely reverse severe acute GVHD in approximately 50% of the patients with steroid-refractory GVHD. In addition, MSCs interfere with coagulation and were found to have a positive effect on hemorrhagic cystitis and were able to stop major hemorrhages in HSCT patients. MSCs produce hematopoietic growth factors and have been used clinically to support hematopoiesis and to treat graft failure. Future related areas of research include prospective randomized clinical trials, determining optimum cell source and dose, identifying the best route of infusion and defining the appropriate number of passages for the MSCs to be used for therapeutic applications.

Keywords Mesenchymal stem cells • Graft-versus-host disease • Graft failure • Hematopoietic stem cell transplantation

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10.1 Acute Graft-Versus-Host Disease

10.1.1 Mechanism

Acute graft-versus-host disease (GVHD) is a major hazard and cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT) [1–3]. GVHD was first described in experimental animals and was called runt disease or secondary disease. Diarrhea and severe skin lesions were seen in allogeneic animals as opposed to syngeneic animals who were unaffected after radiation and marrow transplantation [3]. Alloreactive donor T-cells trigger GVHD and divide after stimulation by recipient major histocompatibility complex antigens or minor antigenic peptides, which may include epithelial cell-associated antigens. In humans, the major histocompatibility complex consists of the HLA-system which includes class I antigens, HLA-A, -B, and -C, and class II antigens which consists of HLA-DR, -DP and -DQ antigens [4, 5]. Antigen-presenting cells including dendritic cells and macrophages present the transplantation antigens to T-cells. Helper T-cells (CD4+) recognize antigens associated with HLA class II molecules [6]. The helper T-cells are stimulated by IL-1 produced by monocytes, which stimulate release of IL-2 which activates cytotoxic T-cells (CD8+ cells). Cytotoxic T-cells react with HLA class I positive targets. Natural killer cells (NK cells) and macrophages also participate in acute GVHD. Interferon-γ enhances the expression of HLA class II on macrophages and epithelial cells and further stimulates T-cell and NK cell activation. B-cells also seem to participate and to be activated during acute GVHD [7, 8]. A major histocompatibility complex disparity between recipients and donor is a major risk factor for GVHD. In humans and also in experimental animals, removal of T-cells from the graft may abolish GVHD (Fig. 10.1) [9–11].

10.1.2 Symptoms

Target organs for acute GVHD in HSCT recipients are skin, gastrointestinal tract and liver. Grading of acute GVHD is from 0 to IV [12, 13]. Grade 0 is absence of GVHD; grade I is a localized skin exanthema; grade II is a skin rash involving >50% of the body or mild-to-moderate symptoms from gut or liver; grade III is severe disease involving skin, gut and liver, and grade IV may be life threatening. During gastrointestinal GVHD, patients have diarrhea, abdominal pain and hemorrhages in severe disease. Liver GVHD is associated with elevated bilirubin and sometimes elevated liver enzymes. Patients with acute GVHD have severe immunological deficiency and frequent bacterial, fungal and viral infections, which may be lethal [12–15]. Patients with severe acute GVHD often die due to infections or severe hemorrhages.

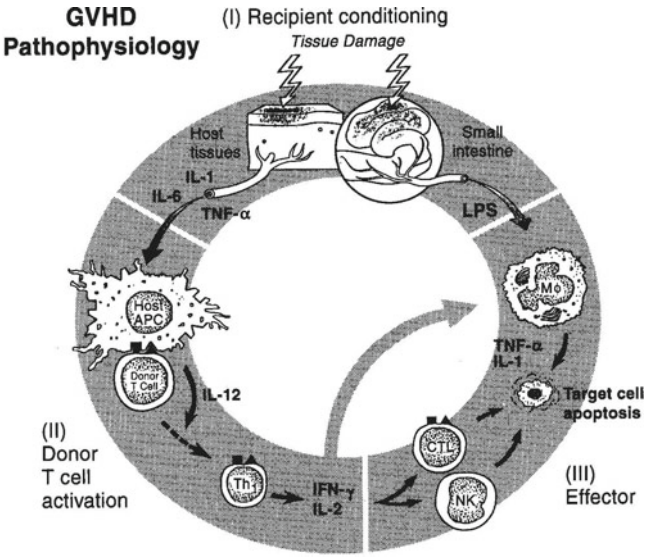

Fig. 10.1 Pathophysiology of acute graft-versus-host disease. There are three phases in the mechanisms of GVHD: (I) recipient conditioning which induce tissue damage; (II) donor T-cell activation, adhesion, co-stimulation and cytokine production, and (III) inflammation and cytolytic effectors. From Ferrara et al. Pathophysiologic mechanisms of acute graft-versus-host disease, originally published in BBMT 1999;5:347–356

10.1.3 Immunosuppression

The golden standard for immunosuppression today to prevent GVHD is cyclosporine combined with a short course of methotrexate [16–20]. Despite the use of HLA-identical sibling donors or genomically well-matched unrelated donors, acute GVHD of grades II–IV occurs in around 40% of HSCT recipients. First line therapy for acute GVHD includes steroids [21, 22]. In most patients with steroid-refractory GVHD, the outcome is dismal. A wide variety of agents used for the treatment of more severe acute GVHD include cyclosporine, tacrolimus, antithymocyte globulin (ATG), various types of monoclonal antibodies against T-cells, for instance anti-CD3 antibodies, IL-2 receptor antibodies, antibodies to tumor necrosis factor-α, recombinant human IL-1 receptor antibodies, psoralene with ultraviolet light (PUVA), thalidomide, denileukin diftitoxin, methotrexate, rapamycine, mycophenolate mofetil, pentostatin, alefacept, and more have been tried with limited success [23–31]. Because of the dismal outcome of severe acute GVHD, there is an urgent need for novel approaches.

10.2 Chronic Graft-Versus-Host Disease

10.2.1 Symptoms and Classification

Chronic GVHD occurs in between 20 and 50% of long-term survivors after HSCT [32–37]. In some rare cases, chronic GVHD may appear early after transplant, but generally it appears after day 100 and later after HSCT [1]. Chronic GVHD may develop as an extension of severe acute GVHD, de novo in patients without previous acute GVHD, or after acute GVHD has resolved [36]. Symptoms include erythema, which may be lichenoid and in severe cases sclerotic. Sicca syndrome is common and may include keratoconjunctivitis, dry mouth, and mucositis. Strictures in esophagus and vagina may appear in severe cases. When the gastrointestinal tract is involved, malabsorption and waisting are seen. Liver disease is diagnosed by elevated liver enzymes. A most devastating form is pulmonary insufficiency with obstructive bronchiolitis [38]. Patients with chronic GVHD have a prolonged immunosuppression and often suffer from gram-positive bacteria with sinuitis and pneumonia as consequences [39]. Patients with chronic GVHD are generally treated with prolonged prophylaxis with co-trimoxazole. Reactivation of herpes viruses is common. Chronic GVHD may be classified as limited or extensive [40]. Based on the judgment of the treating physician, chronic GVHD may also be classified as mild, moderate, or severe [41]. This is better correlated with the outcome among patients with chronic GVHD than categorization of limited and extensive disease [33]. To be able to measure therapeutic response, the National Institutes of Health (NIH) have developed specific criteria for grading of chronic GVHD [42].

10.2.2 Treatment

First line treatment for chronic GVHD includes steroids. A randomized study showed that steroids alone was better than combined with azathioprine [43]. Alternate-day cyclosporine and prednisone was associated with improved survival in patients with high-risk chronic GVHD [44]. For patients who do not respond to steroids combined with a calcineurine inhibitor, there is no good treatment and therefore a variety of immunosuppressive therapies have been elucidated [45]. Therapies include low-dose total body irradiation, thalidomide, mycophenolate mofetil, rapamune, extracorporeal photopheresis, anti-B-cell antibodies, and imatinib [1, 11, 36, 45–50].

10.3 Graft-Versus-Leukemia

Data from experimental animals and also the clinic have found that the immune system can control cancer [51–53]. Thus, Weiden and coworkers reported that patients who underwent HSCT for leukemia had a decreased risk of relapse if they

developed GVHD, especially chronic GVHD [53]. In addition, there is an increased risk of relapse in syngeneic compared to allogeneic HSCT [51, 54, 55]. Relapse was also increased in patients receiving T-cell depleted allografts [56, 57]. Because of the beneficial effect of GVHD on leukemic relapse, mild but not severe chronic GVHD is desired to improve long-term survival [58]. It has also been reported that cytomegalovirus (CMV) infection and/or herpes virus immunity in recipient and donor is correlated with an antileukemic effect [59–63]. The role of herpes virus infection to reduce relapse was recently confirmed by Elmaagacli et al. [64]. It seems like all therapy that decreases acute and especially chronic GVHD also increases leukemic relapse.

The graft-versus-leukemia effect is similar using HLA-identical siblings or well-matched unrelated donors. This may suggest that minor histocompatibility antigens are not targets for the antileukemic attack by donor immune T-cells [65].

10.4 Definition and Properties of Mesenchymal Stem Cells

10.4.1 Isolation and Differentiation

Mesenchymal stem cells (MSCs) may be isolated from tissues such as bone, fat, fetal tissues, cord blood, and placenta [66–70]. Friedenstein and coworkers were the first to describe MSCs [71]. MSCs are rare in the various tissues and in bone marrow they have been estimated to be 1 out of 10,000 nucleated cells. MSCs have raised interest in regenerative medicine because they can differentiate into several cells of mesenchymal cell lineages including bone, cartilage, tendon, cardiomyocytes, muscle, and fat [72–75]. MSCs have been explored for hematopoietic support because they secrete several cytokines that influence differentiation of hematopoietic stem cells [76, 77].

10.4.2 Characteristics

There is no specific marker for MSCs. However, they stain positive for CD29, CD73, CD90, CD105, and CD166 [73, 74, 78]. MSCs are negative for hematopoietic markers, CD34, CD45, and CD14. MSCs have the capacity to differentiate into bone, cartilage, and fat among other tissues after addition of exogenous factors [71, 73, 74]. MSCs do not fulfill the characteristics of true stem cells, because they cannot regenerate and maintain a whole tissue compartment. However, they are multipotent in vivo and were shown to differentiate after in utero infusion into newborn mice and to chicken embryos [79, 80].

10.5 Properties of MSCs Which Make Them Useful in Hematopoietic Stem Cell Transplantation

10.5.1 Immunogenicity and Homing

MSCs express HLA class I molecules and contain intracellular HLA class II that is expressed on the cell surface after interferon-γ stimulation [81]. MSCs seem to escape the immune system. They do not stimulate to strong proliferation of allogeneic lymphocytes. Marked lymphocyte proliferation is not seen after differentiation to bone, chondrocytes or adipocytes, not even after interferon-γ exposure. MSCs are not lysed to the same extent by cytotoxic T-cells that lyse target cells, i.e., leukocytes, from the same individual [82]. NK cells which lyse chronic myeloid leukemia target cells (K562) did not lyse MSCs. Fas ligand or co-stimulatory molecules, such as B7-1, B7-2, CD40 or CD40L, are not expressed on MSCs [83]. Human MSCs were rejected when they were injected into infarcted rat myocardium, which show that xenograft rejection occurs [84]. After injection, MSCs do not seem to be long-lived because they are difficult to detect in vivo after infusion into humans. They have been demonstrated in the circulation shortly after infusion into patients undergoing autologous stem cell transplantation for breast cancer [85]. Gene-marked MSCs were also demonstrated in the bone marrow in children with osteogenesis imperfect [86]. In experimental animals, infused MSCs first home to the lung and thereafter to the liver and spleen and are subsequently detected in small numbers in almost all organs [87, 88]. We could demonstrate MSCs at autopsy in gut, abdominal lymph node, and urinary bladder, associated with gastrointestinal GVHD and hemorrhagic cystitis [89, 90]. We could also demonstrate 7.5%-positive MSC male donor bone cells after birth in a female recipient where we injected HLA-mismatched male fetal MSCs in utero [91]. These anecdotal cases demonstrate how difficult it is to detect MSCs after infusion to patients.

10.5.2 Immunomodulation

MSCs are of great interest because they have immunomodulatory effects, making them useful in transplantation. T-cell alloreactivity induced in mixed lymphocyte cultures (MLC) are inhibited by MSCs [81, 83, 92–94]. MSCs also inhibit lymphocyte proliferation after stimulation with T-cell mitogens such as phytohemagglutinin (PHA). Lymphocyte response to PHA was inhibited in enriched CD3+, CD4+, and CD8+ T-cells [95]. An interesting finding was that MSCs profoundly and constantly inhibited MLC at high concentrations, MSC:lymphocytes (1:10), but variably inhibited and stimulated MLC when used at low concentrations (1:1,000) [94]. We could also demonstrate that MSCs induced suppression in MLC after differentiation to osteocytes, chondrocytes, and adipocytes [81]. Interferon-γ stimulation of MSCs enhanced suppression in MLC using undifferentiated or MSCs differentiated to osteocytes, chondrocytes, or fat. If MSCs were added to the MLC, specific cytotoxic T-cell lysis was inhibited, but no inhibition was seen when MSCs were added in the cytotoxic

phase of the Cr51 release [82]. NK cell-mediated lysis of K562 cells was not inhibited by MSCs. Most likely, inhibition by human alloreactivity in vitro was caused by soluble factors, because MSCs inhibited response in MLC even if they were separated by a transwell membrane. MSCs affect T-cells, B-cells, dendritic cells, and NK cells and more or less the whole immune system [96]. In vitro, MSCs were demonstrated to inhibit the maturation of monocytes, cord blood, and bone marrow-derived CD34+ cells into dendritic cells [97–100]. MSCs decreased cell-surface expression of class II molecules, CD11C, CD83, and co-stimulatory molecules and IL-12 production impairing the antigen-presenting capacity of dendritic cells. MSCs also inhibit the production of TNF-α by dendritic cells [97]. B-cell activation was reported to be inhibited by high concentrations of MSCs (1:1), but MSCs stimulated IgG secretion when used at concentrations of (1:10) [101–103]. MSCs seem to increase regulatory T-cells and activated T-cells [104]. Human leukocyte antigen-G5 secretion by MSCs seem required to induce CD4+, CD25+, FOXP3 regulatory T-cells [105].

Macrophages cocultured with MSCs showed high expression of CD206, a marker of activated macrophages [106]. Such macrophages expressed high levels of IL-10 and low levels of IL-12. Functionally, macrophages cocultured with MSCs showed a high level of "phagocytic activity."

10.5.3 Mechanism of Immunosuppression

Several factors and mechanisms are involved in MSC-mediated immune modulation. This include interferon-γ [94, 107], IL-1B [108], transforming growth factor B1 [83, 93, 109], indoleamine-2,3-deoxygenase (IDO) [110], IL6 [111], IL-10 [97, 103, 112], prostaglandin E2 [97, 103], hepatocyte growth factor [93], TNF-α [113, 114], nitric oxide [115], hemeoxygenase-1 [116], and HLA-G5 [105]. We found that MSCs inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms [117]. MSCs were found to increase IL-2 and soluble IL-2 receptors in MLC, while IL-2 and IL-2 receptors decreased after lymphocyte stimulation with PHA. IL-10 levels increased in MLC, but not in lymphocytes stimulated with PHA and cocultured with MSCs. In contrast, prostaglandin was important for the inhibition of PHA activation of T-cells, but not alloantigens. One of the soluble factors responsible for T-cell inhibition by MSCs seems to be IDO [110]. MSCs upregulate IDO, which depletes the amino acid tryptophan which induces accumulation of the metabolite kynurenine which is toxic for T-cells.

10.6 Safety of MSC Infusion

MSCs secrete cytokines important for hematopoietic stem cell differentiation, and therefore they have been used to support hematopoiesis [76]. MSCs form clusters with megakaryocytes and can expand colony-forming units from CD34+ marrow cells in bone marrow cultures [118]. MSCs from human fetuses or adults promoted

engraftment of immunodeficient mice and fetal sheep [119, 120]. MSCs were also co-transplanted with autologous or allogeneic hematopoietic stem cells to promote engraftment in pilot studies [85, 121]. These studies showed that it was safe to infuse MSCs with no acute side effects. We later found that MSCs, although they are susceptible to CMV and herpes simplex virus type 1, viral DNA from the most common herpes viruses could not be detected in MSCs from healthy seropositive individuals [122]. Thus, it seems unlikely that MSCs will transfer herpes viruses from donor to the recipient.

10.7 MSCs for Acute Graft-Versus-Host Disease

Skin allografts are highly immunogenic. The finding by Bartholomew and coworkers that infusion of MSCs could prolong skin allograft survival in a baboon model inspired me to use MSCs for severe acute GVHD [92]. A 6-year-old boy with acute lymphoblastic leukemia had undergone HSCT from an unrelated donor. He initially had GVHD of the skin that responded to steroids, but GVHD recurred in the gastrointestinal tract and the liver with voluminous hemorrhagic diarrhea and highly elevated bilirubin. The patient progressed in his GVHD despite treatment with cyclosporine, high-dose prednisolone, repeated pulses with methylprednisolone, extracorporeal PUVA, and several infusions of infliximab and daclizumab [123]. Bone marrow from his HLA-haploidentical mother and 2×10^6 MSCs/kg were infused resulting in the normalization of stool and bilirubin within a week of MSC infusion. A subsequent test revealed that he had minimal residual disease of acute lymphoblastic leukemia, and therefore it was decided to discontinue cyclosporine. Following this, GVHD reappeared with voluminous diarrhea and bilirubin increased to 350 mmol/L. Fortunately, 30×10^6 MSCs from his mother were stored frozen in liquid nitrogen. Subsequently, 1×10^6 MSCs/kg were infused and the patient again had a complete response, although this time it took a longer time before bilirubin and stool normalized. Following this dramatic case, seven additional patients were included in this initial compassionate use study [89]. In the first series, we saw dramatic effects in some patients, whereas other patients did not respond at all (Table 10.1). Acute GVHD disappeared completely in six of eight patients. One of these patients developed CMV gastroenteritis and died. Resolution of GVHD was seen in gastrointestinal tract, liver, and skin. Two patients died without any response after MSC treatment. Five patients were alive up to 3 years after transplantation. The eight patients treated with MSCs for gastrointestinal therapy-resistant grades III and IV acute GVHD had a significantly better survival, compared to 16 similar patients, also with biopsy-proven severe gastrointestinal acute GVHD, who were not treated with MSCs. This study prompted the initiation of a larger European phase II study in patients with therapy-resistant acute GVHD treated in five centers [124]. In this study, 55 patients were treated with MSCs at a dose of median 1.4 (range 0.4–9) $\times 10^6$ MSCs/kg. Donors were HLA-identical siblings ($n = 5$), HLA-haploidentical donors ($n = 18$), and unrelated HLA-mismatched

Table 10.1 MSC for treatment of acute GVHD according to published studies

							Outcome			
No. of patients	Pediatrics/ adults	Age, median (range)	Source of MSC	Expansion medium	MSC dose × 10^6/kg	Doses	CR	PR	NR	References
1	1/1	9	BM	FCS	1–2	2	1	–	–	[123]
8	2/6	43	BM	FCS	0.7–9.0	1–2	6	–	2	[89]
6	–/6	40 (22–49)	Adipose	FCS	1–2	1–2	5	–	1	[125]
2	2/–	12, 14	Adipose	FCS	1–2	1–2	2	–	–	[128]
3	–/3	31	BM	FCS	0.9–1.3	1	2	–	1	[127]
55	25/30	22	BM	FCS	0.4–9.0	1–5	30	9	16	[124]
2	2–	4, 14	BM	FCS	0.4–3.0	1	2			
13	–/13	58	BM	Plt-L	0.6–1.5	1–5	1	1	11	[126]
31	–/31	52 (34–67)	BM	FCS	2–8	2	24	5	2	[128]
2	–/2	38	BM	FCS	1.0	1	–	–	2	[132]
12	12/–	0.4–15	BM	FCS	2–8	2–21	7	5	–	[136]
1	–/1	18	BM	FCS	2	2	–	1	–	[130]
3	–/3	(39–64)	BM	Donor serum	0.5	1 i.a.	–	2	1	[138]
9	9/–	10 (4–15)	BM	Plt-L	0.7–2.8	1–5	3	2	4	[129]
1	–/1	42	BM	FCS	0.6–0.9			1		[176]
2	2/–	12	BM	Plt-L	0.9, 2	1	2	–	–	[133]
10	–/10	37 (21–62)	BM	Human serum	0.6–2.9	1–4	1	6	3	[135]
2	2/–	4, 6	UC	FCS	3.3–8.0	1–3	2	–	–	[134]

CR complete response, *PR* partial response, *NR* nonresponse, *BM* bone marrow, *FCS* fetal calf serum, *Plt-L* platelet-lysate medium, *UB* umbilical cord-derived MSCs

donors ($n=69$). The patients received from 1 to 5 infusions of MSCs. Complete response to MSC infusion was seen in 30/55 (55%) and partial response was seen in 9. Children seemed to have a better response (68%) as opposed to 43% in adults ($p=0.07$). HLA-compatibility between the MSC donor and the recipient had no impact on response to therapy. Two-year survival in the complete responders was 52%, as opposed to 16% for partial and nonresponders ($p=0.018$).

Fang et al. treated six patients with a dose of 1×10^6 MSCs/kg of adipose tissue-derived MSCs for steroid refractory acute GVHD (Table 10.1) [125]. In accordance with all other reports, this group saw no side effects. HLA-haploidentical MSCs were given to two patients and third party MSCs were given to four. A complete response was seen in five patients. The resultant nonresponder died from multi-organ failure. One responder had leukemic relapse and subsequently died. Von Bonin and coworkers used platelet lysate medium expanded MSCs for treatment of steroid-refractory acute GVHD [126]. Among 13 adult patients, 2 (15%) had a complete response and some response was seen in 5/11 of the remaining patients after receiving additional immunosuppressive therapy and further MSC infusions.

Three patients were treated with MSCs at a dose ranging from 0.92 to 1.34×10^6 MSCs/kg [127]. Two patients responded and one patient died 12 days after MSC infusion. In a randomized study, patients with grades II–IV acute GVHD were randomized to receive two treatments of MSCs (Prochymal®) in a dose of either 2 or 8×10^6 MSCs/kg [128]. Only adult patients were included in the study and median age was 52 years. Among the 32 patients, 21 had grade II, 8 had grade III, and 2 had grade IV acute GVHD. A complete response was seen in 77% with an initial response to MSC therapy of 94%. The low and the high MSC dose groups did not differ in their response rates. Lucchini and coworkers used platelet-lysate-expanded MSCs for children with severe steroid-refractory acute or chronic GVHD [129]. The children were given a median dose of 1.2×10^6 MSCs/kg. Among nine children with acute GVHD, three had a complete response, two a partial response, and four children did not respond at all (Table 10.1).

Several small studies have reported on the use of MSCs for acute GVHD, often steroid-resistant, with an overall response in a little more than half of the patients [125, 130–134]. Perez-Simon and coworkers reported on ten adult patients treated with MSCs derived from bone marrow and expanded in autologous serum [135]. Three patients had grade II acute GVHD and seven had grades III and IV. One patient had a complete response, six had partial responses, and three patients did not respond at all. In four patients, GVHD recurred between 2 and 5 months after MSC infusion. The patient with a complete response had grades III and IV acute GVHD of the gastrointestinal tract and is the only patient alive.

Prasad and coworkers also reported the use of commercial MSCs (Prochymal®) in 12 children [136]. Ages were from 0.4 to 15 years of age. This pediatric cohort was treated for therapy-resistant grades III and IV acute GVHD. The dose was 8×10^6 MSCs/kg in two patients and 2×10^6 MSCs/kg in ten children. MSC infusion was given twice a week for 4 weeks. Complete response was seen in seven children (58%), partial response in two (17%), and mixed responses were recorded in three (25%) of the children. After treatment with MSCs, 100-day survival was 58%.

In nine (75%) of the children, complete resolution of gastrointestinal GVHD was recorded [136].

Osiris Therapeutics, Inc. has performed a double-blind placebo-controlled phase III study using Prochymal for severe acute GVHD. Patients were given MSCs at a dose of 8×10^6 cells/kg twice, or placebo [137]. Among 192 patients randomized in the trial, the primary endpoint, which was a complete response at 28 days, was 45% in the MSC group and 46% in the placebo group. Thus, the primary endpoints were the same in the Prochymal and the placebo group. However, response of liver GVHD was 76% in the MSC group as opposed to 47% in the placebo group ($p = 0.026$). Complete response of gastrointestinal GVHD was 88% in the Prochymal group as opposed to 64% in the patients receiving placebo ($p = 0.018$). Intra-arterial infusion of MSCs to mesenteria was tried in three patients with steroid-refractory acute GVHD of the GI tract, but this failed [138]. Among the published patients treated with MSCs for severe acute GVHD, a complete response was seen in 52% (79/151), a partial response in 21%, and no response in 27% (Table 10.1).

Long-term reports are sparse, which is due to the limited experience in most centers. However, von Bahr and coworkers from our team reported on long-term follow-up in 31 patients treated with MSCs for acute GVHD ($n = 23$) or hemorrhagic cystitis ($n = 8$) treated between 2002 and 2007 [139]. Two years after GVHD, survival was 61%, but thereafter survival declined substantially. There was a high rate of death from infection, especially invasive fungal infection, among the patients treated with MSCs for severe acute GVHD. An interesting finding was that patients who received MSCs from passage 1 or 2 had a 1-year survival of 75%, as opposed to 21% among patients receiving MSCs from passage 3–4 ($p < 0.01$). This was seen regardless of age. Among the adults receiving early passage MSCs, 1-year survival was 50% as opposed to 8% among adults receiving later passage MSCs ($p = 0.02$). From the experience so far, MSC therapy is promising for moderate-to-severe acute GVHD, although many patients do not respond and long-term survival is not so good. So far, it seems that early treatment seems warranted and one may try for early passage MSCs.

10.8 MSCs for Chronic GVHD

The first patient treated for chronic GVHD had a normalization of slightly elevated liver enzymes, but the lichenoid skin changes did not improve [89]. This patient was judged as nonresponder. Subsequently, four patients with sclerodermatous chronic GVHD were treated with MSC infusions (Table 10.2) [140]. After MSC infusion, a gradual improvement was noted in all four patients. After MSC infusion in these patients, T helper cells 1 increased and T helper cells 2 decreased. This was measured as a gradual decrease in IL-10 and IL-4 producing cells, whereas IL-2 and interferon-γ producing cells gradually increased. This was consistent in all four patients. None of the four patients experienced recurrence of leukemia or myeloma.

There are some additional anecdotal reports of using MSCs for chronic GVHD from different centers (Table 10.2). Thus, Müller and coworkers reported three

Table 10.2 Published studies using MSCs for chronic GVHD

							Outcome			
No. of patients	Pediatrics/ adults	Age median (range)	Source of MSCs	Expansion medium	MSC dose × 10^6/ kg	Doses	CR	PR	NR	References
1	–/1	27	BM	FCS	0.6		–	1	–	[89]
1	1/–		Adipose	FCS			1	–	–	[125]
3	3/–	15 (15–17)	BM	FCS	0.4–3	1–3	–	1	2	[131]
12	–/–	–	–	–	1–2.1	1–3	3	6	3	[143]
4	–/4	42 (38–43)	BM	Plt-L	10–20	4–8 i.b.	4	–	–	[140]
19	–/19	28 (18–39)	BM	FCS	0.2–1.4	1–5	4	10	5	[141]
4	4/–	8 (5–15)	BM	Plt-L	0.7–2.8	1–5		3	1	[129]
1	/1	46	BM	FCS	0.5–1	3		1		[130]
8	–/8	41 (21–66)	BM	Human serum	0.3–3.7	1–3	1	3	4	[135]

CR complete response, *PR* partial response, *NR* nonresponse, *BM* bone marrow, *FCS* fetal calf serum, *Plt-L* platelet-lysate medium

patients with chronic GVHD who were treated with MSCs and one of them had slight improvement [131]. Lucchini and coworkers used platelet-lysate expanded MSCs in four children with chronic GVHD. Median dose was 1.2×10^6 MSCs/kg given as a single dose ranging from 1 to 10 months after HSCT. Five children received one dose and one child received four doses of MSCs at 0.7×10^6 MSCs/kg. Transient benefits were noted. One child had a complete response that subsequently reflaired and two additional patients had partial responses [129].

Subsequently, Weng and colleagues reported 19 patients with refractory chronic GVHD who were treated with a median MSC dose of 0.6×10^6 cells/kg [141]. The response rate was graded according to the NIH criteria [42]. A response was seen in 14 of the 19 patients (74%). In five patients, immunosuppression given for chronic GVHD could be discontinued within a median of 324 days after treatment with MSCs. There were no adverse events after infusion of MSCs in any of these patients. Among the patients with chronic GVHD of the skin, the response rate was 78%. Three patients had sclerodermatous chronic GVHD and one of them had a partial response. Cumulative responses were between 90 and 100% in oral mucosa, gastrointestinal tract, and liver. One patient was treated for obstructive bronchiolitis but did not respond and died of invasive fungal infection. Weng and coworkers also reported that clinical improvement of chronic GVHD was accompanied by an increased ratio of CD5+ CD19+/CD5− CD19+ B-cells and CD8+ CD28−/CD8+ CD28+ T-cells. They suggested that balances of T- and B-cells may be involved in the pathogenesis of chronic GVHD. It is well known that B-cells are involved in the pathogenesis of chronic GVHD [142]. Among these 19 patients treated with MSCs for chronic GVHD, 2-year survival was 78%. A recent study included eight adult patients treated with MSCs at doses ranging from 0.2 to 1.2×10^6 MSCs/kg [135]. Five patients received one dose, two patients received two doses, two received three doses, and one received four doses. A complete response was noted in one patient with chronic GVHD with sicca in the mouth and slight thrombocytopenia. Partial responses were seen in three patients, out of which one had severe chronic GVHD of the gut, one had severe chronic GVHD with sicca in mouth and eyes, exanthema and gut disease. A third patient had severe chronic GVHD with gastrointestinal and musculoskeletal involvement. Four patients did not respond and two died from GVHD and toxicoderma, respectively. Zhang et al. treated 12 patients with chronic GVHD and saw complete responses in three patients (Table 10.2) [143]. Complete resolution was seen in the skin (3/12), lung (1/3), joints (1/5), liver (3/10), oral cavity (4/12), and eye (2/7). The three complete responders could discontinue all immunosuppressive drugs.

Overall complete responders seemed lower in patients with chronic GVHD, 25% (13/53), compared to acute GVHD (Table 10.2). However, overall responses, including complete and partial responses (45%), were similar for chronic (70%) and acute GVHD (73%). So far, the experience of using MSCs for chronic GVHD is much more limited and only the treatment of 53 patients have been reported.

It is certainly logical to treat chronic GVHD as well as acute GVHD [144]. Chronic GVHD resembles autoimmune disorders and MSCs were shown to be effective in autoimmune disease in animal models [140, 145].

10.9 MSCs to Enhance Engraftment and Prevent GVHD and Graft Failure

10.9.1 Prevention of GVHD

In a murine model, co-transplantation of MSCs prevented the development of lethal GVHD [146]. Co-transplantation of MSCs in haploidentical transplant was first reported by Lee et al. who reported engraftment and no GVHD [147]. The largest study was performed by Lazarus et al. with co-transplantation of HLA-identical sibling bone marrow and HLA-identical sibling MSCs in 46 patients [121]. Neutrophil engraftment was achieved on median day 14 and platelet engraftment on day 20. No patient had graft failure and seven patients (15%) had grades III and IV acute GVHD. Chronic GVHD was diagnosed in 22 patients. There were no side effects of infusion of allogeneic MSCs. Whether MSCs reduced GVHD or not is not possible to evaluate because there was no control group. Our group performed co-transplantation of MSCs together with HSCT to three patients with previous graft failure and four patients were included in a pilot study [148]. We observed rapid engraftment and 100% donor chimerism. One patient with aplastic anemia had a graft failure and severe Henoch-Schönlein purpura, which resolved after retransplantation and co-infusion of MSCs. A small randomized study including 25 patients showed that HSCT co-transplantation with MSCs, while decreasing acute GVHD, increased the probability of relapse [149]. The infused MSC dose was median 0.3×10^6 MSCs/kg and neutrophil and platelet engraftment was similar in the MSC and the control groups. Baron and colleagues performed co-transplantation with MSCs in patients receiving HSCT following non-myeloablative conditioning [150]. They saw no death from GVHD and low relapse incidence in recipients of HLA-mismatched grafts. Gonzalo-Paganzo et al. combined cord blood transplants with peripheral blood stem cells and MSCs from the same donor [151]. Severity of acute GVHD and engraftment were similar to control patients. So far, there are too few patients included in the trials including MSCs for co-transplantation with the hematopoietic graft to be able to draw any conclusion if MSCs enhance engraftment or prevent GVHD. A prospective placebo-controlled double-blind randomized study with hematopoietic grafts with or without MSCs is ongoing at our center.

10.9.2 Graft Failure

Primary and secondary graft failure occurring after HSCT may be induced by residual host T-cells [152]. However, NK cells, antibodies, septicemia, viral infections such as CMV and parvo virus, drug toxicity, and a compromised microenvironment may also induce graft failure. In more recent years, graft failure has increased after HSCT because cord blood transplants, haploidentical transplants, and reduced-intensity

conditioning are used more frequently [153]. Therapies for graft failure include granulocyte colony-stimulating factor, donor lymphocyte infusion, boost of hematopoietic stem cells, and re-transplantation [152, 154]. Graft failure is not only associated with mortality, but is also expensive [155]. Ball and coworkers used MSCs for transplantation in recipients of haploidentical grafts [156]. Engraftment was not enhanced, but no patient had graft failure as opposed to 10% among retrospective control patients. Meuleman and colleagues used infusion of MSCs, 1×10^6 cells/kg, for the treatment of threatening graft failure in six patients. After stimulation with granulocyte colony-stimulating factor, none of the patients had a neutrophil count above 1×10^9/L. Two patients showed rapid hematopoietic recovery after MSC infusion. An increase in neutrophils and reticulocytes occurred after 12 and 21 days after MSC infusion in the two patients, respectively. This study suggests that MSCs should be used as first-line treatment for graft failure after HSCT, because they are safe and cheap [157, 158].

10.10 Toxicity and Novel Therapies for Mesenchymal Stem Cells in Hematopoietic Stem Cell Transplantation

10.10.1 Homing

MSCs that were marked with indium^{111} and injected i.v. in rats first showed high activity in the lung and thereafter in the liver [88]. Human MSCs showed slight specific differentiation in multiple tissues after intrauterine transplantation into fetal sheep [79, 159]. In mice with osteogenesis imperfect, normal MSCs were infused and engrafted and normal collagen was demonstrated [160].

10.10.2 MSCs as Enzyme Replacement

Hurler's disease is caused by deficiency of α-L-iduronidase. This disorder may be prevented by HSCT, if performed before 2 years of age [161–163]. In metachromatic leukodystrophy, arylsulfatase-A deficiency is the etiology. Deficiency of these enzymes leads to failure to hydrolyze certain substrates, which leads to accumulation and organ dysfunction, the most disturbing being mental retardation. MSCs express high levels of α-L-iduronidase and arylsulfatase-A [164].

Patients who had undergone HSCT for Hurler's disease and metachromatic leukodystrophy were treated with MSCs given i.v. to enhance enzyme production in patients with symptomatic disease after HSCT [49]. Among the patients with metachromatic leukodystrophy, four of five had improvement in nerve conduction velocity. MSCs have also been used to treat osteogenesis imperfecta, a bone disorder with spontaneous fractures [86, 165]. Five patients with osteogenesis imperfecta

diarrhea and abdominal cramps, where computer tomography showed free-air surrounding the small intestine. The patient had first been treated with 0.06×10^6 MSCs/kg with no effect. After infusion of 0.91×10^6 MSCs/kg, the abdominal-free air disappeared and blood in stools decreased. He was improved, but abdominal pain did not completely disappear. Therefore, infliximab was given. The patients was discharged, but later died of septic shock.

Müller and coworkers reported on a 14-year-old girl who underwent HSCT for T-cell acute lymphoblastic leukemia (T-ALL) with a graft from her father. She suffered from three-lineage failure and severe hemophagocytosis. She was initially treated with steroid pulse therapy and VP-16. Since there was no improvement, she was given three fractions of MSCs from the father, in total 0.5×10^6 MSC/kg. Bone marrow aspirate showed a decrease of hemophagocytosis, suggesting that the microenvironment had improved. Platelet counts started to rise, but there was no effect on leukocytes and reticulocytes. She received a bone marrow boost from her father. After this, she had three lineage hematopoietic reconstitution and is alive after more than 2 years [131]. Our group has also confirmed that MSCs can improve hemophagocytosis (Le Blanc et al., Unpublished observations).

10.11 Alternate Sources of Stromal Cells

10.11.1 Alternate Culture Conditions and Cell Sources

MSCs from bone marrow, cultured and expanded in the presence of fetal calf serum, have been the golden standard for clinical use of MSCs with well-defined surface and differentiation markers [177]. To avoid animal products in the culture system, the use of platelet lysate expanded bone marrow MSCs has been employed [126, 129, 140]. Although expansion is poorer with human serum than fetal calf serum, human serum has also been used to expand bone marrow-derived MSCs [135, 138]. As an alternative to bone marrow, adipose has been used whereby leftovers from plastic surgery has been the source of these third party MSCs [125, 178]. The umbilical cord has also been used clinically as a source of MSCs [134]. Because bone marrow harvesting is an invasive procedure and results in significant discomfort for the donor, alternate MSCs sources are being sought out. An alternative may be MSCs from placenta, which is readily available and normally is disposed after delivery of a baby [179]. The placenta has tissue which protects the fetus from being rejected by the HLA-haploidentical mother. Human placenta-derived multipotent cells have multilineage differentiation potential and strong immunosuppressive properties [180]. Placenta-derived MSCs were demonstrated to inhibit MLC and mitogen-induced CD4 and CD8 lymphocyte proliferation. These cells express IDO and are positive for intracellular HLA-G. Lymphocyte proliferation was restored after addition of neutralizing antibody to IL-10 and TGF-β. We also demonstrated that stromal cells from fetal membrane suppressed MLC [181]. Stromal cells from fetal membrane had stronger suppression in MLC

compared to stromal cells isolated from umbilical cord, placental villi, and bone marrow. Stromal cells from amnion, cord, and placenta were negative for hematopoietic lineage markers, but were positive for bone marrow-derived MSC markers such as CD29, CD44, CD73, and CD105. The cells expressed HLA class I, but not class II. Stromal cells from placenta expressed high levels of the adhesion molecules CD49D and CD54. Fetal membrane-derived stromal cells had no effect on IL-17 production from MLC, in contrast to stromal cells from cord and placenta, which induced IL-17 secretion.

10.11.2 Fibroblasts

It has long been known that fibroblasts have immunomodulatory effects [182–184]. Therefore, it has been suggested that fibroblasts which can be grown from a single punch biopsy may be used as an alternative to bone marrow aspirated MSCs [185]. It was demonstrated that skin fibroblasts inhibited MLC through soluble factors, dependent on interferon-γ from activated T-cells. Interferon-γ induces IDO which is at least partly responsible for MSC suppression of T-cell proliferation. The skin explant model is an in vitro model of human GVHD. For a period of 72 h, a skin fragment is exposed to allogeneic T-cells previously sensitized with dendritic cells from the skin donor. Pathological damage was ameliorated when T-cells were sensitized in the presence of fibroblasts or MSCs. “There is evidence that MSCs and fibroblasts share much more in common than previously recognized” [186]. That said, it remains to be shown whether fibroblasts will have similar in vivo effects as has been demonstrated with MSCs.

10.12 Do MSCs Increase the Risk of Invasive Fungal Infection?

A long-term follow-up of patients treated with MSCs for steroid-resistant acute GVHD showed that among the responders there was a high incidence of invasive fungal infection [139]. Although there is a high incidence of invasive fungal infections in patients with severe acute GVHD regardless of treatment with MSCs, this observation should be given some consideration. One of the immunosuppressive mechanisms by MSCs is the induction of IDO, which depletes the amino acid tryptophan. Tryptophan induces accumulation of kynurenine, which decreases T-cell cytotoxicity [110]. Studies support the crucial role of IDO in limiting the inflammatory response to fungus [187]. By inducing regulatory T-cells and inhibit Th17, IDO and kynurenine pivotally contribute to provide the host with immune mechanisms to protect against fungi [188, 189]. Based on these studies, IDO induction by MSCs would be expected to protect against inflammation by fungi. Despite this, patients treated with MSCs with complete response of GVHD often die from

fungal infection. I therefore would like to speculate that during fungal infection and treatment with MSCs, IDO induction by MSCs results in an over-activation of the inflammatory response, resulting in invasive fungal infection. Patients with severe acute GVHD are especially vulnerable, because of tissue damage and the possibility for fungi to invade into the blood stream from the gastrointestinal tract. Patients with severe acute GVHD, apart from treatment with heavy immunosuppression, have received broad spectrum antibiotics, paving the way for fungal colonization. Therefore, patients with acute GVHD and also those treated with MSCs who recover from GVHD should be given prolonged prophylaxis with antifungal drugs. Coverage for candida species as well as aspergillosis is needed. Liposomal amphotericin B (AmBisome) has such effects, but is not ideal because it needs to be given i.v. [190]. Voriconazole, which is recommended for prevention of aspergillosis, may induce liver toxicity in patients treated with calcineurin inhibitors. Therefore, other drugs should be explored for prophylaxis.

10.13 Discussion

MSCs have generated considerable interest for the treatment of steroid-refractory acute GVHD (Table 10.1). There are several small reports including 1 with up to 55 patients. An overall complete response rate of 52% was achieved. Despite complete response and disappearance of all symptoms of acute GVHD, there is a high mortality in these patients, mainly due to infectious complications [124]. Patients with acute GVHD are severely immunosuppressed, not only because of the immunosuppressive treatment, but also due to the fact that the lymphoid system is a target organ for GVHD. Patients with GVHD are severely immunocompromised [15]. Death in GVHD patients is often due to infections and there is an increased risk of invasive fungal infection [14, 191, 192]. Our group has reported that MSCs decreased the proliferative responses to different herpes viruses in vitro [122]. We also demonstrated that MSCs did not affect interferon-γ production from Epstein–Barr virus or CMV specific cytotoxic T-cells in vitro [193]. This is in contrast to allogeneic cytotoxic T-cells, which are inhibited by MSCs [82, 193]. MSCs had no effect on the expansion of Epstein–Barr virus and CMV pentamer-specific T-cells. It was also demonstrated in two patients who were treated with MSCs for steroid-resistant acute GVHD that the number of CMV cytotoxic T-cells increased after infusion of MSCs. Therefore, it seems unlikely that infusion of MSCs increases the risk of viral infections in treated patients.

Meisel and coworkers also found that after stimulation with inflammatory cytokines that human MSCs, as opposed to those from mice, inhibit broad-spectrum antimicrobial effector function directed against a range of clinically relevant bacteria, including protosoal parasites and viruses [194]. IDO was identified as the underlying molecular mechanism and according to this study, MSCs may inhibit infections.

So far there are 151 patients published in the literature who have been treated with MSCs for acute GVHD (Table 10.1). In addition, there are 163 patients reported

to be treated with prochymal and reported in an abstract [137]. Among those 314 patients, there is no single report of any adverse event during infusion of MSCs. The reason for this is probably due to the low immunogenicity of MSCs [81]. Among the children in the Osiris study, 34 out of 53 (64%) had a complete response as opposed to 46 among 100 adults (46%). This is in line with the findings of the European Group for Blood and Marrow Transplantation Consortium Report, where children had a complete response rate of 68% as opposed to 43% in the adults ($p=0.07$) [124]. From the available data, it is not possible to evaluate if any specific source of MSCs or any specific expansion medium is superior (Table 10.1). It doesn't seem that HLA compatibility between donor and recipient is important for response of GVHD [124]. It seems like early treatment is better than to wait for steroid-refractory acute GVHD [128]. Early treatment with MSCs was reported by Bernardo et al. [195]. In this study, cord blood transplants were co-infused with MSCs to enhance engraftment and hopefully decrease acute GVHD. In the case of grade II acute GVHD, the patients were treated with MSCs. Compared to retrospective controls, co-infusion did not enhance engraftment or prevent graft failure. However, combined with early treatment, no patients in the MSC group died from acute GVHD and no patients developed grades III and IV acute GVHD, as opposed to 26% in the retrospective controls ($p=0.05$).

Although more than 300 patients are reported to be treated with MSCs for acute GVHD, half of them are included in pilot studies. Half of them were included in the randomized study with prochymal. However, the primary endpoint response at 28 days was the same in the prochymal and the placebo arm [137]. However, improved outcome was seen in patients treated for GVHD in the gastrointestinal tract and the liver. A prospective randomized placebo-controlled trial using MSCs for steroid-resistant acute GVHD was started in Europe. However, due to difficulties with regulatory authorities, this intended multicenter trial has only recruited patients at our center. So far, only 23 patients have been included and there was a need for 90 patients in the study to be able to address the primary endpoint, complete response of acute GVHD with sufficient statistical power. Because there are few patients with life-threatening acute GVHD each year in every center, multicenter trials are necessary to evaluate the efficacy of MSCs.

There are now alternate sources than fetal calf serum expanded bone marrow-derived MSCs, such as platelet lysate expanded MSCs, MSCs from adipose tissue, umbilical cord, fetal membrane-derived stromal cells, amniocytes, fibroblasts, and more. To be able to evaluate if any of those cells not only are superior by suppressing MLC in vitro, but also are effective in vivo, the inclusion of hundreds of patients are needed. To be able to achieve this, prospective randomized studies are urgently needed. Other immunosuppressive drugs used for steroid-refractory acute GVHD, such as denileukin diftitoxin, pentostatin, mycophenolate mofetil, sirolimus, extracorporeal PUVA, and alefacept, have shown similar response rates as MSCs [196–201]. Therefore, MSCs may not be compared to placebo, but to the most effective of these drugs. Such comparisons may require even more patients. Other concepts of treating acute GVHD include imatinib, regulatory T-cells, and antiangiogenetic factors [202]. Such treatment may be used alone or combined with MSCs. Despite a decade of research in the field, we still don't know which is the optimal MSC

source or optimal dose. Osiris Therapeutics, Inc. have used a dosing of $2–8 \times 10^6$ MSCs/kg, but responses using academically cultured MSCs have shown efficacy even with doses below 1×10^6 MSCs/kg [124].

It has been suggested that there is a synergistic effect between cyclosporine and MSCs [203]. Withdrawal of cyclosporine in one patient who had responded to MSCs also resulted in a dramatic and immediate recurrence of acute GVHD grade IV [123]. In vitro studies have shown that some immunosuppressive drugs can potentiate the inhibition of alloreactivity by MSCs [204]. Despite all of these findings, additional research is needed.

10.14 Future Challenges

Which effects of MSCs are most important for the treatment and cure of GVHD? Is the immunosuppression by MSCs at the site of injury most important, or is wound healing the major effect? It is likely that both effects are required. Is the effect of MSCs direct at the site of injury, or is it due to MSCs first going to the spleen and that CD11b monocytes are subsequently responsible for wound healing [205]? An interesting finding was that long-term survival was better when early passage MSCs were used [139]. Of course, these results need to be confirmed by other groups. Much more research is needed in this field, including defining the best source of MSCs, the optimum cell dose, the optimum number and intervals of infusions, choosing autologous versus allogeneic or third party MSCs, and to confirm which is the optimal passage of MSCs. MSCs are attractive because they are safe and induce little, if any, toxicity. This is in contrast to immunosuppressive therapy, which have severe side effects, not only with regard to overall immunosuppression and an increased risk of infectious complications, but also due to several side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, and allergic reactions [206]. It is unclear if MSCs pave the way for invasive fungal infection or not. Experimental studies and randomized studies may solve this issue.

Many important mechanistic questions arise regarding MSCs. Regarding homing to the site of tissue damage, it is important to find out which molecules and receptors are crucial. The different sources of mesenchymal stromal cells may have differential homing to various tissues in the body. There are probably several mechanisms of action. It is important to find out how this can be optimally used in GVHD. After infusion into the body, the MSCs do not seem to be long-lived. We do not know where they eventually end up.

To conclude, we can say that infusion of MSCs and probably other stromal cells appears to be safe with no early or late toxic side effects observed. MSCs seem to be an effective treatment for acute and chronic GVHD with dramatic effects in some patients, whereas others do not respond at all. A major challenge is to find out the reason for this. MSCs may also be used to enhance engraftment and prevent rejection. Regarding graft failure, MSCs may be used as a first line treatment, because they are safe and relatively cost effective. Even if a lot of research is ongoing in the field, much more is needed to move this exciting field forward.

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who underwent HSCT had donor osteoblast engraftment, new dense bone, increased total bone mineral content, and improved growth velocity. Reduced frequency of bone fracture was also reported. Six HSCT patients were treated with gene-marked MSCs. MSC engraftment in bone and acceleration of growth velocity were seen. We performed in utero transplantation in a fetus with bilateral femur fractures with severe osteogenesis imperfecta [91]. Using fluorescent in situ hybridization, a bone marrow biopsy showed 0.3–7.4% Y-chromosome-positive bone cells. The patient had fewer fractures than expected after birth.

10.10.3 MSC for Tissue Toxicity and Hemorrhages

Because MSCs may be important for tissue repair, such as cartilage defects, ruptured tendons, and damaged myocardium, they may be used to heal therapy-induced tissue toxicity after HSCT [166–169]. Furthermore, MSCs seem to home to sites of injury [88, 170, 171]. Our group used MSCs for hemorrhagic cystitis, an unwanted complication after HSCT. Hemorrhagic cystitis is induced by the conditioning regimen, especially cyclophosphamide and busulfan [172, 173]. Patients receiving myeloablative conditioning more often have hemorrhagic cystitis compared to those receiving reduced-intensity conditioning. The first two patients we treated with MSCs for hemorrhagic cystitis had grade V. Both died of multiorgan failure, but transfusion requirements were dramatically decreased after MSC infusions [90]. Since then, we have treated 12 patients for severe hemorrhagic cystitis after HSCT [90, 174]. In two patients, treatment with MSCs did not stop the bleeding. In the remaining eight patients, gross hematuria disappeared after median 3 (range 1–14) days. In addition, we gave MSCs to a 61-year-old male who was refractory to platelet transfusions, due to multispecific anti-HLA-antibodies, and who developed life-threatening hemorrhages of the proximal jejunum [174]. Surgery was impossible in this patient. During 5 days prior to MSCs infusion, the patient received a total of 17 units of erythrocytes, 7 units of fresh frozen plasma, and 10 units of HLA-identical platelets. Despite this, his platelet level was below 5×10^9/L. After infusion of 2×10^6 MSCs/kg pooled from two donors, hemorrhages stopped. He experienced additional hemorrhages stopped by MSC infusions, underwent a retransplantation due to graft failure. The described patient is now alive and well 1 year after retransplantation. Apart from healing damaged tissue, MSCs can also stop hemorrhages. MSCs stimulate the clotting system, especially when studied after high passage [175].

Our group has also demonstrated that pneumomediastinum disappeared after MSC infusions in two patients [90]. Furthermore, a patient with steroid-resistant acute GVHD grade III developed colon perforation twice with free gas in the abdomen, peritonitis, and muscle defense. Mismatched and haploidentical MSCs were infused at the two occasions, respectively, and colon perforation was healed twice. The effect by MSCs on colon perforation has also been confirmed by Sato and colleagues [176]. They reported on a patient with aggressive acute GVHD with bloody

Chapter 11
Mesenchymal Stromal Cell Therapy in Crohn's Disease

Ilse Molendijk, Daan W. Hommes, and Marjolijn Duijvestein

Abstract Mesenchymal stromal cells (MSCs) have been shown to possess the ability to home to injured tissues and actively participate in tissue repair. They have the ability to suppress immune responses both in vitro and in vivo and in animal models of experimental autoimmune diseases. In patients with acute and chronic immune diseases, this capacity of MSCs has also been observed. The tendency of MSCs to regenerate damaged tissue combined with their capability to regulate immune and inflammatory responses gives a strong rationale for using MSCs as a new treatment option in diseases characterized by inflammation with severe tissue damage, such as Crohn's disease. This chapter highlights the present knowledge on MSCs in Crohn's disease. The application of MSCs in experimental colitis models and clinical trials with MSCs in luminal and fistulizing Crohn's disease are discussed.

Keywords Mesenchymal Stromal Cell • Mesenchymal Stem Cell • MSC • Crohn's disease

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11.1 Crohn's Disease

11.1.1 Background

Crohn's disease and ulcerative colitis, collectively called inflammatory bowel disease (IBD), are chronic diseases that cause inflammation of the gastrointestinal tract. The etiology of IBD remains unclear, but an inappropriate immune response to microbes in the gut in a genetically predisposed host is thought to be the cause of IBD [1, 2]. The major symptoms of Crohn's disease are abdominal pain, diarrhea, and fatigue. In ulcerative colitis the rectum and part of the colon are affected in a sustained pattern. Crohn's disease can affect any region of the intestine from mouth to anus, but generally involves the ileum and colon. The involved parts are not affected in a sustained pattern as seen in ulcerative colitis, but show healthy tissue between the inflamed regions, better known as skip lesions. Furthermore, Crohn's disease is occasionally associated with strictures and fistulas [1–3]. In both diseases exacerbations and remissions alternate. During exacerbations there is a significant reduction in quality of life [4].

11.1.2 Epidemiology

The peak age of onset for Crohn's disease is 15–30 years [5], with a second peak between the ages of 50–80. There is no gender specificity. The incidence of Crohn's disease is higher in industrialized countries, and in the West compared to the East. A recent systematic review of population-based cohorts estimated the prevalence of Crohn's disease to be 26.0–198.5 cases per 100,000 persons and the incidence to be 3.1–14.6 cases per 100,000 persons/years in North America [6]. In Europe the incidence of Crohn's disease is 6.0–15.0 cases per 100,000 persons/years and the prevalence 50.0–200.0 cases per 100,000 persons [7].

11.1.3 Clinical Manifestation and Diagnosis

The major symptoms of Crohn's disease are abdominal pain, diarrhea (with blood or mucous), and fatigue. Fever, weight loss, nausea, and vomiting are also common complaints. Frequent complications are intestinal obstruction, abscess formation, and fistulas [8]. Fistulas are abnormal passages from the intestines to another organ or to the skin. A population-based estimate of the incidence of fistula in patients with Crohn's disease was determined from a cohort of patients diagnosed in Olmstead County, Minnesota, from 1970 to 1995 [9]. At least one fistula episode was diagnosed in 35% of this cohort during this time interval, of these fistulas 54% were perianal. In approximately 46% perianal fistulas completely heal [10]. Perianal fistulas lead to

substantial physical and emotional distress because of pain, discharge, incontinence, perineal and genital disfigurement, and slow resolution even with treatment [11].

The diagnosis of Crohn's disease is established by the clinical features confirmed by endoscopy. Biopsy specimens from inflamed gut mucosa typically show transmural inflammation, including submucosal edema, granuloma formation, ulcerations, and fibrosis.

11.1.4 Treatment

Crohn's disease is a chronic disease with exacerbations and remissions. Oral 5-aminosalicylic acid (5-ASA) drugs (e.g., sulfasalazine and mesalazine), antibiotic therapy, oral traditional corticosteroids (e.g., prednisone), immunosuppressive therapy (e.g., 6-mercaptopurine, azathioprine and methotrexate), and biological therapies (e.g., infliximab and adalimumab) are available to heal active disease and prevent relapse. The choice of medical treatment depends on the location of disease, its severity, and response to earlier therapy. Most clinicians initially start with 5-ASA, steroids, and antibiotics. Patients who are steroid dependent can be treated with immunomodulating drugs. These drugs are effective in inducing clinical remission, but their widespread use is limited by their toxicity. In steroid refractory Crohn's disease, biological therapies (antibodies to TNF) have been shown to be efficacious, although they are not able to maintain remission in most patients. With time, the disease responds less to medical therapies and 70–90% of the patients will eventually need surgery during the course of the disease [12]. Unfortunately, surgery is accompanied by a high recurrence rate and approximately 39% of the patients with Crohn's disease will require repeated surgery with short bowel syndrome as a threatening complication [12].

Most patients with fistulizing Crohn's disease require a combined medical and surgical approach. The medical approach aims to diminish disease activity, whereas the surgical approach is first aimed at controlling infectious complications by drainage of abscesses and placement of non-cutting silastic setons. Sometimes fecal diversion is needed to attenuate perianal symptoms. When these goals have been reached, surgery is aimed to eradicate the fistula while preserving fecal continence. In this latter phase surgery depends upon the type of fistula and its anatomical extent. Standard surgical approaches are fistulotomy or a mucosal advancement plasty, which are unsuccessful in over 50% of the cases [13].

11.2 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are multipotent cells, capable of differentiating into multiple lineages of the mesenchyme. MSCs have both potent immunosuppressive and tissue regenerative effects. MSCs have been shown to possess the ability to

home to injured tissues and actively participate in tissue repair [14–17]. They have the ability to suppress immune responses both in vitro and in vivo [18] and in animal models of experimental autoimmune diseases [19]. In patients with acute and chronic immune diseases, this capacity of MSCs has been observed [20–21]. The ability of MSCs to suppress immune responses following in vivo transplantation was shown in a case study of severe grade IV graft-versus-host disease (GvHD). Le Blanc et al. [20] reported that repeated administration of purified haploidentical human MSCs (from the patient's mother) following allogeneic stem cell transplantation completely reversed the GvHD. By day 150 colonoscopy was performed and biopsy specimens showed mild GvHD and 4% female epithelium by fluorescence in situ hybridization, implicating that MSCs have had a healing effect on the damaged gut epithelium. One year following treatment, the patient was still free of GvHD and had no minimal residual disease of his leukemia in blood and bone marrow.

MSCs do not express MHC class II or co-stimulatory molecules and are poor antigen presenting cells. Because they do not elicit a proliferative response from allogeneic lymphocytes, it is suggested that MSCs are of low immunogenicity. Currently, both allogeneic and autologous MSCs are under investigation for various disease [22]. Benefits of allogeneic MSCs are their immediate availability and the possibility to control the age and fitness of the donor, as number and functionality have been shown to decrease with age [23, 24].

There is an unmet need for effective medical therapeutics in patients with Crohn's disease not responding to the conventional strategies, including biological therapies. Treatment with MSCs has proven to be feasible, safe, and highly effective in various inflammatory disorders, including Crohn's disease [22]. Accordingly, MSC therapy appears to have the potential to be a safe and effective alternative for these patients.

11.3 Mesenchymal Stromal Cells in Crohn's Disease

11.3.1 Experimental Colitis Models

MSCs have been studied in both dextran sulfate sodium (DSS) and trinitrobenzene sulfuric acid (TNBS) colitis. DSS polymers added to drinking water for several days can induce acute colitis, a condition characterized by bloody diarrhea, ulcerations, and infiltrations with granulocytes. DSS is directly toxic to gut epithelial cells of the basal crypts and affects the integrity of the mucosal barrier. The acute DSS colitis model is particularly useful to study the contribution of innate immune mechanisms to colitis. Colitis can also be induced by intrarectal instillation of the haptenating substance TNBS in ethanol. Ethanol is required to break the mucosal barrier, whereas TNBS haptenizes colonic autologous or microbiota proteins rendering them immunogenic to the host immune system. $CD4^+$ T cells have been shown to play a central role in chronic TNBS colitis, so T helper cell-dependent mucosal immune responses can be studied with this model [25].

In experimental colitis models, MSCs were obtained from the bone marrow (bmMSCs), adipose tissue (atMSCs), and gingiva (gMSCs), and both autologous and allogeneic sources were used. Furthermore, human MSCs were studied in (wild-type) mice (xenogenic). Systemic route of administration was either via the tail vein (rat) or intraperitoneally (i.p.) in mice.

Khalil et al. [26] demonstrated in a DSS-induced colitis model of IBD that systemically administered adult stem cells are effective in reducing both the clinical features and the pathological features associated with IBD. However, in the study by Khalil et al. [26], bone marrow-derived stem cells (not the specific MSC population) were used that were immortalized by retroviral transduction with the SV40 large-T antigen. Despite the interesting biology associated with this study, this is an approach that can likely not be used safely in humans.

Systemic infusion of MSCs obtained from adipose tissue ameliorated the clinical and histopathologic severity of TNBS colitis, abrogating body weight loss, diarrhea, and inflammation along with increasing survival. This therapeutic effect was mediated by down-regulating both Th1-driven autoimmune and inflammatory responses. A wide panel of inflammatory cytokines and chemokines was decreased by the atMSCs and IL-10 levels were increased. They also impaired Th1 cell expansion and induced $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells with suppressive capacity on Th1 effector responses in vitro and in vivo [27]. A second paper from the same group supported these data by showing that systemic infusion of atMSCs protect against experimental DSS colitis and sepsis. The therapeutic effect was associated with down-regulation of the Th1-driven inflammatory responses [28].

Zhang et al. [29] showed that MSCs from human gingiva have similar immunomodulatory and anti-inflammatory properties as bone marrow-derived MSCs. In addition, they showed that a comparable therapeutic effect was mediated in the acute model of DSS colitis. This effect was in part achieved by the suppression of inflammatory infiltrates and inflammatory cytokines/mediators, the increased infiltration of regulatory T cells, and the expression of anti-inflammatory cytokine IL-10 at the colonic sites.

11.3.2 Clinical Trials in Patients with Crohn's Disease

Active luminal disease (for which MSCs are injected intravenously) and fistulizing Crohn's disease (for which MSCs are injected locally) are indications for clinical trials in patients with Crohn's disease. MSCs are from the patient itself (autologous) or from a healthy donor (allogeneic) and are either isolated from bone marrow or adipose tissue.

11.3.2.1 Luminal

Osiris Therapeutics claimed encouraging results of a phase I study in patients with moderate to severe Crohn's disease using Prochymal™. The MSCs in this product

are obtained from the bone marrow of healthy adult volunteer donors. Although in this phase I trial a significant decrease of the Crohn's Disease Activity Index (CDAI) was observed [30], the company recently terminated a phase III trial because of a high placebo response rate.

The feasibility and safety of the intravenous application of autologous bone marrow-derived MSCs to treat moderate to severe luminal Crohn's disease was evaluated in a phase I trial [31]. The bone marrow aspiration procedure performed in ten patients was proven to be feasible and well tolerated. Generated MSCs showed similar growth potential and yield and the same typical spindle-shaped morphology and phenotypical characteristics (positive for CD105, CD73, CD90, and negative for CD34 and CD45) compared to MSCs from healthy donors. Importantly, Crohn's disease MSCs significantly reduced peripheral blood mononuclear cell proliferation in vitro, suggesting that their immunomodulatory capacity is intact. Similar data has been published by Bernardo et al., who demonstrated that bmMSCs expanded in platelet lysate-based medium show biologic characteristics similar to MSCs from healthy donors [32]. In the phase I trial [31], nine patients received two intravenous doses of 1–2 million cells/kg bodyweight, at baseline and 7 days later. MSC infusion was without side effects, besides a mild allergic reaction probably due to the cryopreservant DMSO in one patient. Although primarily designed to study the safety and feasibility of autologous bmMSCs, endoscopic improvement was seen in two patients with extensive Crohn's disease localized in the colon. This suggests that intravenous application of autologous bmMSCs is feasible and well tolerated. However, further studies should be designed to examine the efficacy of MSCs in luminal Crohn's disease.

11.3.2.2 Fistula

Safety of the local application of adipose-derived MSCs in the treatment of fistulizing Crohn's disease was shown in a phase I clinical trial in which in total nine fistulas in four patients were inoculated with atMSCs. Although the results are preliminary and follow-up is short, they are interesting as after 8 weeks 75% of these fistulas were considered healed and no adverse effects were observed in any of these patients [33]. This phase I study was followed by a multicenter, randomized, controlled trial sponsored by Cellerix to evaluate the efficacy and safety of atMSCs in 49 adult patients with complex perianal fistula from cryptoglandular disease ($n=35$) or Crohn's disease ($n=14$). Patients received fibrin glue or 20 million cells plus fibrin glue intralesionally. Fistula healing was evaluated at 8 weeks. If not healed, a second dose of fibrin glue or 40 million cells plus fibrin glue was administered, with healing evaluated 8 weeks later. Healing was defined as absence of drainage (spontaneous and/or by gentle compression) and complete reepithelization of the external openings. The proportion of patients whose fistulas were healed was significantly higher with atMSCs than with fibrin glue. Efficacy was observed in the Crohn and non-Crohn subpopulations [34].

Ciccocioppo et al. [35] enrolled 12 consecutive outpatients refractory to or unsuitable for current available therapies for fistulizing Crohn's disease. MSCs were

isolated from bone marrow and expanded *ex vivo*. MSC expansion was successful in all cases. The intrafistular injections with bmMSCs were scheduled at 4-week intervals, with a median of 20×10^6 cells per time. When autologous MSCs were no longer available or when remission or improvement was achieved, the injections were stopped. In seven of the ten treated patients the fistula tracks closed completely. In all ten patients a parallel reduction of Crohn's disease and perianal disease activity indexes and rectal mucosal healing were induced. There were no adverse effects reported. The percentage of mucosal and circulating regulatory T cells significantly increased during the treatment and remained stable until the end of follow-up at 12 months.

The Leiden University Medical Centre is currently investigating the safety and preliminary efficacy of allogeneic bone marrow MSCs in the induction of response for active fistulizing Crohn's disease in a dose escalation study (ClinicalTrials.gov; NCT01144962).

11.4 Conclusion

In vitro and in vivo animal and human clinical data show a potential for MSCs as a new treatment modality for inflammatory bowel diseases.

Promising initial results have been published, but questions remain about the mechanism underlying the immunomodulating properties of the MSCs and their in vivo survival after exogenous administration. Several clinical studies have demonstrated the clinical efficacy of MSCs for inflammatory bowel disease, but the exact treatment dose, timing and frequency of administration, as well as the optimal source of MSCs, are currently under investigation. Although in only a few patients adverse events have been seen after administration of MSCs, little is known about the possible long-term side effects. Possibility of malignant transformation, ectopic tissue formation, and xenogenic transmission of disease on the long term should be investigated.

Variation in obtained results can be explained by discrepancies in MSC isolation, source and culture protocols, as well as experimental conditions and timing of analysis. However, encouraging preliminary data supports further studies in this new approach in Crohn's disease.

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Chapter 12
Application of Mesenchymal Stem Cells in Amyotrophic Lateral Sclerosis

Josep Gamez

Abstract Mesenchymal stem cells (MSCs) and other cellular therapies are a promising alternative in the treatment of neurodegenerative diseases. Preclinical data suggests that MSCs may have therapeutic potential for ALS, a lethal neurodegenerative disease involving a rapidly progressive death of motor neurons. However, the positive results of cell therapy in animal models have not been confirmed by the phase I/II clinical trials performed to date. The results from patients' off-label cell treatments in clinics worldwide, which are the majority of cases, have also failed to provide grounds for optimism. We reviewed the clinical trials published to date, and social networking tools giving details of ALS patients' experiences with off-label cell treatments. There is no objective evidence that MSC therapy can halt or slow down the course of the disease. This conclusion is supported by a recent meta-analysis of a larger series with intraspinal administration, with a 9-year follow-up, which detected no clear clinical benefits. Future trials should be regulated by an international consortium of stem cell networks to ensure regulatory oversight of these modern therapies.

Keywords Amyotrophic lateral sclerosis • Lou Gehrig's disease • Motor neuron disease • Neurodegenerative disorders • Central nervous system • Stem cells • Mesenchymal stem cells • Stromal stem cells • Multipotent stem cells • Multipotent stromal cells cell therapy • Colony-forming unit fibroblasts • Cytotherapy • Transplant • Neural repair • Regeneration • Neural rejuvenation

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12.1 Are Mesenchymal Stem Cells an Alternative and Effective Therapy for Patients with ALS?

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is the most common neurodegenerative motor neuron disease in adults. As a consequence of a progressive degeneration of the motor neurons in the cortex, brainstem and spinal cord, patients present with progressive weakness, spasticity, and amyotrophy. Involvement of the respiratory muscles, and the diaphragm in particular, leads to respiratory failure and death. As a result of this progressive neuronal cell death, most patients have a survival time of between 3 and 5 years after diagnosis [1–6].

Unfortunately, there is no curative therapy for the almost 30,000 patients currently living with ALS in the USA, and the effect of riluzole — the only drug approved for treatment used to date — is almost imperceptible in terms of the disease's clinical course. None of the many clinical trials undertaken since 1994 has shown that any other drug has any therapeutic effect on ALS. ALS is a devastating neurodegenerative disease, with an unknown etiology, and none of the drugs tested to date has succeeded in curing or halting the progression of the disease. Taking into account that supportive and palliative care — especially assisted ventilation — remain the most effective therapeutic options for prolonging patients' survival time, the development of new therapeutic strategies that can replace the damaged neurons and slow down or halt the disease's course is important [7–9].

In recent years, cellular therapies have become a promising strategic approach for the treatment of neurodegenerative diseases [10–31] and have aroused the interest and hope of the ALS community. According to preclinical data, they have therapeutic potential for ALS and other neurodegenerative disorders due to their capacity to repair damaged CNS tissues and induce neurogenesis, and these data suggest that MSCs are an effective therapy in ALS animal models, improving the clinical course and prolonging survival. Stem cell therapies could potentially replace dysfunctional or dying neurons. These transplanted cells could differentiate into neuronal and glial cells, which could have various therapeutic effects at different sites and times within the lesion, which may protect the motor neurons from ongoing degeneration. Among the possible benefits are the release of neuronal growth factors for the host cells, release of antiapoptotic factors, secretion of neurotransmitters deficient in the host, stimulation of axonal growth, microglial regulation, immunomodulation, differentiation into oligodendrocytes, remyelination of host axons and less probably, differentiation into neurons. Neuronal connections may be formed between disconnected populations, and damaged neuronal circuits replaced [20, 32–35]. The neurodegenerative process would consequently be delayed in overall terms.

12.2 The History of Mesenchymal Stem Cells: From Friedenstein's Seminal Descriptions to the First Experiments in the Lab

Mesenchymal stem cells (MSCs) are stem-like non-hematopoietic multipotential cells able to differentiate into mesenchymal and non-mesenchymal lineages [36]. Since 1966, when Friedenstein observed that it was possible to obtain mesenchymal cells from bone marrow, and that these cells could form bone, cartilage, fat and myoblasts, MSCs have been obtained from adult and fetal tissue, from circulating blood, the umbilical cord, the placenta, amniotic fluid, the heart, skeletal muscle, fat, fibroblasts, synovial tissue, the pancreas, dental pulp and the uterus, among other sources [36–41]. The ability of MSCs to differentiate into neurons and astrocytes, both in vitro and in vivo, makes them very attractive for a possible therapy in ALS [19, 20, 34, 42–49]. They also provide the host tissue with growth factors and modulate the immune system [26, 34, 35, 50–53]. Although there are several sources for MSCs, bone marrow remains the most thoroughly investigated, and bone marrow-derived MSCs have been the most widely used type of stem cell in the preclinical and clinical trials in ALS conducted to date.

In the laboratory, bone marrow MSCs isolated from ALS patients maintain all their distinguishing features, and their expansion in vitro does not lead to chromosomal alterations or cell senescence. Furthermore, under certain conditions, they acquire new morphological characteristics and neural markers suggesting cell differentiation. These multipotential properties of MSCs are of great interest to clinicians due to their potential for repairing tissues and gene therapy. They also have the advantage compared to other stem cell types (especially embryonic and neural stem cells) of few adverse effects, and can be cultivated in vitro with almost no risk of malign transformation [36].

The various routes for stem cell administration studied to date — intraspinal, intrathecal, intramuscular, and intravenous — all appear to lead to improvements in the various animal models of ALS. There is considerable evidence from preclinical and in vitro studies to suggest that unlike most therapies available or being clinically assessed, MSCs may present a real neuroprotective effect. In the absence of an effective treatment for ALS, and despite the lack of preclinical data, ALS could be a target for testing the neuroprotective properties of MSCs [35].

One of the major practical problems with MSCs is the relatively small amount of "self-renewal" cells in the tissues studied. In bone marrow, for example, only ten in every million MSCs are able to self-renew. Numerous changes have been made to Friedenstein's initial methodology for enriching MSCs based on suspension of bone marrow cells. Plastic adherence, medium selection, single-cell cloning, and cell sorting of MSCs have improved the results of large-scale production of stem cells for transplants in patients.

12.3 Trials with Mesenchymal Stem Cells in Transgenic Rodent Models of ALS

Although there are various animal models for motor neuron disorders, including models for ALS, SBMA, SMA, and SMARD1, most preclinical trials using stem cells have been carried out in mice expressing SOD1 mutants, as these transgenic mice, bearing the wild-type $SOD1^{G93A}$, $SOD1^{G37R}$, and $SOD1^{G85R}$ mutants, develop motor neuron disease with clinical and histological changes similar to patients with ALS [54]. Other ALS animal models, such as adult rats with chronic unilateral motor neuron deficiency (through sciatic exotomy), and presymptomatic *nmd* mice [55] are also used in cellular therapies for neurodegenerative diseases and disorders, albeit to a much lesser extent. Mice carrying 25 copies of the G93A SOD1 gene show weakness, tremor and dragging of the hind limbs at approximately 90 days after birth. The disease progresses until 120–150 days after birth, at which point the animals are sacrificed for ethical reasons. Variability of survival is influenced by gender and background, with female mice having a lifespan 4–6 days longer than the males, and mice in the B6 hSOD1 Tg+line surviving longer than those in the SJL hSOD1 Tg+line (143 days compared to 119 days) [56].

The discovery that mesenchymal stem cells in vitro have multi-linear potential [42] and are capable of self-renewal and differentiation into non-mesenchymal lineages including neurons [40, 41, 57, 58] led to experiments in ALS animal models. The first report demonstrating that MSCs ameliorate the phenotype of the $SOD1^{G93A}$ mice used intraperitoneal injections after irradiation in presymptomatic mice 4 weeks after birth. This Milan-based group observed a mean survival time of 12–13 days longer than in the animals' untreated SOD1 littermates [59]. The untreated animals also presented a marked decline in performance 14 days before their transplanted contemporaries. The authors hypothesized that the beneficial effect is due to a "non-neuronal environmental change" which is unlikely to be the result of neuron formation (neurogenesis), and is more probably the result of microglial generation, including cell fusion. Neurogenesis was considered as a possibility when unusual green and yellow fluorescent proteins (GFP+ and YFP+) were observed in the spinal cord and brain. GFP-positive muscle fibers were also found in the quadriceps, tibialis anterior, and paravertebral muscles in the transplanted mice.

Subsequent studies of the same transgenic mice have since replicated these results, and demonstrated that MSCs can survive and migrate after transplantation in the lumbar and spinal cord, release neurotrophic factors, and are able to decrease neuroinflammation by inhibiting astrogliosis and microglial activation [51, 58, 60–64]. Similar delays in disease onset and increased lifespan were observed after intravenous injection of MSCs into irradiated presymptomatic SOD1-G93A mice [65] (see Table 12.1).

The effects of injecting MSCs in cerebrospinal fluid of symptomatic $SOD1^{G93A}$ rats were as positive as those in the presymptomatic rats above. Boucherie's group found that MSCs infiltrated the nervous parenchyma and migrated to the ventral horn. They also observed a differentiation of the MSCs into astrocytes, reducing

Table 12.1 Mesenchymal stem cell therapy in transgenic SOD1 rodent models of ALS

	Animal model	Administration level	Stem cell type	No. of TX cells	No. of animals	Age at TX	Age sacrificed (weeks)	Morphological changes	Effect on the progression of disease	Effect on lifespan
Ende (USA 2000)	Irradiated B6SJL-TgN 1Gur (SOD1G93A) mice	Retroocular	MSCs isolated from bone (femur, tibia) of wild-type mice	5×10^6 cells	6	8 weeks (p/s)	Spared until death	Not specified	Delayed onset (7 days)	Increased (14 days)
Corti (Italy 2004)	Irradiated B6SJL-TgN 1Gur (SOD1G93A) mice	IP	BM	30×10^6 cells	34	4 weeks (p/s)	Spared until unable to right themselves within 30 s	GFP-positive microglial cells in brain and spinal cord	Delayed onset (14 days)	Increased (13 days)
Vercelli (Italy, 2007)	Transgenic B6SJL-TgN (SOD1G93A)^{dl} 1Gur/J mice	Lumbar IS	MSCs from bone (IC) aspirates of healthy human volunteers	1×10^5	25	28 weeks (p/s)	12	Limited proliferation and diffusion. Neural differentiation questioned	Motor tests significantly improved in males	Survival not evaluated
Zhao (China, 2007)	Irradiated B6SJL-TgN 1Gur (SOD1G93A) mice	IV	MSCs from bone (IC) aspirates of healthy human volunteers	3×10^6	29	8 weeks (p/s)	12, 16, 20	Migration into parenchyma of brain and spinal cord. Neuroglial differentiation	Delayed disease progression	Increased (18 days)

(continued)

Table 12.1 (continued)

	Animal model	Administration level	Stem cell type	No. of TX cells	No. of animals	Age at TX	Age sacrificed (weeks)	Morphological changes	Effect on the progression of disease	Effect on lifespan
Morita (Japan, 2008)	Transgenic SOD1-Leu126delTT mice	IT	MSCs isolated from bone of LEW-GFP rats	$3–4\times10^5$	17	14 weeks (p/s)	Spared until unable to right themselves, or eyes highly infected	MSCs on spinal cord surface 10 and 30 days after TX. MSCs invaded spinal cord parenchyma. MSCs or progeny invaded the brain, especially in Purkinje cell layer in cerebellum. GFP-positive MSC-derived cells in spinal cord sections at endpoint. More motor neurons in MSC group than in controls at endpoint; not statistically significant	Beneficial trends in ages of onset and endpoint, and disease duration in MSC-transplanted mice cf. control mice. Not statistically significant	Only statistically longer in females (11 days)
Boucherie (Belgium 2009)	SD-Tg (SOD1G93A) L26H Sprague–Dawley rats	IT	MSCs isolated from bone (femur, tibia) of male wild-type Sprague–Dawley rats	2×10^6	Not specified	90 days (p/s)	125 days	Migration into ventral gray matter. Differentiation into astrocytes. Decreased motor neuron loss	Delayed disease progression (14 days)	Increased (16 days)

Zhang (China 2009)	Irradiated B6SJL-TgN 1Gur (SOD1G93A) mice	IT	MSCs from bone (CI) aspirates of healthy human volunteers	5×10^6	21	8, 10 and 12 weeks (p/s)	When unable to right themselves within 30 s	hMSC delivered through CSF migrated into lumbar spinal cord parenchyma of SOD1 mice	Delayed onset (6 days)	Increased in multiple TX (14 days)

IC iliac crest, *IP* intraperitoneal, *IS* intraspinal, *IT* intrathecal, *IV* intravenous, p/s presymptomatic, *TX* transplantation

neuron loss and therefore prolonging the rats' survival. The authors attributed this to reduced expression of COX-2 and NOX-2 [61, 62].

Survival in the rodent models improved in subsequent studies, as new cell transplantation protocols avoided donors with diminished stem cell capacity, analyzed telomerase activity, and increased the dosage of cells injected [39, 40, 64–77].

Motor neuron survival in $SOD1^{G93A}$ rats is also improved by concomitant intramuscular administration of MSCs due to their ability to release GDNF and the significant increase in neuromuscular connections and motor neuron cell bodies in the spinal cord [24, 33, 78, 79]. This retrograde therapeutic capacity should be taken into account in future clinical trials.

12.4 Description of Clinical Trials

Based on the promising results obtained in animal models of motor neuron disease, some authors decided to administer stem cells in small subsets of ALS patients in early 2000, despite unresolved questions regarding the cells' origin, multipotential capacity, differentiation, source of origin, the number of cells injected, the route of administration, and concomitant administration of neuronal growth factors. Information is available on several small phase I/II clinical trials in ALS published to date. The first human transplantation of stem cells, albeit non-mesenchymal, in this disease, is attributed to Janson et al. This group intrathecally injected three patients with peripheral blood-purified CD34+ cells (the most common components of bone marrow MSCs), which were isolated by leukopheresis and fluorescence-activated cell sorting (FACS) after stimulation with Neupogen® (Filgrastim, G-CSF). Although the authors observed no clinical efficacy, two of the three individuals experienced a slight improvement in their symptoms after transplantation, which persisted for between 4 and 6 months [80].

After this trial, Mazzini et al. carried out studies to verify the safety and efficacy of treatment with bone marrow-derived MSCs. In their first study, nine ALS patients were intraspinally injected at T7–T9 level with autologous BM-derived cells after expansion in vitro. This technique requires dorsal laminectomy and general anesthesia. The adverse effects observed during the follow-up period were mild, and mainly consisted of pain and dysesthesia. However, the final outcome was not as positive, as four patients died after an average of 29.2 months post-transplant (range 9–44 months). Another four patients showed a tendency towards a deceleration in the decline of FVC and ALSRFS-R (the two main clinical markers of progression of ALS) [8, 81, 82]. The remaining patient presented a progression and worsening of the disease. The mean survival time for the deceased patients was 60.5 months (SD 31.5, median 54, range 30–104), while the mean survival time for all nine patients from the onset of the disease until death or the end date of the study was 75.5 months [43, 83].

In 2009, this group reported the results of a further study, involving ten patients (three women and seven men) who received injections of bone marrow-derived

MSCs in the spinal cord at the high thoracic level (T4–T5; T5–T6). At the time of writing, all patients had been monitored for a follow-up period of at least 2 years post-transplant. Seven of the transplanted patients presented severe respiratory failure, four required nocturnal noninvasive ventilation, and the remaining three required tracheotomy [48].

In a 1-year follow-up trial, Deda et al.'s group chose higher intraspinal regions than those used by the Italian group — the anterior part of the spinal cord at C1–C2 level — for injecting autologous bone marrow-derived hematopoietic stem cells in 13 patients in the terminal stage of the disease. This technique requires cervical laminectomy and general anesthesia. Most of them were dependent on mechanical ventilation, and unable to swallow or speak. Three patients died during the 12-month follow-up period (1.5, 2, and 9 months after transplantation), after a mean survival time of approximately 24 months. The patients' initial improvement lasted 3 weeks, and included regained capacity for eating, independent breathing and swallowing, and increased muscle strength, including in the lower extremities. Their neurological findings, nevertheless, remained stable after a follow-up of 1 year. The authors concluded that cellular therapy is an effective and promising treatment for ALS patients [84].

In 2010, an Israeli–Greek group performed an exploratory trial with bone marrow-derived MSCs cultured for 40–60 days before intrathecal + intravenous injection in 19 patients with ALS with a disease duration of 34.3 months. Mild self-limited febrile reaction and headaches were the most commonly reported adverse events. One patient presented aseptic meningitis. ALSFRS-R score underwent no statistically significant changes between the time of transplant and 6 months later [52]. The authors emphasized the immunological effects of MSC transplantation.

A Spanish group used similar methods to those of Dr. Mazzini's group, but injected pluripotential hematopoietic cells from the iliac crest in the spinal cord at T3–T4 level using a dorsal laminectomy and under general anesthesia. Eleven patients were recruited, with seven completing the 1-year follow-up period. The mean ALSFRS-R score was 27.91 at the time of transplantation, and 19.8 one year post-transplant. FVC was 96.3% at the time of transplantation, and 64.0 12 months post-transplant. Two deaths occurred within the follow-up period. An autopsy showed cells with CD34 staining in one case. However, this trial included relatively young patients, with little clinical and functional involvement according to the El Escorial criteria, respiratory muscle function and ALSFRS-R score [85, 86].

In addition to the experiments using bone marrow-derived stem cells mentioned above, there are results of other phase I/II clinical trials using autologous CD133+ stem cells obtained from sources other than bone marrow. One of these studies used allogenic hematopoietic cells from HLA identically matched sibling donors in six ALS patients. Patients received total body irradiation, fludarabine, and horse antithymocyte globulin before intravenous infusion of CD34+ cells. Tacrolimus was the drug selected to prevent graft-versus-host disease. Blood HSCT infusion took place after total body irradiation. Comparison of the progression of the transplanted patients with a matched historic database showed no clinical benefits. Five of the patients died during the follow-up period. The mean survival time between clinical

onset and death was 41.0 months. One of the patients (case 4) required a tracheotomy 39 months post-transplant (106 months after clinical onset) and was still alive at the time the study was reported, 135 months after clinical onset. However, the most important data from this study is the post-mortem observation that two of the 100% engrafted patients demonstrated 16–38% donor-derived DNA at sites with motor neuron pathology, which may correspond to the increase in CD68 or CD1a-positive cells observed [87].

The same type of cell and stimulation with Neupogen (G-CSF) as chosen by Jackson et al. (see above) was used by the group in Monterrey (Mexico) in 2009. In this trial, stem cells were transplanted into the frontal motor cortex in ten ALS patients. Two patients died during the 12-month follow-up period (one 10 days after transplant, and the other 6 months afterwards). The mean survival time between clinical onset and death or the end date of the study was 69.6 months [88].

Another small pilot trial used reinfusion of granulocyte-colony stimulating factor (G-CSF)-mobilized peripheral blood stem cells (PBSC) in eight patients. One of the patients presented deep venous thrombosis. They observed no significant changes in the disease's progression markers (ALSFRS-R and FVC). An autopsy was performed on two of the patients who died, and no evidence of any systemic inflammatory or autoimmune process was observed [89].

The results of the first trial with human CD34 umbilical cells injected in a 63-year-old male patient at T8 level have recently been published [90]. No complications attributable to the cell implantation were observed. Clinical deterioration of the disease appeared to be slowed for a period of 10 months following transplantation (see Table 12.2).

Mazzini has recently performed a meta-analysis of her results in the two consecutive clinical trials mentioned above, including information on survival until death, time elapsed between surgery and gastrostomy, noninvasive pressure ventilation and tracheotomy, during a follow-up of nearly 9 years. She concluded that no clear clinical benefits were detected [91]. In four of six patients in which she observed a slower progression of the disease post-transplant, the effect may have been due to the patients' youth rather than the cell therapy.

Specific clinical information about one of the many stem cell clinics offering direct-to-consumer cell therapy online was available until 2010. This German clinic reported the results of a survey of 53 of their amyotrophic lateral sclerosis patients up to May 2008, 1–6 months post-treatment. Sixteen patients reported no change after the treatment, 19 reported an improvement, with some of these reporting a regain of muscle strength and/or an improvement of balance, sleeping or a reduction of spasms. One patient reported a marked improvement in mobility, breathing, speech and swallowing. Seventeen reported deterioration in the quality of their life. Most patients experienced a reduction in their mobility, strength, speech/swallowing or balance. The treatment failed to halt or reverse the progression of the ALS symptoms. [92].

There have been two observational studies in patients deciding on their own account to attend cell therapy clinics found on the Internet. Gamez et al., in a study of 12 patients, of whom 9 had been treated with bone marrow MSCs administered

Table 12.2 Mesenchymal and other stem cell phase I/II trials reported in amyotrophic lateral sclerosis

Author/year	No of cases	Administration level	Type of stem cells	No. of TX cells ($\times 10^6$)	Mean age at TX	Mean ALSFRS at TX	Mean FVC at TX	Mean time from onset to TX (months)	Follow-up time post-TX (months)	Survival from onset (months)	No. of patients dying during trial	Investigators' subjective impression
Janson (USA), 2001	3	IT	Autologous CD34+ isolated from peripheral blood cells	20–100	43.0	Not specified	Not specified	Not specified	12	Not specified	Not specified	Negative. Mild improvement in first 4 months
Appel (USA), 2008	6	IV	Allogeneic HSCT rich in CD34+	Not specified	45.2	AALS 64	≥60%	23.2	68	53.0	6	Negative
Cashman (Canada) 2008	8	IV	Peripheral blood stem cells rich in CD34+ after G-CSF stimulation⁺	227.5	Not specified	Not specified	Not specified	Not specified	6	Not specified	None; 2 died afterwards	Negative
Deda (Turkey), 2008	13	IS (C1–C2)	Autologous MSCs BM-derived rich in CD34+	20	49.7	Not specified	Not specified	32.3	12	69.5	3	Positive
Mazzini (Italy), 2008	9	IS (T7–T9)	Autologous MSCs BM-derived rich in CD29, CD44, CD90, CD105, CD106, CD166	7–152	45.1	25.6	76.0%	34.5	48	60.5	4	Positive
Martinez (Mexico), 2009	10	IC (frontal cortex)	Mononuclear cells from peripheral blood after MO stimulation with SC filgrastim. CD133	2.5–7.5	45.0	24.6	45.0%	30.1	12	69.6	3	Positive
Blanquer (Spain), 2010	11	IS (T3–T4)	Autologous MSCs BM-derived rich in CD34+, CD117+, C133+.	2.7	47.2	27	96%	20.0	12	Not specified	2	Positive
Karussis (Israel), 2010	19	IT + IV	Autologous MSCs BM-derived rich in CD29, CD73. CD90, CD105, CD166	63.2	53.0	20.8	Not specified	34.3	6	Not specified	Not specified	Positive

(continued)

Table 12.2 (continued)

Author/year	No of cases	Administration level	Type of stem cells	No. of TX cells ($\times 10^6$)	Mean age at TX	Mean ALSFRS at TX	Mean FVC at TX	Mean time from onset to TX (months)	Follow-up time post-TX (months)	Survival from onset (months)	No. of patients dying during trial	Investigators' subjective impression
Mazzini (Italy), 2010	10	IS (T4–T6)	Autologous MSCs BM-derived rich inCD29, CD44, CD90, and CD105	11.4–120	41.8	33.0	93.6%	22	30	Not specified	3	Positive
Andrews (USA), 2011	6	IT	Autologous MSCs BM-derived hematopoietic progenitor rich in CD34+	Not specified	Not specified	34.5	Not specified	Not specified	Not specified	Not specified	Not specified	Negative
Cordes (Germany), 2011	1	IS (T8)	Umbilical cord-derived CD34+	14	65	25	Not specified	22	36	>60	Not specified	Doubtful change in ALSFRS-R score slope

ALSFRS-R ALS Functional Rating Scale revised, *TX* transplantation, *FVC* forced vital capacity, *AALS* Appel ALS scale, *HSCT* hematopoietic stem cell transplantation, *SC* subcutaneous, *IT* intrathecal, *IV* intravenous, *IS* intraspinal, *IC* intracerebral

intravenously and intrathecally, observed no changes in the decline in FVC and the ALSFRS-R score compared with the disease's natural history [82]. Similar results were reported by a team at the Neuromuscular/ALS Clinic in Connecticut, USA, in six of their ALS patients receiving intrathecal administration of autologous stem cells from bone marrow stem cells [93]. They found no significant change in the decline of the ALSFRS-R score caused by the transplant.

ALSUntangled is a group of ALS experts that uses social networking tools to analyze alternative and off-label treatment alternatives [94, 95]. This group has published its opinions on various stem cell clinics (including NuTech Mediworld in New Delhi, India, Xcell-center in Dusseldorf and Cologne, Germany and the Hospital San José Tecnologico in Monterrey, Mexico) in collaboration with Quackwatch, Patients Like Me, and ALS Worldwide [96–102]. As regards unproven cell therapies for ALS, it believes that "it is unethical to charge patients for experimental interventions that are not yet proven safe and effective by properly conducted clinical trials." This position has also been adopted by the International Campaign for Cures of Spinal Cord Injury Paralysis and the International Society for Stem Cell research [103, 104].

12.5 Ongoing Mesenchymal and Other Stem Cell Clinical Trials for ALS

The randomized, double blind "Phase I/II Clinical Trial on The Use of Autologous Bone Marrow Stem Cells in Amyotrophic Lateral Sclerosis (Extension CMN/ELA)" (clinicaltrials.gov identifier: NCT01254539) aims to assess the feasibility and safety of intraspinal and intrathecal infusion of autologous bone marrow stem cells. The trial will include 63 patients, distributed in 3 arms. One will receive T3–T4 laminectomy with autologous bone marrow stem cell intraspinal transplantation. In the second arm, the procedure will consist of intrathecal infusion of 2 ml of autologous bone marrow stem cells. In the third arm, patients will receive intrathecal infusion of 2 ml of placebo (saline solution). This trial is the continuation of another study of 11 patients by the same group, entitled "Phase I/II Clinical Trial on the Use of Autologous Bone Marrow Stem Cells in Amyotrophic Lateral Sclerosis" (NCT00855400), which aimed to assess the feasibility and safety of intraspinal infusion of autologous bone marrow stem cells.

Neuralstem, Inc (USA) is the sponsor of the first-in-human trial of spinal-derived stem cells transplanted into the spinal cord of patients with amyotrophic lateral sclerosis (ALS). The clinical trial "A Phase l, Open-label, First in Human, Feasibility and Safety Study of Human Spinal Cord Derived Neural Stem Cell Transplantation for the Treatment of Amyotrophic Lateral Sclerosis" (NCT01348451) aims to determine the safety of human spinal cord-derived neural stem cell transplantation. The procedure consists of a surgical intraspinal cord implantation of neural stem cells. A sequential design of five groups will be used to reduce the risk to subjects. The first group (Group A) will include six subjects, and the subsequent groups will

include three subjects per group. Each group represents both different inclusion criteria and location of surgery [105].

TCA Cellular Therapy (Louisiana, USA) is the sponsor of the "Phase I, Single Center, Prospective, Non-randomized, Open Label, Safety/Efficacy Study of the Infusion of Autologous Bone Marrow-derived Stem Cells, in Patients With Amyotrophic Lateral Sclerosis" (NCT01082653). Six ALS patients will receive a unique one-time intrathecal infusion of autologous bone marrow-derived stem cells. The study aims to evaluate the safety of the infusion procedure, as assessed by the absence of complications at the site of infusion or the appearance of new neurologic deficit not attributed to the natural progression of the disease.

Corestem, Inc (Korea) is the sponsor of the "An Open-label, Phase I/II Trial for Safety and Efficacy Study of Autologous Bone Marrow Derived Stem Cell Treatment in Amyotrophic Lateral Sclerosis" (NCT01363401) to evaluate the safety and efficacy of autologous bone marrow-derived stem cells ("HYNR-CS inj"), through intrathecal delivery. This study consists of two steps. The first step is a safety study of the intrathecal (IT) transplantation of "HYNR-CS inj" in seven patients with ALS. Safety will be evaluated based on the adverse effects and a clinical laboratory test. The second step is to compare efficacy and safety between the test group and the control group of a total of 64 patients with ALS.

Mayo Clinic (Rochester, Minnesota, USA) is the sponsor of "A Single Patient Treatment Protocol for Autologous Mesenchymal Stem Cell Intraspinal Therapy in Amyotrophic Lateral Sclerosis (ALS)" (NCT01142856), which includes only one ALS patient. MSCs will be isolated from adipose tissue by subcutaneous biopsy and expanded using an FDA-approved protocol. They will then be injected by lumbar puncture into the cerebrospinal fluid. Injection will be completed in the in-patient clinical research unit (CRU). The patient will be monitored for 2 years.

12.6 Conclusions and Future Challenges

The preclinical data suggest that MSCs and stem cells in general have therapeutic potential for neurodegenerative diseases, including ALS. A number of phase I/II clinical trials have been undertaken based on the observation that MSCs are able to differentiate into the mesodermal lineage, and that under certain circumstances, they can transdifferentiate into neurons and glial cells in ALS animal models. Other attractive neuroprotective properties that have aroused interest in the application of stem cells for ALS are the direct release of antiapoptotic and neurotrophic factors, anti-inflammatory capacity, microglial regulation, stimulation of axonal growth, and remyelination.

However, despite the promising results observed in vitro and in ALS animal models, which suggested that it could become an alternative therapy for an incurable disease, its application in ALS patients has not yet replicated those positive findings in the clinical trials conducted. To date, information is available on more than 550 ALS patients undergoing MSCs and other cell therapies (phase I/II trial

and clinics offering off-label direct-to-consumer cell therapy) which have been unable to cure or halt the disease. They have also yet to prove themselves capable of significantly slowing the progression of disability (as measured by the decline in ALSFRS-R scores and spirometry values), and consequently the point at which patients require mechanical ventilation or gastrostomy tube feeding. The reasons for this further failure of translational medicine in ALS (from the laboratory to the patient) are as yet unknown.

Analysis of the causes of this failure includes numerous uncertainties as regards the complexity of the disease and the difficulties involved in the application of stem cell therapy in humans. Among the possible contributory factors related to the disease itself is the fact that motor neurons are post-mitotic cells, as well as the lack of precise knowledge regarding the etiopathogenesis of ALS. Another factor is that patients are treated when they reach the symptomatic phase of the disease, while mice are treated at a presymptomatic stage. It should be borne in mind that patients with ALS remain asymptomatic until they have lost more than 30% of their motor neurons. Finally, there is evidence to suggest that motor neuron death is not cell-autonomous. Microglial activation plays a role in the disease's onset and progression in the SOD1 animal model, with the number of activated cells escalating during progression [54, 106–110].

Possible contributory factors related to therapy with MSCs and other stem cells include lack of knowledge regarding the best source of MSCs, the amount of stem cells required in each injection, the number of injections to be administered per patient, where to inject, how long MSCs survive in the patient, whether this survival time is affected by the site of administration or concomitant neuronal growth factors, whether the stem cells injected emigrate through the nervous system, their capacity for differentiation and induction of neurogenesis, and whether treatment needs to be repeated. In addition, we do not know whether the manipulation protocol for the cells affects the results.

In the literature, the number of transplanted cells varies between 2.5×10^6 and 152×10^6. Autologous CD34+ cells, isolated from both peripheral blood cells and from bone marrow, are the most commonly used stem cells in these transplants. These MSCs are also rich in CD29, CD44, CD90, CD105, CD106, CD117 CD133, CD166, and CD173, although there is no concrete evidence as to which are the best. Furthermore, only a small proportion of these bone marrow-derived MSCs (less than 0.1%) are progenitor cells. Some authors also argue that this proportion decreases in inverse proportion to the donor's age, and is probably lower still when the donor is an ALS patient. Clinical trials using other types of stem cells, such as ensheathing olfactory fetal cells and umbilical cord-derived cells, have also failed to halt the progression of the disease. The optimization of protocols for obtaining MSCs, including isolation, passage selection, in vitro expansion and long-term culture has not substantially altered the results.

We have no knowledge about the survival time of stem cells in ALS patients, or whether this survival may be lengthened by the concomitant use of growth factors or immunosuppressants. The only information available on MSC survival comes from a few preclinical studies. Garbuzova-Davis observed that human stem cells

from the umbilical cord (MNC hUCB) injected intravenously in presymptomatic G93A ALS model mice survive for 10–12 weeks [18, 51]. Survival times of almost 20 weeks have been achieved in irradiated pre-symptomatic SOD1-G93A mice [65], after administration of hMSC injections [65] and when using allotransplantation without immunosuppression [77].

Similarly, there is a lack of consensus as regards the best administration route for MSCs. Intrathecal administration was used for the first stem cell trial in ALS patients in 2001, while a combination of intravenous and intrathecal administration is being used in many clinics and trials. Whether this means of administration is capable of crossing the hematoencephalic barrier and therefore reaching the CNS remains unclear. After intravenous infusion, most MSCs have great difficulty in passing through the pulmonary filter and remain trapped in the lungs, where they are rejected by the host as noncompatible cells, hindering long-term grafting. Nevertheless, the advantage of these intrathecal and intravenous administration routes is that they permit repeated infusion of stem cells, thereby avoiding the risk of a further surgical procedure. In view of the uncertain effectiveness of intrathecal and intravenous administration, some authors have decided to perform trials involving direct intraspinal and intracerebral injections into the CNS. The groups opting for intraspinal administration (T4–T5 and T5–T6 levels) have attempted to preserve the motor neurons used for innervation of the respiratory muscles. Higher spinal cord territories were chosen by the Turkish group, which administered intraspinal injections at C1–C2 level, in order to assess postoperative bulbar scores. The intracerebral route was also used for the administration of stem cells, in order to rescue the upper motor neurons and their axons. Although the groups performing the few clinical trials published to date report that "no severe morbidity was associated with the surgical procedure," the injections are administered intraspinally or intracerebrally, and the general anesthesia required for this type of neurosurgery is a high-risk procedure in ALS patients with severe respiratory muscle or bulbar involvement. Limitations of this technique include the distribution of the motor neurons hindering multiple injections throughout the entire neuroaxis, and the need for general anesthesia, making it impossible in patients with moderate or severe ventilator insufficiency.

There is at present no evidence to suggest that this treatment is beneficial despite the youth of most of the patients recruited in these clinical trials, and the consequent limited functional impact in their ALSFRS-R scores. Notwithstanding the results' failure to show any functional changes in the patients, such as lengthening of survival, or any changes in the decline of the disease's markers (FVC and ALSRFS-R), identification of the transplanted cells in the host's CNS tissue could justify continued work on this therapy. This question is as yet unresolved, as few autopsies of transplanted patients have been performed to date, and the information available from histological studies shows no sign of stem cells differentiating into the neuronal or glial lineage [85, 87, 111]. The only evidence for engraftment of transplanted stem cells was reported by Appel in two examinations of post-mortem tissues, showing 16–38% donor-derived DNA at sites with motor neuron pathology. Despite these findings, the patients presented no changes in progression or survival compared to matched historic database patients [87].

However, the greatest therapeutic doubts vis-à-vis the applications of MSCs concern whether able to repair tissue damage, transdifferentiate into neural cells, migrate to appropriate areas in the CNS, release factors promoting the survival and proliferation of neighboring neural cells, and establish neuronal connections between damaged neuronal circuits. Preclinical studies suggest that MSCs are disappointingly unable to improve the disease's clinical course or help the host's nervous system to recover functions when the neural damage is chronic and the subsequent disability has occurred. This is probably due to its poor integration within the neural tissue and transdifferentiation [34, 35].

An important development that may have a negative impact on the legitimate progress made by scientists involved in stem cell research and its possible future clinical application is the proliferation in recent years of stem cell clinics offering these treatments (which are still at an experimental stage with unproven efficacy) on a direct-to-consumer basis via the Internet or by similar means. Many of these clinics fail to meet scientific and ethical standards, while charging substantial sums of money for unproven therapies. Protecting patients against medical tourism is difficult, as these clinics are insufficiently regulated and ALS patients are understandably anxious to find an effective treatment [104, 112–125].

In conclusion, the results obtained to date in these clinical trials suggest that MSC therapy has not been shown to be sufficiently effective in curing or halting the disease, and its presumed ability to slow down the disease's progress is also as yet unproven. There is consequently not enough clinical evidence to support its use in the treatment of ALS patients. This application remains restricted to research programs, and further insufficiently controlled clinical trials failing to meet scientific standards should be avoided [104, 112, 126].

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Chapter 13
Mesenchymal Stem Cell Therapy for Heart Disease

Massimiliano Gnecchi and Elisabetta Cervio

Abstract Mesenchymal stem cells (MSC) are adult stem cells with capacity for self-renewal and multi-lineage differentiation. Initially described in bone marrow, MSC are also present in other organs and tissues. From a therapeutic perspective, facilitated by the ease of preparation and immunologic privilege, MSC are emerging as an extremely promising therapeutic agent for tissue regeneration and repair. Studies in animal models of myocardial infarction have demonstrated the ability of transplanted MSC to engraft and differentiate into cardiomyocytes and vasculature cells. Most importantly, engrafted MSC secrete a wide array of soluble factors that mediate beneficial paracrine effects and greatly contribute to cardiac repair. Together, these properties can be harnessed to both prevent and reverse remodeling in the ischemically injured ventricle. In proof-of-concept and phase I clinical trials, MSC therapy improved left ventricular function, induced reverse remodeling, and decreased scar size. This chapter reviews the current understanding of MSC biology and mechanism of action in cardiac repair of MSC therapy for cardiac disease.

Keywords Mesenchymal stem cells • Cardiomyocyte • Cardiac repair • Paracrine effects • Exosomes

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13.1 Introduction

The ability to mobilize and activate endogenous stem/progenitor cells in diseased organs or to introduce exogenous stem cells for tissue regeneration/repair may impact many diseases, including those affecting the brain, skeletal muscle, pancreas, and heart. The reports that embryonic and adult stem cells (ASC) can differentiate into cardiomyocytes (CMC), vascular smooth muscle cells (VSMC), and endothelial cells (EC) have stimulated studies investigating the use of stem cells as regenerative therapy for cardiovascular disease. Regenerative and reparative therapies would be particularly important for heart disease since, despite many recent advances in medical therapy and interventional techniques, ischemic heart disease and congestive heart failure (CHF) remain major causes of morbidity and mortality [1, 2]. The current therapeutic approaches to treat congestive heart failure merely delay the progression of the disease [3], thus generating a population of chronically ill patients. Heart transplantation is the only effective therapy for this otherwise deadly clinical condition. However, the limited number of organs donated is not enough to treat all patients who would require a transplant. Consequentially, the disability of a growing number of people with heart disease will continue to place a heavy burden on an already financially strained health-care system, and the socioeconomic costs are incalculable. Cellular therapy for treating these and other heart conditions is a growing field of basic and clinical research. Here, we examine the basic science that is the foundation of future clinical approaches to ASC therapy for heart diseases. In particular, we will focus our attention on mesenchymal stem cells (MSC), describing in detail the mechanisms through which MSC can repair damaged hearts.

13.2 Background

Acute myocardial infarction (AMI) is caused by the abrupt closure of a coronary artery primarily due to thrombus formation. The most effective therapy for AMI is represented by timely revascularization of the infarcted related artery (IRA), obtained with thrombolytic agents, percutaneous coronary intervention (PCI), or bypass surgery. With the advent of reperfusion therapies, the institution of intensive care units and the introduction of effective drugs like beta-blockers and ACE inhibitors, the occurrence of complications in patients with AMI has been reduced and life expectancy improved. Despite all these advances, AMI still produces significant morbidity and mortality especially in those patients who miss the window of opportunity for timely reperfusion. In patients with significant infarct size, ventricular remodeling ensues and often leads to CHF. Recently, stem cell administration has been under investigation as a possible regenerative/reparative therapy for AMI. This strategy is based on the hypothesis that certain multipotent stem cell types, once injected into the heart, would be able to repopulate the necrotic tissue and differentiate into new CMC, thus rescuing contractile function. Stem cell therapy has been tested also in models of chronic myocardial infarction (CMI) and chronic ischemic heart disease (CIHD).

13.2.1 Pathology of Acute Myocardial Infarct: The Traditional View

Following AMI, CMC begin to die starting from the endocardium. If blood supply is not restored within the first 6 h, all the cardiac tissue served by the IRA undergoes necrosis or apoptosis. The loss of myocardium initiates a complex multicellular process to repair the damaged tissue and maintain the structural integrity of the left ventricle. Different cell types are chemo-attracted to the infarcted area and participate in tissue repair. Inflammatory cells rapidly infiltrate the area of injury to remove necrotic and apoptotic CMC, biologically active mediators are activated and released, new blood vessels start sprouting from the native vessels surrounding the infarcted area [4]. This early inflammatory phase is normally followed by a fibrogenic phase. Fibroblast-like cells first appear at the border zone and slowly invade the infarcted region leading to scar formation. The surviving CMC become hypertrophic to compensate the loss of contracting tissue. In humans, the healing process takes from 6 to 8 weeks and leads to progressive changes in ventricular size, shape, and function. Until recently, the remodeling of the left ventricle was believed to be irreversible since the heart was considered a post-mitotic organ without any self-renewal capacity. However, recent evidence of cycling CMC in the postnatal heart [5–7] and the discovery of resident cardiac stem cells (CSC) [8–10] together with the demonstration of bone marrow (BM)-derived stem cells able to home in the heart and transdifferentiate into CMC [11, 12] have challenged the classic dogma that the adult heart is a post-mitotic organ and suggests the fascinating possibility that therapeutic myocardial regeneration might be achieved.

13.3 Adult Stem Cells for Cardiac Repair

The hypothesis that tissue regeneration/repair may be achieved by cells circulating in the bloodstream was proposed as early as the middle of the nineteenth century by Cohnheim [13]. Subsequent studies on wound repair focused on cells resident in the tissues such as pericytes, which are seen to proliferate during repair in most tissues. More recently, resident stem cells were discovered in a variety of tissues including muscle [14], fat [15] and liver [16], strengthening the theory of local repair as the sole mechanism for tissue regeneration. However, the recent observations on stem cell plasticity have largely revitalized Cohnheim's hypothesis and suggested that the stem cells found in most tissues may be replenished by stem cells for non-hematopoietic tissues mobilized from the BM. For these reasons, starting from the late 1990s, the cardiac regenerative capacity of a variety of multipotent ASC harvested from different sources has been experimentally tested both in vitro and in vivo.

Among ASC, CSC seem to possess all the properties required in order to achieve true cardiac regeneration, since they are autologous, can be expanded ex vivo, show proliferative restraint, and, most importantly, show the ability to differentiate into

EC, VSMC, and CMC that appear to become functionally integrated with the surrounding native myocardium [9, 17–19]. Skeletal myoblasts (SM) have been investigated both in experimental and clinical studies. However, their use in cardiac regenerative therapy has been questioned [20–22]. More recently, stem cells resident in other tissues such as fat, cord blood, and placenta have shown to rescue damaged hearts in animal models [23, 24]. However, much of the research in cardiovascular regenerative therapies, both in animals and in human beings, has been conducted using BM-derived stem cells. In particular, it has been demonstrated that administration of BM-MSC can rescue damaged hearts and improve cardiac function in MI animal models and improve vasculogenesis in chronic ischemia models [25]. In the following paragraphs we will analyze basic concepts that explain the therapeutic properties of MSC.

13.4 Mesenchymal Stem Cells

The BM stroma was originally thought to function mainly as a structural support for the hematopoietic stem and progenitor cells in the BM [26]. It is now clear that a heterogeneous population of cells including fibroblasts, adipocytes, EC, osteogenic cells, and adherent stromal cells compose the stroma. In the 1960s Ernest A. McCulloch and James E. Till first revealed the clonal nature of marrow stromal cells [27, 28]. In the 1970s Friedenstein and colleagues reported an in vitro assay for examining the clonogenic potentiality of stromal marrow cells [29–31]. In this assay, stromal cells were referred to as colony-forming unit fibroblasts (CFU-F). Subsequent experiments revealed the multipotentiality of marrow cells and how their fate was determined by environmental cues [32]. For instance, culturing marrow stromal cells in the presence of osteogenic stimuli such as ascorbic acid, inorganic phosphate, and dexamethasone promoted their differentiation into osteoblasts [33]; in contrast, the addition of transforming growth factor-beta (TGF-β) induced differentiation into chondrocytes [34]. Furthermore, it has been shown that these cells can differentiate into adipocytes, tendons, and muscle [35, 36].

Since stromal cells showed self-renewal, differentiation, and characteristics typically associated with stem cells, many investigators referred to cultured stromal cells as MSC. These cells are rare and exist at an estimated frequency of about 1 in 100,000 BM cells [37]. However, the MSC can be isolated and expanded ex vivo, primarily taking advantage of their specific capacity to adhere to plastic surfaces. Briefly, the BM mononuclear cells are isolated using gradient techniques and plated in tissue culture-treated plastic dishes. By changing the culture medium, non-adherent cells are removed so that only the stromal cells remain in the dish. After few days, CFU-F start becoming visible. As for the endothelial progenitor cells (EPC), many investigators use the CFU assay as a method to quantify stromal progenitors. Interestingly, it appears that a strong correlation exists between age and proliferative potentiality, with decreasing progenitor proliferation associated with increasing age. The cells forming the CFU-F have already acquired the majority of the surface

markers thought to be typically expressed by the MSC. These cells can be expanded for many passages without altering their phenotype and biological properties.

MSC in cell culture have been characterized using a panel of specific antibodies; however, there is still a lack of consensus on the definition of MSC since the medium and serum used to culture the cells, the plating density as well as the oxygen tension may affect the phenotype. In general, it is well accepted that human MSC (hMSC) lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules while hMSC do express SH2 (CD105), SH3 and SH4 (CD73), CD90, CD29 and CD166 [32, 38] (Fig. 13.1). Aside from this consensus in terms of hMSC surface antigen expression, the precise phenotype of hMSC in human BM is still debated and the identification of hMSC prior to culture remains ambiguous. Several groups have developed protocols to prepare more homogeneous MSC populations, but none of them has gained widespread acceptance. Interestingly, Prockop and collaborators have reported the existence of a subpopulation of cells in cultures of hMSC that are small, proliferate rapidly, undergo cyclical renewal when the cells are replated, and are precursors of more mature cells in the same cultures. These cells were referred to as recycling stem cells (RS) [39].

Peculiar characteristics make MSC interesting for cell therapy and tissue engineering purposes. For example, MSC can be isolated, expanded ex vivo, and used in an autologous fashion, avoiding the problem of finding a compatible donor. Furthermore, several lines of evidence suggest that MSC may not be subject to allogenic rejection in human and animal models [37, 40, 41]. Three main mechanisms seem to contribute to such immunoprivileged profile. First of all, MSC are hypoimmunogenic since they lack HLA class II and co-stimulatory molecules expression. Secondly, it has been shown that MSC prevent a T-cell response indirectly through modulation of dendritic cells and directly by suppressing natural killer cells as well as $CD8^+$ and $CD4^+$ T-cell function. Thirdly, MSC induce a suppressive local microenvironment through the production of prostaglandins and interleukins. If it was confirmed that MSC truly avoid allogenic rejection, it would be reasonable to start thinking about the institution of an international cell bank of hMSC isolated from the BM of young and healthy subjects. However, other evidence has challenged such an optimistic view and urged for additional experimental studies [42]. Another advantageous characteristic of MSC is that they are easy to modify ex vivo using viral vectors [43]. By overexpressing genes of interest, the functionality of MSC can be increased. For instance, MSC overexpressing antiapoptotic genes have shown to be more resistant to hypoxic stimuli compared with non-modified MSC [44]. Furthermore, MSC might be used as platform to deliver specific soluble proteins to the site of injury. For example, it has been demonstrated that MSC overexpressing VEGF improve vascular regeneration compared with non-modified MSC [45].

Outside the BM, MSC have been recently isolated from many other tissues; among them, fat tissue, cord blood, and placenta are the most common [23, 24]. Circulating MSC have also been described but the results are debated and not always reproducible [46]. Verfaille's group has described a population of multipotent adult progenitor cells (MAPC), that share many of the same characteristics of MSC [47]. However, differently from MSC, MAPC are reported to expand indefinitely and

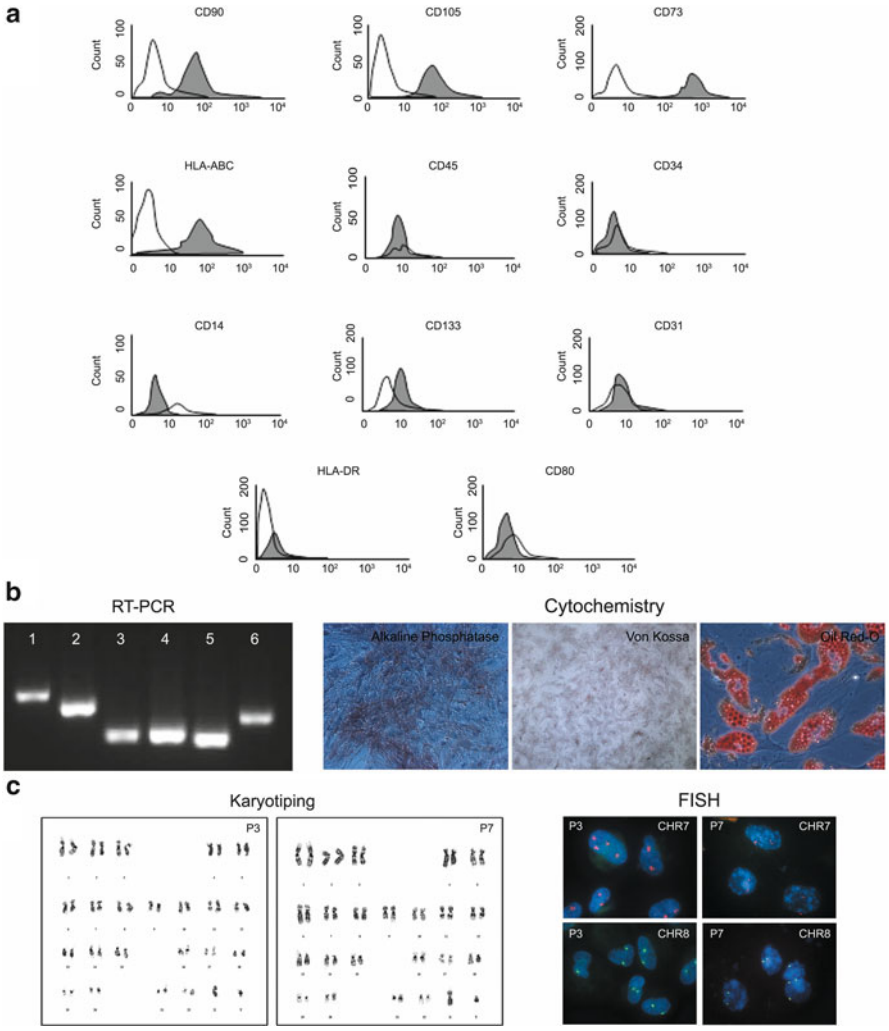

Fig. 13.1 Characterization of mesenchymal stem cells. (**a**) FACS analysis of MSC at P3 showing the typical antigen profile expressed by cells of mesenchymal origin. (**b**) MSC are multipotent. RT-PCR analysis (*left*) for osteocytes markers osteopontin (*1*), Cathepsin K (*2*) and Bone sialoprotein (*3*) or adipocyte markers ADFP (*5*) and PPARγ (*6*), show that using dedicated protocols it is possible to differentiate MSC into osteocytes and adipocytes. Glyceraldeyde 6-phophate dehydrogenase (*4*) was used as endogenous control. Cytochemical analysis (*right*) confirms the differentiation potential of MSC. The pictures show: alkaline phosphatase activity assay (phase contrast—10× magnification), Von Kossa staining (Bright field—2.5× magnification) and Oil Red O staining (phase contrast—40× magnification) after induction of MSC differentiation. (**c**) Karyotyping and FISH analysis of chromosome 7 (*red* signal) and 8 (*green* signal) with subtelomeric-specific probes in MSC at P3 and P7 does not show aberrations

appear to have an extended differentiation potential including ectodermal and endodermal lineages. Finally, a subpopulation of stem cells isolated at single-cell level and referred to as human BM-derived stem cells (hBMSC) has been identified [48]. The hBMSC self-renew without loss of multipotency for more than 140 population doublings and can differentiate into cells of all three germ layer.

13.5 Structural and Functional Effects of Mesenchymal Stem Cells on Infarcted Hearts

BM-MSC isolated from mouse, rat, swine, and humans have been administrated in experimental models of permanent coronary ligation, ischemia/reperfusion (I/R), and cryoinjury. The timing of administration varied from few minutes after injury to 4 weeks, when the acute inflammatory response to ischemia has subsided. Different routes of administration have been tested: direct intramyocardial injection, local coronary delivery, systemic intravenous infusion. A great variety of read-outs have been employed to quantitate the effect of stem cell transplantation into injured hearts. Traditional morphometric analyses documented an overall reduction in infarct size, less severe ventricular remodeling, and improved vascularization [49, 50]. Furthermore, BM-MSC administration ameliorated ventricular function in most cases. Cardiac performance in rodents has been measured both ex vivo, using Langendorff-perfused heart preparations, and in vivo, using intraventricular pressure transducers. Echocardiographic analyses have been performed to monitor function in both rodents and larger animals following cell transplantation. Additionally, ultrasonic crystals have been used to determine regional wall motion across the infarcted region of the heart of larger animals following cell transplantation.

These anatomical and functional assays demonstrated beneficial effects of BM-MSC but were unable to identify the underlying mechanism of stem cell action, that is distinguishing between direct function of the donor cells versus a beneficial effect imparted upon the surviving host myocardium. Understanding the mechanistic basis for limitation of ventricular remodeling and improved cardiac function is of critical importance when attempting to effect modifications aimed at enhancing the intervention. To determine the mechanism of action it is crucial to track the cells after transplantation and follow-up their fate in the heart.

13.6 Mechanisms of Action of Mesenchymal Stem Cells in Cardiac Repair

Transdifferentiation of transplanted stem cells into CMC and into vascular lineage cells has been originally proposed as the principal mechanism underlying the therapeutic action of MSC [37, 51, 52]. More recently, other investigators have failed to

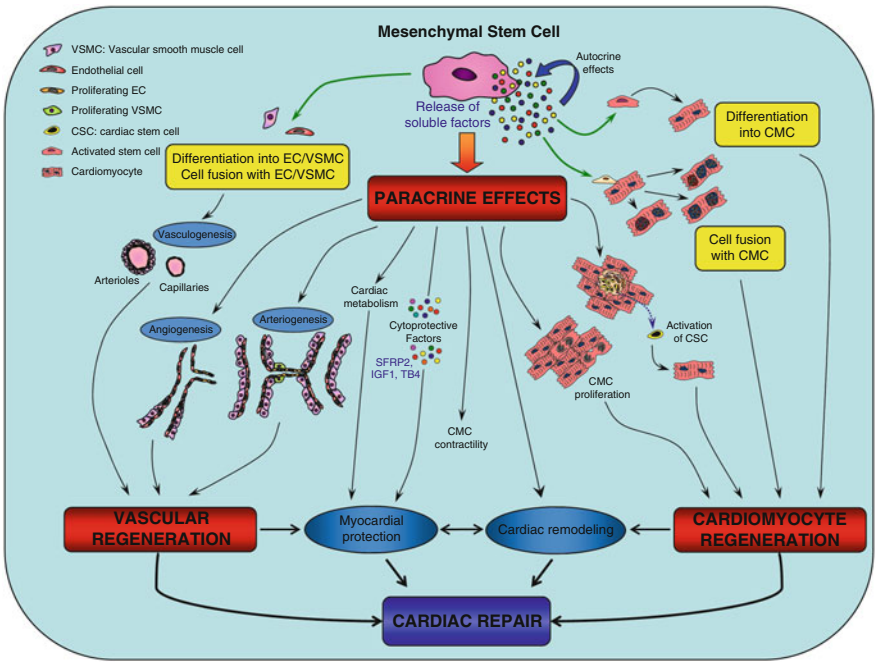

Fig. 13.2 Mechanisms of mesenchymal stem cell action in cardiac regeneration and repair. Cardiomyocyte and vascular regenerations represent the two mechanisms of action originally proposed to explain the reparative effects observed after MSC therapy in ischemic heart disease models. More recently, it has been demonstrated that soluble factors produced and released by MSC determine a series of beneficial paracrine effects, resulting in myocardial repair (see text for details)

detect permanent engraftment of transplanted BM-MSC [53, 54]. Furthermore, cell fusion of BM-derived donor cells with recipient CMC has been reported [55, 56]. Finally, so far it has not been possible to reproducibly induce a functional cardiac phenotype in BM-MSC in vitro using physiological growth factors or nontoxic chemical compounds. These negative results have questioned the plasticity of both endogenous and transplanted BM-MSC.

Regardless of whether stem cells transdifferentiate via a fusion-dependent or -independent mechanism, it has been shown that in many cases the number of newly generated CMC is too low to justify functional improvements. Therefore, it has been proposed that the functional benefits observed after MSC transfer in animal models of cardiac injury might be related to secretion of soluble factors that act in a paracrine fashion, protect the heart, cause attenuation of pathological ventricular remodeling, and promote neovascularization [57–59]. Accordingly, three mechanisms of action have been proposed for adult BM-MSC in heart repair: (1) CMC regeneration, (2) vasculogenesis, and (3) paracrine effects (Fig. 13.2). Regardless of the mechanism of action, there is a general agreement that BM-MSC transplantation is safe and has beneficial effects on infarcted hearts.

13.6.1 Cardiomyocyte Regeneration

Before examining the results supporting the cardiomyogenic potentiality of MSC, it is helpful to do a mathematical exercise in order to better understand what cell number is needed for the regeneration of myocardial infarct in humans. An adult heart contains approximately 20 million CMC per gram of tissue [60]. The average human left ventricle weighs ~200 g and therefore contains approximately four billion CMC. It has been estimated that the loss of 25 % of the left ventricle, corresponding to the loss of one billion CMC, leads to CHF [61]. Therefore, true cardiac regeneration would require restoring approximately one billion CMC synchronously contracting with the host myocardium. We anticipate that with the currently available technology this task is not achievable. However, substantial steps forward have been made and many investigators believe that this goal can be eventually accomplished. Several independent groups have reported cardiomyogenic differentiation of BM-MSC, both in vitro and in vivo [62–66].

13.6.1.1 Cardiac Differentiation In Vitro

Cardiomyogenic differentiation of mouse BM-MSC in vitro has been reported using culture medium supplemented with the demethylating agent 5-aza-cytidine (5-AZA) at a concentration of 3 μmol/l for 24 h [64]. Under these culture conditions, Makino and collaborators reported that the morphology of almost 30 % of the cells changed from fibroblast-like shape to a ball-like form and, with time, to the characteristic rod-shape myofibers. These differentiating cells tended to fuse in a syncytium resembling a myotube and started expressing fetal CMC markers [64]. In particular, the β-isoform of myosin heavy chain (β-MHC) was much more expressed than the α-isoform and specific transcription factors of the cardiac and myocyte lineage, including GATA-4, Nkx2.5, and HAND1/2 [62]. Furthermore, alternative splicing forms of the myocyte enhancer factor 2 (MEF2) gene were expressed by 5-AZA stimulated MSC. Indeed, MEF2A and MEF2B detected in early passage cells were replaced by MEF2C and MEF2D in late passage MSC, suggesting that the developmental program of gene expression would recapitulate the one observed during prenatal life. Electron microscopy revealed a CMC-like structure, such as the presence of sarcomeres, centrally positioned nuclei, and atrial granules. Importantly, it has been shown that MSC-derived CMC-like cells express functionally competent α- and β-adrenergic and muscarinic receptors on the membrane [63]. The differentiated cells are reported to beat spontaneously and synchronously in vitro and the rate of contraction increases after exposure to isoproterenol, whereas the addition of a selective β1 blocker inhibits contractile activity [63]. Other groups proposed that CMC-mediated contact is essential to induce MSC differentiation towards cardiac lineage [65, 66]. In one study hMSC were cocultured with human CMC in a ratio of 1:1 or cultured alone in the presence of medium conditioned by CMC. After 48 h of coculture, immunocytochemistry revealed that differentiating MSC expressed

sarcomeric myosin, β-MHC, cardiac troponin-T (cTnT), and cardiac troponin-I (cTnI) that were not expressed by the hMSC exposed to the conditioned medium [65]. The importance of cell-to-cell contact has been confirmed by Ashraf and collaborators, who cocultured MSC from green florescent protein (GFP)-transgenic mice with rat neonatal CMC [66]. After 7 days of coculture, ~14–32 % of MSC acquired the cardiac phenotype and started contracting synchronously with surrounding CMC. The presence of gap junctions between MSC-derived cardiac cells and neonatal CMC was documented by positive connexin-43 staining. Differentiation was confirmed by transmission electron microscopy analysis, showing a CMC-like ultrastructure, including sarcomeres, abundant glycogen granules, and a number of mitochondria. In a concomitant experiment, MSC separated from CMC by a semipermeable membrane did not differentiate into cardiac cells, confirming the pivotal importance of cell-to-cell contact.

13.6.1.2 Cardiac Differentiation In Vivo

Strong evidence in favor of MSC multipotency is derived from a study testing the fate of hMSC after systemic administration into fetal sheep early in gestation [67]. In this xenogenic system, hMSC engrafted and persisted in multiple tissues for as long as 13 months after transplantation. The cells underwent site-specific differentiation into chondrocytes, adipocytes, BM stromal cells, myocytes, and CMC. The presence of human cells was detected with an antibody specific for β-2 microglobulin or with in situ hybridization for human ALU sequences, and the differentiation into cardiac cells was established both by morphology and by staining with an antibody against SERCA-2. This study not only demonstrated that systemically administered hMSC can migrate across endothelial barriers, stably integrate into the heart, and differentiate into cardiac cells, but also that hMSC have unique immunologic characteristics that allow stable engraftment and the capacity to differentiate in a xenogenic environment. The cardiomyogenic potentiality of hMSC was further tested by Pittenger and collaborators, who injected LacZ labeled MSC into the left ventricular cavity of immunodeficient CB17 SCID/beige adult mice [51]. It was estimated that ~0.44 % of the injected cells survived in the myocardium 4 days after injection but much fewer hMSC were still present at later time points. The engrafted hMSC became with time morphologically similar to the surrounding CMC and aligned with them. Cardiac differentiation was confirmed by double staining with an anti-β-galactosidase antibody and specific cardiac markers. None of the hMSC expressed cardiac proteins 4 days after injection, but started to stain positive for desmin and cTnT at day 14. After 60 days the β-gal$^+$ cells also expressed α-actinin, β-MHC, and phospholamban. High-magnification view showed sarcomeric organization of the α-SA and cTnT positive cells, further demonstrating the ability of adult hMSC to undergo striated muscle differentiation in the heart.

Additional in vivo evidence of the cardiomyogenic potentiality of MSC came from Prockop's laboratory where the integration and differentiation of rat BM-MSC

were examined after transplantation into organogenesis-stage embryos [68]. GFP-tagged MSC were infused into 1.5- to 2-day-old chick embryos and surviving grafted cells were identified as GFP^+ cells 4 days after injection by quantitative PCR and immunohistochemical analysis. MSC expanded 1.3- to 33-fold in one-third of surviving embryos and integrated into multiple host tissues. In particular, the most common site of detection was the heart, even though the site of injection might have played a role in this particular model. Some of the GFP^+ cells found at the heart level expressed α-MHC and some cardiotin, a protein found in the longitudinal sarcoplasmatic reticulum of mature CMC. To exclude cell fusion as a mechanism of differentiation, the GFP^+ cells were retrieved by cell sorting from the dispersed embryos and karyotyping was performed. All the GFP^+ cells contained the normal complement of 42 rat chromosomes, and therefore they were distinctly different from chick cells that contain 78 chromosomes.

Taken together, these results support the concept that MSC can home to the intact myocardium and differentiate into cardiac-like cells. However, can MSC do the same in the presence of myocardial injury and regenerate the lost tissue? One of the most convincing answers to this question came from Fukuda's laboratory [69]. In a first set of experiments, a single hematopoietic $Lin^-CD34^-c\text{-}kit^+Sca1^+$ cell or BM-derived cells harvested from mice ubiquitously expressing GFP were transplanted into lethally irradiated syngeneic mice. AMI was then induced by coronary ligation and BM-derived cells were mobilized by granulocytes colony-stimulating factor (G-CSF) administration. Eight weeks after AMI only three $GFP^+/actin^+$ cells were found in the group transplanted with the single hematopoietic cell, while more than 5,000 $GFP^+/actin^+$ cells were detected in animals receiving BMC. These data suggested that most of the $GFP^+/actin^+$ cells were derived from non-hematopoietic BM-derived cells, most likely from MSC. To confirm this hypothesis, clonally purified MSC were tested using the same protocol. The MSC were transfected with a plasmid encoding GFP under the control of a cardiac-specific myosin light chain promoter. PCR analysis proved the engraftment of MSC in the BM of all recipients. Eight weeks after AMI a total of 1,034 GFP^+ cells were detected in the heart, indicating that MSC mobilized from the BM homed to the infarcted tissue and differentiated into cardiac cells. These data provide strong evidence that MSC can home to the heart after MI, engraft and differentiate into CMC. Furthermore, the results strongly suggest that the majority of BM-derived CMC homing to the heart after myocardial damage is MSC. However, the number of MSC-derived CMC is too low to achieve cardiac regeneration. It is then important to understand if an exogenous administration of MSC can do the job. As already reported, several studies have tested MSC transplantation in experimental myocardial injury models. Overall, the results show that the milieu surrounding the infarcted tissue seems to attract the MSC and promote their engraftment. Indeed, MSC preferentially home to the infarcted area and to the border zone when injected systemically, while their number in the remote areas is limited [70]. Importantly, it has been shown that after a few weeks, MSC engrafted at the border zone and differentiate into cells expressing a variety of cardiac-specific markers [70–73]. These cardiac-like cells seem also to be functionally connected with the native CMC since they express both connexin-43

and N-caderin [73], proteins responsible for cell-to-cell connection and electrical coupling. Unfortunately, even after direct intramyocardial injection, the efficiency of cell engraftment is low and no data are available regarding the replicative potentiality of MSC retained in the heart. Furthermore, the MSC engrafted in the infarct scar appear primarily fibroblast-like and lack the features typical of complete myogenic differentiation such as mature sarcomeric organization and intercalated discs [72]; this may speak to the importance of the local extracellular milieu in driving MSC differentiation. On the other hand, the fact that MSC present at the border zone do turn into cardiac-like cells may support the hypothesis that cell contact with intact CMC is crucial for proper MSC differentiation. Like for other BM-derived stem cell types, cellular fusion may represent a confounding factor when testing the regenerative capacity of MSC. For example, using a *Cre-lox* recombination system, our group has shown that mouse BM-MSC injected into infracted hearts can fuse with resident CMC [55]; however, the frequency of cell fusion was low.

Beside all the mechanistic hypotheses, it is reasonable to conclude that MSC can differentiate into cardiac-like cells in vivo. However, the low efficiency of cardiac regeneration from donor MSC is not sufficient to explain the important beneficial effects observed by the majority of the researchers in terms of both ventricular remodeling and cardiac function after MSC administration. More studies are needed to better understand the signals addressing MSC differentiation towards cardiac lineage in order to be able one day to achieve cardiac regeneration using MSC.

13.6.2 Vasculogenesis

Generating a functional and stable microvasculature network remains one of the major challenges in tissue regeneration and repair. The development of mature vessels relies not only on endothelial proliferation and migration, since cooperation between endothelial cells and pericytes is fundamental for vascular development and maturation. Blood vessels derive from mesodermal precursors called angioblasts early during embryogenesis [74]. In this process, termed vasculogenesis, precursor angioblasts differentiate into EC forming a vascular network. This primordial plexus is refined into a functional network by a process where vessels undergo extensive elongation and maturation [75].

In contrast to the embryonic heart vasculature, the adult heart vessels are quiescent. Only when under stress or pathologic conditions, like MI, the coronary vascular bed expands [74]. Postnatal neovascularization encompasses three different mechanisms: the first is referred to as angiogenesis and consists in the sprouting of new vessels from preexisting vessels. The second mechanism is collateral enlargement and muscolarization, namely arteriogenesis. Recently, a third mechanism has been demonstrated, postnatal vasculogenesis, that consists of the assembly of new blood vessels by differentiation of endothelial precursors originating from the BM [76]. Based on this rationale, administration of BM-MSC has been proposed as a novel strategy to induce therapeutic vasculogenesis. Almost all the experimental

studies testing the potential of MSC to induce vascular regeneration have shown an increase in capillary density and improvement in tissue perfusion. However, it is still debated if differentiation of MSC into EC and VSMC rather than generation of new pericytes and/or release of paracrine mediators represents the main mechanism of action [37, 49, 77]. In support of the first hypothesis, it has been reported that after 15 days in culture, MSC start expressing αSM actin and β-actin filaments, which are, respectively, specific to smooth muscle and non-muscle cells, but they do not express CD31 [78]. Immunofluorescence studies revealed that, once injected into infarcted hearts, some engrafted MSC expressed the smooth muscle phenotype (αSM actin$^+$) while some acquired an endothelial phenotype (CD31$^+$); furthermore, vessel density was augmented in the MSC group in comparison with the control group. In another experimental model of myocardial infarction in rats, MSC differentiation into endothelial phenotype enhanced microvascular density and improved heart function [79]. Also, when tested in chronically ischemic dog hearts, MSC treatment resulted in a trend toward reduced fibrosis and greater vascular density with immunohistological evidence of colocalization of engrafted MSC with EC and smooth muscle cells markers [80]. More recently, it has been confirmed in a pig model that MSC can differentiate into EC as early as 72 h and persist in chimeric vessels at least up to two weeks even though the number of differentiated cells was low [81].

Despite this evidence, some investigators believe that MSC, rather than differentiate into EC and VSMC, participate in vasculogenesis by turning into pericytes that stabilize and favor the maturation of the new vessels [82]. For example, it has been shown that co-implantation of EPC with human MSC into immunodeficient mice results in formation of extensive vascular networks after one week [83]. The presence of human EPC-lined lumens containing erythrocytes throughout the implants indicated not only a process of vasculogenesis from the two cell types, but also the formation of functional anastomoses with the host circulatory system. Importantly, MSC were shown to reside in perivascular locations around the engineered lumens, confirming their active participation in blood vessel assembly. The results are similar and support another report, where human MSC combined with human umbilical vein EC were shown to facilitate blood vessel assembly and adopt a perivascular location and phenotype [84]. Similar to normal pericytes, human MSC-derived perivascular cells contracted in response to endothelin-1 in vivo. Importantly, the authors remarked that they could not detect differentiation of human MSC into endothelial cells in vitro, and that MSC alone could not form conduit for blood flow in vivo [84]. Importantly, there is evidence showing that MSC may have a perivascular origin in multiple human organs; thus, blood vessel wall harbors a reserve of progenitor cells that may be integral to the origin of MSC [85].

Beyond all the controversies, in the majority of the animal studies only a limited number of engrafted MSC stained positive for EC and VSMC markers, suggesting that the direct role of MSC in neo-vasculogenesis is limited. In contrast, as discussed in more detail below, there is solid evidence supporting the key role of MSC as regulators of vascular regeneration via paracrine mechanisms.

13.6.3 Paracrine Effects

There is a growing body of evidence supporting the hypothesis that paracrine mechanisms mediated by factors released by the MSC play an essential role in the reparative process observed after stem cell injection into infarcted hearts. Paracrine secretion has been recognized for more than 15 years, since Haynesworth et al. [86] reported that MSC synthesize and secrete a broad spectrum of growth factors and cytokines such as VEGF, FGF, HGF, insulin growth factor-1 (IGF-1), SDF-1, and thrombopoietin. The mechanisms mediating the effects of these paracrine factors are numerous. Cytokines and growth factors may favor neovascularization, cytoprotection, and endogenous cardiac regeneration. Furthermore, the post-infarction inflammatory and fibrogenic processes, cardiac contractility, and cardiac metabolism may also be influenced in a paracrine fashion (Fig. 13.2).

Despite evidence that BM-MSC incorporate into vascular structures, as discussed earlier, several studies suggest that only a small number of vessels contain donor cells. Nevertheless, BM-MSC lead to a significant increase in capillary density and collateral development when transplanted into ischemic tissues. The molecular processes leading to angiogenesis and arteriogenesis include the pivotal role of nitric oxide, VEGF, bFGF, HGF, angiopoietin, and others. These molecules lead to EC and VSMC migration, proliferation, vessel enlargement and maturation, and synthesis of extracellular matrix. Interestingly, it has been shown that BM-MSC express several pro-angiogenic and pro-arteriogenic factors. Accordingly, it has been proposed that the release of these factors by transplanted stem cells may play an important role in determining the increase in capillary density and collateral development observed in ischemic tissues of animals treated with MSC.

Epstein and collaborators have suggested that local delivery of MSC augments collateral perfusion through paracrine mechanisms [59]. These authors injected 1×10^6 MSC in the adductor muscle of mice 24 h after femoral artery legation. Compared with controls injected with medium or mature EC, distal limb perfusion improved and conductance vessels increased in number and total cross-sectional area. Surprisingly, labeled MSC were tracked dispersed between muscle fibers, but were not seen incorporated into mature collaterals. On the other hand, protein levels of VEGF and bFGF were significantly increased in the muscle of MSC-treated animals compared with controls. Furthermore, colocalization of VEGF and transplanted MSC within adductor tissue was documented. Consequently, the authors concluded that MSC contributed to collateral remodeling through paracrine mechanisms. Gene expression profiling of MSC grown under normal conditions or under hypoxia allowed to document that these cells express a wide range of arteriogenic cytokines at baseline and that several of them are up-regulated by hypoxia [87]. The gene array data were confirmed using ELISA assays and immunoblotting of the MSC conditioned media (CM). Furthermore, it was shown that MSC-CM promoted proliferation and migration of EC and VSMC in a dose-dependent manner in vitro and enhanced collateral flow recovery and remodeling in a model of hind limb ischemia in vivo. Other studies, testing MSC transplantation in experimental

infarcted hearts, reported an increase in capillary density in treated animals compared with controls, despite the presence of few EC of donor origin [70, 88, 89]. In these cases, even though not directly proven, a pro-angiogenic paracrine action seems to be the most reasonable explanation to the effects observed.

Our group expanded the spectrum of stem cell paracrine actions by demonstrating that BM-MSC exert direct cytoprotective action on ischemic CMC. In particular, we clearly showed that cell culture medium conditioned by hypoxic MSC can reduce apoptosis and necrosis of isolated rat CMC exposed to low oxygen tension [57]. The cytoprotective effect was greatly enhanced in MSC overexpressing the gene Akt-1 (Akt-MSC) in vitro. To further validate the protective properties of the Akt-MSC, we studied the effect of the CM in vivo, using a rat experimental model of permanent coronary occlusion. Concentrated CM (C-CM) obtained by ultrafiltration was injected into the heart at the infarct border zone 30 min after left coronary occlusion. After 72 h, the infarct size and the CMC apoptotic index were significantly lower in animals treated with C-CM from Akt-MSC compared to controls. Of note, C-CM from non-modified MSC reduced infarct size compared with saline but the results were not statistically significant, confirming that Akt overexpression enhanced the production of cytoprotective factors. In a follow-up study we confirmed our earlier results and documented how the limitation of the infarct size was matched by preservation of cardiac function. Importantly, the data obtained with C-CM injection essentially replicated the results observed with MSC transplantation in terms of both infarct size and cardiac function, confirming that cytoprotection was the main mechanism of stem cell action in our experimental model. To verify whether Akt overexpression truly up-regulates the expression of secreted factors, we tested by quantitative RT-PCR some candidate genes encoding molecules known to be released by the MSC. Our data showed that VEGF, bFGF, HGF, insulin growth factor 1 (IGF-1), and thymosin ß4 (TB4) were significantly up-regulated in the Akt-MSC at baseline normoxia and increased further after exposure to low oxygen tension. Hypoxic non-modified MSC also up-regulated VEGF, bFGF, HGF, and TB4 even though significantly less than the Akt-MSC. Interestingly, it has been reported that direct injection of TB4 limits myocardial infarct size and induces functional recovery in the same experimental model used in our studies [90]. Our original findings in rodents have been successfully replicated by others also in a large animal model. Indeed, Akt-MSC injected into pig infarcted hearts led to limitation of infarct size and preservation of heart function [91]. Furthermore, other groups have confirmed the paracrine cytoprotective effects exerted by BM-derived stem cells on ischemic CMC [92–94].

Beside cytoprotection, paracrine factors released by transplanted stem cells may alter the extracellular matrix, resulting in more favorable post-infarction remodeling and strengthening of the infarct scar. For example, it has been shown that direct hMSC injection into ischemic rat hearts decreases fibrosis, apoptosis, and left ventricular dilatation while increases myocardial thickness. This resulted in the preservation of systolic and diastolic cardiac function without evidence of myocardial regeneration [95]. It is likely that MSC achieve this preservation of cardiac function, in addition to myocardial salvage, by acutely increasing cellularity and decreasing

production of extracellular matrix protein collagen type I, collagen type II, and tissue inhibitor of metalloproteinase-1 that results in positive remodeling [96]. Furthermore, stem cells may also produce and release local signaling molecules that limit local inflammation when injected into injured tissues. This hypothesis seems to be supported by the fact that expression profiling of adult progenitor cells reveals characteristic expression of genes associated with enhanced DNA repair, up-regulated antioxidant enzymes, and increased detoxification systems.

It has also been suggested that ASC may positively influence cardiac metabolism and contractility. Feygin and collaborators demonstrated that the border zone of infarcted pig hearts is affected by profound bioenergetic abnormalities which are partially attenuated after MSC transplantation [97]. Because of the low cell engraftment, the authors postulated that MSC did not provide a structural contribution to the damaged heart and concluded that the observed beneficial effects likely resulted from paracrine repair mechanisms. We have recently reported that Akt-MSC prevent metabolic remodeling in infarcted rat hearts [98]. Treatment with Akt-MSC spared phosphocreatine stores and significantly limited the increase in 2-DG uptake in the residual intact myocardium compared with the saline- or the MSC-treated animals. Furthermore, Akt-MSC-treated hearts had normal pH, whereas low pH was measured in the saline and MSC groups. We have also observed that cell shortening, maximal rate of relengthening ($+dL/dt$), and maximal rate of shortening ($-dL/dt$) of isolated adult rat CMC are significantly improved in the presence of CM from hypoxic MSC, particularly Akt-MSC, compared with standard conditions. Results from other groups seem to confirm that ASC can release inotropic factors [99].

Other evidence suggests a further intriguing hypothesis: exogenous stem cell transplantation may activate resident CSC and/or stimulate CMC replication via paracrine action, thus improving endogenous cardiac regeneration. For example, it has been shown that intramyocardial administration of HGF and IGF-1 at the infarct border zone induces CSC migration, proliferation, and differentiation [9]. Since MSC release both HGF and IGF-1, particularly under hypoxic stimulation [58], it is reasonable to hypothesize that MSC injected into ischemic hearts may attract and activate resident CSC. Indirect evidence supporting this hypothesis has been documented in a study in which MSC were injected into infarcted pig hearts [71]. Immunohistochemical analysis performed after 10 days revealed the presence, only in MSC-treated animals, of newly formed CMC, some of which stained positive for c-kit and others for Ki67. Unfortunately, the co-staining for c-kit and Ki67 was not performed, so that the true origin of those replicating CMC could not be determined. However, the authors concluded that endogenous cardiac regeneration was present. Also, the administration of hBM-MSC seems to determine proliferation of host CMC [48]. The RNA levels of HGF, IGF-1, VEGF, and bFGF were significantly up-regulated in hBM-MSC-treated hearts compared with controls. Interestingly, in the same study the paracrine cytoprotective and pro-angiogenic actions exerted by BM-derived stem cells were further confirmed. Recently, more direct evidence that BM-MSC stimulate proliferation and differentiation of endogenous CSC has been produced [81]. Transendocardial injection of GFP-MSC was performed in a pig model of ischemia/reperfusion injury: a detailed tissue analysis clearly showed that

MSC stimulate endogenous CMC turnover in two likely related ways: by stimulating endogenous c-kit^{+} CSC and by enhancing CMC cell cycling.

13.7 Discovery of Mesenchymal Stem Cells-Derived Therapeutic Molecules

The demonstration that BM-MSC, particularly those which are genetically modified, may secrete therapeutic molecules provides a potential breakthrough in that, rather than administering cells, one may be able to administer specific proteins or other soluble factors produced by these cells for cardiac therapy [43]. In this contest, Akt-MSC revealed themselves as a new model to identify possible novel cytoprotective molecules. In general, testing the properties of CM is particularly straightforward, both in vitro and in vivo. However, identifying the nature of specific factors involved in cardiac repair is a much more demanding task, although extremely relevant and worth pursuing (Fig. 13.3). Hypothesizing that the paracrine mediators are proteins or peptides, there are basically two approaches to identify them: transcriptomics and

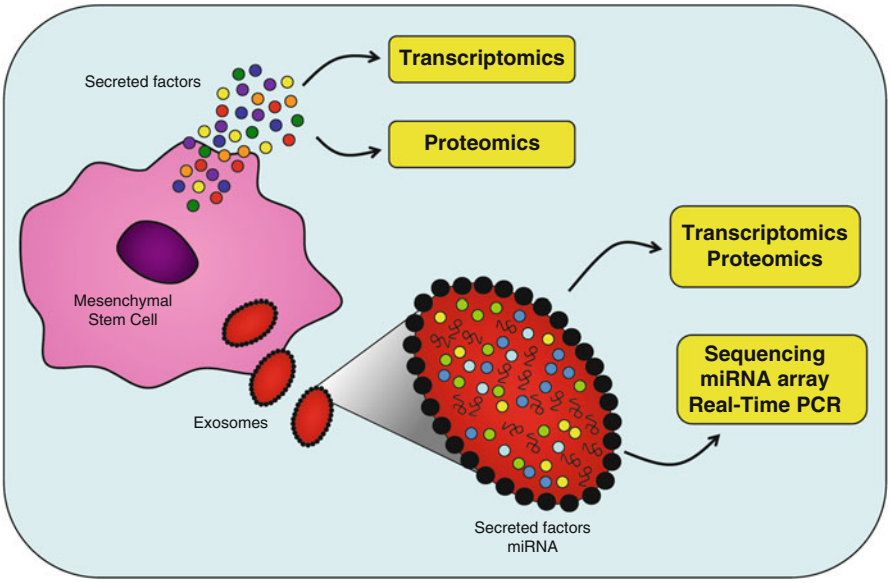

Fig. 13.3 Different approaches to identify putative paracrine mediators. To identify secreted paracrine factors, multiple experimental approaches can be used. The most common are transcriptomics and proteomics. The discovery that exosomes are involved in paracrine-mediated effects, has opened new scenarios. It is thought that exosomes act either by activating specific cell receptors or by releasing proteins and miRNA inside the target cells. Accordingly, to profile the content of exosomes it is possible to use transcriptomics, proteomics, or miRNA array profiling

proteomics. Each of these methods has pros and cons that will be discussed together with specific examples of paracrine factor discovery.

Among the different approaches possible, our group chose to perform a wide microarray expression analysis of Akt-MSC vs native MSC [100]. Although the genomic approach, as compared with a proteomics, can overlook important post-transcriptional events such as alternative splicing, it is currently more comprehensive, technically less demanding, and enables easier identification of novel genes with previously unknown cell survival effects. Approximately 650 transcripts were differentially regulated between Akt-MSC and control MSC. A sub-analysis of the data revealed 62 transcripts encoding for 51 unique genes potentially contributing to the paracrine effects of Akt-MSC. Among these up-regulated genes, the secreted frizzled related protein 2 (Sfrp2) was the most dramatically up-regulated. Microarray data were confirmed by quantitative RT-PCR and by Western blot analysis. We have demonstrated that Sfrp2 exerts cytoprotection on ischemic CMC and that the pro-survival effect of Akt-MSC was markedly attenuated upon knockdown of Sfrp2 with siRNA. Furthermore, we have shown that the mechanism of action of Sfrp2 is through modulation of Wnt signaling pathway. The cardioprotective and additional beneficial properties of Sfrp2 on damaged hearts were recently confirmed by other investigators [101].

Transcriptomic analysis was used to identify factors responsible for tissue repair observed after intravenous (iv) injection of hMSC in a mouse model of MI [102]. Administration of hMSC results in a high number of cells trapped predominantly in the lung. Data from microarray gene expression analysis indicated that after embolization in lung, 451 human transcripts of hMSC were up-regulated. These transcripts were examined to identify putative genes of interests and TNFAIP6 (TSG-6), previously shown to be a powerful anti-inflammatory factor, emerged as one of the most solid candidates, with a 47-fold increase. Knock-down experiments showed that iv injection of hMSC, but not hMSC transduced with TSG-6 siRNA, limits inflammatory responses and infarct size [102]. Moreover, iv administration of recombinant TSG-6 also reduced inflammatory response and infarct size, confirming the importance of this soluble factor in tissue repair.

Proteomic analysis is an alternative approach to identify putative paracrine factors, although sample preparation still represents a major hurdle. One of the problems with this approach is that proteins are usually secreted at low concentration in the culture media, making it difficult to recover these factors. In addition, culture media are rich in salts and other compounds that interfere with most proteomic techniques, making selective precipitation of proteins almost mandatory for a correct analysis. In addition, the presence of serum proteins, even in trace amounts, dramatically influences the dynamic range of the sample and consequently the identification of secreted proteins. Thus, the development of reliable methods for profiling secretory proteins is highly desirable. Efficient methodologies should be developed for the enrichment and analysis of the secretome of different cell lines, free of essential contaminants. These new methods should encompass the optimization of cell incubation conditions in serum-free medium, the sub-fractionation of the CM with appropriate chromatographic techniques, the establishment of

biochemical assays to monitor the paracrine effects of the isolated protein, and the use of the CM in heterologous cell systems for biological assays.

Despite these limitations, several interesting results have already been obtained with proteomics. For instance, two-dimensional liquid chromatography tandem mass spectrometry (LC-MS/MS) has been used to globally profile the proteome of murine MSC (mMSC) [103]. Using this technique, it was possible to identify 258 proteins specifically expressed by mMSC, 54 of which were classified as secreted proteins. In another study, a chemically defined serum-free medium was conditioned by MSC derived from hESC using a clinically compliant protocol [104]. The CM was analyzed by multidimensional protein identification technology and cytokine antibody array analysis and revealed the presence of 201 unique proteins. Computational analysis predicted that these factors are involved with three major groups of biological processes: metabolism, defense response, and tissue differentiation, including vascularization, hematopoiesis, and skeletal development. Furthermore, several of these proteins are known to be activators of important signaling pathways in cardiovascular biology, bone development, and hematopoiesis such as Jak-STAT, MAPK, Toll-like receptor, TGF-β, and mTOR.

The administration of soluble factors instead of stem cells may be more easily translated into the clinical arena since it has several obvious advantages. Indeed, this strategy would bypass most of the issues associated with cell-based therapy, i.e., immune compatibility, tumorigenicity, xenozootic infections and waiting time for ex vivo expansion of autologous cell preparations. Such an approach would have a greater potential for the development of "off-the-shelf" stem cell-derived products. However, there are technical problems related to protein/peptide administration. The most obvious limitation of protein therapy is represented by the necessity to maintain therapeutic concentrations in order to induce the desired effect for the necessary length of time. Establishing the threshold concentration and the necessary time remains to be determined and represents a difficult task. Different actions may require different concentrations and timing. Substantial differences between animal models and humans further complicate the scenario. For example, it has been shown that a single dose of specific growth factors is effective in enhancing neovascularization in animals but not in humans [105]. Other possible hurdles are represented by protein stability and pharmacokinetic. To overcome these problems, a variety of strategies have emerged for manipulating protein properties, stability, specificity, immunogenicity, and pharmacokinetic [106]. Mechanisms for altering these properties include manipulation of primary structure, incorporation of chemical and post-translational modifications, and utilization of fusion partners. The protein and peptide therapeutics have already become an important class of drugs due to advancements in molecular biology and recombinant technologies. Currently, most therapeutic proteins are administered by the parenteral route which has many drawbacks. Various delivery strategies have evolved over the past few years to improve delivery of proteins and peptides, including the use of biopolymers and nanomaterials for controlled release of proteins [107], and delivery via noninvasive routes such as subcutaneous release or dermal patches. Noninvasive approaches remain challenging due to poor absorption and enzymatic instability, pharmacokinetics and

pharmacodynamics of protein therapeutics. Development of an oral dosage form for protein therapeutics is still most desirable, although it presents a greater challenge. Even though the road to reach optimal protein therapy has numerous hurdles, we anticipate that the constant development and application of rational protein design technology will enable significant improvements in the efficacy and safety of existing protein therapeutics, as well as allow the generation of entirely novel classes of proteins and modes of action. In this case, curing AMI with a single protein or, most likely, with a cocktail of proteins may become reality.

13.8 Exosomes

Recently, it has been proposed that the beneficial paracrine effects observed after MSC therapy are mediated by exosomes [108] (Fig. 13.3). Lai and colleagues demonstrated, through size fractionation studies, that the active component in CM was a large complex 50–200 nm in size. They purified exosomes from CM of MSC derived from human embrionic stem cells (hESC) by size exclusion using high-performance liquid chromatography and demonstrated that exosomes reduce infarct size while the CM deprived of exosomes do not [108]. The secretion of cardioprotective exosomes is not unique to hESC-MSC and was also found in MSC derived from different sources [108]. Altogether, these observations suggest that the secretion of protective exosomes is a characteristic of MSC and may be a reflection of the stromal support role of MSC in maintaining a microenvironmental niche for other cells such as hematopoietic stem cells. Besides cytoprotection, it has also been suggested that exosomes can act directly through the interaction ligand/receptor or indirectly on angiogenesis by modulating soluble factor production involved in endothelial and progenitor cell differentiation, proliferation, migration, and adhesion [109]. For instance, exosomes generated from platelets play an interesting beneficial pro-angiogenic role in a model of myocardial ischemia by delivering a cocktail of pro-angiogenic proteins, such as VEGF, basic fibroblast growth factor, and PDGF, has been demonstrated [110].

Compared with other secreted vesicles, exosomes have much better defined biophysical and biochemical properties. They are small membrane vesicles (between 30 and 100 nm in diameter) of endocytotic origin that are secreted by most cells in culture. They seem to form by invagination and budding from the limiting membrane of late endosomes, resulting in vesicles limited by a lipid bilayer containing cytosol from the producing cells and exposes the extracellular domain of various transmembrane proteins at their surface. As a bi-lipid membrane vesicle, exosomes not only have the capacity to carry a large cargo load, but also protect the contents from degradative enzymes or chemicals: proteins, RNA and microRNA (miRNA) contained in MSC exosomes are protected from degradation by trypsin and RNase as long as the lipid membrane is not compromised [108, 111]. Most exosomes have an evolutionary conserved set of proteins, but they have a unique tissue/cell type specific proteins that reflect their cellular source. They have been shown to be

secreted by many cell types [112–119]. They are also found in physiological fluids such as normal urine [120], plasma [121], and bronchial lavage fluid [122].

Exosomes are known to bear numerous membrane proteins that have binding affinity to other ligand on cell membranes or the extracellular matrix, such as the transferrin receptor, tumor necrosis factor receptors, integrins, and tetraspanin proteins (e.g., CD9, CD63, and CD81). These membrane bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment. For example, integrins on exosomes could home exosomes to CMC that express ICAM1, a ligand of integrins after myocardial I/R injury [123], or to VCAM-1 on EC [124]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion and fusion events, could facilitate cellular uptake of exosomes by specific cell types.

Several studies have analyzed the biological activities of exosomes, but little is known about their possible functions in vivo. They are believed to be important for intercellular communication. Exosomes may also facilitate the uptake of therapeutic proteins, RNA or miRNA into injured cells. It was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [125]. This may be a mechanism by which MSC exosomes exert their cardioprotective effects on ischemic CMC that have a low intracellular pH [126]. During myocardial I/R injury, the restoration of blood and oxygen to ischemic myocardium paradoxically exacerbates the ischemia-induced cellular insults. It has been hypothesized that with their complex cargo, exosomes would have adequate potential to participate in a wide spectrum of biochemical and cellular activities, simultaneously target and correct the various ischemia-induced cascades, and prevent occurrence of the paradoxical reactions induced by reperfusion. In addition, many of the proteins in the exosomes are enzymes. Since enzyme activities are catalytic rather than stoichiometric and are dictated by their microenvironment (e.g., substrate concentration or pH), the enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which in turn, is proportional to the severity of disease-precipitating microenvironment.

Despite this encouraging evidence, the translation of cardioprotective MSC-derived exosomes into a therapeutic agent presents several unique challenges. The major challenge would be to produce good manufacturing practices (GMP) grade exosomes from non-autologous cell sources. Finding a robust scalable and highly renewable cell source will be central to the development of a commercially viable manufacturing process for the production of MSC exosomes in sufficient quantity and quality to support clinical testing or applications. To address this issue, it has been proposed to immortalize ESC-derived MSC by *Myc* [127]; as this modification is thought to not compromise the quality or yield of exosomes. The translation of MSC-derived exosomes into clinical applications is also complicated by the relative novelty of exosomes with few precedents in the regulatory and safety space of biopharmaceuticals. This will require the formulation of new standards for manufacture, safety, and quality control.

13.9 Future Perspectives

Although MSC therapy holds promise in the future treatment of heart disease such as AMI, CIHD, and CHF, its current use is significantly hampered by biological and technological challenges. One of the major problems is represented by the extensive loss of cells after transplantation. Many studies have shown that the majority of cells successfully delivered to the heart die within the first weeks [77]. The causes of cell death in AMI setting are multifactorial and are influenced by the ischemic environment, which is devoid of nutrients and oxygen, coupled with the loss of survival signals from matrix attachments and cell–cell interactions. We and others have conceptualized the idea of improving cell survival by overexpressing protective genes [128]. To optimize this approach, one may consider the use of viral vectors encoding multiple cytoprotective genes, acting on different cell death and apoptosis pathways. Recently, it has been proposed that preconditioning of stem cells with different cytokines may result in improved cell engraftment [129]. The combination of genetic modification and preconditioning may further enhance cell survival and engraftment. An alternative method would be to seed cell ex vivo on a biodegradable polymeric scaffold, followed by in vivo engraftment instead of injecting the cells directly into the site of injury [130]. Improvement of MSC survival will ultimately allow us to address cell scalability and to make cell-based therapies more easily applicable to humans.

Safety concerns regarding the proarrhythmic effects following transplantation of MSC for cardiac repair must be taken into consideration. So far, BM-derived stem cells have not shown proarrhythmic effects in early clinical trials. However, it is not known whether increasing the number of cells that survive or the number of the cells injected will provoke an arrhythmogenic reaction. Tailored preclinical studies should be carried out in small and large animal models with the specific aim to rule out completely that MSC can induce arrhythmias.

Other unresolved issues are the efficacy and safety concerns surrounding the changes in gene expression and functional properties of MSC with advancing age and disease [131]. The properties of self-renewal and lineage potentiality of cells harvested from high-risk patients may be affected. We know that both the age and the presence of disease status adversely influence several aspects of the intrinsic characteristics of ASC. For instance, EPC from patients with cardiovascular disease display varying degrees of functional impairment and an inverse correlation has been reported between the number of circulating EPC and the prevalence of risk factors for CAD [132]. If these deficiencies were also demonstrated for MSC, they may limit the therapeutic application of individualized treatment using a patient's own isolated MSC.

Importantly, the morphological and histological complexity of the myocardium should not be overlooked when designing cell-based protocols for cardiac grafting. The expectation that injecting a stem cell may result in the regeneration of new functionally competent cardiac tissue may be excessively simplistic. Even though the anatomy and the physiology of the heart are not as complicated as other organs

such as the pancreas or the kidney, rebuilding new cardiac tissue may not be an easy task. The myocardium consists of a variety of cell types including CMC, fibroblast, EC, and other vascular cells embedded in a complex extracellular matrix that provides the scaffolding for the three-dimensional alignment of the various components which is required for proper mechanical and structural function. This level of complexity raises caution against designing overly simplistic grafting protocols based on a single cell type. It may well be that the optimal grafting procedure for cardiac repair requires more than one cell type and/or biomaterials to produce a graft that is able to recapitulate normal cardiac function. In this regard, we strongly believe that tissue engineering will likely play a pivotal role and will advance the field of regenerative medicine [133].

Finally, the demonstration of paracrine/autocrine mechanisms improves our understanding of MSC biology and action in tissue repair and regeneration. It is evident that the improvement in cardiac function following MSC therapy can be attributed mainly to the release of key paracrine factors by stem cells in the injured myocardial microenvironment. A growing body of evidence strongly suggests that these secreted molecules mediate a number of protective mechanisms including cell survival, neovascularization, remodeling, and proliferation. The regulatory machinery governing paracrine factor release appears to be complex and dependent on spatiotemporal parameters. Advances in profiling technologies continue to identify significant secreted factors that mediate cardiac repair mechanisms. The potential for magnifying stem cell-mediated paracrine effects using "engineered," "conditioned," or other ex vivo manipulated stem cells will significantly propel this type of therapy forward and provide invaluable information regarding stem cell biology.

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Chapter 14
Advances in Lentiviral Vector-based Cell Therapy with Mesenchymal Stem Cells

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Abstract The field of possible application of mesenchymal stem cells in medicine and research expanded tremendously with the advent of improved Lentiviral-vectors capable of inserting stable copies of genes of interest and expressing proteins or biologically active RNA species ad libitum, performing delicate gene editing or active gene silencing or serving as advanced drug delivery systems utilized in ex vivo cell therapy. The combination of these two fields has created a number of new areas of research in the landscape of modern medicine which are now extensively studied and discussed here. These areas include tissue engineering, tissue repair, wound healing and tissue implants, anticancer therapies, angiogenesis, myocardial infarction and repair as well as understanding and treating acute lung damage and injury. In addition, genetically modified, tagged MSCs are being intensively deployed in research and therapeutic attempts of the various ailments of the central nervous system including Parkinson's disease, Alzheimer's disease, various phases of acute ischemia and trauma. The emergence of new and important data for type II diabetes research is being followed with treatment suggestions and studies of senescence to find novel applications for genetically engineered MSCs. We find in general that genetically modified MSCs are at the cusp of breaking through from basic research into the next phase of clinical trials.

Keywords Alzheimer's disease • Angiogenesis • ARDS • CD105(+), CD90(+), CD73(+), CD14(−), CD19(−) or CD79a (−), HLA-DR1 (−) • CD105 (+), CD90 (+), CD73 (+), CD34 (−), CD45 (−), CD11b (−) • Cell therapy • Cerebral ischemia • Chronic granulomatous disease • Cystic fibrosis • Diabetes • Drug delivery • Duchenne muscular dystrophy • Gene editing • Gene silencing • Gene therapy • Lentiviral vector

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safety • Lentiviral vector • Lysosomal storage diseases • Myocardial infarction • Parkinson's disease • Periodontal stem cells • Progeria • Pseudotyping Lentiviral vector • Tissue engineering • Traumatic spine injury • Wilson disease • Wound healing

14.1 MSCs as Targets for Lentiviral Vector

Stem cells, including the mesenchymal stem cells (MSCs), are very close manifestations of Plato's imagery of the shadows on the cave wall, since they are difficult to study outside their intimate interactions with their microenvironment [288]. Our observation methods change their responses and characteristics [9, 74, 82, 103, 145, 252], as in quantum physics, when the observation changes the observed. With that caveat, we can admire the rapid development in stem cell research. Alas, the difficulties in research are faithfully reflected in the confusion in the nomenclature used for describing and classifying stem cells, including the classes of stem cells of mesodermal origin. The recent definition of MSCs by Dominici states that MSCs are a stromal cell type, possessing the following characteristics and markers: plastic adherence in cell culture, specific surface antigen expression of CD105(+), CD90(+), CD73(+), CD34(−), CD45(−), CD11b(−) or CD14(−), CD19(−) or CD79a(−), HLA-DR1(−) and multi-lineage in vitro differentiation potential (osteogenic, chondrogenic, and adipogenic) [59]. However, this definition would neatly exclude CD34+ hematopoietic stem cells (HSCs), while one also could argue that the hematopoietic stem cells are just a specialized subclass of the mesenchymal progenitors [180]. Another subset of MSCs that express hyaluronan (CD44), an adhesion molecule important for stem cell homing [14, 125, 281], would also be excluded, but their perivascular equivalents could be considered to be true MPCs [180]. It becomes even more complicated if we include the results of (stem) cell reprogramming, when more or less differentiated cell types are regressed into less differentiated, pluripotent cell types [91, 185, 192, 239], providing us with a never ending stream of novel biomarkers, more often represented by whole proteome analysis [1, 203]. Time will tell, what are the biomarkers and criteria for properly characterizing the particular stem cell populations, but there is a functional definition lingering around as a firm conceptual handle on the idea of cell plasticity of which stem cells are prominent representatives [21, 22]. The plasticity indicates the ability of matured or not fully differentiated cells to differentiate into novel cell types, or more accurately, it describes the existence of cells specialized into becoming progenitors of differentiated cells while sustaining their own type and maturation level. Combining this with the embryology and origins of cell lineages from the three primordial "dermata" (ecto-, endo-, and mesoderm) provides us with a useful and generalized definition of MSCs being the pluripotent, self-renewing stromal cells of mesenchymal origin and allowing us to determine the specific biomarkers later, at our convenience, as the state of affairs in mesenchymal stem cell biology progresses and solidifies. There is no doubt that we will find the appropriate placement for the specialized subtypes as well as the proper and practical placement of some of the induced pluripotent cells (iPCs) in the realm of MSCs.

Regardless of the exactitude of the classification, the (omni-) presence of the mesenchymal cell lineages in all of the organs and tissues [11, 102, 116, 132, 180] renders them good candidates not only for general stem cell therapy [22, 73, 102, 235], but even more promising is the potential use of MSCs for gene therapy [35, 45, 176, 196], cell reprogramming [9, 36, 252], delivery of bioactive molecules [163, 196], and tissue engineering [4, 82, 153]. In addition, a number of new issues are arising from the results describing the importance of stem cells in inducing and sustaining the malignant phenotype and the potential therapeutic targeting of a wide range of the elusive cancer stem cell types [27, 183, 217, 244]. The genetic modification of MSCs and associated cell types with Lentiviral vectors opens their application beyond reliance upon the innate properties of the cells. Expression of proteins that can modulate their biology or therapeutic properties enormously expands their utility for therapy.

The lack of a crisp definition of all the stem cell types affects targeting of Lentiviral vectors to specific subsets of stem cells. However, recent successful efforts in the pseudotyping of Lentiviral vector is a step in the right direction. The use of the VSVg pseudotype expanded the tropism of the Lentiviral vectors and, as a result, practically any cell type can be targeted and the narrowing of the tropisms by developing novel vector-pseudotypes will be addressed. The emergence of single chain antibodies as pseudotype indicates that we can expect a rapid expansion of this technology in the near future and will result in a precise tool for studying cell lineages.

We focus and limit our review on the recent progress made in stem cell research using Lentiviral vector-based gene delivery, a method that is emerging as the safest and most effective way to modify (stem) cells permanently or temporarily, if using non-integrating versions of the novel generations of Lentiviral vectors, both of which have clear potential for a wide range of research application in preclinical studies as well as therapeutic applications.

14.1.1 Lentiviral Vectors: Overview of Design and Safety Issues

The most commonly used Lentiviral vector framework is HIV-1 based although HIV2, SIV (Simian Immunodeficiency virus), FIV (Feline immunodeficiency virus) have been successfully tested; see review by Dropulic [62]. The native HIV-1 is a human pathogen; but it had been modified to eliminate pathogenicity and increase safety before considering it as a broadly available tool for gene transfers.

Typically, Lentiviral vector are generated by trans complementation, a process that separates the essential components of HIV (the genes encoding Gag-Pol, Rev, and Env) into separate plasmids, which lack the packaging signal and, therefore, can never end up in a packaged vector unless they appear in a recombinant sequence [63]. The components (Tat, Vif, etc.) responsible for pathogenicity by upregulation of transcription [54] and export of genomic RNA to the cytoplasm have been successively removed from the constructs. The potential for a recombination event is minimized, and for all practical purposes avoided, by carefully editing the genes

and using codon degeneracy to reduce the chances of recombination with the wild-type virus. These separate plasmids are used to co-transfect a packaging cell, typically HEK293, along with a payload plasmid that carries the packaging signal necessary for starting the envelope formation and encapsidation of the mRNA that carries the payload gene (s) as well as the 5′ and 3′ long terminal repeats (LTRs) necessary for integration into the transcriptionally active regions of the host chromosomes. Packaging is a delicate process, which ensures that with the RNA, appropriate tRNAlys, protease, integrase, and reverse transcriptase enzymes are carried by the vector with the packaging elements necessary for successful cell entry, reverse transcription of vector RNA to DNA, transport of that DNA into the nucleus, and the permanent integration of the DNA into host chromosome. The ENV gene encodes the protein GP160 that is cleaved into trimer-forming GP120, which appears as spikes decorating the vector particle; and GP41, that carries a transmembrane region and a carboxy terminal sub-domain that interacts with the nucleocapsid within the envelope. The N-terminal domain has a fusogenic domain that facilitates cell entry by fusing the outer membrane of the vector with the cell membrane. A region further down to from the amino terminal region also binds to GP120 which in turn binds to primary HIV-1 receptors on the target CD4+ of T lymphocytes. This property if left unmodified would significantly limit the usability of the Lentiviral vector, as very few cells types can be directly infected by HIV-1. Pseudotyping overcomes this limitation and permits targeting to any mammalian cell.

Pseudotyping essentially replaces the original HIV ENV gene with a corresponding molecule from other viruses and carries over the cell-targeting specificity (i.e., the tropism of the virus) and obviates some of the safety concerns related to GP120 [258]. The list of successful pseudotypes and cell tropism is rather lengthy [18, 76–79, 105, 222] and growing. The most successful pseudotype so far uses the ENV from vesicular stomatitis virus (VSVg) that successfully broadens the tropism to cells in the brain, kidney, and liver amongst other. It extends to mesenchymal (stem) cells, even those in nondividing (resting) state [77, 136, 280]. Filovirus ENV pseudotypes shift the tropism to a more limited set of cells, airway epithelial and endothelial cells [130, 148]. Baculovirus GP64 and Hepatitis C virus E1 and E2 pseudotypes redirect the vectors toward liver cells targeting their respective receptor, CD81 (tetraspanin) [19]. Rabies virus ENV has been shown to efficiently retarget the vectors to neuronal cells [162, 262]. RD114 ENV pseudotyped Lentiviral vector show preference for hematopoietic cellular compartment [20, 56, 85, 115, 221, 268]. However, some applications require targeting a specific cell type, which is not necessarily covered by the available pseudotypes listed above. In those cases, new targeting methods have been developed to further tighten the tropism of Lentiviral vector by co-expressing cell-specific coreceptors that recognize one of the cell-type specific markers.

The payload plasmid components providing the backbone for the transfer vector in the early HIV vectors were composed of a 5′ LTR, followed by a major splice donor site, a packaging signal site encompassing the packaging signal components of the 5′ region of Gag (necessary for high efficiency packaging and high vector titer) and a deletion of the rest of the gag gene. Deletion of the U3 region from the 3′ LTR promoters became also possible by relaying on constructing genes with their

own promoter(s). The latest generation of Lentiviral vectors carry an additional safety element, the self-inactivating LTR (SIN Lentiviral vector, [114]) replacing the LTR with an HIV-independent promoter from Cytomegalovirus (CMV). In these vectors the LTRs are modified in a way that upon integration they lose their intrinsic promoter ability reducing genotoxic potential. In addition the irreversible changes that occur during integration diminish the ability to mobilize after integration and to recombine with other elements to form a full-fledged, replication capable virus [25, 279]. The formal proof of increased safety is still lacking, ironically, because of the inability to create and detect RCL capable viruses from Lentiviral vector-treated cells [25], indicating that this risk is mainly theoretical.

The removal of RRE and the associated splice donor and acceptor elements results in significant loss of transduction efficiency of the vector [127], while adding a 100 nucleotide central polypurine tract (central DNA flap) restores the transduction efficiency by improving the reverse transcription and nuclear transport efficiency [47, 283]. The woodchuck hepatitis virus transcriptional regulatory element (WPRE) is another widely used regulatory element added to the Lentiviral vector backbone to stabilize the transcription transgene mRNA levels and improve transgene expression [292]. However, an open reading frame of the oncogenic WHV-X element has been found within the native WPRE sequence [128], so the sequence has been modified to remove the translation start site [282].

Further optimization continues to improve the safety of Lentiviral vector, such as isolating the integrated vector DNA to prevent translation beyond the vector boundaries by adding isolating elements. However, the insulators have themselves proven to be genotoxic in some instances, and no proof has emerged that such isolators are truly needed [100]. Gene switches such as Tet-On and -Off have been added to subsequent generations of Lentiviral vector and proven to be highly functional, operating with very low leakage [194, 260]. The Cre-Lox system has also been successfully implemented in the Lentiviral context allowing high efficiency engineering and sophisticated, site-specific recombination techniques including the delivery and irreversible switching by small hairpin RNA (shRNA) expression [135], a tool extensively used in gene function analysis [37, 193].

A major concern regarding the safety of Lentiviral vectors has been the potential genotoxicity resulting in oncogenesis, as observed previously during clinical trials for treating X-linked severe combined immunodeficiency (X-SCID) with transplanted HSCs treated with murine oncoretroviral vectors carrying the gamma chain of IL2R genes [96–99]. The preferential insertion of oncoretroviral vectors in proximity of the LMO2 proto oncogene and the subsequent constitutive activation of the proto-oncogen driven by the enhancer element (LTR) in the vector resulted in uncontrolled cell proliferation. However, in a series of studies comparing oncoretroviruses and Lentiviral vector, it has been shown that while the oncoretroviral vectors trigger a dose dependent acceleration of cancer onset in a mouse transplantation model sensitive to cancer-triggering genetic changes (CDkna2−/−), Lentiviral vectors lacked such activity [172, 175] even though the vector integration rate was significantly higher. This important observation implying that a low level of insertional mutagenicity has been confirmed independently by several groups indicating

a favorable safety profile for Lentiviral vectors while emphasizing the importance of vector design and avoidance of strong enhancers in the vectors [32, 169, 171].

Recent clinical trials have supported the good safety profile of Lentiviral vectors. There have been no oncogenic effects reported in any of trials using Lentiviral vectors to date [30, 83, 123, 143, 161, 195, 210, 276].

14.2 Development of Gene Therapy Techniques Using Lentiviral Vectors

14.2.1 Gene Silencing with RNA Interference

Gene silencing by small interfering RNA (RNAi) is based on duplex formation between the mRNA and a short complementary micro RNA or small inhibitory RNA, each having the ability to interfere with the protein synthesis and downregulate the expression levels of the targeted protein. A major problem with the inhibitory RNA technologies is the short half-life and delivery of the RNAi. This can be resolved using Lentiviral vectors encoding artificial genes with appropriate micro RNA sequences that can be integrated into the host cell DNA and efficiently transcribed into primary micro RNA that utilizes the natural intracellular processing by microprocessor complex formation with Drosha to form small hairpin RNA (shRNA) in the nucleus. The exported miRNA is subsequently cleaved by Dicer and produces the complex forming inhibitory RNAi. The process is rather complex, but efficient to produce a significant blockade of protein expression that may be incomplete but readily achieves significant reduction, that is adequate for gaining insight into the function of the targeted protein and efficient enough for phase I and II clinical trials, though no therapeutic use has been approved by the FDA. It is interesting to note that MSCs are capable of secreting cholesterol-rich phospholipid microparticles encapsulating miRNA, and therefore have the potential to facilitate intercellular communication and act as regulatory agents in their microenvironment [38]. An excellent review has been published on the biogenesis and clinical applications of small RNA compounds by Davidson and McCray [49].

Hematopoietic or general pluripotent stem cells are often selected targets for RNA interference-based interventions and one of the promising efforts deal with creating artificial virus resistance genes and virus resistant somatic cells. Preventing HIV infection by reconstituting the immune system with such stem cell-derived virus-resistant progeny has been used as model system with significant clinical relevance [121]. The idea is that an efficient HIV infection requires virus entry through the CD4 surface antigen and one or more virus co-receptors, among which CCR5 has been shown to play an essential role in the case of R5 tropic viral strains involved in primary HIV infection. Clinical data indicate that CCR5 deficiency or certain mutations in this co-receptor protect the infected individuals from the onset of full blown AIDS, and the hope is that the artificial knockdown of CCR5 using gene

therapy and RNAi will achieve similar protection [8, 57, 121, 146, 233]. The relative inefficiency of the CCR5 suppression remains a significant issue, but major improvement and complete knockdown of CCR5 have been achieved with somewhat longer (28 base instead of 23) shRNA [7].

Mesenchymal stem cell research is taking full advantage of the shRNA techniques by characterizing the subtle, and not so subtle, changes induced by individual gene knockdowns. It is a long held view that mechanical stresses and mechanical characteristics of stem cells, as well as the microenvironment, can affect stem cell proliferation and differentiation. Lentiviral vectors are excellent and efficient targeting tools for these stem cells, even resting ones, and can deliver the shRNA without causing major changes and stress that would otherwise change the stem cells on its own. Chowdhury et al. studied the spreading response of MSCs and showed that myosin II, F-actin, Src, or CDC42 were essential for cell spreading and changes in the mechanical characteristics ("softening") of the stem cells led directly to the downregulation of the OCT3/4 gene. This indicates the possibility that small mechanical events may affect the embryo and developing tissues and even transplanted stem cells [41].

Another area of efficient use of Lentiviral vectors and RNAi technology in stem cell research is the production of transgenic embryos which carry knockdown genes. Production of transgenic embryos is highly efficient, and if the fertilized egg is transduced at a single cell stage, the entire germ line is affected, or partial chimerism can be achieved if multicellular embryos are treated with Lentiviral vectors. An example of such a study is that by Wang et al., in which they showed that the knockdown of RunX1 in embryonal tissues and MSCs by Lentiviral vector-delivered interfering RNA blocked chondrogenesis in limb buds [257]. The technique has been shown to be very efficient for transgenesis, as high as 44% average rate of germ-line transmission can be achieved [227], providing a new source of gene-modified MSCs. A recent comprehensive review of the use of naturally occurring regulatory miRNA technology in mesenchymal stem cell research has been written by Guo et al. [93], indicating that stem cells have discrete and distinct expression profiles that can account for intrinsic stem cell properties such as self-renewal and pluripotency, a property that cannot longer be overlooked by experts dealing with MSCs. The accumulating data indicate that the progenitors and terminally differentiated mesenchymal cells can be tracked and defined by function-related miRNAs in addition to the already established sets of surface markers. The miRNAs already identified affect osteogenic differentiation, chondric differentiation, adipogenic differentiation, myogenic differentiation, neuronal differentiation, wound healing, and replicative senescence. These advances open a wide array of possibilities to direct the differentiation patterns of the stem cell population temporarily by using non-integrating Lentiviral vectors that are automatically lost from dividing cell populations and lead to the natural disappearance of control signal after a few cell division but potentially giving a push to the original stem cell population to develop in a preferred direction.

Extensive progress has been made in regards to the elucidation of the Hedgehog signaling pathway in MSCs using RNA interference delivered with Lentiviral vectors. The data suggest that at least some of the elements indeed act through the

regulatory miRNA network, by downregulating the cellular miRNA levels. The data, however, also suggest significant off-target effects of the interfering RNA molecules and indicate that we are a long way from the potential clinical use of the elucidated networks [124].

An ingenious method was devised by Hu et al., to prepare the brain for traumatic interventions (surgery, extensive stem cell transplantations, etc.) by downregulating the cerebral Matrix Metalloproteinase 9 (MMP9) using Lentiviral vector and MMP-9 shRNA 2 weeks before the trauma. The knockdown of MMP-9 with the shRNA proved to be an effective way to preserve the blood–brain barrier, and they achieved significant reduction of brain infarction volumes, reduction of brain water content and evans blue/IgG extravasation (measure of edema formation) as well as a reduction in the neurobehavioral deficit in their rat brain trauma model [108] implying a potential for improved protocols for traumatic brain interventions needed for more extensive type of intracranial stem cell implantations.

14.2.2 Gene Transfer into MSCs

As mentioned earlier, Lentiviral vectors provide a very efficient method for generating transgenic embryos, significantly reducing the need for the generation of a high number of embryos to establish new sources of gene-modified stem cell lines, embryonic, or other [227]. The Lentiviral technology is able to deliver a payload of 6–8 kB very efficiently, but payloads of 10kB can be handled and delivery of 12–13 kB is possible, at a cost of lower efficiency. This payload-carrying capacity allows the delivery of very large genes such as the gene encoding blood clothing factor VIII, a 2,351 amino acid long protein together with its stabilizer, the von Willebrand factor (2,813 amino acids in its native form) simultaneously or, one may need to use domain-engineered and shortened version of both; similarly it can be used to deliver all three chains of an IgM molecule in a single, tri-cistronic complex. The implication is that the Lentiviral vector system has sufficient payload capacity to deliver a number of relevant genes together with several supporting molecules envisioned for highly complex gene therapy scenarios currently outside the scope of monogenic gene therapy as practiced today. It may be used to target diseases with multi-gene disorders such as high blood pressure, arthritis, or diabetes in the future.

14.2.3 Gene Editing Using Zinc Finger Nucleases Encoded Within Lentiviral Vectors

Zinc-finger nucleases (ZFNs) have the remarkable ability to (a) bind to a specific location in the double-stranded DNA; (b) break the double-stranded DNA at that specific location and, if an endogenous repair template is provided, (c) initiate homology-directed repair, restoring the integrity of the newly edited double-stranded DNA. As their name implies, there is a specific DNA-binding part of this class of

enzymes that consists of a tandem repeat of DNA-binding zinc-finger motifs, hence the DNA binding specificity and a catalytic domain, FokI. For DNA cleavage to occur, FokI has to dimerize, one on the sense and the other on the antisense strand, while the zinc-finger domains attach to the right target half site and the left target half site. Upon binding, a nick with a 5′ overhang is initiated by FokI between the target sites and the homology-directed DNA repair mechanism is activated. What makes this configuration useful is that the spacer between the two target half sites can be several hundreds or even thousands of base pairs long and by providing a template for the activated repair mechanism, a novel DNA sequence of equal length can be introduced into the DNA; see a recent reviews by Caroll [29] and others [50, 101, 117, 134, 246].

Fundamentally, two factors determine the efficacy of the DNA editing or repair that the technology allows. The first is the specificity of the zinc-finger binding, which also determines the length of the spacer and the proper specificity and uniqueness of the binding site and allows the minimization of the off-target effects that may be introduced by similar sites far away from the desired and targeted locus [101]. Huge efforts are being made to tailor the zinc-finger nucleases for particular applications and improving the selectivity by successfully engineering the DNA-binding specificity of the binding domain [3, 101, 158, 201, 220]. The second factor is the efficient delivery of the ZFNs and the template DNA by vectors. While the early attempts relied on retroviral vectors, adeno and adeno-associated vectors, and even baculovirus vectors, the recent advances in the field clearly indicate that the Lentiviral delivery system is considered to be a safer and more efficacious route. As high as 50% conversion rate can be achieved with lentiviral delivery in a variety of cell lines and human embryonic stem cells [154] as compared with the earlier best rates of 18% with other methods in human and other species [3, 101, 197, 198, 201, 220, 245, 264].

14.2.4 Using Lentiviral Vectors in Tissue Engineering and Repair

One of the many Holy Grails of medicine, the ability to replace diseased tissue or even entire organs, seems to be hovering at the not too distant horizon. There is rapid progress in a wide range of areas, but at the center of the solution is almost always biocompatible scaffolding that is populated with a wide variety of cells. The strategically positioned cells find their place within the 3D structure, propagate, differentiate, and fill the available space, while producing a structure that can replace or enhance the damaged tissue in the form of various implants or prosthetics. As for scaffolding, the options are quite numerous, including those obtained from cadavers or live organs (animal or human origin), by removing the cells while preserving the fibrous tissue that maintains the basic morphology of the organ. Alternatively, a scaffold can be printed with various 3D printers [126, 159, 214–216, 229, 255, 261]. Processed cartilage can also result in scaffold and it can be used to rebuild and regrow an implantable ear, nose, or cartilage for trachea reconstruction [174].

The culture, expansion, and differentiation of human MSCs into artificial tissues represent a very complex series of events and Lentiviral vectors often serve as excellent research tools for marking, visualizing, and tracking the process [253], or modifying the gene or protein expression patterns [68]. A number of tissue engineering attempts have reached the clinic and Lentiviral vector have played various roles in the advancement of the technology. A very promising technology is the use of these scaffolded artificial tissues employing MSCs and Lentiviral vectors for delivering biologics for prolonged times.

Van Damme succinctly described the potential of these artifical tissues built on scaffolds and providing artificial implants for drug delivery. Lentiviral vectors were used to transduce mesenchymal cells to express green fluorescent protein (GFP) or FVIII. Expression was superior compared to oncoretroviral transduction, showing consistently higher transduction rates and expression remained high for several months post-transduction. The transduced cells retained their stem/progenitor cell properties, and they were still capable of differentiating along adipogenic and osteogenic lineages in vitro, while maintaining high GFP and FVIII expression levels. Implantation of Lentiviral vector-transduced human bone marrow mesenchymal cells using collagen scaffolds into immunodeficient mice resulted in efficient engraftment of gene-engineered cells and provided sites for transgene-expression in vivo. In addition to the bone marrow-derived stem cells, adipose tissue-derived mesenchymal stem cells have been shown to be amenable to populate implantable scaffolds and retain the potential to differentiate into osteogenic cells. Some of these scaffolds have been engineered for use in reconstructing craniofacial bone defects. Lentiviral vector have been used to deliver fluorescent proteins to track cells during manipulation such as osteogenic differentiation. The GFP-marked stem cells and their progeny remained fluorescent over the 8 weeks of the study period. The GFP-marked stem cells were successfully induced into osteogenic cells both in monolayers and three-dimensional scaffolds. Quantification showed no decrease in staining of the osteoinduced stem cells indicating the efficiency and durability of the labeling [256].

14.3 Aortic Implants

Tissue engineered vascular grafts built on bilayered elastomeric poly (ester-urethane) urea scaffolds and seeded with pericytes have shown promise in the past. However, in vitro endothelialization is still an issue for the use of these types of grafts. Doebis et al. reported in 2006 enhanced endothelialization using allogeneic endothelial cells or their precursors, expressing recombinant anti-alpha-MHC I single chain antibody to prevent rejection. The recombinant antibody was delivered efficiently ex vivo using Lentiviral vector, and has significantly reduced the MHC-1 expression levels as well as the killing of allogeneic cells by MHC-1 specific CD*+T cells [58]. The results suggest that these allogeneic cells may provide a suitable alternative supply for the lining of vascular prostheses.

Endothelial cells and their precursors are attractive targets for gene therapy, both for the treatment of cardiovascular disease and for the systemic delivery of recombinant gene products directly into the circulation. There have been a few reports which show Lentiviral vector-mediated gene transfer efficiency. Sacoda and colleagues compared the effectiveness of Lentiviral vector compared to adeno and oncoretroviral vectors. Bovine aortic endothelial cells (BAECs) were infected, in vitro, with these viral vectors. Transduction efficiency of beta-Gal gene transfer in BAECs by adenovirus, Lentiviral vector, or retrovirus at a multiplicity of infection (MOI) of 10 (determined on HeLa cells) was 69 ± 11, 33 ± 8, or 22 ± 6% respectively. At higher MOI [50] both adenovirus and Lentiviral vectors achieved an almost 100% transduction rate. However, retroviral vectors showed only 48 ± 6% at MOI 50 and no increase at MOI 100. The percentage of beta-Gal positive cells decreased rapidly at longer passage of cells after being transduced by adenovirus. In contrast, Lentiviral vector and retrovirus vectors mediated transductions showed sustained higher percentage of positive cells. Furthermore, the transductions by Lentiviral vectors had no significant effect on viability of BAECs suggesting that for long-term cell therapy the Lentiviral vectors have overall the best features [219]. Expressing IL10 in similar settings in the early, initiation phase, also inhibited and delayed the onset of the rejection process [287].

One of such cases in which the performance of the endothelial cells may need to be boosted is to increase the resistance to ischemia–reperfusion injury of the vascularized transplants and implants or normal tissues undergoing prolonged surgery. This is a condition which occurs too frequently and is responsible for devastating tissue injury caused by systemic activation of the complement system. Lentiviral vectors can be used to force the over-expression of the anti-apoptotic gene, Bcl-xL and indeed, it has shown significant protection from early apoptotic loss of vascular endothelial cells [286].

14.4 Periodontal Stem Cells

Recently, tooth tissue engineering has attracted more and more attention. Stem cell-based tissue engineering is thought to be a promising way to replace a missing tooth. The potential MSCs for tooth regeneration mainly include stem cells from human exfoliated deciduous teeth (SHEDs), adult dental pulp stem cells (DPSCs), stem cells from the apical part of the papilla (SCAPs), stem cells from the dental follicle (DFSCs), periodontal ligament stem cells (PDLSCs), and bone marrow-derived MSCs (BMSCs). A recent review by Peng et al. shows promising progress [190]. However, in practice, tissues other than bone marrow can serve as stem cell donors, including adipose tissue, periodontal ligament, and pulp for oral tissue regeneration [206]. The experimental data suggest that not only the stem cells ex vivo, but cells in the osteogenic tissue are amenable to direct transduction by Lentiviral vector [259]. This opens up the periodontal reconstruction interventions to the beneficial effects of gene therapy enhancing the wound healing and improving

engraftment by expressing growth promoters at low and slowly decreasing concentrations. Estrela published an excellent review on the potential of MSCs in regeneration of dental tissues [68] and Rodrigues-Loza reviewed the mesenchymal cell types recovered from dental tissues [208]. Other data clearly show that the primary osteogenic cells are efficiently transduced by Lentiviral vector, and that their infusion into the mandible is a feasible method for locally delivering DNA to primary osteogenic and bone cells in rat models [259], indicating that future applications in vivo dental implant enhancement, using dental scaffolding, bone healing, and tooth regeneration may be feasible. Recent efforts extend toward engineering dental repair by changing the expression of growth factors and bone morphogenic proteins leading to dentin formation, as discussed in a 2011 review by Casagrande [31] and which seem to be amenable to cell therapy efforts with non-integrating Lentiviral vector.

14.5 Wound Healing

One of the tissues that is often injured but that presents difficulties when it comes to healing and repairs is the tendon. Enhancing the healing process by in situ overexpression of helper factors such as IL10 could reduce recuperation time and perhaps improve the quality of the repair. Richetti et al. reported promising results in a murine model of patellar tendon injury after direct injection of an IL10 transgene using Lentiviral vector. Although the tendons showed no obvious histological difference, the IL-10-treated groups had superior mechanical characteristics by day 42 [205]. Although the mechanism of wound healing in tendons is not yet understood, the involvement of MSCs is suspected and delivery of additional factors that partake in healing process is discussed by Meyerose and Ashlan [13, 163].

Recent findings by Shamis and colleagues [230] demonstrated that embryonic stem cells could be directed to specified and alternative mesenchymal cell fates whose function could be distinguished in engineered human skin equivalents. Lentiviral shRNA-mediated knockdown of hepatocyte growth factor (HGF) resulted in a dramatic decrease of HGF secretion from cell lines (EDK cells) that led to a marked reduction in their ability to promote keratinocyte proliferation and re-epithelialization of cutaneous wounds. In contrast, H9-MSCs demonstrated features of MSCs but not those of dermal fibroblasts, as they underwent multilineage differentiation in monolayer culture, but were unable to support epithelial tissue development and repair and produced significantly lower levels of HGF. Characterization of these induced mesenchymal cells in 3D, engineered human skin equivalents demonstrated the utility of this tissue platform to predict the functional properties of stem cell-derived fibroblasts before their therapeutic use in reconstructive skin transplantation and wound healing.

Inhibition of hyper-keratinization by expressing a mutant form of TCGf Beta3 that has lost its binding site for latency-associated peptide, reduced the re-epithelialization density and fibroblast/myofibroblast trans-differentiation within the wound

area [251] in a mouse skin wounding model. The expression of this mutated gene was achieved by injecting Lentiviral vectors encoding the mutTCGF Beta3, into the regenerating tissue and the changes induced by this intervention predict a significant decrease in keloid formation and provide a potential model for preventing the painful disfigurement that follows the abnormally strong skin remodeling and scar tissue formation that oftentimes accompanies wound healing. The data indicate that future stem cell therapy with carefully designed interventions for patients prone to scar tissue formation could find wide spread application.

14.6 Corpus Cavernosum

One of the causes of erectile dysfunction is the damaged penile cavernous smooth muscle cells (SMCs) and sinus endothelial cells. Song reports that it may be feasible to restore these cells by applying MSCs to penile cavernous ECs or SMCs. For this purpose immortalized (via Lentiviral vector encoding v-myc) human bone marrow mesenchymal stem cell line B10 cells were transplanted into the cavernosum of Sprague–Dawley rats and harvested 2 weeks later. The expression of CD31, von Willebrand factor (vWF), smooth muscle cell actin (SMA), calponin, and desmin was determined immunohistochemically in rat penile cavernosum. Multipotency of B10 to adipogenic, osteogenic, or chondrogenic differentiation was found. Expression of endothelial cell-specific markers (CD31 or vWF protein) and expression of smooth muscle cell-specific markers (calponin, SMA, or desmin protein) were demonstrated in grafted B10 cells indicating that human MSCs may be a good candidates in the treatment of penile cavernosum injury [238].

14.7 Rules of Attraction: Angiogenesis and the Mesenchymal Stem Cell Migration

Angiogenesis requires the presence and active involvement of MSCs and therefore MSCs are ready to be recruited into the areas when there is a need for novel blood vessels: the inflamed, hypoxic, tumor infested locations. Gehmert et al. described an interesting model to study the migration of MSCs. In their work, immunodeficient mice were engrafted with human breast cancer cells (4T1) in the left mammary pad. A day later, the mice were injected IP with luciferase-labeled adipose tissue-derived MSCs (using Lentiviral vector technology). The MSCs were found to rapidly migrate into the tumor, confirming the previous observations that MSCs can be found within the tumor stroma and vasculature, even if the inflammation is not present, as the immunodeficient mice lacked the inflammation signaling pathway. Based on this result, it can be suggested that MSCs can be attracted solely by the cytokines produced by the tumor. However, the power of inflammation has been clearly

demonstrated in control animals, which received *E. coli* injections at contralateral locations and attracted all the MSCs leaving the tumor implant MSC-free [84]. Elucidating the migratory mechanisms of the MSCs seems to be an important step toward finding a delivery system to inflammatory sites and finding the conditions for clear migration into established tumors. Even the simple marking of tumor tissue with fluorescent proteins (such as GFP) holds important promise for surgeons, as delineating a breast cancer in situ during surgery would be possible by applying UV light and tracing the contours of the tumor. The technique already allows sophisticated molecular imaging combined with stem cell therapy [254].

Wang and colleagues used the ability of MSCs to differentiate into endothelial cells in vivo to establish whether the differentiated MSCs persist in vivo and to determine if this potential persistence contributes to functional improvement after experimental myocardial infarction. They generated a Lentiviral vector encoding two distinct reporter genes, one driven by a constitutive murine stem cell virus promoter and the other driven by an endothelial-specific Tie-2 promoter. The endothelial specificity of the Lentiviral vector was validated by its expression in endothelial cells but not in undifferentiated stem cells. The Lentivirus-transduced MSCs were injected into peri-infarct areas of the hearts of severe combined immune-deficient mice. Persistence of injected cells was tracked by bioluminescence imaging (BLI) and verified by immunohistochemical staining. The BLI signal from the endothelial-specific reporter revealed that the stem cells differentiated into endothelial cells 48 h after injection. However, both the constitutive and endothelial-specific signals disappeared by day 50. Nonetheless, the improvement in left ventricle ejection fraction with therapy persisted for up to 6 months. Immunohistochemical staining showed that stem cell-derived endothelial cells integrated into endogenous CD31+ vessels. Furthermore, stem cell-transplanted hearts had more CD31+ vessels and a lesser degree of cardiac fibrosis compared with the controls at 6 months. Increased angiogenesis and decreased fibrosis were associated with cardiac functional improvement. Similarly MSCs double-marked with GFP-Lentiviral vector and superparamagnetic iron oxide could be followed by MRI for up to 8 months in a porcine model of infraction and revascularization [274].

14.8 Myocardial Infarction

Endothelial cells respond to mild injurious stimuli by upregulating anti-apoptotic gene expression to maintain endothelial integrity. EC dysfunction and apoptosis resulting from ischemia/reperfusion injury may contribute to chronic allograft rejection. Under optimized conditions for Lentiviral vector transduction of rat aortic endothelial cells (RAEC) the delivery of the anti-apoptotic gene, Bcl-xL, via Lentiviral vector, protects RAEC from apoptotic death. The authors confirmed the damaging effect of the reperfusion phase. Endogenous Bax expression increased with I/R injury, whereas endogenous Bcl-xL remained constant. RAEC transduced with Lentiviral vector expressing Bcl-xL were protected from early apoptosis caused

by I/R injury, correlating with reduced cytochrome c release into the cytosol. This protective effect may be attributed to altering the balance of pro- and anti-apoptotic proteins, resulting in sequestration of the harmful Bax protein, and may open up new strategies for controlling chronic allograft rejection [286].

Inhibition of Na+/H+ exchanger 1 (NHE1) reduces cardiac ischemia-reperfusion (I/R) injury as well as cardiac hypertrophy and cardiac failure. Although the mechanisms underlying these NHE1-mediated effects suggest delay of mitochondrial permeability transition pore (MPTP) opening, and reduction of mitochondrial-derived superoxide production, the possibility of NHE1 blockade targeting mitochondria has been incompletely explored. A short-hairpin RNA sequence mediating specific knock down of NHE1 expression was incorporated into a Lentiviral vector (shRNA-NHE1) and transduced into the rat myocardium. NHE1 expression of mitochondrial lysates revealed that shRNA-NHE1 transductions reduced mitochondrial NHE1 (mNHE1) by approximately 60%, supporting the expression of NHE1 in mitochondria membranes. Electron microscopy studies corroborate the presence of NHE1 in heart mitochondria. Immunostaining of rat cardiomyocytes also suggests colocalization of NHE1 with the mitochondrial marker cytochrome c oxidase. To examine the functional role of mNHE1, mitochondrial suspensions were exposed to increasing concentrations of $CaCl_2$ to induce MPTP opening and consequently, rat heart mitochondrial swelling. shRNA-NHE1 transduction reduced the $CaCl_2$-induced mitochondrial swelling by $64 \pm 4\%$. Whereas the NHE1 inhibitor HOE-642 (10 μM) decreased mitochondrial Ca^{2+}-induced swelling by only 37 ± 6. Because mitochondria from rats injected with shRNA-NHE1 present a high threshold for MPTP formation, the beneficial effects of NHE1 inhibition in I/R resulting from mitochondrial targeting should be considered as a future target for cell therapy [250]

Oxidative stress is important in a number of pathologies, including cardiovascular diseases, such as atherosclerosis and cardiac ischemia–reperfusion injury. An important mechanism for adaptation to oxidative stress is the induction of genes through the antioxidant response element (ARE) which regulates the expression of antioxidant and cryoprotective genes via the transcription factor Nrf2 (nuclear factor E2-related factor 2). As Nrf2-regulated genes are induced during oxidant stress, occurring for example in reperfusion after ischemia, Hurttila et al. took a novel approach to exploit ARE for the development of oxidative stress-inducible gene therapy vectors. To this end, one, two, or three ARE-containing regions from human NAD(P)H: quinone oxidoreductase-1, glutamate-cysteine ligase modifier subunit and mouse heme oxygenase-1 were cloned into a vector expressing luciferase under a minimal SV40 promoter. The construct, which was the most responsive to ARE-inducing agents, was chosen for further studies in which a Lentiviral vector was produced for an efficient transfer to endothelial cells. Heme oxygenase-1 (HO-1), which has well-characterized anti-inflammatory properties, was used as the therapeutic transgene. In human endothelial cells, ARE-driven HO-1 overexpression inhibited nuclear factor-kappa B activation and subsequent vascular cell adhesion molecule-1 expression induced by tumor necrosis factor-alpha. They concluded that the ARE element is a promising alternative for the development of oxidative stress-inducible gene therapy vectors [111].

Progenitor cell therapy is a potential new treatment option for ischemic conditions in the myocardium and skeletal muscles. However, it remains unclear whether umbilical cord blood (UCB)-derived progenitor cells can be therapeutic in ischemic muscles and if yes, whether the ex vivo gene transfer can be used for improving the effect. The use of Lentiviral vector led to efficient transduction of both UCB-derived HSCs and MSCs resulting in long-term transgene expression. Moreover, it did not alter the differentiation potential of either HSCs or MSCs. In addition, the therapeutic potential of CD133+ and MSC progenitor cells transduced ex vivo with Lentiviral vector encoding the mature form of vascular endothelial growth factor D (VEGF-D) or the enhanced green fluorescent protein (eGFP) marker gene achieved permanent gene expression. The transplantation of the progenitor cells into nude mice serving as mouse model of skeletal muscle ischemia enhanced the regeneration of ischemic muscles, but notably, without a detectable long-term engraftment of either CD133+ or MSC progenitor cells. The results show that rather than directly participating in angiogenesis or skeletal myogenesis, the UCB-derived progenitor cells indirectly enhance the regenerative capacity of skeletal muscle after acute ischemic injury. However, rather counter-intuitively, the VEGF-D gene transfer into the progenitor cells did not improve the therapeutic effect in ischemic muscles [131].

Another cell type with improved adult stem cell functions has been discovered and cells have been isolated from the peripheral blood of young children. This clonally expandable, telomerase expressing progenitor cell type is distinct from hematopoietic or mesenchymal stromal cells and resembles that of embryonic multipotent mesoangioblasts. Cell numbers and the proliferative capacity correlate with donor age, and express the pluripotency markers Klf4, c-Myc, as well as low levels of Oct3/4, but lack Sox2. Overexpression of Sox2 by Lentiviral transduction of Sox2 (Sox-MABs) enhances pluripotency and facilitates differentiation to cardiovascular lineages. Furthermore, the number of smooth muscle actin positive cells was higher in Sox-MABs. In addition, pluripotency of Sox-MABs was shown in a mouse model by demonstrating the generation of endodermal and ectodermal progenies and injection of Sox-MABs into nude mice after acute myocardial infarction resulted in improved cardiac function compared to mice treated with control cells (cMABs). Furthermore, cell therapy with Sox-MABs resulted in an increased number of differentiated cardiomyocytes, endothelial cells, and smooth muscle cells in vivo [133].

14.9 Lung Damage and Lung Repair

Mesenchymal stem cell therapy emerges as a viable therapy in the context of acute lung injury/acute respiratory distress syndrome and chronic disorders, such as lung fibrosis and chronic obstructive pulmonary disease. There is evidence for beneficial effects of MSCs on lung development, repair, and remodeling. The engraftment in

the injured lung does not occur easily, but several studies report that paracrine factors can be effective in reducing inflammation and promoting tissue repair. MSCs release several growth factors and anti-inflammatory cytokines that regulate endothelial and epithelial permeability and reduce the severity of inflammation, as reviewed by Arboreau et al. [2], suggesting that carefully controlled expression of these factors using transduced stem cells could enhance the beneficial effects of the mesenchymal stem cell therapy. This may be a risky proposal, however, since constitutive expression of TGF beta/TGF alpha in epithelial MSCs generated breast cancer stem cells [12]. Acute respiratory distress syndrome (ARDS) is a crippling disease with no effective therapy, and characterized by progressive lung damage followed by dyspnea. MSCs have been proposed as a new therapeutic modality for ARDS because the stem cells can attenuate inflammation and repair the damaged tissue by differentiating into several cell types. The beneficial effect of the stem cells is still a minor mystery, as it is known that macrophages participate in the development of ARDS and that MSCs can only weekly modulate macrophage function. The chemokine CCL2 is a potent inducer of macrophage recruitment and activation, and its expression is elevated in patients with ARDS. A set of MSCs have been generated by transducing the cells with a Lentiviral vector expressing 7ND, a dominant-negative inhibitor of CCL2, expecting enhanced therapeutic function of the MSCs if the hypothesis is valid. The transduction was effective, and the stem cells produced a large amount of 7ND. After inducing lung injury by bleomycin treatment, the iv-injected MSCs readily migrated into the site of injury as confirmed by immunostaining 24 h postinjection. This finding suggests that MSCs could work as a drug delivery tool. Mice treated with 7ND-expressing MSCs showed significantly milder weight loss, suffered less severe lung injury, lower collagen content, lesser accumulation of inflammatory cells and inflammatory mediators, and ultimately showed significant gains in survival [218]. No evidence of 7ND-mesencymal stem cell-induced toxicity was observed during or after treatment. Thus, inhibiting the effects of macrophages may greatly enhance the ability of MSCs to affect lung repair in ARDS.

Direct transduction of lung tissues for gene therapy has always been an attractive proposal. The reoccurring problem, however, is that the airways are far less accessible to vector particles than hoped for and the depth of penetration of inhaled substrate ends in the branches which are larger than 100 μM in diameter [48, 263]. An attractive alternative delivery of gene therapy components could be the intrapleural injection of MSCs. To enable tracking, the cells were labeled with green fluorescent protein (GFP) using a Lentiviral vector, and were found readily attached to the pleura of Sprague–Dawley rats. The isolated and recovered cells preserved the typical mesenchymal stem cell phenotype and could differentiate into adipocytes, osteoblasts, and chondroblasts in vitro. The highest number of the labeled cells was found to be adhered to the mediastinal pleura, but no labeled cells were detected in the lung parenchyma or other tissues/organs, such as the liver, kidney, spleen, and mesenterium, a remarkable compartmentalization of a stem cell transplant [200].

14.10 Neurological Disorders

14.10.1 Alzheimer's Disease

Alzheimer's disease (AD) is one of the most devastating conditions and its prevalence is still rising paralleling the increase of average life expectancy. A hallmark of the disease is the accumulation of amyloid plaques and extensive neurodegeneration in the context of an intracerebral inflammation, leading to progressive dementia. Over the years, a tripartite set of goals crystallized, when the potential treatments of AD were considered: (a) stop the progression of the disease by reducing/reversing the plaque formation; (b) stop the neurodegeneration that seems to be a consequence of both internal changes (neurofibrillary tangle formation and related issues) and changes external to the cells, related to plaque formation and degeneration of the neuronal microenvironment; and (c) recover neurological function by replenishing the lost neuronal compartment [71, 81, 94, 122, 152, 188, 291]. Interestingly, MSCs and stem cell therapy are increasingly considered a potentially important part of the toolset to achieve these goals.

The symptoms that are collectively categorized as AD often have different backgrounds, some of which seem to have roots implying genetic causes, such as improper processing of beta amyloid peptide. Consequently, a disease-modifying therapeutic approach in Alzheimer's disease aims to reduce the accumulation of neurotoxic beta amyloid aggregation peptides. Habish et al. report new findings for a potential autologous stem cell-based strategy for delivery of enzymatic activities against beta amyloid formation in the brain. F-spondin and neprilysin (CD10), genes expressed in adult MSCs, are known to be involved in the formation and degradation of beta amyloid peptides, respectively. Coincubation of the converted MSCs with HEK-293 cells stably expressing amyloid precursor protein (APP) lead to a significant cell dose-dependent decrease of amyloid peptide release and deposition, indicating that MSCs might be useful for delivering anti-amyloid activity to treat AD [95]. This direction of research is gaining new momentum from the discovery of a new beta amyloid secretase and the tremendous progress gained in recent years in the field of amyloid formation, its contribution to neurodegenerative diseases [122] and allowing new gene therapies to be conceived and tested.

One effort has utilized human umbilical cord blood-derived MSCs (hUCB-MSCs) which were transplanted into amyloid precursor protein and presenilin1 double-transgenic mice. This experiment resulted in significantly improved spatial learning and a decrease in memory decline. Furthermore, beta amyloid peptide deposition, beta-secretase 1 (BACE-1) levels, and the hyper-phosphorylation of the Tau proteins were dramatically reduced in hUCB-MSC transplanted APP/PS1 mice. Interestingly, these effects were associated with reversal of disease-associated microglial neuroinflammation, as evidenced by decreased microglia-induced pro-inflammatory cytokines, reduction in the number of alternatively activated

microglia, and decrease in anti-inflammatory cytokines. Combining these findings with the potential cell therapy targeting, these MSCs are expected to produce a sustained neuroprotective effect by establishing a feed-forward loop engaging the alternative activation of microglia, thereby ameliorating disease pathophysiology and reversing the cognitive decline associated with amyloid deposition [139]. Peng and colleagues report additional details on the use of Lentivirus-expressed siRNA as a method to ameliorate Alzheimer disease neuropathology in APP transgenic mice by reducing the levels of beta-site APP cleaving enzyme 1, or BACE1 [189].

A series of experiments demonstrated the potential of neural stem cells transduced by a multigenic Lentiviral vector stably expressing recombinant human nerve growth factor in relevant amounts to exploit their ability for therapeutic applications. The multigenic Lentiviral vector contained a tricistronic cassette to express simultaneously up to three independent genes: (1) rhNGF (beta subunit); (2) EGFP (enhanced green fluorescent protein); and (3) Neo (R) (neomycin antibiotic resistance gene). Lentiviral vectors were released in culture media and subsequently used to transduce mouse stem cells. Remarkably, the subsequent test revealed that engineered NSCs were all positive for EGFP and after 30 passages in vitro engineered cells maintained their multipotentiality to differentiate into neurons, astrocytes, and oligodendrocytes. Furthermore, it was found that rhNGF-stem cell-derived neurons expressed choline acetyltransferase and displayed an enhanced axonal growth. The stem cells showed an altered sphere forming frequency either in rhNGF-NSC or in both groups of control NSC. Lentivirus-mediated rhNGF gene transfer into NSC was achieved without changes in the expression of neural differentiation markers, like microtubule-associated protein 2 (MAP2) (a/b), glial fibrillary acidic protein (GFAP) and chondroitin sulfate proteoglycan [34]. Secreted rhNGF increased axonal sprouting by rhNGF-NSC-derived neurons, which was associated with ChAT expression. rhNGF-NSCs may prospectively be a good candidate for the treatment of neurodegenerative diseases.

A protein that has been shown to promote APP accumulation is beta-secretase (beta-site APP cleaving enzyme 1, or BACE1). Typically, a marked increase in the level of BACE1 is found in the cerebrospinal fluid of those affected with Alzheimer's disease. Through in vivo studies using APP transgenic mice, it has been demonstrated that decreasing the expression of BACE1 via Lentiviral vector delivery of BACE1 siRNA has the potential for significantly reducing the cleavage of APP, the accumulation of these products, and the consequent neurodegeneration. As such, Lentiviral-expressed siRNA against BACE1 is a therapeutic possibility in the treatment of AD.

Neprilysin has recently been implicated as a major extracellular beta amyloid degrading enzyme in the brain. A unilateral intracerebral injection of a Lentiviral vector expressing human neprilysin (Lenti-Nep) was tested in transgenic mouse models of amyloidosis reduced amyloid-beta deposits by half relative to untreated mice, indicating that neprilysin may have a role in Alzheimer's disease treatment. That said, a more efficient delivery system is likely required, a property that a neprilysin expressing stem cell could potentially provide [160].

14.10.2 Parkinson's Disease

Gene transfer to the central nervous system provides a powerful methodology for the study of gene function and gene–environment interactions in vivo, in addition to a vehicle for the delivery of therapeutic transgenes for gene therapy. Research has been significantly aided by successfully targeting specific regions of brain, and for Parkinson's disease, the substantia nigra. The key to success is the ease of pseudotyping Lentiviral vectors, which makes it possible to change the patterns of tropism. Cannon et al. used Isogenic Lentiviral vector particles encoding a GFP reporter and pseudotyped with envelope glycoproteins derived from vesicular stomatitis virus (VSV), Mokola virus (MV), lymphocytic choriomeningitis virus (LCMV), or Moloney murine leukemia virus (MuLV). Adult, male Lewis rats were injected unilaterally with stereotactic infusions of vector into the substantia nigra. Three weeks later, patterns of viral transduction were determined by immunohistological detection of GFP. Different pseudotypes gave rise to different sites of transgene expression. VSV and MV pseudotypes transduced midbrain neurons, including a subset of nigral dopaminergic neurons. In contrast, LCMV- and MuLV-pseudotyped Lentiviral vector resulted in transgene expression exclusively in astrocytes. The restricted transduction of astroglial cells was not explained by the cellular distribution of receptors previously shown to mediate entry of LCMV or MuLV. The availability of neuronal and astrocyte-targeting vectors will allow dissociation of cell autonomous and cell nonautonomous functions of key gene products in vivo. Similar tissue and cell-specific patterns can be achieved in stem cells using cell/tissue-specific promoters and miRNA [43, 79, 86, 177, 186, 199, 213, 232, 242, 266, 290].

Multipotent mesenchymal stromal cells have raised great interest for brain cell therapy due to their ease of isolation from bone marrow, their immunomodulatory and tissue repair capacities, their ability to differentiate into neuronal-like cells, and for their ability to secrete a variety of growth factors and chemokines. A subpopulation of human MSCs, the marrow-isolated adult multilineage inducible (MIAMI) cells, when combined with pharmacologically active microcarriers (PAMs) have shown great promise in a rat model of Parkinson's disease. PAMs are biodegradable and non-cytotoxic poly (lactic-co-glycolic acid) microspheres, coated by a biomimetic surface and releasing a therapeutic protein, which acts on the cells conveyed on their surface and on their microenvironment. In this study, PAMs were coated with laminin and designed to release neurotrophin 3, which stimulate the neuronal-like differentiation of MIAMI cells and promotes neuronal survival. After adhesion of dopaminergic-induced (DI)-MIAMI cells to PAMs in vitro, the complexes were grafted in the partially dopaminergic-deafferented striatum of rats, which led to a strong reduction of the amphetamine-induced rotational behavior together with protection/repair of the nigrostriatal pathway. These effects were correlated with the increased survival of DI-MIAMI cells that secreted a wide range of growth factors and chemokines. Moreover, the observed increased expression of tyrosine hydroxylase by cells transplanted with PAMs may contribute to this functional recovery [52] and provide an excellent new delivery system for genetically modified/enhanced cells into substantia nigra.

Lewy body disease is a heterogeneous group of neurodegenerative disorders characterized by alpha-synuclein accumulation and includes gradually worsening dementia with Lewy bodies (DLB) accumulating in neurons followed by advanced Parkinson's Disease (PD). Recent evidence suggests that impairment of the lysosomal pathways (i.e., autophagy) involved in alpha-synuclein clearance might play an important role. For this reason, the expression levels of members of the autophagy pathway in brains of patients with DLB and Alzheimer's disease and in alpha-synuclein transgenic mice were examined by immunoblot analysis. In DLB cases, the levels of mTor were elevated and Atg7 were reduced compared to controls and AD. Levels of other components of the autophagy pathway such as Atg5, Atg10, Atg12, and Beclin-1 were not different in DLB compared to controls. In DLB brains, mTor was more abundant in neurons displaying alpha-synuclein accumulation. These neurons also showed abnormal expression of lysosomal markers such as LC3, and ultrastructural analysis revealed the presence of malformed autophagosomes in abundance. Similar alterations were observed in the brains of alpha-synuclein transgenic mice. Intracerebral infusion of rapamycin, an inhibitor of mTor, or injection of a Lentiviral vector expressing Atg7 resulted in reduced accumulation of alpha-synuclein in transgenic mice and amelioration of associated neurodegenerative alterations supporting the notion that defects in the autophagy pathway, and more specifically in mTor and Atg7, are associated with neurodegeneration. This supports the possibility that modulators of the autophagy pathway might have potential therapeutic effects using genetically altered stem cells [44, 270].

Although the advances in Parkinson's disease research to date are significant, the lack of clinical use of genetically modified cells is a bit surprising and may indicate an oversight and underuse of the advanced tools provided by the combination of stem cells and Lentiviral vectors.

14.10.3 Focal Ischemia, Disruption of Blood–Brain Barrier, and Neuronal Damage

Lasting cerebral ischemia is a frequent (~80%) consequence of stroke and, as a result, most of the stroke research is focusing on ameliorating the devastating consequences of ischemic events: endothelial damage, neurodegeneration, and breakdown of the blood–brain barrier (BBB) leading to difficult-to-treat cerebral edema [108]. Data indicate that transplantation of human umbilical cord stem cells helps to protect ischemic brain [149], and the protection is partially attributed to cytokines and protective factors produced by these stem cells [10, 149]. Another promising finding is that the mesenchymal and neuronal stem cells preserve their ability to differentiate into glial and neuronal cells [51, 119, 149, 231, 249, 275]. Various studies on focal cerebral ischemic models have implicated the direct activation and expression of matrix metalloproteinases (MMPs), especially MMP-9, as a key orchestrator of BBB disruption. Moreover, studies have shown that MMP-9 siRNA

can protect the BBB from ischemia/reperfusion injury. One study investigated the neuroprotective role of a Lentiviral vector-mediated MMP-9 shRNA following focal cerebral ischemia [108], indicating that it is possible to deliver MMP-9 inhibitors by genetically enhanced stem cells. This study also showed the ability to deliver the target deeper into the affected area normally not accessible by direct Lentiviral vector infusion. The forerunner of such interventions is a study testing the hypothesis that transplantation of human neurotrophin-3 (hNT-3) over-expressing neural stem cells into rat striatum after a severe focal ischemia would promote functional recovery. The rat neural stem cells were transduced with a Flag-tagged hNT-3 gene in a Lentiviral vector. The stem cells were transplanted into the striatum ipsilateral to the injury of adult rats 7 days after 2 h occlusion of the middle cerebral artery from 3 days to 2 weeks after transplantation. The modified cells (NSCs-hNT3, as defined by Flag immunofluorescence staining) that survived the transplantation procedures could secrete significantly higher levels of neurotrophin-3 protein in the graft sites than controls ($P < 0.001$). Furthermore, the rats that accepted NSCs-hNT3 exhibited enhanced functional recovery on neurological and behavioral tests, compared with control animals transplanted with saline or untransduced stem cells, indicating that they might have value for enhancing functional recovery after stroke [285].

Recovery from ischemic events is slow and rather unpredictable. However, there seem to be new therapeutical opportunities that could enhance the process such as using VEGF-induction therapy [16]. There is accumulating evidence indicating that VEGF has direct neuroprotective effects on various cultured neurons of the central nervous system. Interestingly, in vivo VEGF controls the correct migration of facial branchiomotor neurons in the developing hindbrain and stimulates the proliferation of neural stem cells in enriched environments and after cerebral ischemia. On the other hand, transgenic mice expressing reduced levels of VEGF develop late-onset motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (ALS). Also, reduced levels of VEGF have been implicated in a polyglutamine-induced model of motor neuron degeneration. Intracerebroventricular delivery of recombinant VEGF protein delays disease onset and prolongs survival of ALS rats, whereas intramuscular administration of a VEGF-expressing Lentiviral vector increases the life expectancy of ALS mice by as much as 30%. Deciphering the precise role of VEGF at the neurovascular interface promises to uncover new insights into the development and pathology of the nervous system and should be helpful to the design of novel strategies to treat (motor) neurodegenerative disorders [137]. VEGF-expressing MSCs have also been found beneficial in Parkinson's disease [16, 271]. The development of Lentiviral particles engineered for macrolide-responsive human vascular endothelial growth factor 121 (VEGF121) expression will bring closer the in vivo use of inducible growth factor cell therapies, expressing the factors only in ischemic conditions using hypoxia-inducible erythropoietin promoter [6]. Alternatively, the inducible VEGF121 promoter system also compared favorably with isogenic streptogramin- and tetracycline-responsive configurations and showed excellent growth-factor fine-tuning following transduction into a variety of mammalian cell lines and different human primary cells. Chicken embryos transduced for macrolide-controlled VEGF121 production can be fine-tuned to prime a dose-dependent neovascularization [168].

Expression of survivin (SVV) using an SIN Lentiviral vector carrying vascular endothelial growth factor further improved the expression of VEGF and basic fibroblast growth factor in male Sprague–Dawley rats under hypoxic conditions. The in vivo experiment that produced this observation consisted of three groups of rats, one receiving intravenous injection of 500 μL of phosphate-buffered saline without cells (control group) and two groups administered the same volume solution with either three million GFP-MSCs (group GFP) or SVV/GFP-MSCs (group SVV). All animals were submitted to 2 h middle cerebral artery occlusion followed by reperfusion. Modification with SVV further increased secretion of both factors. The survival of the transplanted cells in the SVV group was 1.3-fold higher at 4 days after transplantation and 3.4-fold higher at 14 days after transplantation, respectively, when compared with group GFP and reduced the cerebral infarct volume by 5.2% at 4 days after stroke and improved post-stroke neurological function at 14 days after transplantation. Modification with SVV could further enhance the therapeutic effects of MSCs possibly through improving the MSCs survival capacity and upregulating the expression of the protective cytokines in the ischemic tissue [151].

The identification of the genes differentially regulated by ischemia will lead to an improved understanding of cell death pathways such as those involved in the neuronal loss observed following a stroke. Furthermore, the characterization of such pathways could facilitate the identification of novel targets for stroke therapy. One such novel approach was the amplification of the differential gene expression patterns in a primary neuronal model of stroke, by employing a Lentiviral vector system to specifically bias the transcriptional activation of hypoxically regulated genes. Over-expression of the hypoxia-induced transcription factor subunits HIF-1 alpha and HIF-2 alpha elevated hypoxia-mediated transcription of many known HIF-regulated genes well above control levels. Furthermore, many potentially novel HIF-regulated genes were discovered that were not previously identified as hypoxically regulated. Most of the identified novel genes were activated by a combination of HIF-2 alpha over-expression and hypoxic insult. These included several genes with particular importance in cell survival pathways and of potential therapeutic value. Hypoxic induction of HIF-2 alpha may therefore be a critical factor in mediating protective responses against ischemic injury. Further investigation of the genes identified in this study may provide increased understanding of the neuronal response to hypoxia and may uncover novel therapeutic targets for the treatment of cerebral ischemia [202] and the genes need to be considered as useful targets in future mesenchymal stem cell therapies. However, the use of hypoxia-induced gene therapy has to be evaluated carefully in the light of recent provocative observations indicating that the hypoxic phenotype contributes to appearance of highly malignant cancer forms from the initial epithelial–mesenchymal transition to the ultimate organotropic colonization, and that can potentially be regulated by hypoxia, suggesting a master regulator role of hypoxia and HIFs in metastasis [6, 155]. Furthermore, modulation of cancer stem cell self-renewal by HIFs may also contribute to the hypoxia-regulated metastasis program. The hypoxia-induced metastatic phenotype may be one of the reasons for the modest efficacy of anti-angiogenic therapies and may well explain the provocative findings that anti-angiogenic therapy increased metastasis in preclinical models [155].

14.10.4 Traumatic Spine Injury

The image of a wheelchair-bound superman exemplified for all of us the tragedy that affects many of the victims of traumatic spinal cord injury and motivated research into protecting and restoring spinal-cord functionality beyond and above the usual efforts. The results are promising on many fronts [236]. On one hand, the intervertebral disk, cartilage, and bone injuries that threaten the integrity of the spinal cord can be almost completely healed and the healing can be facilitated and enhanced by stem cell therapies in most of the experimental models. The treatment often includes stem cells engineered with Lentiviral gene transfer for enhancing and promoting wound healing and tissue restoration [13, 87, 89, 109, 247]. Significant success has been achieved by expressing bone morphogenic proteins in the injured tissue [80] and observations that mechanical stimulation has a multiplying effect in bone regeneration will hopefully carry the research into clinical trials [140] sooner than later. Probably, the first trials will be done in well-designed spinal surgery, allowing even risky interventions, currently not practiced [13, 88, 163].

The progress is significantly slower when it comes to restoring the functionality of severed spinal cord, but successful demonstration that MSCs migrate into the site of injury and differentiate into proper cell types needed for the healing [224] predicts potential breakthroughs. In this set of experiment, mesenchymal cells were labeled with green fluorescent protein using Lentiviral vector, were injected into the subarachnoid space, and their migration and differentiation was observed. Cells were found on the surface of the injured spinal cord parenchyma, in deeper area of the perivascular spaces and some of them had been found deeply integrated into the parenchyma. Immunostaining for nestin demonstrated that some GFP-positive cells differentiated into neural stem cells and mature neurons or glial cells in situ. Lentiviral vectors pseudotyped with rabies env were successfully used to deliver genes into spinal cord and site of injury and showed successful retrograde transfer into deeper areas, indicating that gene therapy is possible and factors necessary for further differentiation of stem cells can be delivered [224, 241]. Further advances in pseudotyping with Rabies virus glycoprotein has a promise for more efficient motor neuro-specific delivery of transgenes and restoration of neuronal functions.

14.11 Drug Delivery by MSCs

As the examples indicate above, MSCs have been recognized as promising delivery vehicles for gene therapy in the CNS. A particularly unmet need is delivery of compounds that could help patients suffering from a particularly aggressive form of cancer, gliomas. A glimpse into a possible future can be gained from experiments in which stem cells were used to evaluate the antitumor effect of cytosine deaminase (CD) in a rat C6 glioma model. Lentiviral vectors expressing CD and enhanced green fluorescent protein (eGFP) were constructed and transduced into rat MSCs

which were intracranially injected alone or in combination with C6 glioma cells supported by unlabeled parental MSCs. The presence and effect of the engineered stem cells were then correlated with the possible effects on tumor growth, tumor cell apoptosis, tumor size, and rat survival in the presence of 5-fluorocytosine (5-FC). Fei et al. found that the CD/eGFP cells were largely localized at the junction of the tumor with normal tissue. The mean survival time of rats co-injected with C6 glioma cells and MSCs-CD/eGFP cells was significantly extended to 45.9 days with tumor size reduction when compared with rats injected with C6 glioma cells alone surviving an average of 15.3 days, or those co-injected with C6 glioma cells and parental cells surviving only for 16.0 days. In addition, data suggest that MSC-CD/eGFP-mediated gene therapy promoted tumor cell apoptosis in rat C6 gliomas [72].

Without going into detail, hypoxia-induced genes seem to play an important role in the fate of MSCs and require further studies, as modifying and preconditioning as well as changing their effects temporarily by gene therapy indicates a plethora of important insights into the potential use of this complicated class of stem cells in tumor therapy [142, 147, 150, 155, 267, 272], and we expect rapid progress in this area in the near future. The rational is that tumor cells have significantly altered metabolism with a shift toward the anaerobic pathway and changes in the respective gene expression patterns providing novel targets and delivery methods for cancer therapy.

Transplantation of HSCs to correct a series of lysosomal storage diseases and peroxisomal disorders has almost 25 years of history and involves over 20 diseases [23]. However, the success was limited to only a small subclass of diseases such as Hurler syndrome, X-ALD, and infantile Krabbe disease. Detailed studies are now available suggesting that hematopoietic stem cells are suitable only for a carefully selected cases, leaving open the field for a more versatile mesenchymal stem cell therapy, especially those instances having neurological symptoms [69]. Bone marrow-derived MSCs are another promising platform for cell- and gene-based treatment of inherited and acquired disorders including a whole range of lysosomal storage diseases. Several animal models exist to run preclinical studies [164]. Human MSCs distribute widely in a murine xenotransplantation model, and the human stem cells are amenable to Lentiviral vector-mediated transduction to obtain expression of therapeutic levels of enzyme in xenotransplantation models of human disease (non-obese diabetic severe combined immunodeficient mucopolysaccharidosis type VII [NOD-SCID MPSVII]) [164]. Transduced MSCs persisted in the animals that underwent transplantation and comparable numbers of donor MSCs were detected at 2 and 4 months after transplantation. The level of circulating enzymes were sufficient to normalize the secondary elevation of other lysosomal enzymes and reduce lysosomal distention in several tissues providing additional evidence that transduced human MSCs retain their normal trafficking ability in vivo and persist for at least 4 months, while able to deliver therapeutic levels of proteins in an authentic xenotransplantation model of human disease.

Similar results have been reported by Muller and colleagues, who were able to restore aryl sulfatase and beta galactosidase levels in genetically deficient bone marrow MSCs, and showed that untransduced cells from patients with metachromatic leukodystrophy,

who are ASA deficient, took up a substantial amount of ASA that was released into the media from MSCs [173], an important milestone for future attempts to try stem cell therapy of metachromatic leukodystrophy. GM1 ganglyosiosys was successfully treated with MSCs in a mouse beta-galactosidase knockout model indicating that autologous transplantation may be feasible using Lentiviral-transduced MSCs [228].

Fabry disease affects an estimated 1 in 40,000–60,000 males, and far less frequently females. It is an inherited lysosomal disorder caused by a deficiency of alpha-galactosidase A (alpha-gal A). The systemic accumulation of globotriaosylceramide (Gb3) results in gradual tissue deterioration leading to organ failure. There is a limited mouse model of the disease showing Gb3 accumulation in an alpha-gal A-deficient mouse model. However, most of the important clinical manifestations are absent and the lack of relevant large animal model hinders the development of proper cell therapy. When compared to the human alpha-gal A, the porcine alpha-gal A showed a high level of homology in the coding regions. Cell lysate and supernatants from Fabry patient-derived fibroblasts transduced with a Lentiviral vector carrying the porcine alpha-gal A cDNA (LV/porcine alpha-gal A) showed high levels of alpha-gal A activity, and its enzymological stability was similar to that of human alpha-gal A. Even more importantly, uptake of secreted porcine alpha-gal A by non-transduced cells was observed. Furthermore, Gb3 accumulation was reduced in Fabry patient-derived fibroblasts transduced with the LV/porcine alpha-gal A. The finding that the porcine version of the gene is also X-linked (X22q) provides hope that a large animal (porcine) model of Fabry disease can be constructed in the near future for use in testing a novel application of cell therapy using MSCs [278]. The success of such model and eventually the feasibility of the treatment depends on the "bystander phenomenon," i.e., the transduced mesenchymal cells intended for delivering the enzyme secrete the enzyme in abundance, but the defective cells in their microenvironment also must be able to take up the enzyme and utilize it. To facilitate the uptake, a fusion protein between Gb3 and HIV Tat protein has been made [104]. If successful, the range of enzyme replacement therapy approach could widen significantly. The data published by Higuchi et al. indicate that indeed the Tat's ability to penetrate the cell membrane was maintained in the recombinant fusion protein and it enhanced the enzyme uptake, as expected. Since the different manifestations of the disease produce problems in different organs (brain, kidney, and heart), it seems to imply that MSCs will be the best candidates for this enzyme replacement therapy as the earlier attempts to perform enzyme replacement therapy in mouse model showed insufficient efficiency [277].

14.12 Diabetes and the Hope for a Breakthrough in Mesenchymal Cell Transplantation

The enormity of the problems posed by diabetes is reflected by the statistics published on the NIH website (http://diabetes.niddk.nih.gov/dm/pubs/statistics/#dd). By the age of 65, almost one in four Americans suffers from diabetes. The at-risk population of

prediabetics is 37% of the population older than 20 years. The sheer number of patients indicates that restoring glucose metabolism by pancreas or pancreatic islet transplantation, even in the most severe cases, is just impractical if not impossible. The low engraftment rate makes the prospects of such treatment even worse, especially, as there never will be a sufficient number of donors. That leaves the stem cell technology as the major source of hope for solving the relevant issues in recovering regulated insulin production and glucose regulation functionality in diabetes. A large number of clinical trials using MSCs are under way [75, 106, 120], and a rather confusing sets of stem cell markers are listed in these studies indicating that there is a plurality of stem cells residing in different tissues, all of which have the potential to help pancreatic tissue regeneration. Not surprisingly, the most obvious source of these stem cells could be the pancreas itself, from which the resting stem cells can be isolated, reactivated, and expanded by variety of stimulants. The data are still being evaluated, and need further confirmation, reproduction, and lineage tracing. The currently available datasets could not firmly substantiate the claims when using different markers (*Carbonic anhydrase II* vs. *hepatocyte nuclear factor 1 beta*) [61, 113, 237, 243]. Since then, neurogenin 3 also was considered as a marker for endocrine type differentiation of proto beta cells [273], leaving the subject as to whether well-defined adult beta islet cell progenitors truly exist in significant numbers rather murky. The phenomenon of in vitro trans-differentiation of the acinar cells into beta cells upon exposure to EGF, LIF, notch1-inhibitors [15] looks promising, and recently Zhou and colleagues added a more extensive study on in vivo reprogramming of adult pancreatic exocrine cells into beta cells [289]. However, the reported efficiency was low and the progenitor cells remained elusive. This left the field searching for other sources, including MSCs from bone marrow, liver, intestine, and neural tissue (reviewed by Efrat [64–66] and Jones [118]), that are capable of trans-differentiating into insulin-producing beta cells. With the available results, their ultimate hope was that these cells could be used to seed the pancreas with new sets of insulin producing islands. Since lineage tracing was often omitted and the reproducibility of the results remained unsettled, the field, despite its high importance, seems to be somewhat in shambles [106, 107], ready for deployment of the novel, Lentiviral vector supported techniques.

Szabat et al. report a significant set of results on beta-cell maturation using Lentiviral vector-based lineage-study examining a novel Pdx1/Ins1 dual fluorescent reporter vector. They confirmed that individual adult human and mouse beta-cells exist in at least two differentiation states, distinguishable by the activation of the Ins1 promoter. They performed real-time imaging of the maturation of individual cultured beta-cells and followed the kinetics of the maturation process in primary human and mouse beta-cells and collected gene expression profiling data as well. The gene expression profiling of FACS purified immature Pdx1+/Ins1 (low) cells and mature Pdx1 (high)/Ins1 (high) cells from cultures of human islets, mouse islets, and MIN6 cells revealed that Pdx1+/Ins1 (low) cells are enriched for expression of multiple genes associated with beta-cell development/progenitor cells, proliferation, apoptosis, as well as genes coding for other islet cell hormones such as glucagon [240]. It turns out that trans-differentiation can be successfully performed using MafA. MafA is a leucine zipper transcription factor from the Maf family that can be

activated by p38 MAP kinase. This protein is a known pancreatic transcriptional factor controlling the beta-cell-specific transcription of the insulin gene [40]. Expressing it using Lentiviral vectors in placenta-derived multipotent stem cells (PDMSCs) that constitutively expressed Oct-4 and Nanog resulted in significantly upregulated expression of a series of pancreatic development-related genes (Sox17, Foxa2, Pdx1, and Ngn3), similar to that of native pancreas and islet tissues. MafA increased the expression levels of the mRNAs of NKx2.2, Glut2, insulin, glucagons, and somatostatin, and further facilitated the differentiation of PDMSCs into insulin + cells. Importantly, the expression of MafA in PDMSCs xenotransplanted into immunocompromised mice improved the restoration of blood insulin levels to control values and greatly prolonged the survival of graft cells in immunocompromised mice with STZ-induced diabetes [40].

Another successful lineage analysis and monitoring the induced trans-differentiation was reported by Cheng et al., in which a relatively abundant epithelial cell source, fetal human pancreas, was used to assess the proliferation potential, changes in lineage markers during culture, and capacity for generating insulin-expressing beta cells from fetal epithelial cells. The fetal epithelial cells readily formed primary pancreatic progenitor cultures, although their replication capacity was rather limited. This was overcome by introduction and expression of hTERT (human Telomerase Reverse Transcriptase) which greatly enhanced cellular replication in vitro. However, during culture the hTERT-modified pancreatic progenitor cells switched their phenotype gaining additional mesodermal properties. This phenotypic switching was inhibited when a pancreas-duodenal homeobox (Pdx)-1 transgene was expressed with a Lentiviral vector, along with inductive signaling through activin A and serum deprivation. This restored endocrine properties of hTERT-modified cells in vitro and were able to express insulin in vivo in immunodeficient mouse model [39]. The complexities of these result indicate that a sophisticated multi-gene cell therapies may be needed to solve the issues of proper modulation of transdifferentiation pathways.

Other strategies using a Lentiviral vector-based approach to achieve beta-cell proliferation through the beta-cell-specific activation of the hepatocyte growth factor (HGF)/cMet signaling pathway are also being explored. One of these methodologies is based on the beta-cell-specific expression of a ligand-inducible, chimeric receptor (F36Vcmet), under transcriptional control of the promoter from the human insulin gene, and its ability to induce HGF/cmet signaling in the presence of a synthetic ligand (AP20187) and result in specific proliferation of human pancreatic beta-cells [182]. The selective, regulated beta-cell expansion may help to increase the availability of cells for transplantation in patients with advanced diabetes.

These recent studies show that rapid progress may be achieved in this field and Lentiviral vectors may provide the necessary tools to analyze the issues. However, some of the notable efforts are made to avoid stem cell therapy altogether in certain types of diabetes. Instead, choosing a more direct route, applying in vivo gene therapy for expressing insulin gene in cell types other than beta cells. Ren et al. successfully restored near normal insulin levels for 500 days by expressing insulin in resting liver cells transduced with Lentiviral vector using a rat diabetes model [204].

14.13 Other Monogenic Diseases

Although monogenic disease appears to be the most obvious human diseases to treat with gene therapy, since they are caused by a single-gene defect, the progress in clinical studies has thus far been rather limited. Explanations for the lack of success include inefficiency of transductions in vivo, dangers posed by vectors, the failure to permanently correct the gene defect in sufficient number of cells, or the rapid turnover of cells. Alternative approaches therefore involve the search for and use of stem cell populations and depleting the active stem cell compartments ablation using cytostatic drugs to give chance if increased engraftment by transplanted stem cells. Combining the versatility and availability of MSCs, their ability to engraft, the use of autologous instead of allogeneic sources for safe transplantation, and the fact that the stem cell population can be expanded in vitro allows highly efficient ex vivo gene therapy relying on latest generation of Lentiviral vectors.

14.13.1 Cystic Fibrosis

Cystic fibrosis (CF) is caused by a mutation in the gene for the cystic fibrosis transmembrane conductance regulator protein (CFTR). The mutant form of the protein causes severe defect in mucus metabolism in the lungs and intestinal track that deteriorates into a life-long, deadly disease. CF is theoretically amenable to gene therapy. In spite of intensive research and a large number of clinical trials in the last 18 years, little practical success can be shown for treating cystic fibrosis [191]. The explanations include the fact that the deeper regions in the inner surface of the lung are not accessible to direct inhalation and direct treatment [48, 263]. In light of this finding, stem cells remain the most promising delivery vehicles. Castellani et al. reviewed the recent attempts to identify lung- or bone marrow-derived populations of stem cells or progenitor cells and application of such cells, allogenic or gene-corrected autologous cells, to colonize the airways, while differentiating into functional respiratory columnar epithelial cells [33]. When the reporter gene expression was analyzed in trachea-lungs and bronchoalveolar lavage, 0.4–5.5% of stem cells survived in injured airways, but no stem cells survived in control, healthy airway, or in the epithelial lining fluid [138]. The most successful approaches thus far appear to be obtained with bone marrow-derived MSCs, although the trans-differentiation rate thus far has been limited to below 10–14% [26]. As an alternative, the proven multipotent nature of bronchoalveolar stem cells isolated from lung tissue may provide another promising approach for stem cell therapy. Some additional improvement is expected from more efficient targeting of Lentiviral vectors. Mitomo and colleagues built a Sendai virus Env-pseudotyped SIV Lentiviral vector that can be manufactured at high enough titer and is capable of transducing respiratory epithelium of the murine nose in vivo at levels that may be relevant for achieving clinical benefit to cystic fibrosis patients [167]. Availability of novel cystic fibrosis gene-carrying

stem cell lines derived from placental mesenchymal cells certainly will help to speed up the research [53]. However, much more needs to be known about the normal differentiation and functioning of the airway's basal cells and the differentiation and lineages of stem cells to have more efficient treatment options both for gene therapy and for stem cell therapy [207]. We expect that the intensity of research and push for clinical trials will remain high as the outline of directions will become clearer. Also the methods to derive respiratory cell types from stem cells will remain a critical piece [181].

14.13.2 Duchenne Muscular Dystrophy

This disease is an X-linked recessive disorder caused by a mutation in the dystrophin gene that destabilizes muscle cell membranes and causes muscle dystrophy in approximately 1 of 3,600 boys. The musculoskeletal abnormalities deteriorate to a fatal level and the average life expectancy is no more than 25 years, even with high quality care. The research is facilitated by the availability of dystrophin-deficient transgenic mice (mdx-mice) and double knockout (utrophin/dystrophin-deficient mice) that can be used as experimental disease models [144, 269]. Human immortalized pluripotent cell lines expressing the mutant dystrophin gene are also available [187]. The ability of MSCs to differentiate into muscle cells places them on the top of the list of candidates that could be used to treat Duchenne muscular dystrophy [157].

Lentiviral vectors have been used in this field for conditional immortalization of human cells for basic biologic studies. Cudre-Maroux et al. demonstrated that the Lentiviral vector-mediated transduction of immortalizing genes into human primary cells is an efficient method for obtaining such cell lines. For Duchenne muscular dystrophy, the muscle satellite cell model was used to examine the impact of the transduced genes on the genotypic and phenotypic characteristics of the immortalized cells. The most commonly used immortalizing gene, the SV40 large T antigen (T-Ag), was extremely efficient at inducing the continuous growth of primary myoblasts, but the resulting cells rapidly accumulated major chromosomal aberrations and exhibited profound phenotypic changes. In contrast, the constitutive expression of telomerase and Bmi-1 in satellite cells from a control individual and from a patient suffering from Duchenne's muscular dystrophy yielded cell lines that remained diploid and conserved their growth factor dependence for proliferation. However, despite the absence of detectable cytogenetic abnormalities, clones derived from satellite cells of a control individual exhibited a differentiation block in vitro. In contrast, a Duchenne-derived cell line exhibited all the phenotypic characteristics of its primary parent, including an ability to differentiate fully into myotubes when placed in proper culture conditions. This cell line should constitute a useful reagent for a wide range of studies aimed at this disease [46]. A realistic source of stem cells would be the adipose tissue-derived stem cells that can be enhanced for muscle repair. Forced expression of MyoD using Lentiviral vector

in vitro strongly induced myogenic differentiation, while the adipogenic differentiation was inhibited. Moreover, MyoD-expressing human multipotent adipose-derived stem cells had the capacity to fuse with DMD myoblasts and can restore dystrophin expression. Importantly, transplantation of these modified human, multipotent, adipose-derived stem cells into injured muscles of immuno-depressed Rag2(−/−)gammaC(−/−) mice resulted in a substantial increase in the number of human multipotent adipose-derived stem cell-derived fibers [92]. Goncalves and colleagues went a step further and devised a technique to monitor the fusion events necessary for myoblast formation by using an elaborate bipartite genetic switch that relays on recombinase-inducible genetic switch that is activated after two cell types, one of which expresses Cre and the other the rest of the elements with LoxP1 sites, that switch on only upon fusion. This provides a sensitive tool to study the lineages and process of myocyte fusion in transgenic system [90].

Ikemoto et al. used high transduction-efficiency Lentiviral vector-mediated gene transfer into freshly isolated autologous satellite cells. Freshly isolated cells have better myogenic capability than satellite cell-derived myoblasts, and expansion of the satellite cells does not affect their regenerative potential. The transduced cells successfully regenerated the targeted muscle groups in mdx mice [112]. However, the VSVg pseudotyped Lentiviral vector are inferior in transducing nondividing murine cells, and Shunchang et al. demonstrated that by pseudotyping with feline immunodeficiency virus ENV better transduction rates can be achieved [234].

14.13.3 Chronic Granulomatous Disease

We include this disease because of the challenges researchers faced in attempts to use cell-based therapies. Granulomatous disease is a rather rare X-linked immunodeficiency disorder caused by mutations in the CYBB gene encoding the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase catalytic subunit gp91(phox). Earlier attempts to restore the gene function with oncoretroviral vectors failed due to (a) gene silencing common for retroviral inserts, (b) high risk of genotoxicity that these oncoretroviral vectors pose [165], and (c) low transduction efficiency and inability to target appropriate cell lineage and practice differentiation-restricted gene expression [17]. The solution for the compound problem seems to lie in using a safer and more efficiently targeting Lentiviral vector system [17, 223].

It has been demonstrated and repeatedly confirmed that by using Lentiviral vector it is possible to transduce HSCs as well as differentiated neutrophiles from patients with X-linked chronic granulomatous disease (X-CGD) and correct the X-CGD-phenotype in the NOD/SCID model. The Lentivector was a VSV-G-pseudotyped, third-generation, self-inactivating (SIN) Lentivector encoding gp91 (phox). Lentiviral vector efficiently transduced CD34+ peripheral blood stem cells under ex vivo conditions nonpermissive for cell division and resulted in 54% of the cells expressing gp91 (phox). Lentivector also achieved significant correction of

differentiated human X-CGD neutrophils arising in vivo in NOD/SCID mice that underwent transplantation (20% and 2.4%, respectively). Thus, third-generation SIN Lentivector-gp91 (phox) performs well as assessed in human X-CGD neutrophils differentiating in vivo, and the studies suggest that the NOD/SCID model is generally applicable for in vivo study of therapies evaluated in human blood cells expressing a specific disease phenotype [165, 209, 226]. However, long-term solution can be expected from transducing HSCs, and not the fully differentiated neutrophils that have only limited lifespan left and the lack of genotoxicity safety of the third-generation SIN v Lentiviral vector seem to address these requirements perfectly.

14.13.4 Wilson's Disease

Wilson's disease is a genetic disease caused by a spectrum of mutations in the ATP7B gene, whose product is a liver transporter protein responsible for coordinated copper export into bile and blood. Zhang and colleagues reported in 2011 an attempt to restore the normal phenotype by directed hepatocyte differentiation from human-induced pluripotent stem cells. The phenotype correction was achieved by chaperone drug curcumin that can reverse the functional defect in vitro in the case of the R778L Chinese hotspot mutation in the ATP7B gene. They propose this model system for correcting the gene using Lentiviral technology [284]. ATP7B gene seems to be relevant for the wider mesenchymal stem cell field as over-expressing it protects MSCs form copper toxicity. This in turn could be used as a selection advantage of transduced MSCs over the non-transduced ones in copper-rich environment for enriching the transduced mesenchymal cell compartment before transplantation [225].

14.14 Senescence, the Associated Diseases and the Role of Lentiviral vector-Modified MSCs

The image of the "fountain of youth" represents a mirage deeply engraved in the human psyche and expresses our fear and resentment of one of the inevitabilities of life: if we are lucky, we will get old, decrepit, suffer a lot from series of painful, chronic diseases, and finally succumb. The irony is that those who we consider unlucky, die young, but are saved from the long lasting predicaments of aging. Certainly, the intricacies of the factors leading to longevity, or the lack thereof, keep generations of stem cell researchers awake and busy and for good reasons. A model for aging has been found in the condition known as Progeria, or more precisely the Hutchinson–Gilford Progeria Syndrome, a rare disease affecting children of both sexes and which is caused by a mutant prelaminin A gene, encoding the lamin A-processing enzyme. Prelaminin A that retains a Farnesyl group, subsequently expressing its abnormal form, Progerin. Progerin in turn is anchored to the nuclear

membrane and destabilizes the nucleus, limiting the ability of cells to divide and leading to premature cell death. Unlike other accelerated aging diseases affecting DNA repair (Werner's and Cockayne's syndrome), progerin may play role in normal aging process [211] and its production is slowly turned on in cells that have uncapped chromosomes, i.e., have truncated telomeres, resulting in premature depletion of stem cell compartments. See the popular UCSF website for details: http://www.ucsf.edu/news/2011/10/10766/aging-disease-children-sheds-light-normal-aging.

Additional upregulation of multiple genes in major inflammatory pathways indicated an activated inflammatory response in progeria patients. This response has also been associated with normal aging, emphasizing the importance of studying progeria to increase the understanding of the normal aging process [178]. The progressing disease shows a pattern of tissue and organ degeneration that correlates with the depletion of a variety of stem cell compartments, a correlation first pointed out by Favreau [70]. The insight into the role of stem cell depletion in Progeria accumulated rapidly in the last couple of years [170, 178, 179, 211]. This leads to the establishment of an animal model by creating Zmpste24 knockout mice [67] in 2008, which showed premature senescence and progeroid symptoms. With the role of stem cells in aging and in Progeria, the doors opened for studying stem cell renewal via dedifferentiation. Autologous or heterologous transfer of native or Lentivector-enhanced cells [129, 184, 212, 265] are being actively considered as a possible interventions to slow down progeria as well as natural aging [28]. However, in both cases, the changes are systemic and murine gene therapy data indicate that the therapy in lysosomal storage disease models, affecting large segments of the body, is more efficient if done at an earliest possible age [28, 129]. This may have something to do with the limited availability of the microenvironment for the modified or transplanted stem cells. For this, the preexisting ones, even when "old" and malfunctioning, already occupy the microenvironment appropriate for stem cells. We already know that wound sites create new sites and attract MSCs [5]. Also, it is possible that cancer growth is able to generate and maintain an appropriate microenvironment for cancer stem cells [24] as well as MSCs [42] (potentially for use as anticancer agents) but the normal tissue, even in aging, seems to be resilient in accepting externally provided stem cells. Experiments are under way to create artificial microenvironments using nanotechnology to deliver stem cells that produce therapeutical factors [52], and 3D scaffolding mimicking bone marrow niches are being designed for similar purpose [55] and Lentiviral vector are often used to deliver the genes of interest [60, 141, 166, 248].

14.15 Clinical Trials Using Lentiviral Vectors and MSCs

VIRxSYS pioneered the use of lentiviral vectors in Phase I clinical trials to deliver antisense HIV genes as an Antisense RNA therapy for AIDS [110, 156]. This established an initial safety profile for the ex vivo use of Lentiviral vectors (see http://

ClinicaTrials.gov: identifier VRX496-USA-05-002). This Phase I trial demonstrated the safety and tolerability of a single dose of approximately ten billion autologous HIV infected CD4+ T cells transduced with the Lentivector VRX496 carrying a 937-base antisense targeting the HIV envelope. These encouraging results have led to design of a Phase II clinical trial to evaluate the safety, tolerability, and biological activity of four or eight repeated infusions of five to ten billion autologous VRX496-modified HIV+, CD4+ T cells. A major obstacle to completing this Phase II trial was manufacturing enough cells to administer multiple infusions in patients. For this study the safety issues were cleared successfully, opening the way for more extensive use of Lentiviral vectors in clinical trials.

Currently, 16 lentiviral clinical trials are listed at the ClinicalTrials.gov website. All of these trials are in early stage, Phase I or II. Three of these trials have not yet started and 11 trials are still recruiting patients. Most of the clinical trials are focused on hematopoietic stem cells, which are outside of the scope of this work. One trial targets Netherton Syndrome (ClinicalTrials.gov: identifier: NCT01545323) and attempts to restore LEKTI serine protease levels in an affected 5 cm^2 skin area, a proof of principle study that has the potential to utilize mesenchymal stem cells in the future.

14.16 Conclusions and Future Directions

We witnessed a tremendous progress in characterizing and understanding stem cells, the factors needed for maintaining the stem cell phenotype as well as changing it in a predictable mode forcing the mesenchymal stem cells into various differentiation pathways. This progress provides a test bed for a higher level of bioengineering when the genetic buildup of the stem cells is changed to achieve well-defined therapeutical goals. The overview of the recent literature presents a long list of "proof of concept experiments" in which tantalizing possibilities are validated as things that can be accomplished in a wide range of fields representing different pathologies: from the debilitating Alzheimer's, Parkinson's diseases; various traumatic neuronal injuries, diabetes, neuronal and cardiac ischemia; to age-related tissue degeneration and tissue engineering or delivery of biologics for therapeutical purposes. In parallel, Lentiviral vectors are becoming highly valued tools in this tedious work as they are highly efficient vehicles for gene delivery to mark cells, express genes of interest, proteins, and various inhibitory RNA species in stem cells. Consequently these stem cells, especially the various forms of MSCs, have been shown to be highly effective in delivering the targeted genes to difficult-to-reach tissues, including the CNS. Manipulating genes and gene expression, gene transfer has been made safe and efficient by the recent progress in Lentivector technology and merged successfully with the stem cell technology. This field has reached an advanced stage, at which it has become feasible to use them safely in a clinical environment. More and more researchers as well as clinicians are becoming familiar with the power of these technologies both for ex vivo and in vivo cell therapy. It

does not take a prophet to predict that advanced stem cell therapy has gaining a strong foothold, and even though a tremendous amount of work is needed to be done for it to become a everyday intervention, it is here to stay and will become a routine treatment for the next generation of patients.

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Chapter 15
Genetically Engineered Mesenchymal Stem Cells for Cell and Gene Therapy

Yunjoon Jung and Jan A. Nolta

Abstract Stem cells have enormous potential for regenerative medicine to treat fatal diseases and injuries that cannot otherwise be healed. In particular, adult stem cell-based therapies have been studied for several decades. Mesenchymal stem cells/marrow stromal cells (MSCs) have shown safety and therapeutic efficacy in preclinical models of various diseases such as cardiovascular disease, cancer, bone defects, renal failure, and neurodegenerative disorders. In spite of the great potential, several factors including low survival rate, low efficiency of MSC homing to injured sites, and poor levels of engraftment and retention have been major

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technical challenges to be overcome before MSC-based therapy can be applied to clinical applications in a consistently therapeutic manner. Genetically modified MSCs can be one option to overcome some of these problems and to deliver therapeutic agents. MSCs are powerful delivery vehicles and potent protein synthesis factories, and therefore the use of gene-modified MSCs to provide growth factors and other signals to improve the repair of damaged or diseased tissues holds much promise. Here we review the basic biology of human MSCs and the current status of preclinical and clinical trials using genetically engineered MSCs.

Keywords Mesenchymal stem cells • Gene therapy • Cellular therapy • Preclinical models • Growth factor production • Revascularization • Cancer • Renal disease • Bone disease • Neurodegenerative disorders • Clinical trials • Regenerative medicine

15.1 Human Diseases and Stem Cells

15.1.1 Degenerative Diseases

The human body is made up of millions of cells. When cells are injured, we may experience disease or disability. Those with type I diabetes have damaged or decreased numbers of islet cells, which are unable to produce sufficient amounts of insulin. Such individuals must substitute what is missing by taking frequent, daily insulin injections. Those with spinal cord injuries have damaged nerve cells which are no longer able to conduct messages from limbs to the brain and back and as a result have lost the ability to move some part of their body. Some organs like the liver and skin are excellent at repairing and regenerating themselves, while other organs or tissues have far less capacity to do so.

Islet or whole organ transplantation is one of the methods to cure these types of diseases but immune rejection is a major problem and the patients must often remain immunosuppressed, increasing risk of infections and cancers. Organ transplantation is also hampered by severe donor shortages. In spinal cord injury, transplantation is not even an option to consider. One promising new way to treat some diseases and disabilities is to regenerate injured or missing cells with stem cells, either by replacing those that have been damaged in the tissue or organ (e.g., pluripotent cell-derived differentiated cell types), or to provide cells that deliver factors that can encourage endogenous recovery (e.g., mesenchymal stem cells).

15.1.2 Pluripotent Stem Cells and Their Therapeutic Potential

Both embryonic stem cells (ESCs) and adult stem cells (e.g., hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs)) are under examination in clinical trials of cell therapy. Bone marrow (BM) transplantation, which has provided great success in transplantation for more than 50 years, has shown a great therapeutic benefit for the blood-forming system and this may extend to promoting healing of other tissues [1].

Recently, researchers' attention has been focused on human (h) ESCs, which theoretically have the potential to differentiate into all types of adult human tissues (pluripotency) and can grow indefinitely (self-renewal) [2]. Since their initial derivation, hESCs have become a promising tool for developmental biology and regenerative medicine. However, concerns related to ethical objections regarding the use of human embryos for hESC derivation have dramatically restricted research using these cells and therefore have set back the development of hESCs for clinical trials, although recent first-in-human phase I clinical trials from the company Geron were initiated in 2011 [3]. Later, Geron announced that they pulled out their entire program due to the financial reasons, but to date, safety has been demonstrated [4].

Due to their allogeneic nature, immune rejection of transplanted cells or tissues derived from hESCs is another potential drawback to therapeutic applications. The immune system of the patient recognizes transplanted "foreign" cells or tissues and escalates a rapid response, attacking the graft. This attack can result in the loss of the graft, which can lead to the death of patients if it was an organ on which the patient was reliant (e.g., heart). Immunosuppressive drug regimens, similar to those used for current human tissue and organ transplant procedures, might lessen the severity of the anticipated immune rejection, but at the same time may put the tissue recipient at an increased risk for infections. This risk can be lessened by application of human leukocyte antigen (HLA)-matched tissue, as is currently being practiced in organ transplantation, or could be completely eliminated by the use of the patient's own tissue. The latter possibility might, in the future, be achieved by reprogramming the patient's own somatic cells to induced pluripotent stem cells (iPSCs) [5].

Takahashi and Yamanaka pioneered methods to generate iPSCs by virally transducing four transcription factors into human somatic cells and showed that the resultant iPSCs have similar characteristics to ESCs [5]. Recent reports, however, have indicated that iPSCs are not exactly the same as ESCs and may be more prone to genetic mutations during the reprogramming and expansion phases [6]. Also, abnormal gene expression in some differentiated cells from iPSCs can induce T-cell-dependent immune responses in autologous transplantations done in mice [7]. Differentiated progenies from iPSCs and hESCs still have an immature status similar to early human fetal tissues (<6 weeks), and it is too early to tell whether it will be appropriate to apply these cells for transplantation therapies in humans [8].

15.1.3 Mesenchymal Stem Cells and Clinical Trials

Over the last half century, adult stem cell therapies in the form of bone marrow, mobilized peripheral blood, and umbilical cord blood transplantations have rescued thousands of patients from induced or genetic disorders [9]. After the first human hematopoietic stem cell transplantation in 1956, the technique gradually evolved to become a standard clinical procedure (reviewed in [10]). Mesenchymal stem cells (MSCs) were first described as adherent "marrow stromal cells" and were studied for their role in supporting hematopoiesis and were then engineered to provide

factors for other cells (reviewed in [11]). Later, these cells were found to differentiate into cartilage, bone, fat, tendon, and fibroblasts [9]. Over the past three decades of study, MSCs have become a tool for regenerative applications either through direct differentiation into specific tissues (e.g., bone), or indirectly through protein or cytokine secretion and immune suppression [9, 11–14]. MSCs have become a promising cell-based therapeutic because they are easily accessible from various tissues (e.g., bone marrow, fat and umbilical cord tissue) and are easily grown in culture. MSCs can be expanded in vitro to a clinical scale and can be cryopreserved without the loss of their integrity.

MSCs have demonstrated systemic migration after intravenous injection, in particular to areas of hypoxia or tissue damage [15]. The systemic administration of allogeneic MSCs has not been observed to cause any adverse effects in numerous treated patients, in part due to immunomodulatory effects [16, 17]. Also, MSCs have been considered safe as they do not show tumor formation after transplantation [18] and have been widely tested and shown efficacy in preclinical and clinical studies for cardiovascular, neurodegenerative, graft-versus-host (GvHD), and autoimmune diseases [9, 11]. Due to the MSC's osteoblast differentiation potential, Caplan and colleagues applied allogeneic MSCs to osteogenesis imperfecta patients [19]. In addition, LeBlanc et al. investigated the immunomodulatory effects of transplanted MSC for steroid-resistant GvHD [16], and similar methods were applied to other diseases [9]. These early studies have established a good clinical record of safety for systemic MSC administration.

15.2 Genetic Modification of Mesenchymal Stem Cells

Even though there have been remarkable advances in demonstrating safety in MSC clinical trials, therapeutic efficacy is still debated [9]. Unsolved problems such as low cell survival and engraftment efficiency after MSC transplantation still remain to be resolved [13, 14]. Genetic engineering of MSCs is a potential means to improve their therapeutic potential. MSCs can be modified to express therapeutic agents, to improve cell survival or to possess an enhanced ability to home to a disease site. In the following section, we will briefly discuss the pros and cons of several gene modification methods.

15.2.1 Choice of Vector Systems

Genetic modification of MSCs can be achieved by permanent integration or episomal expression of target genes via viral vector transduction or by transient expression of specific genes using nonviral delivery [20]. Viral delivery of desired genes to MSCs is one of the most utilized methods in preclinical studies due to the ability to achieve high infectivity with broad tropism. However, the transduction efficiency

Table 15.1 Summary of viral vectors

Characteristics	Retroviral vector	Lentiviral vector	Adenoviral vector	AAV vector
Cloning capacity (kb)	8	9	8-10	4.9
Chromosomal integration	Yes	Yes	No	No (yes if rep is included)
Transgene activity	Long-term	Long-term	Weeks	>1 year
Infect nondividing cells	No	Yes	Yes	Yes
Oncolytic activity	No	No	Yes	No

Abbreviation: *kb* kilobase

depends on the target cells (Table 15.1). Clonal analysis of transduced MSCs has shown that these cells often contain several thousand copies of transgene RNA per cell and can maintain transgene expression for 6 months or longer [11, 21–23]. In MSC studies, retroviral, lentiviral, adenoviral, and adeno-associated virus (AAV) vectors are generally used [20]. Adenoviral and AAV vectors do not integrate into the host genome but can express transgenes in an episomal manner. In non-dividing cells, these viral particles can sustain long-term transgene expression but would be diminished as cells proliferate in dividing cells due to the dilution of viruses. However, it is well documented that the capsids of adenoviral vector can be recognized by the patient's innate immune system and can cause adverse events [24]. Even though success has been shown with the delivery of factor IX with AAV into the hepatocytes of human hemophilia patients, transduced hepatocytes were cleared due to an immune reaction to capsids from the AAV [25]. To achieve short-term transgene expression in MSCs for applications such as angiogenic growth factor expression after myocardial infarction or surface antigen modification for increase cell survival, it may be beneficial to choose AAV vectors.

MoMuLV-based retroviral and HIV-based lentiviral vectors can offer long-term expression of transgenes in target cells due to their permanent integration into the host genome. While lentiviral vectors can transduce both dividing and non-dividing cells, retroviral vectors can only transduce dividing cells (Table 15.1). In terms of chromosomal integration sites, there are differences in the integration hot spots between these two viral vectors, but most integration is into the active regions of chromosomes. Retroviral vectors tend to favor integration into transcriptional start sites, promoter regions, or CpG islands, while lentiviral vectors do not appear to have preferential integration regions [26, 27]. Numerous papers, including work from our own lab, have demonstrated that MSCs can be transduced with retroviral or lentiviral vectors and can retain transgene expression for many passages. In addition, transduced cells retain in vitro lineage-specific differentiation and in vivo engraftment, with no detectable complications caused by viral integration [11, 18, 22, 23, 28–31].

Although a promising method, nonviral gene transfer must overcome low efficiency (discussed in [32]). To test gene delivery methods, McMahon et al. tested GFP expression via adenoviral, AAV, lentiviral, plasmid transfection, and electroporation in rat MSCs [33]. Lentiviral delivery showed the highest GFP expression with minimal cell death. Adenoviral vectors provided effective GFP expression but with

a reduced transduction efficiency and an increased cell toxicity compared to lentiviral vectors. In that study, AAV vectors could not effectively deliver transgenes into rat MSCs, although another study showed that AAV vectors could infect human MSCs [34]. To improve the transduction efficiency for AAV vectors, specific serotypes of AAV must be chosen as only serotype 2 AAV vectors were shown to have a high MSC transduction efficiency [35]. Transfection and electroporation of plasmids were not as effective as viral delivery of GFP and were harmful to MSCs due to the associated cytotoxicity. Furthermore, transfected plasmids can randomly integrate as concatamers into host chromosomes at a frequency of 1/3000 to 1/5000 [36].

15.2.2 Safety Considerations for Genetic Modifications

Even though long-term gene expression can be achieved by retroviral and lentiviral vectors, the risk of insertional mutagenesis remains a concern when considering genetically engineered MSC for clinical trials. In the hematopoietic stem cell (HSC) clinical trial that used retroviral vector for X-linked severe combined immunodeficiency disease (X-SCID) in France, four out of eleven children developed leukemia [37, 38]. One out of ten patients treated by hematopoietic stem cell gene therapy for Wiskott–Aldrich syndrome also developed acute lymphocytic leukemia [39]. Later, it was discovered that the leukemia in both cases was due to the long-terminal repeat (LTR) of the retroviral cassette having integrated in the proximity of the *LMO2* proto-oncogene promoter. As a result, the integrated LTR acted as a promoter to drive LMO2 expression and lead to leukemia, in cells that were greatly expanded already due to the selection process in both trials, and delivery of a growth factor receptor in the case of XSCID.

Naldini and colleagues compared the in vivo tumor induction capacity by both retroviral and lentiviral vectors [40]. Using the tumor-prone p16 knockout mouse strain, this group found that retroviral vectors triggered a dose-dependent induction of tumor onset, while lentiviral vectors showed low genotoxicity upon integration. Later, the same group also showed that the retroviral LTR is the major component capable of generating unregulated cell growth in this tumor-prone mouse model, by swapping between retroviral vector LTR and self-inactivating (SIN)-LTR in lentiviral plasmids [41]. Indeed, additional gene-modified stem cell clinical trials using SIN-LTR lentiviral vectors have avoided this outcome [42]. The possibility of adverse events can be monitored by serial transplantation experiments in vivo [18, 40].

In contrast to hematopoietic stem cells which are capable of long-term self-renewal and differentiation in vivo, gene-modified MSCs have not been reported to cause tumors, using in vivo assays. We have completed a comprehensive decade-long study of the biosafety of MSCs stably transduced by retro- and lentiviral vectors and did not observe adverse events arising from the human cells, in sensitive xenograft assays [18]. However, it should be noted that we did not perform serial transplantations and the follow-up of the human MSCs in immune-deficient mice was limited to 18 months, due to the natural lifespan of the mice.

A way to potentially avoid risks from random gene insertion would be to use human embryonic stem cell (ESC) or human induced pluripotent stem cell (hiPSC)-derived MSCs [43] which have safe harbor integrations of the transgenes and have been subsequently expanded in vitro. The lifespan of primary MSCs are limited up to 40 doublings during in vitro expansion [44]. Aging significantly reduces the in vitro and in vivo survival and differentiation potential of primary MSCs [45]. This could be potentially overcome in the future by the use of hESC or hiPSC-derived MSCs, since these cell types could theoretically be expanded indefinitely after transduction and prior to differentiation to the MSC lineage (discussed in [46]).

15.3 Gene-Modified MSCs in Pre-clinical Models

MSCs genetically modified to secrete cytokines and other growth factors have been successfully used in animal models for various diseases, and are therefore poised to be tested in human clinical trials [11]. Accumulating evidence indicates that genetically modified MSCs have therapeutic potential in various disease models and that genetic engineering of the cells can improve cell survival, homing to the disease sites, secretion of therapeutic agents and differentiation into different cell types. Here we will describe the current status of gene-modified MSCs in various preclinical models.

15.3.1 MSC Survival After Transplantation

The majority of intravenously transplanted MSCs appear to die within several hours or lodge nonspecifically in the lung, spleen, liver, or kidney [11, 14, 22, 47]. To improve MSC survival after transplantation, several approaches have been examined including the overexpression of proliferation-related or anti-apoptotic-related genes or preconditioning using hypoxia or other approaches prior to transplantation (Table 15.2).

Several laboratories have focused on the overexpression of Akt, a protein which inhibits apoptosis. MSCs engineered to overexpress Akt survived longer than unmodified MSCs after transplantation in a variety of animal models. Dzau and colleagues documented that Akt-overexpressing MSCs had a higher survival rate after transplantation into an ischemic heart model and showed that the improvement of cardiac function following transplantation was due to the paracrine factors secreted from the surviving MSCs [48]. Another paper from the same group further demonstrated that transplanted Akt-modified MSCs balanced the metabolism and pH of the myocardium [49]. Recently, a swine myocardial infarction model also added to the evidence showing that Akt transduced MSCs survived longer and showed greater efficacy than unmodified MSCs [50]. Our group has shown that, rather than performing gene modification, hypoxic preconditioning of human MSCs at 3% oxygen

Table 15.2 Summary of survival-related gene expressing MSCs in preclinical models

Transgene	Model	Route of administration	Effect	Ref
Akt	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[48]
Akt	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[49]
Akt	Swine infarcted myocardium	Intra-myocardial	Improved cardiac function	[50]
Connexin43	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[51]
Hsp20	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[52]
Bcl-2	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[53]
Bcl-xL	Rabbit cartilage defect	Intra-articular	Improved cartilage healing	[54]
HO-1	Rat infarcted myocardium	Intra-myocardial	Improved cardiac function	[55–58]
HO-1	Mouse infarcted myocardium	Intra-myocardial	Improved cardiac function	[59]
HO-1	Swine infarcted myocardium	Intra-myocardial	Improved cardiac function	[60]

Abbreviation: *Hsp20* heat shock protein 20, *Bcl-2* B-cell lymphoma 2, *Bcl-xL* B-cell lymphoma-extra large, *HO-1* Heme oxygenase 1

for 24 h prior to transplantation will upregulate AKT activity, enhance cell homing and survival, and enhance their in vivo capacity to promote revascularization in a xenograft model of hindlimb ischemia [61].

Overexpression of connexin43, a gap junction protein, also showed a higher MSC survival rate and improvement of cardiac function. This result was shown to be due to the fact that these cells expressed more Bcl-2, one of the negative regulators in the apoptotic pathway, along with phosphorylated Akt. These cells also expressed less Bax, a pro-apoptotic protein [51]. In the same fashion, Hsp20 overexpression in MSCs has been shown to increase the cell survival rate along with reduced fibrosis. Hsp20 can protect other proteins against heat-induced cellular stress. The beneficial effects of these gene-modified MSCs were associated with enhanced Akt activation and increased secretion of growth factors such as VEGF, FGF-2, and IGF-1 [52].

MSCs engineered to express Bcl-2, which is one of the key anti-apoptotic proteins, have shown better survival and improved cardiac function due to reduced apoptotic events and increased VEGF secretion [53]. Recent studies using Bcl-xL-(one of the Bcl-2 family members) modified MSCs also supports this concept [54]. Direct intra-articular injection of Bcl-xL overexpressing MSCs into a rabbit articular cartilage defect model improved MSC survival and increased cartilage healing. Several studies mediated Heme oxygenase (HO-1), which is known for protection against apoptosis in rat [55–58], mouse [59], and swine cardiac ischemic models [60]. It is well known that HO-1 itself has therapeutic potential in the treatment of cardiac disease [62].

Although overexpression of pro-proliferative and anti-apoptotic genes in MSCs improved cell survival after transplantation, there must be extreme caution for any consideration of application to human clinical trials as these genes are related to

Table 15.3 Summary of homing-related gene expressing MSCs in preclinical models

Transgene	Model	Route of administration	Effect	Ref
CXCR4	Rat infarcted myocardium	Intra-myocardial	Improved MSC homing and cardiac function	[69]
CXCR4	Mouse infarcted myocardium	Intra-myocardial	Improved MSC homing and cardiac function	[70]
CCR1	Mouse infarcted myocardium	Intra-myocardial	Improved MSC survival, homing and cardiac function	[71]

Abbreviation: *CXCR4* C–X–C chemokine receptor type 4; *CCR1* C–C chemokine receptor type 1

cancer activity and overexpression may lead to cell transformation. Therefore it is most prudent to learn from the overexpression data in these instances and apply this knowledge to developing novel ways to precondition cells prior to transplantation to achieve similar effects. For example, our group others have explored the use of hypoxic prestimulation prior to transplantation [61, 63, 64].

15.3.2 MSC Homing

Efficient MSC homing to the tissue of interest is one of the most important aspects of effective MSC therapy. Extensive studies have shown that MSC migration is mediated by growth factor or chemokine/receptor pairs such as SDF-1/CXCR4, HGF/c-MET, VEGF/VEGFR, MCP-1/CCR2, and others (detailed review in [14]). The well-known homing receptor CXCR4 is a chemotactic receptor for stromal cell-derived factor-1α (SDF-1). CXCR4 is absent in populations of in vitro expanded MSCs, but freshly isolated MSCs have a small positive population [65–67]. It has been shown that hypoxic (tissue normoxic) preculture can induce CXCR4 expression [64]. Surface antigen modification of CD44 by the FUT VI enzyme improved homing efficiency of BM-MSCs into the bone in NOD/SCID mice without the expression of CXCR4 [67]; ex vivo engineered E-selectin, which is not expressed naturally in MSCs, was also shown to be sufficient to home MSCs into bone[67]. Also, our group has also shown that modification of MSCs with bone-homing ligands tethered to bisphosphonate has also resulted can result in homing of MSCs back to the bone [68].

In a rat myocardial infarction model, MSCs engineered to overexpress CXCR4 showed greater numbers of cells that had homed to ischemic sites and improved left ventricular function, as compared to unmodified MSCs [69] (Table 15.3). Huang et al. further analyzed that CXCR4-overexpressing mouse MSCs migrated to the infarction site and released the collagen degrading enzyme, matrix metalloproteinase-9 (MMP-9), which lead to a reduction of the remodeling of infarcted myocardium [70]. Dzau and colleagues turned their focus on a different chemokine receptor, CCR1 [71]. This receptor is one of the G protein-coupled receptors known to bind to CCL7 and is usually expressed by MSCs at low levels.. This group noticed that

infarcted hearts have higher expression levels of CCL7. To better guide MSCs to ischemic sites, they overexpressed CCR1 in murine MSCs. These cells had better survival, reduced cardiac remodeling and increased cardiac functions in comparison with non-engineered MSCs.

Since permanent expression of E-selectin is not required to home MSC to bone, transient expression of these homing proteins can be considered [67]. Nonviral methods such as plasmid transfection, cytokine treatment, hypoxia, and others that can increase levels of homing receptors can be an alternative method to improve MSC localization to bone, to the perivascular space, and to damaged tissues in general.

15.3.3 Cardiovascular Diseases

Cardiovascular diseases are the leading cause of death in the USA. An estimated 79 million American adults (1 in 3) have one or more types of cardiovascular diseases [72]. Ischemia and hypertensive heart failure cause irreversible loss of cardiomyocytes. Potent pharmacological treatments have significantly improved morbidity and mortality [73]. These methods along with the development of implantable cardioverter-defibrillators [74] and left ventricular assist devices have all significantly increased survival rates [75]. Despite all these improvements in clinical management, the prevalence of heart failure remains. The current best therapy for cardiac failure, heart transplantation, is hampered by the shortage of organ donors. Stem/progenitor cell transplantation for curing cardiac diseases remains an attractive concept that is studied in numerous preclinical and clinical trials.

So far, most gene-modified MSC studies using anti-apoptotic and proliferative genes showed improvement of cardiac function in acute cardiac infarction cases due to better survival of transplanted MSCs and secretion of various growth factors (Table 15.4). Among the growth factors, the most heavily studied is vascular endothelial growth factor (VEGF). VEGF-overexpressing MSCs administered to treat acutely infarcted heart in mouse [76] and rat [77, 78] significantly increased vascular density, reduced the infarcted area and improved cardiac function. Human MSCs genetically modified to secrete VEGF were also found to significantly enhance blood flow recovery in an immune-deficient mouse model of hindlimb ischemia [83]. Hepatocyte growth factor (HGF) is also one of the promising options to improve cardiac ischemia. Ectopic expression of HGF in MSCs improved cardiac function, reduced ventricular remodeling, and enhanced vascular density in rat models [79–81]. Another study confirmed that HGF or VEGF-expressing MSCs also improved cardiac function [82].

In coronary or peripheral artery diseases, bypass surgery or angioplasty is popular solution. Our laboratory examined cell fates, proliferation of growth factor overexpressing MSCs and angiogenesis using VEGF-overexpressing human MSCs in an immune-deficient mouse ischemic hind limb injury model [30]. Other studies have focused on the therapeutic potential of factor releasing MSCs, but cell fate decisions and the proliferation potential of vector containing MSCs are less well illustrated.

Table 15.4 Summary of growth factor expressing MSCs in cardiovascular preclinical models

Transgene	Model	Route of administration	Effect	Ref
VEGF	Mouse infarcted myocardium	Intra-myocardial	Increased vascular density and cardiac function	[76]
VEGF	Rat infarcted myocardium	Intra-myocardial	Improved MSC homing and cardiac function	[77, 78]
HGF	Rat infarcted myocardium	Intra-myocardial	Improved MSC homing, reduced remodeling, increased cardiac function	[79–81]
HGF/VEGF	Mouse infarcted myocardium	Intra-myocardial	Increased vascular density and cardiac function	[82]
VEGF	Mouse ischemic hind limb model	Intravenous injection	Enhanced blood flow restoration in ischemic hind limb model	[30]

Abbreviation: *VEGF* Vascular endothelial growth factor, *HGF* Hepatocyte growth factor

We showed that bFGF or PDGF-B overexpression in MSCs increased proliferation. When cultured in differentiation conditions, both bFGF and PDGF-B overexpressing MSCs showed enhanced osteogenesis, but strong inhibition was shown for adipogenesis in MSC overexpressing PDGF-B and only mildly affected in the MSCs overexpressing bFGF. Overexpression of TGF-β_1 blocked both osteogenic and adipogenic differentiation but VEGF overexpression did not vary in any of these differentiation assays, most probably due to the lack of VEGF receptor expression on MSCs. Therefore, due to the lack of autocrine effects on the MSCs that would produce it, we further examined the role of MSCs engineered to produce VEGF165a in vivo. VEGF overexpressing MSCs were demonstrated to significantly enhance blood flow restoration in a xenograft model of hind limb ischemia, without adverse events [30].

15.3.4 Cancers

Cancer is the second leading cause of death in the USA, accounting for 1 in every 4 deaths over all ages in 2010 [84]. It is estimated that approximately 1 in 2 men (44%) and 1 in 3 women (38%) have a lifetime probability of being diagnosed with an invasive cancer. Since the declaration of "War on Cancer" in 1971, there have been tremendous advancements in cancer biology and successful drug treatments. In consensus, metastatic cancer is the major cause of deaths, not the primary cancer. Metastatic cells spread to the bones, lung, kidney, liver, brain, and other organs, and it is very difficult to locate these metastases by established diagnostics. The short half-life of some drugs limits their delivery to some metastatic tumor sites and side effects on non-tumor cells is one of the major impediments to curing cancers.

MSCs have been proposed as one of the several treatment modalities for cancer therapy due to supposed antitumor effects, but this is still highly controversial. Some

papers claimed that MSCs had antitumor properties such as inhibiting the proliferation of glioma, melanoma, lung cancer, hepatoma, and breast cancer [85]. Others showed that MSCs secreted IL-6 and this increased proliferation [86] or production of CCL5 from MSCs and increased metastasis of breast cancer cells [87]. One thing that both sides agreed on is that MSCs migrate into cancer sites with not fully understood mechanisms [85]. With this notion in mind, many groups modified MSCs as delivery vehicles for therapeutic reagents; categorized as immunostimulatory agents, cytotoxic agents, prodrug activators, and viral vector delivery (detailed review in [85]).

15.3.4.1 Immunostimulatory Agents

Cancers have an ability to modulate their environments to hide their identity [88]. Stimulating endogenous immune systems by cytokines is one of the interesting options to treat cancers. Interleukins are known to regulate inflammatory and immune responses [89]. IL-12 and IL-18 are known to kill tumors directly and to recruit T cells and natural killer cells and those cells can eradicate tumors [90]. Administration of MSCs expressing IL-12 compared to adenoviral delivery of IL-12 every 5 days for 4 times showed reduction in the spread of metastatic melanoma, breast cancer, and hepatoma [91] (Table 15.5). IL-12 delivered by adenoviral vector showed toxicity and the levels of IL-12 were only elevated in the serum, but not the intratumoral environment. However, MSC overexpressing IL-12 showed increased apoptosis of tumor cells and higher levels of IL-12 in the intratumoral samples.

The same concept to eradicate renal carcinoma was applied by Gao et al. [92]. They injected MSCs bearing IL-12 once in xenografted nude mouse models and showed reduction of tumor growth and prolonged survival compared to systemic administration of adenoviral delivery of IL-12. Other teams reported that IL-12 expressing MSCs showed therapeutic efficacy on melanoma and cervical cancers [93] as well as intracranial glioma [94]. Similarly, IL-18 modified MSCs also have been investigated to treat glioma in a rat model [95]. IL-18 expressing MSCs were systemically administered and showed inhibition of glioma growth and prolonged survival of rats bearing glioma. IL-2 expressing MSCs also showed similar efficacy in a rat glioma model [96].

Interferons (IFNs) are cytokines released from the host cells and have functions to activate natural killer cells or macrophages and to increase antigen presentation to be recognized by T cells [90]. IFNα and IFNβ were pursued to treat various tumors using MSCs as the vehicle to deliver them, because systemic administrations of IFNs cause toxicity in vivo. As described earlier, MSCs are prone to migrate into tumor sites and IFN expressing MSCs recruit cells of the host immune system. IFNα overexpressing MSCs were evaluated in mouse melanoma lung metastasis models [97] and a mouse plasmacytoma model [98]. Both studies showed that intravenously [97] and subcutaneously [98] injected MSCs producing IFNα increased tumor apoptosis and decreased cancer proliferation along with prolonged survival of mice bearing tumors. Several laboratories utilized IFNβ expressing MSCs to treat

Table 15.5 Summary of immune-stimulatory gene expressing MSCs in cancer preclinical models

Transgene	Model	Route of administration	Effect	Ref
IL-12	Metastatic mouse model	Subcutaneous injection every 5 days for 4 times	Prevention of metastatic melanoma, breast cancer, and hepatoma	[91]
IL-12	Renal carcinoma in xenograft nude mouse model	One time tail vein injection	Reduction of tumor growth and prolonged survival of mice	[92]
IL-12	Melanoma and cervical cancer mouse model	Intravenous injection	Anti-metastatic effects	[93]
IL-12	Intracranial glioma mouse model	Ipsilateral hemisphere injection	T-cell infiltration in intracranial gliomas, and anti-angiogenesis	[94]
IL-18	Rat glioma model		Inhibition of glioma growth and prolonged survival of glioma-bearing rats	[95]
IL-2	Rat glioma model	Injection to contralateral hemisphere	Inhibition of glioma growth and prolonged survival of glioma-bearing rats	[96]
IFNα	Mouse melanoma metastasis model	Intravenous injection	Increase in tumor apoptosis and a decrease in proliferation and blood vasculature and prolonged survival of mouse bearing tumors	[97]
IFNα	Mouse plasmacytoma model	Subcutaneous injection	Increase in tumor apoptosis and a decrease in proliferation and prolonged survival of mouse bearing tumors	[98]
IFNβ	Melanoma nude mouse model	Intravenous injection	Inhibition of the growth of malignant cells *in vivo*	[99]
IFNβ	Melanoma and breast cancer mouse model	Intravenous injection	Inhibition of tumor cells growth and prolonged survival of mice bearing tumors	[100, 101]
IFNβ	Prostate cancer lung metastasis model	Tail vein injection	Increased tumor cell apoptosis and natural killer cell activity, decreased tumor cell proliferation and blood vessel counts	[102]
IFNβ	Metastatic pancreatic cancer model	Intraperitoneal injection	Increased tumor growth inhibition. anti-inflammatory drug reverses the MSC-mediated effects	[103]

Abbreviation: *IL-12* Interleukin-12, *IL-18* Interleukin-18, *IL-2* Interleukin-2, *INFα* Interferon α, *IFNβ* Interferon β

various tumors in rodent models [99, 100, 102, 103]. Studeny et al. showed that IFNβ-modified MSCs inhibited melanoma growth in vivo [99]. Interestingly, therapeutic efficacy was only shown when MSCs had migrated to tumor sites but systemically delivered IFNβ or that produced by MSCs at a site distant from the tumors did not. Similar approaches were applied to a breast cancer model [100, 101], prostate cancers [102], and pancreatic cancers [103].

15.3.4.2 Cytotoxic Agents

Tumor necrosis factor-related apoptosis-induced ligand (TRAIL) is a pro-apoptotic protein that will enter cancer cells but normal cells are not affected [104, 105]; TRAIL-induced apoptosis occurs via the caspase pathway. A major drawback of systemic administration of TRAIL is a large amount of TRAIL needed to kill cancers due to the fast clearance of TRAIL by the kidney [106]. Several groups investigated MSCs expressing TRAIL as a vehicle to deliver locally to tumor sites and to sustain the TRAIL expression enough to kill the cancer (Table 15.6). Szegezdi et al. showed that MSCs were not sensitive to TRAIL-induced apoptosis because TRAIL receptors in MSCs were inactive and downstream genes of the TRAIL pathway were rarely expressed [119]. Another report also confirmed that TRAIL did not affect the MSC characteristics such as cell proliferation and differentiation into osteogenic and adipogenic lineages, and that it enhanced the migration of MSCs [120]. With these characteristics, the therapeutic efficacy of TRAIL-secreting MSCs was evaluated in various cancer models. MSC-mediated TRAIL delivery in a human/mouse xenograft model was performed by Mohr et al. [107]. They showed that TRAIL-expressing MSCs could reduce the growth of human lung carcinoma xenografted into immune-deficient mice. TRAIL-expressing MSCs were evaluated in different cancer types including glioma [108, 109, 111, 112, 116, 117], lung cancer [110], breast cancer [110, 113], squamous cancer [110], cervical cancer [110, 113], pancreatic cancer [113, 115], and colon cancer [113, 114, 118].

Several papers have shown that some cancers have a subset that are resistant to TRAIL-mediated apoptosis due to low levels of TRAIL receptors. To address this problem, a variety of methods to sensitize the cancer cells to TRAIL were applied; several laboratories conducted combination studies using TRAIL secreting MSCs in conjunction with drugs [113], RNAi [114], irradiation to the cancers [115], or 5-fluorouracil (5-FU) [118]. Grisendi et al. found that BT549 breast cancer cell lines survived in the high concentration of TRAIL due to the lack of the expression of TRAIL receptor, DR4 and DR5 [113]. They treated BT549 with the proteosome inhibitor PS-341, also known as Bortezomib, which upregulates expression of the DR5 receptor. With the combination of Bortezomib and TRAIL-producing MSCs, tumor apoptosis was increased. Mohr et al. investigated the use of RNAi in combination with TRAIL secreting MSCs to treat metastatic pancreatic carcinoma to treat TRAIL-resistant cells [115].

X-linked inhibitor of apoptosis protein (XIAP), which prevents apoptosis by inhibition of caspase-3 and caspase-9 activation, leading to the resistance to TRAIL treatment, was silenced by the shRNA technique and in combination with TRAIL-

Table 15.6 Summary of TRAIL releasing MSCs in cancer preclinical models

Transgene	Model	Route of administration	Effect	Ref
TRAIL	lung carcinoma xenograft	Direct injection into tumor site	Reduction of tumor growth	[107]
TRAIL	Human glioma nude mouse model	Direct injection into tumor site or opposite hemisphere injection	Reduction of tumor mass and prolonger mice survival	[108]
TRAIL	Glioblastoma multiforme mouse xenograft model	Stereotactic injection	Antitumor effects	[109]
TRAIL	Lung (A549), breast (MDAMB231), squamous (H357), and cervical (Hela) cancer mouse xenograft model	Subcutaneous injection	Reduced the metastatic lung cancer	[110]
TRAIL	Glioma mouse xenograft model	Intravenous injection	Prolonged survival of xenograft mice bearing tumors	[111]
TRAIL	Glioma mouse xenograft model	Ipsilateral injection	Inhibition of tumor growth in vivo	[112]
TRAIL	Human cervical carcinoma, pancreatic cancer, colon cancer, and, with bortezomib to TRAIL-resistant breast cancer xenograft model	Flank injection	Increased apoptosis of cancers in vivo	[113]
TRAIL	Colorectal carcinoma xenograft model	Flank injection	Reduction of tumor growth in vivo	[114]
sTRAIL RNAi toXIAP	Pancreatic carcinoma xenograft model	Intravenous injection	Growth retardation on treatment with sTRAIL-MSCs Remission by sTRAIL-MSCs with RNAi to XIAP	[115]
TRAIL Irradiation	Glioma xenograft model	Stereotactic injection	Sequential treatment of irradiation and TRAIL-MSCs enhanced therapeutic efficacy to kill TRAIL-resistant glioma	[116]
TRAIL	Brainstem glioma rat model	Stereotactic injection	Short- and long-term prolonged survival of rats bearing tumor	[117]
TRAIL	Colorectal carcinoma xenograft model	Flank injection	Showed colon cancers were resistant to TRAIL-MSCs Intraperitoneally injected 5-FU with TRAIL-MSCs overcome the resistance	[118]

Abbreviation: *TRAIL* Tumor necrosis factor-related apoptosis induced ligand, *RNAi* RNA interference, *XIAP* X-linked inhibitor of apoptosis protein, *5-FU* 5-fluorouracil

expressing MSCs and RNAi, metastatic pancreatic cancer in human to mouse xenograft models went into remission. TRAIL-secreting MSCs alone showed reduced growth of tumor but could not block the tumor growth enough. Sequential treatments of irradiation and TRAIL expressing MSCs showed killing of TRAIL-resistant glioma cells [116]. Mueller et al. found that a subset of colon carcinoma cells were resistant to TRAIL-induced apoptosis [118]. They injected 5-FU, which is an active form of prodrug to kill cancers, with TRAIL-expressing MSCs, and showed improved efficacy.

15.3.4.3 Prodrug Activators

Prodrug systems, which convert nontoxic prodrugs into cytotoxic materials, are also utilized to treat various types of cancer (detailed review in [121]). Currently, several types of prodrug activation systems are available. Cytosine deaminase (CD) converts nontoxic 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) and herpes simplex virus (HSV) thymidine kinase (TK) is sensitive to ganciclovir. Activated prodrugs are known to kill cancers through the bystander effect [121]. Several papers have exploited the CD-mediated prodrug activation approach (Table 15.7). MSCs producing CD can convert 5-FC to 5-FU, then 5-FU diffuses out from MSCs to kill rapidly dividing cells. Several cancer models such as colon cancer [122], melanoma [123], gastric cancer [124], prostate cancer [125], glioma [126], and a rat glioblastoma model [127] have been evaluated with intravenously injected CD-producing MSCs and have shown inhibition of tumor growth. In the prostate cancer [125] and rat glioblastoma model [127], cytosine deaminase::uracil phosphoribosyltransferase (CD::UPRT), which is a better converter than CD alone, has been exploited.

For TK-mediated cancer treatment, TK-expressing MSCs were able to deliver cytotoxic effects to human glioblastoma cells, but delivery to HeLa cells and MCF7 breast cancer cells was not achieved with the same efficacy [128]. It turned out that cytotoxic effects were transferred into adjacent cells by gap junctions, and HeLa and MCF7 cells did not form gap junctions with MSCs, making TK-mediated induction of apoptosis less effective. Intravenously injected TK-expressing MSCs were effective in reducing tumor volume in the nude mouse model. The same approach was applied to prostate cancer [129] and glioma [130, 131]. Huang et al. reconstructed the gap junction connection by overexpressing Connexin43 in combination with TK [131]. Using this approach, they showed enhanced inhibition of tumor growth as compared to MSC therapy with TK-alone.

15.3.4.4 Viral Vector Delivery

Oncolytic viruses such as adenovirus are able to replicate and selectively kill cancer cells, sparing normal cells [132] (Table 15.1). Direct injection of oncolytic viruses to intratumoral sites showed efficacy and tumor regression in clinical trials, but intravenous injection did not [132]. However, it is well known that adenoviral

Table 15.7 Summary of prodrug converter gene expressing MSCs in cancer preclinical models

Transgene	Model	Route of administration	Effect	Ref
CD	Colon cancer xenograft model	Intravenous injection	MSCs migrated into tumor sites and reduced tumor growth	[122]
CD	Melanoma xenograft model	Intravenous or intraperitoneal injection	MSCs migrated into tumor sites and reduced tumor growth	[123]
CD	Gastric cancer xenograft model	Intravenous injection	MSCs migrated into tumor sites and reduced tumor growth	[124]
CD::UPRT	Prostate cancer xenograft model	Intravenous injection	MSCs migrated into tumor sites and reduced tumor growth	[125]
CD	Glioma xenograft model	Stereotactic injection	Multiple injections of MSCs reduce the preexisting tumor size	[126]
CD::UPRT	Rat glioblastoma model	Intracerebral injection	Complete tumor regression	[127]
TK	Human glioblastoma nude mouse model	Intravenous injection	Reduction of tumor volume	[128]
TK	Prostate cancer nude mouse model	Intravenous injection	Reduction of tumor volume and prolonged survival of tumor-inoculated mice	[129]
TK	Glioma xenograft model	Intravenous injection	Inhibition of tumor growth and prolonged survival of tumor-inoculated mice	[130]
TK/Connexin43	Glioma rat model	intracerebral injection	MSCs migrate into tumor sites and enhanced inhibition of tumor growth compared with TK-MSCs	[131]

Abbreviation: *CD* Cytosine deaminase, *UPRT* uracil phosphoribosyltransferase, *TK* thymidine kinase

vectors cause a host immune reaction. High concentrations of adenovirus can cause a transient elevation in liver enzymes, a sign of an immune reaction, and has been associated with severe adverse events.

Since MSCs are used as a vehicle to deliver to tumor sites and as a reservoir for the adenovirus, it is feasible to apply this approach for cancer treatment (Table 15.8). Komarova et al. first utilized MSCs to deliver oncolytic adenovirus [133]. Several papers also showed efficacy with adenovirus-loaded MSCs using a breast cancer lung metastasis xenograft model [134], intracranial glioma [135–137], ovarian cancer [133], and metastatic neuroblastoma [138]. Instead of adenovirus, Mader et al. utilized oncolytic measles virus-loaded MSCs to treat ovarian cancer in a xenograft model [139]. The measles virus is known to induce cytopathic effects on cancers, but native viruses are neutralized by preexisting antiviral antibodies. MSCs bearing

Table 15.8 Summary of oncolytic virus containing MSCs in cancer preclinical models

Transgene	Model	Route of administration	Effect	Ref
Adenovirus	Ovarian cancer mouse xenograft model	Intraperitoneal injection	Reduction of tumor size and prolonged survival mice bearing tumor	[133]
Adenovirus	Breast cancer metastatic lung cancer xenograft model	Intravenous injection	MSCs migrated to tumor site and reduced the tumor growth in vivo.	[134]
Adenovirus	Intracranial glioma	Stereotactic injection	MSCs migrated to tumor site and release viruses	[135]
Adenovirus	Intracranial glioma	Intra-arterial injection	MSCs migrated to tumor site and reduced the tumor growth in vivo and prolonged survival of mice bearing tumor	[136]
Adenovirus	Malignant glioma	Stereotactic injection	Prolonged survival of mice bearing tumor	[137]
Adenovirus	Metastatic neuroblastoma in human patients	Intravenous infusion to patients	One out of four children had complete remission	[138]
Measles virus	Ovarian cancer xenograft model	Intraperitoneal injection	Prolonged survival of mice bearing tumor	[139]

measles viruses formed syncytia in the presence of antiviral antibodies and enhanced the survival of mice bearing tumors.

In Spain, there has been progress towards the clinical application of adenovirus-loaded MSCs to treat neuroblastoma [138]. In this study, 4 children from ages 2 to 5 with metastatic refractory stage IV neuroblastoma were infused at least twice with MSCs bearing oncolytic adenovirus. The clinical team followed the patient's renal and liver functions, white and red blood cell and platelets counts, and they checked the adenovirus concentration in serum and urine every 2 weeks. One of the four patients showed that metastatic tumors had disappeared and is now in complete remission for 36 months after the first treatment.

15.3.5 Bone-Related Diseases

It is not surprising that one of the first human clinical trials using MSCs was to treat osteogenesis imperfecta, a genetic bone disease, because MSCs can form bone [9]. Several laboratories tried to enhance osteogenesis by overexpressing bone morphogenetic protein 2 (BMP2), insulin-like growth factor 1 (IGF1), VEGF, and human telomerase reverse transcriptase (hTERT) (Table 15.9). Shi et al. examined whether hTERT overexpression can maintain MSC proliferation and osteogenic differentiation potential in ex vivo culture [147]. An in vitro osteogenic differentiation assay in hTERT overexpressing MSCs showed more mineralized bone structure than

Table 15.9 Summary of pro-osteogenesis gene expressing MSCs in bone defect preclinical models

Transgene	Model	Route of administration	Effect	Ref
hTERT	Beige mouse	Subcutaneous injection	More osteogenic cells and osteogenic potential	[140]
Wnt-4	• Nude rat periodontal defect model • Craniofacial Defect Model	Embedded with Polylactic co-glycolide polymer scaffolds (periodontal defect model)	Enhanced osteogenesis and showed extensive periodontal bone regeneration and improved the repair of craniofacial defects in vivo	[141]
BMP2	Ulnar bone defects in the canine model	MSCs mixed with β-tricalcium phosphate ceramic granules and placed onto the defect area	Significant increase of newly formed bone area and healed or partly healed all of the bone defects	[142]
BMP2	Ovariectomized, female C57BL/6 mice	Intravenous injection	Significant increase in bone mineral density and bone mineral content and more trabecular bone following MSC-BMP2 therapy	[143]
BMP2	Periodontal defects rabbit model	Not available	Regenerated cementum with Sharpey's fiber insertion and bone formation	[144]
BMP2 Runx2	Nude mouse model	Subcutaneous injection with PLGA	Enhanced bone formation compared to BMP2 expressing MSCs	[145]
IGF1	Insulin-receptor-substrate knock-out mice	Intravenous injection	Restored the fracture by new bone formation and promoted the occurrence of a well-organized callus	[146]

Abbreviation: *hTERT* human Telomerase reverse transcriptase, *Wnt-4* wingless-type MMTV integration site family, member 4, *BMP2* Bone morphogenetic protein 2, *Runx2* runt-related transcription factor 2

unmodified MSCs. In their following paper, hTERT overexpressing MSCs were subcutaneously injected into beige mice and showed more osteogenic cells than MSCs alone, along with an increased osteogenic potential due to the upregulation of CBFA1, osterix, and osteocalcin [140].

Chang et al. evaluated the possibility of whether non-canonical Wnt-4 regulates the osteogenic pathway [141]. They tested two different bone defect models, a periodontal bone defect model and a craniofacial defect model with Wnt-4 expressing MSCs embedded in polylactic co-glycolide polymer scaffolds. Wnt-4 overexpressing MSCs increased osteogenesis and showed extensive periodontal bone regeneration and improved the repair of craniofacial defects in vivo. Several papers used BMP2 expressing MSCs to enhance bone formation. Li et al. evaluated BMP2-expressing canine MSCs on ulnar bone defects in the canine model [142]. Sixteen weeks after

the transplantation, BMP2 overexpressing MSCs increased the area of newly formed bone and healed or partially healed all of the bone defects.

Ponnazhagan and colleagues looked at bone regeneration in an osteopenic mouse model with BMP2 expressing MSCs [143]. Intravenously injected BMP2 secreting MSCs enhanced bone mineral deposits and more trabecular bone formation than MSCs alone. Chen et al. engineered MSCs to express BMP2 and implanted them into a periodontal defect rabbit model [144]. BMP2 overexpressing MSCs regenerated cementum with Sharpey's fiber and enhanced bone formation where it attached to periodontal fibers. To enhance bone formation compared to BMP2 expressing MSCs, Runx2, one of the master regulators of osteogenesis, was co-expressed [145]. Runx2/BMP2 co-expressing MSCs were embedded within a PLGA scaffold and implanted subcutaneously into athymic nude mice. BMP2/Runx2 expressing MSCs showed enhanced bone formation compared to MSC only and BMP2 expressing MSCs. Instead of BMP2, efficacy using IGF1 overexpressing MSCs was recently evaluated in a tibia fracture model [146]. IGF1 secreting MSCs were intravenously injected into the tail of insulin-receptor-substrate knock-out (Irs(−/−)) mice, which lack the ability to repair fractures. Authors claimed that IGF1 expressing MSCs improved new bone formation and restored the tibia fracture in Irs(−/−) mice. From the in vitro and in vivo assays, they showed that IGF1 induced osteogenesis via the Irs1-PI3K signaling pathway, with autocrine and paracrine effects.

15.3.6 Renal Failure

Most kidney diseases are related to the characteristics of ischemic, inflammatory and immunologic injury. MSC-mediated treatments were pursued as cellular therapy to improve these problems. It is known that erythropoietin (EPO) is downregulated at the end stage of renal failures. Eliopoulos et al. transduced EPO into murine MSCs and injected them subcutaneously into syngeneic mice with chronic renal failure [148] (Table 15.10). Among various doses, higher doses showed increased hematocrit levels to normal compared to controls and better survival of the mice. In a follow-up study, the same group co-introduced IGF-1 and EPO secreting mouse MSCs subcutaneously to the renal failure mouse model. An enhanced hematocrit level was achieved and cardiac function was improved [149]. Our group has also overexpressed EPO from human MSCs, in the late 1990s, and found a significant increase in hematocrit and differentiation of co-transplanted human hematopoietic stem cells to the red cell lineage [31]. However, toxicity occurred from the very high RBC counts resulting from the high unregulated dosages of EPO that MSCs can produce if the transgene is not under the control of a regulated inducible promoter (reviewed in [11]). These studies confirmed that MSCs can be powerful in vivo delivery vehicles, but suggest that, with growth factor expression, it will be important to regulate the amounts of protein produced.

For the acute renal failure model, Hagiwara et al. examined the production of kallikrein, which makes cells resistance to oxidative stress-induced apoptosis, from

Table 15.10 Summary of MSCs expressing kidney-related genes in renal failure preclinical models

Transgene	Model	Route of administration	Effect	Ref
EPO	Mouse chronic renal failure	Subcutaneous	Increased hematocrit and activity of mice	[148]
IGF1/EPO	Mouse chronic renal failure	Subcutaneous	Increased hematocrit and activity of mice	[149]
Kallikrein	Rat acute renal failure	Carotid artery injection	Protection against apoptosis in acute renal failure model	[150]
VEGF	Nude mouse renal failure	Intravenous tail injection	Protection against apoptosis in acute renal failure model and better renal function	[151]

Abbreviation: *EPO* Erythropoietin, *IGF1* Insulin-like growth factor 1

gene-modified modified MSCs. Kallikrein engineered rat MSCs showed significant reduction of apoptosis induced by H_2O_2 and inhibition of neutrophil and monocyte infiltration [150]. A recent paper showed VEGF-mediated protection and improvement of acute renal failure in a nude mouse model [151]. In that report, VEGF-engineered human fetal MSCs also showed better survival of renal epithelium by increased cell proliferation and reduced apoptosis, better renal function, and increased peritubular capillary density [151].

15.3.7 Neurological Diseases

MSC therapies have shown efficacy in preclinical models of various neurological diseases such as Parkinson's disease, Huntington's disease, multiple sclerosis, and stroke (detailed review in [152]). Here we review genetically engineered MSCs in preclinical models of Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis (Table 15.11). Stroke models are reviewed in [157].

15.3.7.1 Huntington's Disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease, which is caused by the excessive expression of cytotoxic polyglutamine (poly-Q) in the mutant huntingtin protein HTT and death of medium spiny neurons due to HTT toxicity and the lower expression of brain-derived neurotrophic factor (BDNF) [158]. Unfortunately, there is currently no cure for HD [159]. Canals et al. showed that disease onset is dependent on BDNF expression levels in the R6/2 HD mouse model and BDNF can improve symptoms and extend life span [160]. With this notion in mind, efficacy of MSCs expressing BDNF and nerve growth factor (NGF) transplantation in the YAC128 HD mouse model were evaluated [153]. For the YAC128 HD model, hyperkinesis starts at 3 months of age with progressive motor

Table 15.11 Summary of neuroprotection-related gene expressing MSCs in neurodegenerative disease preclinical models

Transgene	Model	Route of administration	Effect	Ref
BDNF	YAC128 Huntington's mouse model	Intrastriatal injection	Ameliorated symptoms of HD	[153]
GDNF	SOD1 ALS rat model	Intramuscular injection	Increased the number of neuromuscular connections and motor neuron cell bodies in the spinal cord and prolonged survival	[154]
GDNF	6-OHDA rat Parkinson's disease model	Intrastriatal injection	Decreased amphetamine-induced rotation and rejuvenated tyrosine hydroxylase-immunoreactive fibers in short-term treatment	[155]
BDNF	6-OHDA Parkinson's disease model	Intrastriatal injection	Decreased clinical symptoms and rejuvenated DA neurons	[156]

Abbreviation: *BDNF* Brain-derived neurotrophic factor, *GDNF* Glial cell-derived neurotrophic factor, *HD* Huntington's disease, *YAC128* yeast artificial chromosome containing 128 CAG repeats, *SOD1* superoxide dismutase 1, *ALS* Amyotrophic lateral sclerosis, *6-OHDA* 6-hydroxydopamine, *DA* dopaminergic

neuron impairment at 6 months of age and neurodegenerative features showing at 9 months of age. In the report by Dey et al., they transplanted BDNF secreting MSCs in a preventive manner at 4 months of age, which is ahead of the onset of motor neuron impairment. As expected, BDNF expressing MSCs transplanted into the striatum of HD mice showed significant improvement in motor function, as measured by performance on the rotarod, and significant reduction in levels of hindlimb clasping, a hallmark phenotype of affected HD mice. The least amount of neuronal loss within the striatum of the YAC128 mice at 13 months of age was observed in those transplanted with the growth factor-producing MSCs.

The underlying mechanisms for the beneficial effects from BDNF overexpressing MSCs are not completely known. These effects may represent a combination of the anti-apoptotic and axon-extending properties of MSCs, combined with the effects of the naturally produced neurotrophins in conjunction with the additional BDNF expression. BDNF therapies for HD have been extensively reviewed by Zuccato and Cattaneo [161–163]. It will be interesting to see if the effects of BDNF expressing MSCs can help to prevent the worsening of the symptoms or reverse the course of disease progression in this currently untreatable severe neurodegenerative disorder.

15.3.7.2 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a neurodegenerative disorder caused by the loss of motor neurons connected to muscle, and failure of this neuromuscular junction leads to paralysis of patients [152]. Most ALS cases are sporadic events and only 10% of them are familial cases. Currently,

the underlying disease etiology of sporadic ALS is unknown but in familial cases, it is linked to point mutations of cytosolic Cu^{2+}/Zn^{2+} superoxide dismutase 1 (SOD1). Currently, there are no treatments available that prevent neuromuscular decline to significantly delay the progression of ALS.

Glial cell-derived growth factor (GDNF) has been shown to have neuroprotective function in motor neurons of the SOD1 ALS mouse [164]. Following intramuscular transplantation of MSCs engineered to express GDNF in a rat model of familial ALS, Svendsen and colleagues showed an increased number of neuromuscular connections and motor neuron cell bodies in the spinal cord at mid-stages of the disease [154]. Furthermore, they showed that GDNF secreting MSCs could delay the disease progression and significantly extended lifespan in the SOD1 rat model [154].

15.3.7.3 Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disease caused by the progressive degeneration of dopaminergic (DA) neurons in the midbrain [165]. Currently, there is no cure for PD, although implantation of deep brain stimulation devices and pharmacological agents can ameliorate clinical symptoms [166]. Different growth factors have been evaluated in an attempt to recover the damaged DA neurons, or to delay the rate of decline. GDNF secreting MSCs promoted rejuvenation of host striatal DA fibers and improvement in DA-dependent behavioral function in a rat model of PD [155]. Similar results were confirmed using BDNF secreting MSCs, where intrastriatally injected BDNF overexpressing MSCs showed improved clinical symptoms and rejuvenated striatal DA fibers in a rat model of Parkinson's disease [156].

15.4 Beyond the Preclinical Models: Future Directions for Genetically Engineered MSC Therapy in Working Toward Human Clinical Trials

In human clinical trials, safety is one of the major concerns and it is critical to identify and minimize risks associated with treatment [46]. In current MSC culture protocols, MSCs are cultured in fetal bovine serum-containing media. To generate safe and clinically acceptable MSC expansion protocols, xeno-free cell culture media should be better developed to allow optimized growth and subsequent in vivo function, while minimizing the risk of transmitting pathogens or causing human immune reactions [167]. In the case of hESC culture, Martin et al. found that xenogeneic serum replacement is the source of nonhuman sialic acid Neu5Gc, which causes immunological reactions involving human antibodies [168]. Therefore, human clinical applications of MSCs would best employ chemically defined media.

Karyotypic stability is a highly important criterion for any cell type expanded in culture, prior to consideration of clinical trials. In contrast to murine MSCs which

can easily undergo transformation in culture, there has been no documented evidence that human MSCs can be transformed during short-term in vitro expansion due to the development of chromosomal abnormalities. There has been one report that was retracted because it was found that the MSC cultures were contaminated with HeLa cells [169–171]. This type of contamination can occur readily in laboratories that use aspiration flasks to remove media from culture flasks, since all cell cultures are eventually mixed by regurgitation from the hose line, in spite of new sterile pipettes for each culture. Aspiration flaks must never be used in Good Laboratory Practices or in Good Manufacturing Practice Facilities, to ensure the identity of the culture. A second group, de la Fuente et al. retracted their paper because they could not reproduce the transformation data [172]. Nevertheless, well characterized in vitro MSC culture protocols and carefully adhered to standard operating procedures must be followed, along with the establishment of sensitive techniques to investigate chromosomal abnormalities.

Even though genetically engineered MSCs showed enhanced efficacy in the various preclinical disease models discussed here (including cardiovascular, cancer, bone formation defects, renal damage, neurological diseases, and others), there are so far no studies approved to move forward with human clinical trials. Currently, there are 123 human clinical trials registered using MSCs worldwide and all of the studies are utilizing unmodified MSCs [173].

There are barriers toward human clinical trials using genetically modified MSCs as there is no safe standard protocol to engineer MSCs to express transgenes. Each vector system has its own advantages and limitations with regard to efficacy and safety for the planned human clinical trials. As discussed earlier, most preclinical studies have utilized permanently integrating viral vectors as a delivery method for the gene of interest in order to continually express the transgene. Transient expression of genes of interest are not effective in most cases, but can be used to modify surface antigens of MSCs in order to increase MSC homing capacity and survival after transplantation. The risk of insertional mutagenesis caused by viral cassette integration into the host genome must be considered prior to the planned clinical trial. The potential risk to benefit ratio for that disorder or disease must be carefully evaluated, as is currently done for hematopoietic stem cell gene therapy applications [20].

Site-specific integration can be one of the options to eliminate insertional mutagenesis. It is well known that AAV vectors including rep protein integrate into chromosome 19 [174]. With rep, however, the cloning capacity of AAV vectors is reduced significantly. Annealing two inverted repeats (ITRs) can extend its cloning capacity to double the size of the insert [175]. These modified AAV vectors can be one of the options to avoid insertional mutagenesis with sustained expression of the genes of interest. Zinc-finger nuclease (ZFN)-mediated homologous recombination (HR) could be another option to modify MSCs safely, if success rates can be improved [176–179]. Zinc fingers have specific binding sites to DNA and engineered zinc-fingers with nucleases can cut the specific genomic regions of DNA. After the cleavage of specific DNA by ZFNs, the therapeutic cassette can be inserted by Homologous Recombination to create safe site-specific integration. Even though there are tremendous efforts to optimize ZFNs, nonspecific cleavages by ZFNs are still problematic [180].

Benabdallah et al. investigated whether ZFN-mediated targeted gene addition to safe harbor sites are possible [181]. They inserted Epo into the C–C chemokine receptor 5 (CCR5) gene loci, a putative safe harbor site, in MSCs by ZFN-mediated HR. Up to 40% of MSCs were successfully modified with EPO in CCR5 loci. Then, they injected these modified MSCs into NOD/SCID interleukin-2Rγ null (NSG) mice and these mice showed higher hemocrit levels in comparison with unmodified MSCs.

In the practical setting, identification of genetically engineered MSCs, which have safe harbor integrations of the transgenes, is restricted due to the limited lifespan of primary MSCs during in vitro expansion. Aging, moreover, significantly reduces the survival and differentiation potential of BM-MSCs [45]. hESC or hiPSC-derived MSCs can be considered in this case. With human pluripotent stem cells (hESC or hiPSC), a vector integration site could be mapped and cells with safe harbor integrations could potentially be expanded nearly indefinitely to generate differentiated MSCs with safe harbor integrations. Our group and others are working toward this future goal [46]. We have shown that pluripotent stem cell-derived MSCs can perform in vivo in a manner analogous to adult MSCs, by homing into areas of hypoxic injury [43].

With current techniques, it is difficult to track where the transplanted MSCs go in humans and to evaluate their long-term survival and function [14, 46]. Gene marking studies, using non-therapeutic genes such as eGFP or luciferase to track transplanted cells, are prohibited in clinical trials. Therefore, mysteries remain and can only be deciphered from large animal models. To ensure integrity and safety of the transplanted MSCs, suicide genes can be utilized to eliminate gene-modified MSCs if they are found to cause problems in patients [182, 183]. Schuldiner et al. showed that HSV-TK expressing hESCs had self-renewal and pluripotency and were sensitive to ganciclovir to kill cells [182]. They, moreover, could ablate teratoma that had arisen from the subcutaneous injection of undifferentiated TK-hESCs by intraperitoneal injection of ganciclovir. However, caution must be used when considering this strategy for MSCs, since TK has bystander effects on nearby cells and MSCs are known to effectively transfer the protein.

15.5 Conclusions

MSCs have been shown to be safe and have early evidence of efficacy in various clinical trials for heart attack, stroke, graft-vs.-host disease, and multiple sclerosis, among others [184–188], but some problems still need to be solved and efficiency and reproducibility need to be improved. Genetically modified MSCs can potentially overcome these barriers to increase the efficiency of therapy for many disorders. Given the possibility of immune reaction or insertional mutagenesis for vector transduced MSCs, long-term observation of modified MSCs must be followed carefully to meet safety regulations. The field looks to future applications of gene delivery to safe harbor sites to improve biosafety. Since they are powerful delivery

vehicles and potent protein synthesis factories, the use of gene-modified MSCs to provide missing enzymes or growth factors and other signals to improve the repair of damaged or diseased tissues holds almost unlimited potential.

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Chapter 16
Optimal Tissue Sources of Mesenchymal Stromal Cells for Clinical Applications

Celena Heazlewood and Kerry Atkinson

Abstract Mesenchymal stromal cells (MSCs) are multipotent cells that can differentiate into the mesenchymal lineages of fat, bone, and cartilage. They are of particular interest because they can preferentially migrate to sites of inflammation and injury and can be transplanted into patients without the need for immune suppression. These remarkable qualities have allowed MSCs to be used for a range of differing medical conditions within the clinic and they are proving to be a promising candidate for an allogeneic "off-the-shelf" cellular therapy ready for immediate use in acute or chronic medical settings. Although these cells were traditionally derived from the bone marrow, investigators have found that MSCs can be derived from alternative sources that are younger, more easily accessible and usually regarded as "biological waste material." While MSC-like populations have been isolated from almost every tissue in the body, it has been found that MSC populations retain a "memory of tissue origin" resulting in different functional abilities. This suggests that differences in MSC populations may be important for the development of future MSC therapeutic approaches for tissue and organ repair and that careful consideration is required when choosing a source of MSCs for clinical trials.

Keywords Tissue source • Mesenchymal stem/stromal cells (MSC) • Clinical applications

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16.1 Introduction

Stem cell biology has become a critical area for the understanding of tissue regeneration and for effective implementation of the underlying principles of regenerative medicine [1]. Since the discovery and characterization of multipotent mesenchymal stem/stromal cells (MSCs) from bone marrow (BM), MSC-like populations from other tissues have now been characterized by the International Society for Cell Therapy [2].

MSCs show promise as a biological therapeutic agent for a diverse range of medical situations. They have also been demonstrated to improve cerebral function in animal models of stroke and spinal cord injury, myocardial function (after myocardial infarction), as well as improved liver and joint damage [3–11]. Until recently, it was thought that MSCs could only differentiate into cells of their germ layer of origin, the mesoderm. However, some studies have suggested these stromal cells show plasticity beyond their traditional mesodermal lineage by differentiating in vitro into cells of ectoderm and endoderm germ layers [12–15]. Some studies have reported that MSCs have been induced to generate, at least in vitro, into tissues of both ectodermal (neurons) and endodermal (hepatocytes) nature [12–16], although this remains controversial. MSCs also demonstrate remarkable immunosuppressive properties on both T cell, B cell, NK cell, and dendritic cell function and can be transplanted across major histocompatibilty complex (MHC) barriers in adult immune competent recipients without the need for immune suppression [17–20]. Our studies have found that one mechanism for this may be their ability to secrete indoleamine dioxygenase (IDO), which in turn depletes tryptophan, leading to T-cell suppression [17]. As indicated above, there appears to be no need to match the MSC donor to the recipient for antigens of the major histocompatibilty complex. An example of this is in the treatment of acute graft-versus-host disease (GVHD) following allogeneic hematopoietic stem cell (HSC) transplantation, in which there is significant evidence that MSCs from third party unrelated MHC-unmatched donors are therapeutically effective in both preclinical models and in human studies of GVHD [21].

Furthermore, MSCs are known to preferentially migrate to sites of inflammation [22, 23]. In both preclinical and clinical studies, MSCs have been shown by some to have the ability to preferentially migrate to sites of injury, facilitating their use in tissue repair and regeneration because they can be injected intravenously rather than requiring direct injection into the target organ of interest [24]. The mechanism for such repair is unknown but increasing evidence suggests that they exert their effects through paracrine mechanisms rather than transdifferentiation. It is likely that increased inflammatory chemokine concentration at the site of inflammation is a major factor causing MSCs to migrate to these sites. Chemokines are released after tissue damage and MSCs are known to express the receptors for several of these [25–27], including the chemokine receptor CXCR4, which is thought to be important in MSC migration [26, 28–31]. It is also well established that stem/progenitor cells secrete many other soluble factors that may directly or indirectly have

reparative qualities, and these factors may signal through pathways that act to promote angiogenesis, decrease apoptosis, and regulate formation of scar tissue [32]. Taken together, these characteristics support the use of MSCs as a candidate cellular therapeutic agent.

Over the past decade, the search for MSC-like cells in specific tissues has led to the discovery of MSCs in every organ and tissue in the body [1, 33–35]. Of these, tissue sources such as placenta and liposuction fat have been shown as promising alternative MSCs sources to the traditional source, bone marrow [36, 37]. Therefore, this chapter will discuss the isolation and characterization of MSCs from several tissue sources and potential therapeutic applications for the clinic.

16.2 Isolation of MSCs from Bone Marrow

MSCs were originally derived from the bone marrow over 40 years ago by Friedenstein and colleagues [38]. They described an adherent, fibroblast-like population that could regenerate rudiments of normal bone in vivo [38, 39]. Originally termed CFU-F (colony forming unit-fibroblast), MSCs are a type of postnatal stem cell that originate from, and generate, mesodermal tissues. Their many advantages include their relative ease of isolation, expansion potential, stable phenotype and compatibility with different delivery methods and formulations, reviewed in "Therapeutic applications of mesenchymal stromal cells" [1].

Bone marrow (BM)-derived MSCs are a rare cell population (~0.001% of BM mononuclear cells) in vivo, resulting in a low MSC yield on initial isolation. Hence, ex vivo expansion is required to gain sufficient numbers for clinical applications. Briefly, whole marrow is either subjected to fractionation using a density gradient such as Percoll or Ficoll or alternatively a red blood cell lysis is performed and cells then cultured in basal medium such as Dulbecco's modified Eagle's medium and 10–20% fetal calf serum (FCS) [22, 33, 36, 40]. Cells are subsequently maintained in culture for several days during which contaminating, non-adherent hematopoietic cells such as macrophages are depleted. In some instances, the property of plastic adherence alone is not sufficient to allow purification of MSCs, as in the case of cells from mouse bone/bone marrow. Phinney and colleagues [41] reported the presence of hemapotoietic $CD45^+$ $CD11b^+$ granulocytic and monocytic precursors in these cultures. Due to the lack of specific phenotypic markers, these observations led to the development of new methods involving CD34/CD45/CD11b immunodepletion to generate purified MSC preparations [42]. It has been reported that cell isolates can be enriched for MSCs based on the expression of either Stro-1 in humans, and Sca-1 in mice, and the absence of CD45 in MSCs from each species [43]. Human MSCs are further characterized according to the characteristic (but not unique) cell surface phenotype of $CD90^+$, $CD105^+$, $CD73^+$, $CD44^+$, whilst murine MSCs are $CD90^+$ and $CD44^+$ [22, 44].

Despite enriching for MSCs by cell surface phenotype, our group and others have found *heterogeneity* within the ex vivo expanded population. These cells

display a tri-, bi-, or mono-potential mesodermal differentiation ability. Bulk populations of MSCs in ex vivo expansion cultures are mostly transit amplifying cells, although recently two papers provided the first evidence for a true mesenchymal stem cell (ability to both self-replicate and to differentiate) using serial transplantation experiments in mice [45, 46]. Thus, true multipotent mesenchymal stem cells likely make up only a small fraction of the expanded cells using current expansion methodologies. Further, it has been proposed that current tissue culture methods used to expand MSCs reduce multipotency and result in lower migratory/engraftment capacity of the expanded MSCs.

It has also been shown that humans and animals show a decreased rate of production of bone marrow mesenchymal stem and progenitor cells with increasing age [47]. Several studies have provided evidence of a strong correlation between age and the proliferative potential exhibited by MSCs in vitro [48–50]. Thus, the progenitor pool may be depleted following extensive proliferation. Consequently, this results in a reduced ability to ensure regeneration after injury or disease depending on MSC age [51]. A decline in the quality of cells is obviously not ideal for therapeutic applications.

For such reasons, sources of MSCs other than bone marrow are being investigated for their potential clinical use for regenerative potential and immunomodulatory functions [24, 36]. Ideally, a readily available source that can be obtained by a noninvasive procedure yields large numbers of MSCs for ex vivo expansion and a younger source of cells with less decline in their quality would be an ideal alternative. There have been investigations of MSCs derived from tissues normally regarded as waste, such as adipose tissue from liposuction fat and other alternative tissues such as dental pulp and fetal derived tissues including the placenta and cord blood [36, 37, 52–58].

16.3 Alternative Tissue Sources

MSCs have been isolated from almost every tissue in the body including, fat (adipose) tissue [37, 53], teeth (dental pulp) [54, 59], lymphoid organs (thymus and spleen) [34, 60], scalp tissue [61], endometrium and menstrual blood [62–64]. The amount of material required depends on the abundance of MSCs within that tissue. However, in all cases, careful removal of the tissue is important, followed by enzymatic digestion in collagenase with continuous shaking at 37°C. The cell suspension is collected and filtered, followed in some cases by centrifugation. The final cell pellet is then counted and plated in culture. Initial seeding density depends on the tissue of origin, for example, 10×10^6 cells/cm^2 for cells isolated from the thymus or lymph nodes, 5×10^6 cells/cm^2 from the spleen and 1×10^6 cells/cm^2 for cells isolated from fat [37, 60].

Similar to bone marrow MSCs, cells from these alternative sources are usually washed in PBS 48–72 h after initial plating. Once cells have adhered in culture, they initially form colonies and cells exhibit a spindle-shape, fibroblast-like morphology.

Usually 7–14 days after initial plating colonies begin forming. Once 80–90% confluence is reached, cells require harvesting for continued ex vivo expansion.

16.3.1 Adipose Tissue

Adipose tissue is a source that is relatively easy to obtain in large quantities. It covers a widespread area of the human body and often regarded as waste material after lipectomy (liposuction) surgery. Cells that are MSC-like can be derived from adipose tissue and are useful not only for their potential therapeutic applications, but also for the low cost of their harvest and delivery. The stromal cells isolated from this tissue exhibit characteristics common to mesodermal tissues, including adherence to plastic, formation of fibroblast-like colonies, extensive proliferative capacity, ability to differentiate into several mesodermal lineages (including bone, cartilage, muscle, and fat), and expression of several common cell surface antigens typical of BM MSCs.

Kern and colleagues [37] described a method to obtain adipose-derived MSCs from liposuction procedures. Briefly, the raw lipoaspirate (50–100 ml) was washed intensely and digested with 0.075% collagenase type 1 for 30–60 min at 37°C with gentle agitation. The digest was centrifuged and filtered through a 100 μm strainer. The filtered cells were centrifuged again and resuspended cells were plated at a seeding density of $1 \times 10^6/cm^2$ into T75 or T175 flasks. Non-adherent cells were removed after 12–18 h and the resulting fibroblast-like cells were termed adipose-derived fibroblastoid adherent cells. These cells were cultivated under the same conditions as described for bone marrow-derived MSCs. When cell colonies reached confluence, cells were harvested and reseeded at a mean density of $1.8 \pm 3.1 \times 10^3/cm^2$. Using this isolation technique, 100% success rate was described for isolating adipose-derived MSCs [37].

Using this isolation technique, a comparative study between adipose-derived MSCs and bone marrow-derived MSCs by Kern and colleagues found that at an initial plating density of 1×10^6 cells/cm^2, adipose-derived MSCs formed more colonies than bone marrow MSCs (557 ± 673 vs. 83 ± 61) [37]. This indicated that a greater number of MSCs reside within fat tissue compared to bone marrow. In addition, no cellular morphological differences were found between the two sources with both cell populations displaying an adherent, fibroblast-like morphology. Interestingly, when expansion characteristics were analyzed, it was found that bone marrow MSCs displayed lower population doublings than adipose tissue-derived MSCs with adipose tissue having the ability to be cultivated longer than bone marrow MSCs. The cell surface phenotype of cultured cells were assessed and found no expression of the hematopoietic markers, CD14, CD34, and CD45 in both of the MSC populations and more than 90% of MSCs from both sources expressed the characteristic MSC phenotype of CD44, CD73, CD29, and CD90. HLA-I was also expressed on both populations with none of the cell populations expressing HLA-II.

Mesodermal differentiation potential was also compared between each source. No differences in differentiation potential between the adipose and bone marrow-derived MSC populations were detected, with all donors from each cell population displaying multilineage potential into adipocytes, osteocytes, and chondrocytes. This was also observed at the single cell level by Rodriguez and colleagues who could maintain the characterization of adipose-derived MSCs throughout long-term culture [65].

MSC-like populations from these two tissue sources showed some similarities and differences in gene expression. Wagner and colleagues reported up-regulation of 25 genes expressed in both populations compared to fibroblasts [66]. Many of these genes were involved in extracellular matrix, morphogenesis, and development. Despite some similarities in gene expression, these cells were unique displaying a tissue-specific profile. Whether these differences in the transcriptome have a significant impact on their functions as a therapeutic agent remains to be determined [66].

However, due to the ease in accessibility of adipose tissue (regarded as waste from liposuction surgeries), their large expansion potential, and the differentiation and immunosuppressive properties that these cells possess, adipose tissue represents a candidate tissue source for large-scale manufacturing of MSCs for clinical applications. In particular, MSCs from this tissue could be used for plastic surgery.

16.3.2 Dental Tissue

Dental tissues are specialized tissues that do not undergo continuous remodeling. Therefore, MSCs derived from these tissues may be more committed or restricted in their differentiation potency in comparison with BM MSCs [67]. Interestingly, several populations of MSCs have been isolated from different sections of the tooth. These include cells from the pulp of both exfoliated (children's) and adult teeth, from the periodontal ligament that links the tooth root with the bone, from the tips of developing roots and from the tissue (dental follicle) that surrounds the unerupted tooth [68]. The first type of dental MSC was isolated from the human pulp tissue and termed "post-natal dental pulp stem cells" (DPSCs) [54]. Subsequently, three alternative dental MSC-like populations were isolated and characterized from: exfoliated deciduous teeth (SHED) [69], periodontal ligament stem cells (PDLSCs) [70], and stem cells from apical papilla (SCAP) [71]. Recent studies have identified a fifth dental tissue-derived progenitor cell population, referred to as "dental follicle precursor cells" (DFPCs) [67, 72].

During the characterization of these newly identified dental "stem" cells (MSCs), their properties have been compared with those of BM MSCs. In general, dental-derived stem cells display a similar cell surface phenotype to BM MSCs. They have multidifferentiation potential, with the capacity to give rise to at least three distinct cell lineages—osteo/odontogenic, adipogenic, and neurogenic lineages. Differences

have been noted between the dental stem cell populations and BM MSCs, in that dental stem cells appear to be more committed to an odontogenic lineage rather than the osteogenic lineage, reflecting a bias towards the tissue in which these MSC-like populations reside [67]. Although many similarities of gene expression between DPSCs and BM MSCs was found for more than 4,000 known human genes, there were several differentially expressed genes between the two sources. Collagen type XVIII alpha1, insulin-like growth factor-2 (IGF-2), discordin domain tyrosine kinase 2, NAD(P)H menadione oxidoreductase, homolog 2 of Drosophila large disk, and cyclin-dependent kinase 6, were found to be highly expressed in DPSCs, whereas insulin-like growth factor binding protein-7 (IGFBP-7) and collagen type I α2 were highly expressed in BM MSCs [55, 67].

A microarray study by Yamada and colleagues [73] demonstrated high expression levels of alkaline phosphatase (ALP), dentin sialophosphoprotein (DSPP), and dentin matrix acidic phosphoprotein 1 (DMP-1) in dental MSCs after osteogenic induction compared with BM MSCs. Interestingly, these proteins are critical for proper mineralization of bone and dentin and are present in diverse cells of bone and tooth tissues. This finding is exciting and is particularly significant for the oral maxillofacial field because the repair and regeneration ability of these bones, which aid in orofacial functions like speech, chewing, swallowing, and facial expressions are intricate and complex.

In addition, the study also focused on differences between induced dental MSCs and BM MSCs on a cluster that contains genes which are up-regulated in DPSCs and down-regulated in MSCs after osteogenic induction. A notable feature of this cluster was the cooperative regulation of genes for cell signaling, cell communication, or metabolism [67, 73]. Even though DPSCs and BM MSCs are regulated by similar factors, and share a common protein expression profile, these populations differ significantly in their proliferative ability and developmental potentials in vitro, although current evidence suggests that biochemical pathways involved in the differentiation of DPSCs into functional odontoblasts are similar to the differentiation pathway of BM MSCs into osteoblasts [55, 67].

Differences were also noted in the ability of dental-derived MSCs to differentiate into chondrogenic and adipogenic lineages, with DPSCs displaying weak or limited ability in comparison with BM MSCs [71, 74]. However, DPSCs can develop into distinct tissues representative of the micro-environments from which they were derived in vivo. In comparison, BM MSCs formed only bone tissue in the mouse model when treated in the same manner [54, 75]. Additionally, dental-derived MSCs expressed neural characteristics that were not expressed in bone marrow-derived MSCs, which may reflect the former's possible derivation from neural crest cells. These data indicate that dental-derived MSCs may be a more potent and better suited source for neural applications than bone marrow MSCs, although further preclinical experiments are required to understand this fully [67]. Therefore, while DMSCs display similar phenotypic characteristics to BM MSCs, we conclude from this evidence that dental-derived MSCs are a distinct cell population that may provide a remarkable therapeutic application that BM MSCs cannot.

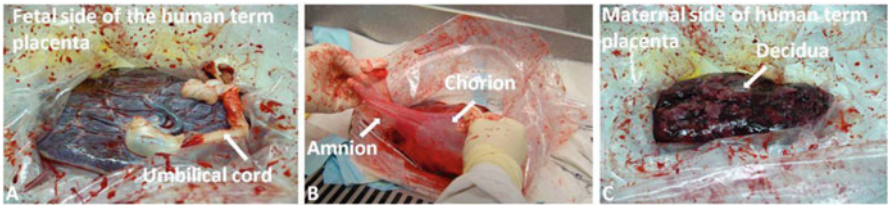

Fig. 16.1 Human term placenta. (**a**) The fetal side of the human placenta with the umbilical cord still attached. (**b**) Mechanical separation of the fetal membranes, the amniotic membrane and the chorionic membrane. (**c**) The portion of the placenta that embeds into the mother's uterus, the decidua

16.3.3 The Term Placenta

The human term placenta represents an attractive source of MSCs due to its ready availability, easy access without invasive procedures, and lack of the ethical issues that surround the use of embryonic stem cells. It consists of both fetal (amnion and chorion) and maternal (decidua) components (Fig. 16.1) [35].

Our group compared whole term placental-derived MSCs (pMSCs) and BM MSCs under good manufacturing practice conditions with the intention of using pMSCs for clinical trials [36]. We discovered pMSCs differed in their proliferation and gene expression profiles to BM MSCs. Placental-derived MSCs show greater expansion capability than BM MSCs. Hence, pMSCs had a greater long-term growth ability than BM MSCs and displayed an equivalent immunosuppressive capacity of T-cell alloproliferation in mixed lymphocyte reactions. Furthermore, neither pMSCs nor BM MSC caused any acute toxicity in healthy mice when injected intravenously at the same, or higher, doses than those currently used in clinical trials of BM MSCs. Therefore, our study suggested human placenta is an acceptable alternative source for human MSCs and with this knowledge we are currently investigating placental-derived MSCs effects in several ongoing trials in idiopathic pulmonary fibrosis, treatment-refractory Achilles tendinopathy and trials in non-healing bone fractures, asthma and acute myocardial infarction are currently being planned [24, 36, 76].

16.4 Fetal Tissue

Fetal-derived MSCs are theoretically attractive because they generally have not been exposed to viruses and toxins, may contain less genetic abnormalities than adult tissue-derived MSCs, and may have greater proliferative capacity and a greater retention of "stemness" memory [35]. It has been suggested that they have proper-

ties intermediate between embryonic and adult stem cells [35, 58, 77]. Thus, they may be a superior MSC source for clinical trials than traditional sources such as adult bone marrow.

Over the past decade much has been discovered about MSCs in the fetal environment. Over the last several years, it has been discovered that MSCs can be isolated from the placenta [36, 56], fetal blood [58], fetal liver [78], fetal bone marrow [79], amniotic fluid [12, 80], and umbilical cord blood [81, 82]. However, MSCs from some of these fetal sources need to be obtained during the first and second trimesters of gestation, resulting in difficulty in obtaining these tissues. Therefore, the human term placenta represents an attractive source of MSCs due to its ready availability (in that it is generally considered a biological waste product after delivery of the baby), its easy access without invasive procedures, and lack of the ethical constraints. This organ consists of both fetal (amnion and chorion) and maternal (decidua) components, but for the remainder of this chapter, we will focus on fetal-derived products only.

16.4.1 Term Amniotic and Chorionic Membranes

The amnion is the innermost membrane, in contact with the amniotic fluid that encases the fetus during gestation. The amnion is a thin, avascular membrane composed of an epithelial layer and an outer layer of connective tissue. The amniotic epithelium is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It is attached to a distinct basal lamina which is, in turn, connected to the amniotic mesoderm [35, 83, 84]. In the amniotic mesoderm, a network of dispersed fibroblast-like mesenchymal cells and macrophages are observed [83]. The chorion is the outer fetal membrane composed of layers of polygonal cells also consisting of both mesoderm and trophoblast regions [84]. Recent studies have reported that fetal-derived MSCs can be isolated from these tissues [35, 85].

Briefly, to isolate MSCs from these tissues, the amniotic and chorionic membranes are removed from the remaining placental tissue and washed in phosphate-buffered saline (PBS) to remove blood clots. The tissue is then sliced into approximately 5 mm pieces and again washed to remove any remaining blood. The tissue is enzymatically digested in either trypsin and/or collagenase type I and DNase for 1–2 h (or until the tissue has been digested) and placed on a shaker at 37°C. The cell suspension is filtered and either a ficoll density gradient or red blood cell lysis can be performed before the cells are cultured in basal medium such as DMEM, with fetal calf serum and antibiotics [35, 85–87].

Using this technique, human term amniotic and chorionic MSCs have been isolated, ex vivo expanded and characterized [85]. It has been shown that amnion mesenchymal cells (AMCs) and chorion mesenchymal cells (CMCs), isolated by mechanical separation and subsequent enzymatic digestion, demonstrate plastic adherence and fibroblast-like morphology and are able to form colonies that could

be expanded for at least 15 passages. Using flow sorting techniques, these cells have also been shown to be phenotypically similar to bone marrow MSCs and, when cultivated in specific induction media, both cell populations can differentiate into bone and cartilage. However, differences in their adipogenic differentiation potential were apparent with amnion and chorion MSCs showing weak adipogenic potential [85, 88]. This may indicate the "primitiveness" of these cells in that they are too young to form fat and require additional signals for differentiation. Despite the poor adipocyte differentiation ability, these cells have been shown to be immunosuppressive by their ability to inhibit T-cell proliferation [35, 89, 90]. Further studies are now investigating the use of these tissue sources for therapeutic applications in many human diseases and injuries as an alternative to bone marrow MSCs [91, 92].

16.4.2 Amniotic Fluid

Amniotic fluid helps protect the fetus throughout gestation. This unique environment allows the fetus to move freely within the uterus and protects the fetus from mechanical injury [35].

Amniocentesis is a diagnostic procedure that samples amniotic fluid from 14 weeks gestation until birth. This can be used to isolate amniotic fluid MSCs (AF-MSCs) [35]. It has been found that amniotic fluid-derived MSCs express pluripotency genes such as SSEA-4, NANOG, and OCT4 [93]. They have been reported to proliferate faster with over 250 population doublings, exhibit a higher colony-forming efficiency compared to other MSC populations and exhibit a higher osteogenic differentiation ability [12, 94]. Although these properties seem theoretically more advantageous, it is debatable whether amniotic fluid is a practical and reliable source for generating MSCs on a regular basis for clinical trials. Only a few studies have successfully isolated single cell-derived MSC clones from amniotic fluid. It has been estimated that approximately 1% of cells in culture obtained from human amniocentesis are MSCs [95]. This suggests these cells may be more primitive than adult BM MSCs but is not an ideal MSCs source [35].

16.4.3 Cord Blood

There has been controversy on the presence of MSCs in umbilical cord blood, with few laboratories being able to report successful repeated isolations between different donors [96, 97]. In general, the success rate for isolating MSCs from umbilical cord blood has been reported at only 29% as opposed to that of bone marrow and other tissue sources with 100% success rate. Thus, this is one limitation in the use of cord blood as a reliable therapeutic MSCs source. Nevertheless, Kern and

colleagues [37] and Kogler and colleagues [82] reported the collection and isolation of umbilical cord blood MSCs. They collected cord blood from the unborn placenta of full-term deliveries in a multiple bag system containing 17 ml of citrate phosphate dextrose buffer and processed blood within 24 h of collection. Prior to density gradient centrifugation, the anticoagulated cord blood was diluted with EDTA-PBS. The mononuclear cell fraction was then seeded at a density of 1×10^6 mononuclear cells/cm^2 into pre-coated FCS plates. Twelve to eighteen hours after initial plating, non-adherent cells were removed and adherent cells began to appear as colony-forming unit-fibroblasts. CFU-Fs were harvested and cells replated at a mean density of $3.5 \pm 4.8 \times 10^3$/cm^2.

When comparing bone marrow and umbilical cord blood-derived MSCs at an initial plating density of 1×10^6 cells/cm^2, Kern and colleagues found that umbilical cord blood-derived MSCs formed CFU-Fs that could be detected during the first 2–4 weeks after initial seeding [37]. This was in contrast to bone marrow-derived MSCs which formed a monolayer 4–5 days after this initial seeding. This indicated that umbilical cord blood contains fewer MSCs/unit volume than bone marrow. At a dose of 1×10^6 initially plated cells, bone marrow MSC formed more colonies (83 ± 61) than umbilical cord blood (0.002 ± 0.004). Thus, it is evident that there are few MSCs within cord blood.

An adherent, fibroblast-like morphology was observed with no cellular morphological differences described between cord blood and bone marrow MSCs. Interestingly, it was found that cord blood-derived MSCs possessed higher population doublings in all passages compared to bone marrow MSCs. Consequently, umbilical cord blood MSCs can be cultured for longer than bone marrow MSCs, which is theoretically useful for the manufacturing of MSCs for clinical trials. Cell surface phenotyping of umbilical cord blood MSCs showed no expression of hematopoietic cell but more than 90% of MSCs from both sources expressed the characteristic MSCs phenotype of CD44, CD73, CD29, and CD90. HLA-I was also expressed in both populations but neither populations expressed HLA-II.

Despite similarities in morphology and cell surface phenotype, differences have been noted between the two cell populations. A comparative study performed by our group found umbilical cord blood MSCs did not differentiate into the adipogenic lineage [98]. These data suggest, that as with amnion and chorion MSCs, cord blood MSCs are restricted in their mesodermal differentiation potential: if they are an earlier lineage, they may require additional signals to initiate adipogenic differentiation. Supporting our observations, Chang and colleagues showed that umbilical cord blood MSCs have strong osteogenic potential and reduced adipogenic potential [99]. In addition, it was also found that cord blood MSCs displayed a weak inhibitory effect on T-cell alloproliferation in comparison with BM MSCs. However, Winter and colleagues showed that co-incubation of INF-γ and TNF-α enhanced the immune suppressive responses of cord blood MSCs [100]. This suggests that while unstimulated cord blood MSCs are weakly immune suppressive, they may be able to be activated to become more immune suppressive in an inflammatory environment. Additionally, gene expression data demonstrated a unique transcriptome profile for

each MSC population and ingenuity pathway analysis showed contributions of each MSC population to different functions [98]. These results reenforce the notion that MSC populations from different tissues are not identical and retain memory of their tissue of origin.

Despite cord blood MSCs having a remarkable ex vivo expansion potential, this source is not recommended as a reliable or ideal therapeutic source due to the low frequency of MSCs within the cord blood.

16.5 MSC-Based Therapeutic Applications

As outlined in this chapter, MSCs are likely be useful in a diverse range of therapeutic applications (reviewed in "Therapeutic applications of mesenchymal stromal cells") [1] and can be used to exploit either their immune suppressive capability or their tissue repair capability. The use of fetal-derived MSC in orthopedic applications appears ideal due to their ability to engraft and differentiate into bone and cartilage. Patients suffering with diseases and injuries such as osteogenesis imperfecta, spinal disease, joint disorders (osteoarthritis), and nonunion bone fractures may benefit from MSC administration. Promising results from preclinical studies in mice with osteogenesis imperfecta have shown first trimester fetal blood MSCs markedly reduced fracture rates and skeletal abnormalities [77]. Furthermore, in a single case study, Le Blanc and colleagues showed the potential of unrelated fetal liver MSC transplanted in utero to a developing fetus with osteogenesis imperfect [101]. In this instance, donor MSCs engrafted into the bone which showed normal trabecular development.

The use of MSCs in conjunction with hematopoietic cell therapy and graft-versus-host disease (GVHD) is also promising. It has been reported in a study of 28 breast cancer patients who had received high dose of chemotherapy and autologous peripheral blood stem cells (PBSC) transfusion that MSC facilitated hematopoietic recovery [102]. Furthermore, enhanced hematopoietic recovery was also reported in a study with seven patients who received PBSC, bone marrow or cord blood transplant and MSC infusion. We are conducting our own phase I study using placenta-derived MSC co-transplanted with cord blood cell transplantation in patients with chemotherapy–refractory hematological malignancies. We found no adverse effects from the MSCs [24]. A recent phase II study by Osiris Therapeutics Inc. treated patients with severe acute GVHD (http://www.osiristx.com). With the use of adult MSCs in combination with corticosteroids, they reported a higher complete response rate than those not treated with MSCs. Additionally, MSCs appeared to accelerate lymphocyte engraftment when infused with or shortly after HSC transplantation. These results are promising and Osiris Therapeutics is currently conducting a phase III study for the treatment of newly diagnosed severe acute GVHD.

The effect of MSCs has also been extensively studied in other applications such as cardiovascular, central nervous system, pancreatic, renal, and hepatic diseases.

Osiris Therapeutics Inc. recently completed a phase I placebo-controlled case study of bone marrow-derived MSCs in patients with acute myocardial infarction. It was found that the MSCs were well tolerated and patients were less likely to experience an arrhythmic event and had improved cardiac ejection fraction and lung function at 6 months after infusion. They are now following this up with a phase II trial. Studies by Mesoblast PTL have shown similar results (http://www.mesoblast.com).

16.6 Discussion

It is now apparent that MSCs can be isolated from almost every organ in the human body. We described in this chapter the use of several alternative tissue sources to bone marrow MSCs as a cellular therapeutic agent. These sources were younger than adult bone marrow, obtained by relatively noninvasive procedures and generally classified as biological waste material, making them convenient tissue sources for manufacturing MSCs for clinical trials. Despite similarities that were found among the different tissue sources such as morphology and cell surface phenotype, differences were discovered in differentiation ability, gene expression, and immunosuppressive capacity. These differences may contribute to differing functional abilities in vivo and may result in variable ability in mediating specific clinical outcomes.

There is evidence that suggests that there is a reparative and differentiation bias of MSC populations depending on the tissue of origin. In fact, it has been reported that both fetal and bone marrow MSCs can induce repair after myocardial infarction, although by different mechanisms [103]. The higher osteogenic differentiation ability of amniotic fluid MSCs suggests that MSCs from amniotic fluid may be an optimal source for orthopedic applications [104, 105]. MSCs derived from the fetal-derived placenta also show enhanced migratory ability but less adipogenic potential [106]. Similarly, umbilical cord blood MSCs show no such adipogenic capacity [98, 107]. In contrast, adipose-derived MSCs show a greater adipocyte differentiation potential [107]. A comparative gene expression study between adipose, umbilical cord, and bone marrow MSCs compared to mature fibroblasts found 25 consistent genes in all the MSC populations [37, 66]. However, differences were discovered in the phenotype and functional capacity varied depending on their tissue of origin [33, 98, 107]. This is also supported by a study performed between amniotic fluid, amniotic membrane, and cord blood-derived MSCs that found specific biological functions for MSCs from each different gestational tissue [108]. This suggests that distinct tissue populations of mesenchymal stromal cells have specific functional roles depending on their tissue of origin. These differences in MSC populations may be important for the development of future MSC therapeutic approaches for tissue and organ repair and suggest careful consideration when choosing a source of MSC for clinical trials. Taken together, these remarkable qualities have allowed MSCs to be explored in a range of differing medical conditions within the clinic and are proving to be promising candidates for an allogeneic “off-the-shelf” cellular therapy ready for immediate use.

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Chapter 17
Regulatory Considerations Applicable to Manufacturing of Human Placenta-Derived Mesenchymal Stromal Cells (MSC) Used in Clinical Trials in Australia and Comparison to USA and European Regulatory Frameworks

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Abstract Independent development of regulatory frameworks in Australia, Europe and the USA has led to differences in their regulatory approach to biologics (or biologicals). Some of these were favourable for the conduct of early clinical trials (i.e. TGA CTN and CTX). Others have been affected by external factors (i.e. UK membership in the EU) or have expanded their scope (i.e. CBER emergence within the FDA). Recently efforts have been made to harmonise the three frameworks via joint guidances to industry and researchers and memoranda of understanding and cooperation among the regulatory bodies from the regions. We present our own experience in manufacturing and use of human placenta-derived mesenchymal stromal cells (hpMSC) in phase 1 clinical trials conducted in Australia according to the new Biologicals Framework established by Therapeutic Goods Administration (TGA) as from 1 July 2011. We also present similarities and differences with some other regulatory frameworks (USA and EU) that may be of interest to us in the future.

Keywords Mesenchymal stromal cell • MSC • Placenta • Regulatory requirements • Biologicals framework • Clinical trials • TGA • FDA • EMA

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Abbreviations

ARTG	Australian Register of Therapeutic Goods
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drugs Evaluation and Research
CFR	Code of Federal Regulation
CTN	Clinical Trial Notification
CTX	Clinical Trial Exemption
DDXs	Doctors and Dentists Exemptions
EMA	European Medicines Agency
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GMP	Good Manufacturing Practice
HREC	Human Research Ethics Committee
ICH	International Conference on Harmonisation
IND	Investigational New Drug Application
IRB	Institutional Review Board
MHRA	Medicines and Healthcare Products Regulatory Agency
PMDA	Pharmaceutical and Medical Devices Agency
TGA	Therapeutics Goods Administration

17.1 Introduction

Taking newly created biomedical discoveries from the bench to the bedside has proven to be a highly challenging and costly exercise. It has been defined as Translational Research and recognised by researchers, funding agencies and, recently, by regulatory agencies across the globe, as an imminent need. Success in translational research requires not only highly trained experts and complex skill sets within a team but also an understanding of the application of data in different regulatory frameworks. Independent development of regulatory authorities in Australia, Europe and the USA has led to differences in their approach to pharmaceutical manufacturing as well as regulation of biologic drugs (or biologicals). Some of these frameworks were favourable for the conduct of early clinical trials with minimal supporting data; others have been affected by external factors or have expanded their scope through the years. Recent efforts have been made to harmonise the three frameworks.

Although therapeutics derived from biological sources have been subjected to regulatory oversight for some time (i.e. monoclonal antibodies) the biologic products used in transplantation procedures have historically been exempted from this oversight. The unique source of the "active ingredients" renders cell and tissue therapies difficult to be assessed by the traditional regulatory system which has been geared to pharmaceutical quality control. It has been recognised that therapeutic claims for biologics needed to be supported. New considerations have led the existing

regulatory agencies of the developed world to propound new regulatory approaches for biologics (or biologicals). Furthermore, in addition to considerations of regulation of medicines and medical devices manufactured in the traditional manner, the regulation of cell and tissue products is closely linked to sensitive areas of public health policy and funding. This positions regulators in a challenging position as they attempt to reconcile their roles as independent assessors with public health needs and perceptions.

Cell and tissue biologic therapies must be developed and manufactured through disciplined and specific mechanisms, even when full compliance with traditional concepts of pharmaceutical manufacturing practice is not possible, particularly for life-saving therapies. These systems incorporate considerations of risk–benefit ratios and include mechanisms for transparent and accountable release of products, usually based on urgent medical need.

This chapter explores regulatory considerations applicable to manufacturing and use of human placenta-derived mesenchymal stromal cells (hpMSC) in clinical trials in Australia and comparison to USA and European regulatory frameworks. Sections 17.2, 17.3 and 17.4 present the regulatory frameworks provided by the Therapeutic Goods Administration (TGA), the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in Australia, the USA and the EU, respectively. In Sect. 17.5 we present our own experience in manufacturing and use of hpMSC in phase 1 clinical trials conducted in Australia. Section 17.6 gives an overview of information required in applications for clinical trials and ICH GCP. Finally, in Sect. 17.7 a comparison is given taking into account regional drug regulations and clinical trial approval processes.

17.2 Therapeutic Goods Administration: Australia

In Australia the Therapeutic Goods Administration (TGA) was established in 1990 as a division of the Commonwealth Department of Health and Aged Care. Its charter is to ensure that therapeutic goods are assessed and controlled for safety, quality and efficacy at a standard equal to that of the comparable countries. Its role is also to provide the Australian community with access, within reasonable time, to modern therapeutic advances. It is based on the Therapeutic Goods Act, 1989 (the Act), which is approved by the Federal Parliament and establishes a uniform, national system of regulatory control to ensure the quality, safety, efficacy and timely availability of therapeutic goods for human use. Although there are certain applicable territories/state regulations involved, responsibility for the regulatory control lies with the TGA as the national regulatory authority [1–5].

The TGA controls the supply of therapeutic goods through three main processes (pre-market evaluation and approval, licencing of manufacturers and post-market surveillance).

Therapeutic goods for human use that are imported, manufactured in Australia, supplied by a corporation, supplied interstate or to the Commonwealth, or exported

must be included in the Australian Register of Therapeutic Goods (ARTG) unless specifically exempted. Access to unapproved therapeutic goods (items exempted from entry to ARTG) is controlled through a few different mechanisms: Special Access Scheme (Cat. A and B), Clinical Trials (CTN and CTX Schemes), authorised prescriber/s, and importation for personal use [3–5]. Human Research Ethics Committees (HREC) also play an important role in the regulation of the supply of unapproved goods under the Act in relation to Clinical Trials (both CTN and CTX schemes), the Special Access Scheme and approval of Authorised Prescribers [2]. Separate branches of the TGA are responsible for prescription drug evaluation, regulation of therapeutic advice, over-the-counter drug regulations and compliance issues, and general administration. Another branch consists of the TGA Laboratories (previously the National Biological Standards Laboratory) [3–5].

The TGA regulatory framework is based on a risk management approach to ensure public health and safety, while at the same time trying to free industry from any unnecessary regulatory burden. Australian manufacturers of all medicines must be licenced under Part 4 of the Act, and their manufacturing process must comply with the principles of Good Manufacturing Practice (GMP). In assessing the risk, factors such as the strength of a product, side effects, potential harm through prolonged use, toxicity and the seriousness of the medical condition for which the product is intended to be used are all taken into account. Medicines assessed as having a higher level of risk (prescription medicines and some non-prescription medicines) are evaluated for quality, safety and efficacy and registered on the ARTG. Medicines having a lower risk (i.e. over-the-counter medicines and complementary medicines including vitamins) are assessed for quality and safety. Once approved for marketing in Australia, medicines are included in the ARTG and can be identified by the AUST R number (for registered medicines) or an AUST L number (listed medicines) that appears on the packaging of the medicine [1–5].

An important part of TGA control and activities is through Clinical Trials. There is no requirement that application to the TGA to market medicines must contain data from clinical trials conducted in Australia. However, the Australian CTX (Clinical Trial Exemption) and CTN (Clinical Trial Notification) Schemes offer considerable benefits by providing the momentum to research and developing new medicines locally. The choice of which TGA Clinical Trial Scheme (CTN or CTX) to follow lies primarily with the sponsor (sponsoring organisation) and then with HREC. Notification under the CTN or application under the CTX is required for any medicine not entered on the ARTG (including a new formulation of an existing product or any new route of administration), or the use of a registered medicine beyond the conditions of its marketing approval (including new indications extending the use of the product to a new population group and the extension of doses or duration of treatment outside of the approved range) [1–5].

There are a number of Regulatory Requirements, Standards and Guidelines applicable to novel cell therapies used in Clinical Research in Australia (Table 17.1) [25–29]. The new Biological Framework was established by the Australian Therapeutic Goods Administrations (TGA) after long and careful consideration (http://www.tga.gov.au/bt/hct.htm) to improve the regulation of human tissue

Table 17.1 Regulatory requirements, standards and guidelines applicable to novel cell therapies used in clinical research in Australia

Name[a] and issuing authority	Abbreviated Name	Purpose and Context
Australian Code of Good Manufacturing Practice—Human Blood and Tissues By Therapeutic Goods Administration	cGMP	To meet the requirements of the Therapeutic Goods Act 1989, blood and tissue banks must meet the requirements of the Manufacturing Principles, which reference the Australian Code of Good Manufacturing Practice—Human Blood and Tissues. In July 2002 the Australian Health Ministers' Conference recommended that the Therapeutic Goods Administration (TGA) develop a new regulatory framework for human cell and tissue therapies and other emerging biological therapies.
Access to Unapproved Therapeutic Goods—Clinical Trials in Australia By Therapeutic Goods Administration	TGA	This document describes the regulations for allowing patients access to unapproved medicines or medical devices by participation in a clinical trial. It is primarily directed at sponsors and investigators, but will also provide useful guidance to Human Research Ethics Committees (HRECs). HRECs are also directed to the TGA publication Human Research Ethics Committees and the Therapeutic Goods Legislation.
Good Clinical Practice By Therapeutic Goods Administration	GCP	The Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) is an internationally accepted standard for the designing, conducting, recording and reporting of clinical trials.
National Statement on Ethical Conduct in Human Research By The National Health and Medical Research Council	NHRMC	This Statement entitled the National Statement on Ethical Conduct in Human Research ("the Statement") consists of a series of Guidelines made in accordance with the National Health and Medical Research Council Act 1992 ("the Act").
Human Research Ethics Committees and the Therapeutic Goods Legislation By Therapeutic Goods Administration	TGA	This document describes the role of Human Research Ethics Committees (HRECs) in relation to the supply of unapproved therapeutic goods.
Australian Red Cross Blood Services (ARCBS) and National Transplantation Service (NTS)	ARCBS-NTS	Before the Blood Service came into formal existence in 1996, the collection, processing and distribution of blood products throughout the country's health system was managed by individual State and Territory Red Cross Transfusion Services. The establishment of a national blood service has facilitated new levels of national and international co-operation, resulting in improved consistency, quality and safety across Australia.

(continued)

Table 17.1 (continued)

Name[a] and issuing authority	Abbreviated Name	Purpose and Context
Australian Bone Marrow Donor Registry Guidelines By Australian Bone Marrow Donor Registry	ABMDR	The Australian Bone Marrow Donor Registry is the tenth largest registry in the world. The registry is guided by people committed to help any person in need of a haemopoietic stem cell transplant. The ABMDR Board provides oversight and guidance to the ABMDR and its executives.
AusCord Guidelines By Australian national network of umbilical cord blood banks and cord blood collection centres	AusCord	AusCord is the Australian national network of umbilical cord blood banks and cord blood collection centres. AusCord aims to provide greater opportunities to patients who need a life-saving procedure through cord blood transplantation.

[a]Name of Standard/Regulation or Guidelines and Regulatory Agency/Issuing Authority

and cellular therapies and to 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 (http://www.tga.gov.au/ct/index.htm#med). Human placenta-derived mesenchymal stromal cells (hpMSC) are categorised as Class 3 products within the new framework. As a result, any clinical trial utilising Class 3 hpMSC can be conducted under the TGA's Clinical Trial Notification (CTN) Scheme. The CTN scheme is designed to combine rapid approval of clinical trial protocols with ongoing monitoring and supervision by HRECs acting in accordance with nationally agreed guidelines developed by the National Health and Medical Research Council (NHMRC). It is important to note that the TGA does not review any data relating to the trial under this scheme (http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/ComplianceActivities/Enforcement/ComplianceProgram s/ucm095207.htm).

17.2.1 TGA Exemptions and "Special Schemes" for Investigational Drugs (Including Biologicals)

Access to unapproved therapeutic goods (items exempted from entry to ARTG), other than Clinical Trials (CTN and CTX Schemes) is controlled in Australia by TGA through several mechanisms: Special Access Scheme (Cat. A and B), authorised prescriber/s, and importation for personal use [3–5].

Under the Therapeutic Goods Act (the Act), therapeutic goods/medicines can be exempted to allow for their use for experimental purposes in humans (clinical trials). The same regulatory Act (mostly in Sections 18 and 19 of the Act) allows some other exemptions, such as exemption of therapeutic goods by the Federal (National)

Minister of Health, if the Minister is satisfied that, in the national interest, the goods may be stockpiled as quickly as possible in order to deal with a potential threat to public health (that may be caused by a possible future emergency) or to deal with an actual threat to public health (caused by an emergency that has occurred). According to the Act, it is possible to allow exemptions for special and experimental uses, if the Secretary of the Federal Department of Health by notice in writing, grants an approval to a person for use in the treatment of another person, or solely for experimental purposes in humans (and such approval may be given subject to such conditions as are specified in the notice of approval). Medicines (except for gene therapy) that are dispensed or extemporaneously compounded for a particular person can be exempt from TGA regulation. Similar exemptions may apply to medicines individually dispensed by traditional Chinese medicine and homoeopathic practitioners [3–5].

According to the Act, it is possible to allow exemptions due to unavailability of therapeutic goods. In this case the Secretary may grant approval, by notice in writing, for registered goods that could act as a substitute for goods that are unavailable, goods that are in short supply, or registered goods that could act as a substitute for goods that not exist. All of these actions under specified circumstances may require provision of particular information about the goods storage, handling, use, monitoring, records, and disposal of unused goods [5].

17.3 Food and Drug Administration (CDER vs. CBER): USA

The Centre for Drug Evaluation and Research (CDER) and its Office of New Drugs are divisions of the Food and Drug Administration (FDA) responsible for investigating the quality, efficacy and safety of drug products, including clinical trials materials, in the USA. CDER is divided into five subdivisions (known as Offices of Drug Evaluation or ODEs), each responsible for a particular therapeutic area of drug control [6–8].

The Centre for Biological Evaluation and Research (CBER) regulates biological and related products including blood, vaccines, allergens, tissues, and cellular and gene therapies. Biologics, in contrast to drugs that are chemically synthesised, are derived from living sources (such as humans, animals and microorganisms), are not easily identified or characterised and many are manufactured using biotechnology. These products often represent cutting-edge biomedical research and, in time, may offer the most effective means to treat a variety of medical illnesses and conditions that presently have few or no other treatment options [6–8]. CBER's review of new biological products, and for new indications for already approved products, requires evaluating scientific and clinical data submitted by manufacturers to determine whether the product meets CBER's standards for approval. After a thorough assessment of the data, CBER makes a decision based on the risk–benefit for the intended population and the product's intended use. CBER's authority resides in the Public Health Service Act and in specific sections of the Food Drug and Cosmetic Act [9–13].

The American system for clinical trials approval bears some similarities to that in Australia, but there are some notable exceptions. Unlike the Australian system of TGA approval through the CTX scheme and HREC approval through a CTN, all clinical trials applications in the USA are considered by the FDA. Simply stated, the American system does not allow an HREC to approve a clinical trial in isolation, as can occur under the Australian system. The formal application for a clinical trial in the USA is known as an Investigational New Drug application (IND). The information contained in the IND is similar to that which might constitute a CTX application in Australia (i.e. biological and toxicological information, chemistry and manufacturing information and clinical trials protocol and investigator information) [9–13].

17.3.1 FDA Exemptions and "Special Schemes" for Investigational Drugs (Including Biologics)

Access to unapproved drugs in the USA can occur through a number of legal mechanisms (apart from clinical trials), such as a special exception or compassionate exemption, an emergency Investigational New Drug (IND), and a Treatment IND [6–9].

If the eligibility criteria in a study protocol are not suitable for a particular patient, it may still be possible to get that patient treated according to a special exception (also called compassionate exemption). This process depends on the decision of investigator and sponsor, and requires written request to the FDA, modifying the consent form and obtaining permission from the local institutional review board (IRB) (ethical committee). The requesting letter should state the rationale for the exception and provide a brief patient history. It is sent as general correspondence to the appropriate IND application [9].

There is also an exception available through a mechanism called a treatment IND. Under this program, a sponsor of a drug that has shown clinical promise and is still under review by the FDA may charge for the drug during the review process if permission is granted by the FDA. This provides expanded access to the drug prior to commercial distribution [9]. According to the Code of Federal Regulation (21CFR312.34), in general, a drug that is not approved for marketing may be under clinical investigation for a serious or immediately life-threatening disease condition in patients for whom no comparable or satisfactory alternative drug (or other therapy) is available. During the clinical investigation of the drug, it may be appropriate to use it in the treatment for patients not in clinical trials (in accordance with specific IND application). The purpose is to facilitate the availability of promising new drugs to desperately ill patients as early as possible, and also to gain additional data on the drug's safety and effectiveness. There are specific requirements applicable to certain phases of the trials (Phases 2 and 3), and specific criteria (i.e. if the drug is intended to treat a serious or immediately life-threatening disease, and there is no comparable or satisfactory alternative drug) [6–9].

17.3.2 Outline of the FDA Framework for Human Cells, Tissues, and Cellular and Tissue-Based Products

In the early 1990s, the Center for Disease Control and Prevention (CDC) reported that human immunodeficiency virus (HIV) had been transmitted through transplantation of human tissue. Information was also reported which suggested that potentially unsafe tissue was being imported into the USA for transplantation into humans. Prompted by reports that potentially unsafe bone was being imported, the Commissioner of Food and Drugs ordered an immediate investigation. Information resulting from this investigation identified an immediate need to protect the public health from the transmission of HIV and hepatitis B and C through transplantation of unsuitable tissue. Concerns that disease transmission could occur, coupled with information derived from these investigations, prompted the Food and Drug Administration (FDA, the Agency) to publish an interim rule in December 1993 that specifically required certain communicable disease testing, donor screening, and record-keeping for human tissue intended for transplantation. A final rule was issued in July 1997 [14]. The FDA chose to regulate tissues under the legal authority of Section 361 (Sec. 361) of the Public Health Service Act (hereafter, PHS Act) [42 USC 264]. This section authorises the Surgeon General, with the approval of the Secretary, Department of Health and Human Services, to make and enforce such regulations as judged necessary to prevent the introduction, transmission, or spread of communicable diseases from foreign countries into the USA or from State to State. Section 361 of the PHS Act focuses on preventing the introduction, transmission and spread of communicable diseases [14].

In 1997, the agency announced its plans for human cells, tissues, and cellular and tissue-based products (HCT/Ps) in two documents: "A Proposed Approach to the Regulation of Cellular and Tissue-Based Products" (62 FR 9721, March 4, 1997) and "Reinventing the Regulation of Human Tissue". FDA requested written comments on its proposed approach and, on March 17, 1997, held a public meeting to solicit information and views from the interested public. Since that time, the Agency has published three final rules and one interim final rule to implement aspects of the proposed approach [14]. On January 19, 2001, the FDA issued regulations to create a new unified system for registering HCT/P establishments and for listing their HCT/Ps (registration final rule, 66 FR 5447). The registration rule became effective in two stages. The first effective date, April 4, 2001 was applicable to establishments that were already regulated under 21 CFR Part 1270. The second effective date was originally January 21, 2003, and was applicable to establishments that manufacture HCT/Ps currently regulated as biological products, drugs, or devices, haematopoietic stem cells from peripheral and cord blood, and reproductive cells and tissues. On January 21, 2003, the FDA announced that the registration requirements for these establishments would be further delayed until January 21, 2004 [14].

On January 27, 2004, the FDA issued an interim final rule to except human dura mater and human heart valve allografts from the scope of that definition until all of the tissue rules became final. On May 25, 2004, the FDA promulgated regulations

requiring most cell and tissue donors to be tested and screened for relevant communicable diseases (donor-eligibility final rule, 69 FR 29786). On November 18, 2004, FDA issued regulations that require establishments that manufacture HCT/Ps to comply with Current Good Tissue Practices (CGTP), which would include, among other things, proper handling, processing, labelling and record-keeping procedures. The regulations require each establishment to maintain a quality program to ensure compliance with CGTP. In addition, with the implementation of CGTPs, human dura mater and human heart valve allografts are now included in the scope of HCT/Ps regulated under the 21 CFR 1271. On May 25, 2005 the FDA published an interim final rule to revise certain regulations regarding the screening and testing of HCT/P donors and related labelling (interim final rule, 70 FR29949). This action was taken by the FDA in response to comments from interested persons regarding the impracticability of complying with certain regulations as they affect particular HCT/Ps [14].

The CGTP and other regulations are contained in 21 CFR Part 1271, along with provisions relating to establishment registration. These regulations will apply to HCT/Ps recovered on or after the rule's effective date, May 25, 2005. HCT/Ps that were recovered before the effective date of the new rules are subject to 21 CFR 1270, and subparts A and B of Part 1271, as appropriate. In addition, 21 CFR Part 1271 subparts A, B, C, F, 21 CFR 1271.150(c), and 21 CFR 1271.155 of subpart D apply to reproductive HCT/Ps. The new Part 1271 is made up of six subparts:

- General provisions pertaining to the scope and purpose of Part 1271, as well as definitions.
- Registration and listing procedures.
- Provisions for the screening and testing of donors to determine their eligibility.
- Current Good Tissue Practice (CGTP) requirements.
- Certain labelling and reporting requirements.
- Inspection and enforcement provisions.

21 CFR 1271.10(a) sets out the criteria that form the foundation of our tiered, risk-based approach to regulating HCT/Ps. HCT/Ps that meet all of these criteria are subject only to regulation under section 361 of the PHS Act. These HCT/Ps are subject to the regulations in Part 1271, and no pre-market approval is required. HCT/Ps that do not meet all of the criteria in 21 CFR 1271.10(a) are regulated as drugs, devices and/or biological products. The HCT/Ps are subject to the regulations specific to drugs, biological products, or medical devices, in addition to applicable sections of Part 1271. Bone (including demineralised bone), Ligaments, Tendons, Fascia, Cartilage, Ocular Tissue (Corneas and Sclera), Skin, Arteries and Veins (except umbilical cord veins), Pericardium, Amniotic membrane (when used alone, without added cells for ocular repair), Dura mater, Heart valve allografts, Semen, Oocytes, Embryos and Haematopoietic stem/progenitor cells derived from peripheral and cord blood [14].

The above HCT/Ps are regulated solely under section 361 of the PHS Act and the regulations in 21 CFR Part 1271 if they meet all of the following criteria:

- Minimally manipulated; -Intended for a homologous use only as reflected by the labelling, advertising, or other indications of the manufacturer's objective

intent; -Not combined with another article (except for water, crystalloids, or a sterilising, preserving, or storage agent, if the addition of the agent does not raise new clinical safety concerns with respect to the HCT/P); and Either:
- Do not have a systemic effect and are not dependent upon the metabolic activity of living cells for the primary function; OR
- Have a systemic effect or are dependent upon the metabolic activity of the other cells for the primary function, AND:
- Are for autologous use; -Are for allogeneic use in a first- or second-degree relative; OR -Are for reproductive use [14].

Those HCT/Ps that do not meet all 21 CFR 1271.10(a) criteria and are regulated as drugs, devices, or biological products are covered under separate compliance programs [14], such as:

- Blood and Blood Products are covered under CP 7342.001 "Inspection of Licenced and Unlicenced Blood Banks, Brokers, Reference Laboratories, and Contractors"; and CP 7342.002 "Inspection of Source Plasma Establishments"
- HCT/Ps that do not meet all 21 CFR 1271.10(a) criteria, and are regulated as Medical Devices are covered under CP 7382.845 "Inspection of Medical Device Manufacturers"
- HCT/Ps that do not meet all 21 CFR 1271.10(a) criteria, i.e. Autologous, Allogeneic, or Xenogeneic Cells whose biological characteristics have been altered (propagate, pharmacologically treated, etc.); Ex Vivo and Gene Therapy products are regulated as biological drugs and are covered under CP 7345.848 "Inspection of Biological Drug Products"
- HCT/Ps recovered before May 25, 2005 and regulated under 21 CFR 1270 and subparts A and B of Part 1271 are covered under CP 7341.002A "Inspection of Tissue Establishments"

HCT/P establishments must follow CGTP requirements to prevent the introduction, transmission, or spread of communicable diseases by ensuring that the HCT/Ps do not contain communicable disease agents, that they are not contaminated, and that they do not become contaminated during manufacturing [14]. The following are Core CGTP requirements as referenced in 21 CFR 1271.150(b) [14]:

- Requirements relating to facilities (21 CFR 1271.190(a) and (b))
- Requirements relating to environmental controls (21 CFR 1271.195(a))
- Requirements relating to equipment (21 CFR 1271.200(a))
- Requirements relating to supplies and reagents (21 CFR 1271.210(a) and (b))
- Requirements relating to recovery (21 CFR 1271.215)
- Requirements relating to processing and process controls (21 CFR 1271.220)
- Requirements relating to labelling controls (21 CFR 1271.250(a) and (b))
- Requirements relating to storage (21 CFR 1271.260(a)–(d))
- Requirements relating to receipt, pre-distribution shipment, and distribution of an HCT/P (21 CFR 1271.265(a)–(d)).
- Requirements relating to donor eligibility determinations, donor screening and donor testing (sections 1271.50, 1271.75, 1271.80 and 1271.85).

All establishments engaged in manufacture (as defined in 21 CFR 1271.3(e)) of an HCT/P must register with and submit to the FDA, a list of each human tissue product manufactured unless being exempt by 21 CFR 1271.15. New establishments must register and list within 5 days of beginning operations [14]. CBER maintains an alphabetic listing of currently registered HCT/P establishments that is accessible on the CBER Internet web site at http://www.fda.gov/cber/tissue/hctregestabl.htm [14].

17.4 European Medicines Agency: Europe

The European Medicines Agency (formerly known as EMEA, now abbreviated to EMA) is a decentralised body of the European Union with headquarters in London. The European Medicines Agency is headed by the Executive Director, who is appointed by the Agency's Management Board. The Agency is divided into five Units, each with between two and four sectors. Most sectors are further divided into a number of sections. In addition to its staff, EMA is composed of a Management Board and six scientific committees. The committees and working parties (some including patients' and doctors' representatives) are supported by more than 4,500 European experts and conduct the main scientific work of the Agency [15, 16].

The EMA's main responsibility is the protection and promotion of public and animal health, through the evaluation and supervision of medicines for human and veterinary use. The European Medicines Agency coordinates the evaluation and supervision of medicinal products throughout the European Union. The European Medicines Agency brings together the scientific resources of some 40 national competent authorities in 30 EU and EEA-EFTA countries (Iceland, Liechtenstein and Norway). It cooperates closely with international partners, reinforcing the EU contribution to global harmonisation [15, 16].

The EMA began its activities in 1995, when the European system for authorising medicinal products was introduced, providing for a centralised and a mutual recognition procedure. The Agency has a role in both, but is primarily involved in the centralised procedure. Where the centralised procedure is used, companies submit one single marketing authorisation application to the Agency. A single evaluation is carried out through the Committee for Medicinal Products for Human Use (CHMP) or Committee for Medicinal Products for Veterinary Use (CVMP). If the relevant Committee concludes that quality, safety and efficacy of the medicinal product is sufficiently proven, it adopts a positive opinion. This is sent to the Commission to be transformed into a single market authorisation valid for the whole of the European Union [15, 16].

The Committee for Advanced Therapies (CAT) was established in accordance with Regulation (EC) No. 1394/2007 on advanced therapy medicinal products (ATMPs). It is a multidisciplinary committee, gathering together some of the best

available experts in Europe to assess the quality, safety, and efficacy of ATMPs, and to follow scientific developments in the field. The main responsibility of the CAT is to prepare a draft opinion on each ATMP application submitted to the European Medicines Agency, before the Committee for Medicinal Products for Human Use (CHMP) adopts a final opinion on the granting, variation, suspension or revocation of a marketing authorisation for the medicine concerned. At the request of the EMA Executive Director or of the European Commission, an opinion is also drawn up on any scientific matter relating to ATMPs [15, 16].

The Regulation on Advanced Therapies (Regulation (EC) 1394/2007) defines advanced therapy medicinal products (ATMPs) such as gene therapy, somatic cell therapy and tissue engineered products in Article 2 of Regulation (EC) 1394/2007. Article 29 of the same Regulation foresees that ATMPs on the EU market in accordance with national or EU legislation will have to comply with the new legislation by 30 December 2011 for ATMPs other than tissue engineered products or 30 December 2012 for tissue engineered products. In EMA announcement from July 2008, the European Medicines Agency raised awareness among manufacturers, companies and hospitals having ATMPs legally on the market that these products will have to undergo a marketing authorisation procedure (in line with Regulation (EC) 1394/2007), unless they are exempted in accordance with Article 28 (2) of the same Regulation. This exemption applies to ATMPs prepared on a non-routine basis according to specific standards, and used within the same EU member state in a hospital under the exclusive professional responsibility of a medicinal practitioner, in order to comply with an individual medical prescription for a custom-made product for an individual patient.

Further definition of advanced therapy medicinal products (ATMPs) and a set of specific instructions (i.e. for somatic cell therapy medicinal and tissue engineered products, for advanced therapy medicinal products containing devices or for gene therapy medicinal products) were provided in the Commission Directive 2009/120/EC amending the Directive 2001/83/EC [15, 16].

The European Medicines Agency's Committee for Advanced Therapies (CAT) has unveiled a Work Programme to 2015, intended to help increase the number of advanced therapy medicinal products (ATMPs) that make it from early research stage to the market. With this 5-year program, the CAT aims for an environment that encourages the development of ATMPs while recognising that the traditional regulatory framework for medicines does not currently fully address the needs of companies and organisations (including hospitals) that develop these medicines. The training and early dialogue between the CAT and relevant stakeholders play a central role. In this context, the CAT will also look at the current regulatory framework and at how it can be made more accessible for small and medium-sized enterprises, academia, patient groups, hospitals, charity foundations, and trusts developing ATMPs. Proposed actions are also taking into account that new and emerging science is an important driver for progress and change in the health-care field [15, 16].

In March 2011 an EMA-FDA pilot program for parallel assessment of Quality by Design applications was established. The assessment of Marketing Authorisation Application (MAAs)/New Drug Applications (NDAs) including Quality by Design

(QbD) or enhanced pharmaceutical development approaches, requires a good understanding of statistical, analytical and risk assessment development methods that have not been systematically used by pharmaceutical industry or regulators in the past. This program provides advice to applicants on the background and objectives of the pilot study, as well on the operational steps that will be taken to coordinate a parallel review and related GMP inspections by EMA and FDA, following ICH guidelines developed (ICH Q8, 9, 10) in order to facilitate the implementation of Quality by Design in pharmaceutical industry on a global level [15, 16].

It is reasonable to expect development of similar initiatives between regulatory agencies in the future, including those in area of advanced therapy medicinal products (ATMPs).

17.5 Our Own Experience in Manufacturing and Use of Human Placenta-Derived Mesenchymal Stromal Cells in Phase 1 Clinical Trials Conducted in Australia

17.5.1 Manufacturing Human Placenta-Derived MSC for Clinical Trials

Human mesenchymal stem cells (MSC) derived from a number of different organs and tissues, such as placenta, are increasingly being used in clinical trials for a range of regenerative and inflammatory diseases. At the Mater Mother's Hospital, Brisbane, there are approximately 10,000 deliveries/year, making term placenta an attractive and readily available tissue for the isolation and manufacturing of clinical grade MSC. Placentas are normally disposed of after delivery, are obtained without invasive procedures, and their use does not elicit ethical debate. Placental MSC (hpMSC) show a classical MSC cell surface phenotype and mesodermal differentiation properties as well as potent immunosuppressive properties [17, 18].

We have been unable to find major differences between hpMSC and human bone marrow MSC (bmMSC) in terms of morphology (Fig. 17.1), cell surface phenotype [19], chemokine receptor display [20], mesodermal differentiation capacity [19] or immunosuppressive capacity [21]. Yen and colleagues [22] found hpMSC to express the embryonic antigens SSEA4, Tra1-60 and Tra1-81, whereas bmMSC did not. However, we found only low level expression of SSEA4 and Tra1-60 on the hpMSC generated from our placental samples [20].

Due to concerns about the sterility of tissue at the time of collection, the placenta is obtained from healthy mothers undergoing elective Caesarean sections as they are performed in a relatively sterile environment. Full informed consent is obtained prior to delivery. The placenta is subsequently double bagged, placed in a cool box and transferred to our manufacturing facility for processing.

Our protocol for the isolation of hpMSC from term placenta utilises a collagenase (GMP grade)-based digestion of tissue which has been dissected and washed

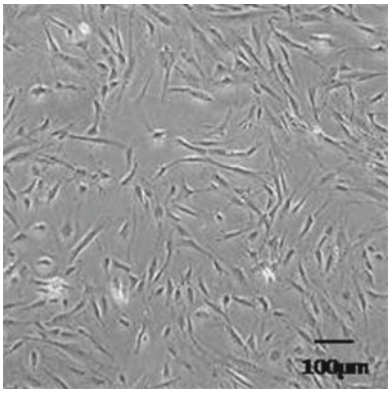

Fig. 17.1 hpMSC, passage 4 (phase contrast microscopy, original magnification 40×, scale bar is 100 µm)

to remove blood before isolation of cells. After digestion, large particulate matter is removed by low speed centrifugation and cell suspensions are collected and filtered into fresh tubes using 70 µm filters. The cells are then pelleted by centrifugation, resuspended and erythrocytes are subjected to rapid lysis with water. The cells are washed with Hank's Balanced Salt Solution (HBSS) and the final cell pellet is resuspended in low glucose-containing Dulbecco's Modified Eagle Medium (DMEM-LG), 25% foetal calf serum (FCS) and 50 µg/ml gentamycin. Cells are initially seeded into eight T175 (175 cm^2) tissue culture flasks and cultured in a humidified incubator at 37°C, 5% CO_2 [23]. A scheme of the production schedule is shown in Fig. 17.2.

We used part (300–500 g) of one placenta for each of our first two production manufacturing runs (Fig. 17.3). This represented over 50% of the total mass of the placenta and was used to seed 6× T175 flasks (termed passage 0). This protocol yielded approximately 40×10^6 cells at the first passage, which were then split between 90 flasks at 4.38×10^5 cells/flask (2.5×10^3 cells/cm^2). The average yield for each subsequent passage was 7.42×10^8 cells (standard deviation of 8.26×10^7). At each passage, 4.0×10^7 cells were held back for the next passage and used to seed a further 90 flasks at 4.38×10^5 cells/T175 (Fig. 17.4) the remaining cells were cryopreserved.

The cell yields and proliferation rates at each passage were generally consistent, as follows (due to leucocyte contamination proliferation rate was not calculated at passages 0 and 1):

Placenta 1. Cell yield ($\times 10^6$): 95(P0), 63(P1), 1160(P2), 400(P3), 960(P4) and 640(P5).
Population doublings: –(P0), –(P1), 4.9(P2), 3.3(P3), 4.6(P4) and 4.0(P5).
Doubling rate per day: –(P0), –(P1), 0.8(P2), 0.6(P3), 0.8(P4) and 0.7(P5).

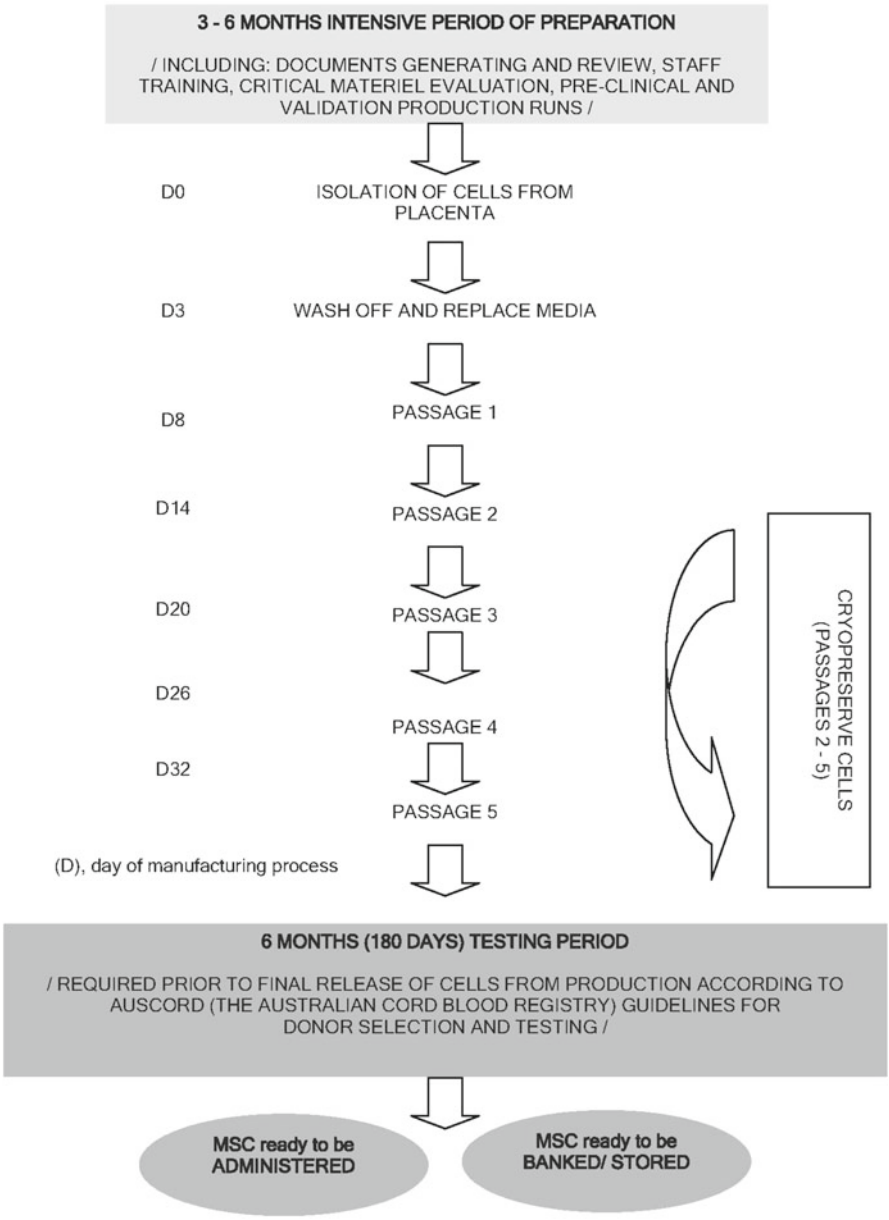

Fig. 17.2 Manufacturing of hpMSC for clinical trials

Fig. 17.3 hpMSC processing

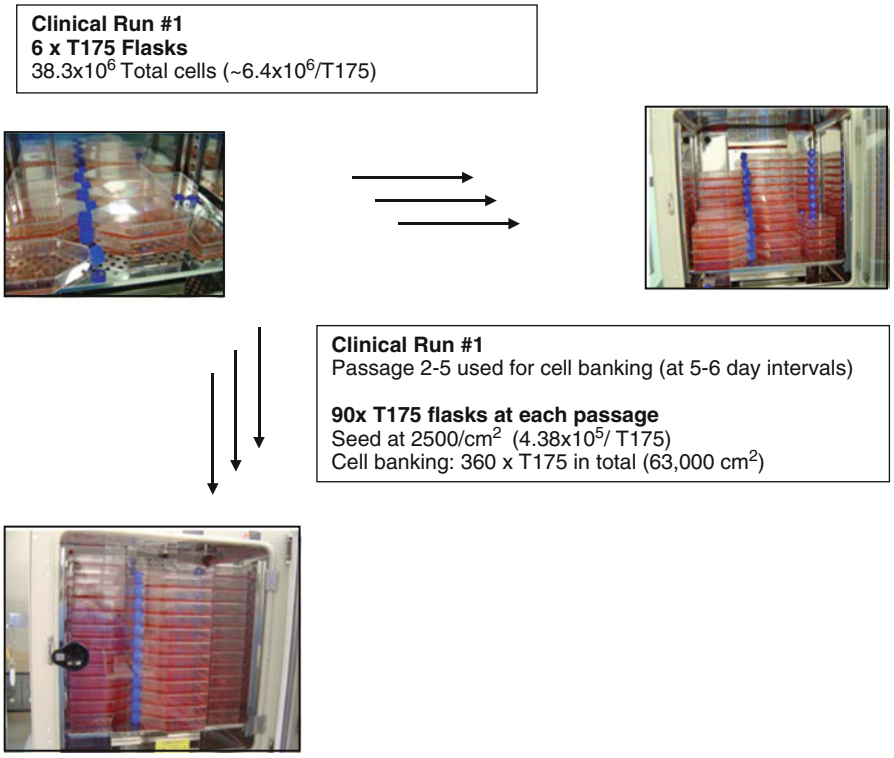

Fig. 17.4 hpMSC passaging (passage 1 to 5)

1. Min 6 months (180 days) storage

2. Release process consists of two phases:
a) Production release according to production release criteria (in Table).
b) *Release prior to infusion* obtained at the clinical site, externally or internally, with the testing of sterility 14-day microbiology culture and viability >70% by Trypan Blue exclusion.

Cells have to pass both of these sets of testing in order to be administered to a patient.

Cellular product testing	Release Criteria
14 days microbiology culture	Sterile
Viability	>70% by Trypan Blue exclusion
Purity by flow cytometry	>85%CD73+, >85% CD105+, <1% CD45+
Karyotype	Cytogenetic analysis normal
Gram stain	Negative
Mycoplasma test of final product	Negative
Endotoxin test of final product	< 2 EU/ml
Day 180 donor serology for infectious disease markers	Negative
Day 180 repeat infectious disease testing on mother and baby health ques onnaire	Normal

Fig. 17.5 Manufacturing two-tier MSC release criteria

Placenta 2. Cell yield ($\times 10^6$): 562(P0), 38(P1), 760(P2), 720(P3), 631(P4) and 637(P5).
Population doublings: –(P0), –(P1), 4.3(P2), 4.2(P3), 4.0(P4) and 4.0(P5).
Doubling rate per day: –(P0), –(P1), 0.7(P2), 0.7(P3), 0.7(P4) and 0.7(P5).

Release criteria (Fig. 17.5) for cryopreservation were fulfilled: 14 days microbiology culture in passages P0–P5 (sterile); viability >70% by Trypan Blue exclusion; purity by flow cytometry (required to be >85% CD73+, >85% CD105+, <1% CD45+) was as follows: P2: 0.4% CD45, 95% CD73/CD105; P3: 0.0% CD45, 99%CD73/CD105; P4: 0.0% CD45, 91% CD73/CD105; and P5: 0.0% CD45, 96% CD73/CD105; karyotype (P2–P5: cytogenetic analysis normal); Gram stain (P2–P5: negative); mycoplasma test of final product (P2–P5: negative); endotoxin test of final product (P2–P5: <2 EU/ml); pre-donation screening and day 180 donor serology for infectious disease markers (both negative); and pre-donation health questionnaire for mother and day 180 health questionnaire for mother and the baby (both clear).

Although cell recoveries were generally excellent, it was noted that cell recovery at passages 4 and 5 of placenta 1 was only 60%. These cells were still acceptable as they did not fall below our trypan blue-determined viability threshold of 70%. Some of this variability may have been due to the fact that only small aliquots of cells are stored separately (in cryovials) for counting purposes. In our experience, the yield from cryovials is generally slightly lower than for bags. However, viability as determined by trypan blue is generally equivalent.

Sterility testing and cell recovery criteria from the pilot vials for placenta 1-derived hpMSC thawed for infusion into the first patient in our phase I trial of hpMSC were fulfilled at the clinical site testing: sterility 5-day culture (no growth) and cell recovery from pilot vial 96% (P2), 100%(P3), 60%(P4) and 60%(P5), respectively.

17.5.2 Conducting Clinical Trials with Human Placenta-Derived MSC

The Biological Framework was established by the Australian Therapeutic Goods Administrations (TGA) to improve the regulation of human tissue and cellular therapies and provides improved clarity by applying different levels of pre-market regulation to biological products based on the risks associated with the use of each product (TGA 2011). Human placenta-derived mesenchymal stromal cells (hpMSC) are categorised as Class 3 products within the new framework (http://www.tga.gov.au/ct/index.htm#med). As a result, any trial utilising Class 3 hpMSC can be conducted under the TGA's Clinical Trial Notification (CTN) Scheme. The CTN scheme is designed to combine rapid approval of clinical trial protocols with ongoing monitoring and supervision by HRECs acting in accordance with nationally agreed guidelines developed by the NHMRC (TGA 2004). It is important to note that the TGA does not review any data relating to the trial under this scheme. The Human Research Ethics Committee (HREC) reviewing a new clinical trial protocol utilising hpMSCs must have sufficient experience amongst its committee members in order to effectively review the protocol ensuring scientific validity, and that participant's rights and well-being are protected according to the Australian National Health and Medical Research Council's (NHMRC) 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).

Along with the study protocol, a Participant Information Sheet and Consent Form (PISCF) and other supporting documents including an Investigators Brochure is submitted for review by the HREC and the TGA, and is used by study personnel to facilitate their understanding of the key features of the protocol, in particular, the dosing and methods of administration of the hpMSCs.

In 2007, for our first phase 1 clinical trial, CT4-MSC-UCB-001, a phase I multicentre open label dose-escalation study of unrelated, MHC-unmatched placenta-derived mesenchymal stromal cells (MSC) in recipients of unrelated umbilical cord blood haematopoietic stem cell (HSC) transplants, the total time from submission of the application until approval was received by the HRECs at the Mater Health Services (MHS), Westmead Hospital (Sydney Western Area Health Service) and the Sydney Children's Hospital (Sydney South Eastern Area Health Service) was 1 year in total. During this time, a request was made by the MHS HREC for an external audit to be conducted on the manufacturing processes outlined in the study protocol which was conducted by staff from the Australian Red Cross Blood Service (ARCBS). Two-way clinical trial agreements were put in place whereby MHS

indemnified each of the participating sites for any adverse events related to the administration of the hpMSCs.

One patient was enrolled in this study who later died from pneumonitis related to CMV reactivity [18]. It is important to note that the donor of the hpMSCs was CMV negative prior to the collection of the placenta and again at the follow-up screening period. This study is now closed at all sites, as umbilical cord blood transplants are no longer performed in adults at Westmead Hospital and no recruitment occurred at Sydney Children's Hospital.CT4 may not have yielded significant clinical results; however, it did highlight some of the impracticalities in incorporating the manufacturing protocol within a clinical trial protocol. As a result, MMRI# CM1: A protocol for manufacturing a human placenta-derived mesenchymal stromal cell bank for use in Mater Health Services Human Research Ethics Committee (HREC)-approved clinical trials was established in 2009. This manufacturing protocol allowed us to continue manufacturing hpMSCs independent of a clinical trial and therefore establish a master bank of hpMSCs. This type of protocol was new to the MHS HREC. After careful consideration, the protocol was approved under the provision that any clinical trial utilising the hpMSCs as the investigational product was to be reviewed by the MHS HREC.

All the hpMSC donors are subject to screening requirements as per the AusCord (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). The same process is 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.

Upon establishing the master bank of hpMSCs (CM1), respiratory physicians at The Prince Charles Hospital (TPCH) in Brisbane collaborated with our team to submit a clinical trial protocol entitled MSC IPF: A phase 1 study to evaluate the potential role of mesenchymal stromal cells in the treatment of idiopathic pulmonary fibrosis. This study first received HREC approval in September 2009 from the TPCH HREC; however, the clinical trial agreement and research governance aspects were not agreed to until October 2010, more than a year later due to the fact that two completely separate health-care providers were involved—Mater Health Services and Queensland Health. Thus far, 2 patients have received hpMSCs generated by our laboratories with no related adverse events. The novel concept of using hpMSCs in different clinical applications is slowly transferring into the norm. Our most recent clinical trial protocol MSC TEN: A phase 1 trial to evaluate the potential role of MSC in the treatment of chronic refractory tendinopathy only took 4 months to approve. This study is now open for recruitment for patients with chronic refractory Achilles tendinopathy.

ClinicalTrails.gov search listed a total of 192 MSC clinical studies worldwide, including 171 studies with known status (i.e. updated on a regular basis); four clinical trials using bone marrow-derived MSC (bmMSC) are completed or in progress in Australia (Table 17.2), while we currently conduct three clinical trials with human placenta-derived MSC (hpMSC) (Table 17.3).

Table 17.2 MSC clinical studies conducted in Australia (Source: http://www.clinicaltrials.gov, July 2011)

Clinical trial title	Sponsor/phase	Condition	Intervention(s)	ClinicalTrials.gov Identifier	Current status
Efficacy and Safety of Adult Human Mesenchymal Stem Cells to Treat Steroid Refractory Acute Graft Versus Host Disease	Osiris Therapeutics/ Phase III	Graft Versus Host Disease	Mesenchymal Stem Cells	NCT00366145	Completed
A Study to Evaluate the Potential Role of Mesenchymal Stromal Cells to Treat Obliterative Bronchiolitis After Lung Transplantation	The Prince Charles Hospital/Phase I	Bronchiolitis Obliterans Lung Transplantation	Placental MSC	NCT01175655	Recruiting
Safety and Efficacy Study of MSB-CAR001 in Subjects 6 Weeks Post Anterior Cruciate Ligament Reconstruction	Mesobalst Ltd/Phase I	Anterior Cruciate Ligament Injury	MSB-CAR001 Combined with Hyaluronan	NCT01088191	Recruiting
An Australian Study of Mesenchymal Stromal Cells for Crohn's Disease	Royal Perth Hospital/ Phase II	Crohn Disease	Mesenchymal Stromal Cells (MSC) for infusion	NCT01090817	Recruiting

Table 17.3 Preclinical and clinical studies conducted by the Stem Cell Biology, Regenerative Medicine and Stem Cell-based Anti-Cancer Therapeutics Group MHS/MMRI

Abbreviated name	Research Register Reference (Other Ref.)	Title	Current status	Related publications
Preclinical studies				
P054	2015 (1541)	Exploration of therapeutic potential of mesencyhmal/stromal cells from bone marrow	Ongoing	Brooke et al. [24]
P055	2016 (1542)	Exploration of therapeutic potential of mesencyhmal/stromal cells from umbilical cord blood and placenta	Ongoing	Barlow et al. [19]
Clinical studies				
MSC-UCB-001		A phase I multicentre open label dose-escalation study of unrelated, MHC-unmatched placenta-derived mesenchymal stem cells (hpMSC) in recipients of unrelated umbilical cord blood haematopoietic stem cell (HSC) transplants	Closed	Brooke et al. [20]
CM1-MSC Manufacturing	1733 (1292A)	A protocol for manufacturing human placenta-derived mesenchymal stem cell bank for use in Mater Health Services Human Research Ethics Committee (HREC) approved clinical trials	Ongoing	Brooke et al. [17]
MSC IPF	1733 (1425E)	A phase 1 study to evaluate the potential role of mesenchymal stem cells in the treatment of idiopathic pulmonary fibrosis	Open and recruiting	Brooke et al. [18]
MSC TEN	2136 (1658A)	A phase 1 trial to evaluate the potential role of hpMSCs in the treatment of chronic refractory Achilles tendinopathy	Open and recruiting	Ilic et al. [23]

17.6 Information Required in Applications for Clinical Trials and ICH GCP

In the last 5–10 years much has happened internationally in clinical trials regulation and this is having a direct impact on clinical trial operations on a regional level as well as worldwide.

Different activities including the 2004 agreement between Australia and New Zealand to establish a joint therapeutic goods regulatory agency (which was subsequently postponed), the May 2004 EU Directive implemented in a majority of member states, the April 2004 Japanese merging of three organisations into a single body called Pharmaceutical and Medical Devices Agency (PMDA), many activities in India as it revamps its regulations to make it more attractive for clinical trials, a number of Central and Eastern European countries looking at their new EU member status or non-member status perspectives, and many others, require sponsoring organisations from the developed world to constantly review their strategies and procedures.

However, there is some common practice to pursue, and the majority of regulatory bodies of developed world countries require sponsors to provide most of (or all) the listed information below when submitting an application. To begin any kind of testing on humans, applicant/s must submit the following:

- Ethical Approval [either by an organisational Human Research Ethics Committee (HREC), Institutional Review Board (IRB) or any other Competent Authority/ Ethics Committee].
- Clinical Trial Protocol and applicable Amendments (if any).
- Clinical Trial Administrative Details and Clinical Trial Agreements.
- Introductory Statement and General Investigational Plan.
- Detailed Information on the investigational drug—chemical, pharmaceutical and biological documentation (chemical formula, method of synthesis and substantiation of its structure, quality specifications for the active compound, if any excipients, information regarding manufacture of the clinical dosage, stability data, suitability for the use proposed); pharmaco-toxicological documentation (preclinical efficacy, toxicology, pharmacokinetic and pharmacodynamic data); previous clinical experience/human experience with that particular investigational drug (if any); any severe adverse events (if applicable).

All above mentioned data may be part of the Investigator Brochure (as required by the FDA and TGA) or separate documents.

- Detailed Information on Principal Investigator and Clinical Trial Staff.
- Detailed Information on Laboratories and/or other Facilities to be used.
- Financial Disclosure (for Medical Staff) and Indemnity Documents may be required at a later stage, but not necessary for the initial application process.

Finally, according to the ICH, GCP is defined as an international, ethical and scientific quality standard for designing, conducting, recording and reporting trials

that involve the participation of human subjects. Compliance with this standard provides public assurance that the rights, safety and well-being of trial subjects are protected. It is consistent with the principles that have their origin in the Declaration of Helsinki, and that the clinical trial data are credible [6].

However, these guidelines may be overridden by national legal requirements and by the requirements of individual regulatory agencies as appropriate, to address matters relevant to local conditions or cultures.

17.6.1 Australia

The Therapeutic Goods Administration (TGA) has adopted the International Conference on Harmonisation (ICH) in principle, to replace the Guidelines for Good Clinical Research Practice (GCRP), but at the same time has recognised that some elements are, by necessity, overridden by the National Statement on Ethical Conduct in Human Research (and therefore not adopted), and that others require explanation in terms of "local regulatory requirements" (see: Notes for Guidance on Good Clinical Practice [CPMP/ICH/135/95]) [7].

The objective of the ICH GCP guideline is to provide a unified standard for the European Union (EU), Japan and the USA to facilitate the mutual acceptance of clinical data by the regulatory authorities in these jurisdictions. The ICH GCP guideline was developed within the Expert Working Group (Efficacy) of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. This guideline was developed after consideration of the codes of good clinical practice of the jurisdictions of the EU, Japan and the USA, as well as those of Australia, Canada, the Nordic countries and the World Health Organization (WHO), in consultation with the relevant government regulatory authorities, in accordance with the ICH process. Generally, it is mandatory that the ICH GCP guideline is followed when generating clinical trial data that are intended to be submitted to regulatory authorities in the developed world.

Approval and authority to conduct clinical trials, such as a first-in-man phase I clinical trial, in Australia rests ultimately with the Secretary of the Federal Department of Health. The approval for this is granted by the Therapeutic Goods Administration (TGA) following successful application process (1). Clinical trials conducted in Australia (of any phase) may be performed under two separate but interrelated programs—known as the Clinical Trials Exemption (CTX) or Clinical Trials Notification (CTN) schemes (1). The difference between CTN and CTX is the level of involvement of the TGA in reviewing data about the therapeutic good involved in the trial before the trial begins (2).

In general, the following information is required:

- Part 1: Administrative information about the sponsor and a brief overview of the application.
- Part 2: Chemical, pharmaceutical and biological documentation.

Information in this section includes the chemical formula of the investigational drug and its method of synthesis and substantiation of its structure. Quality specifications must be provided, as well as information regarding the manufacture of the clinical dosage.

- Part 3: Pharmaco-toxicological documentation.
  Preclinical efficacy, toxicology and pharmacokinetic information (particularly data showing the preclinical toxicity testing that was performed and how this relates to the clinical trial proposed). Pharmacokinetic and pharmacodynamic data must also be presented to assure the TGA of the safety of the trial.
- Part 4: Clinical documentation.
  Information detailing any previous clinical experience with the drug in question (if available).
- Part 5: Documentation of fatal or life-threatening adverse events.
  This information is not usually available for drugs in Phase I clinical trial. However, if there is previous clinical experience with the compound, any severe adverse events must be detailed
- Part 6: Summary information for human research ethics committees (HREC).
  Section designed to assist the HREC in their consideration. The TGA also reviews this information to ensure that the HREC is being supplied with relevant data to allow an informed decision-making process to occur with the following documentation generally contained in this section: Summary statement, Status of the medicine in overseas countries, Overview of Chemical, pharmaceutical and biological documentation (Summary of Part 2), Overview of Pharmaco-toxicological documentation (Summary of Part 3), Clinical documentation (Summary of Parts 4 and 5), and Usage guidelines.

17.6.2 USA

Unlike the Australian system of agency approval through the CTX and HREC approval through a CTN, all clinical trial applications in the USA are considered by the FDA. The American system does not allow an HREC to approve a clinical trial, as can occur under the Australian system. In comparison with Australian TGA requirements, the following information is required by the FDA as the USA authority:

1. A cover sheet with administrative information about the sponsor.
2. An introductory statement and general investigational plan.
   A brief overview of the objectives of the proposed study and how it fits into the broader development plan for the investigational drug.
3. Investigator brochure.
   The Investigator Brochure (IB) is a compendium of all the scientific and clinical data collected to date on the investigational drug and is used by the principal clinical investigators as their primary reference source.
4. Clinical protocol.

5. Chemistry, manufacturing and control information (CMC)
   The requirements of this section are essentially identical to those of an Australian regulatory submission. The FDA, like the TGA, recognises that limited information on the manufacturing process, formulation and stability of the investigational drug product may be available early in its development. Information required in this section includes a description of the investigational drug; where and how it is manufactured; quality control limits on its manufacture and its stability, and the manufacture of the clinical dosage form. Additional information (not required in Australian applications) is the requirement to show how the investigational drug (and any placebos) is to be labelled
6. Pharmacology and toxicology information
   The format and content of information in this section is very similar to one in the Australian application. A brief overview of the biological and pharmacological properties of the investigational drug is required in addition to summary of the toxicological information collected to date. The summary should contain: brief information about the design and execution of the individual toxicology studies, information discussing how the toxicological information collected in animals relates to and influences the design of the clinical trial and what (if any) pointers it gives to potential human toxicity
7. Previous human experience with the investigational drug.
   The application contains information regarding previous clinical experience with the investigational drug, including tabulations of any adverse reactions observed.

The application contains information regarding previous clinical experience with the investigational drug, including tabulations of any adverse reactions observed.

17.6.3 European Union

After more than 10 years of discussions about the need and content of a harmonised approach to clinical trials in Europe ("Directive 2001/20/EC on the approximation of the laws, regulations and administrative provisions of the Member States relating to the implementation of good clinical practice in the conduct of clinical trials on medicinal products for human use"), the "GCP-Directive", finally came into force on April 4, 2001. For several aspects the Directive referred to guidelines which were to be released before May 1, 2003. At this date, the Member States had to adapt and publish the laws, regulations and administrative provisions necessary to comply with the Directive which came into force on May 1, 2004.

The GCP-Directive applies to all Phase I to IV trials intended to discover or verify clinical, pharmacokinetic or dynamic effects of investigational medicinal products and/or to identify any adverse reactions. Non-commercial trials with marketed drugs are covered by the Directive as well. They only can benefit from simplified drug manufacturing requirements, if they deal with indications covered by the marketing authorisation. The Directive does not apply to non-interventional trials.

In March 2010 the European Commission issued a communication "Detailed guidance on the request to the competent authorities for authorisation of a clinical trial on a medicinal product for human use, the notification of substantial amendments and the declaration of the end of the trial" (2010/C 82/01). This document refers to the Directive 2001/20 and brings clarification of clinical trials authorisation and the type of information required in Member States as well as the "ICH Countries".

In addition, all Member States regulatory authorities' requirements apply to clinical trials conducted on their territory.

17.6.4 United Kingdom: How Has the UK System of Drug Regulation Been Affected by Its Membership of the European Union?

The main aim of the EU Directive on Clinical Trails, Directive 2001/20/EC (the Directive) was to simplify and harmonise administrative provisions governing clinical trials across the European Union. The aim was also to provide an environment for conducting clinical research that protects participants without obstructing the discovery of new essential medicines. It applied equally to all commercial and non-commercial trials. Although it was generally considered as a positive development, there were some frustrations expressed in terms of trying to keep up to date with the latest publications and the status at both national and pan-European levels. The UK regulatory framework is implemented through the Medicines and Healthcare Products Regulatory Agency (MHRA). The EU regulations that came into force on 1 May 2004 introduced new procedures for the authorisation of clinical trials (Clinical Trials Authorization, CTA) by MHRA. These new Regulations only applied to trials of medicinal products. Clinical studies involving medical devices, food supplements or other non-medicinal therapies (such as surgical interventions) were not covered by the Directive.

The new regulations have not changed the civil liabilities of the NHS, of universities, or of medical funding bodies in the UK. However, it changed the whole aspect of planned and ongoing clinical trials since it was illegal to start a clinical trial of a medicinal product even if MHRA has issued the authorisation and ethics committee has given a favourable opinion. It was necessary to have a sponsor (either recognised in the EU or with a legal representative recognised in the EU). Many people wanted one general body to take overall responsibility for publicly funded clinical research in the UK, but it was not the case. The Directive specified that it was without prejudice to the civil and criminal liability of the sponsor and of the investigator. Although the proposed Regulation did not change the civil liabilities of NHS, universities or others undertaking clinical trials under the legislation, there were certain changes to reduce the risk of unauthorised clinical research resulting in unexpected liabilities.

One of the requirements of the Directive was that all drug trials are to be conducted according to Good Clinical Practice (GCP). DDXs (Doctors and Dentists Exemptions) and other exemption schemes were not valid from 1 May 2004. If such an exemption certificate was held and the study proceeded beyond April 2004, it required application for a CTA certificate (approval) from MHRA. Approval of the institutional ethics committee was not sufficient any more. The MHRA introduced a waiver system whereby DDXs, CTXs, CTCs and CTMPs could be rolled-over to a CTA. DDXs were specific to a medicinal product whereas CTAs are specific to a trial and the Investigational Medicinal Products (IMPs) used in it.

Under the UK Regulations, investigational medicinal products now need to be supplied and packaged according to Good Manufacturing Practice (GMP), reducing the risk of product liability. The EU Directive and the new UK Regulations clarified specific legal duties of sponsors, investigators and others in clinical trials of medicines (including the national body, MHRA), based on internationally recognised principles. These brought the conduct of clinical trials in the UK much more in line with the USA, since it changed the UK two-tiered system (similar to the Australian CTX/CTN schemes) as mentioned above.

Although a significant amount of information was available, the new legislation caused concerns and it was not always easy to interpret. It was a useful reminder of the need for implementing high standards in clinical research governance—to protect individuals and ensure reliable findings. After already existing criticism on the UK regulatory framework, one may expect further changes in the UK regulatory framework. These criticisms included the fact that virtually all research on drugs and up to 70% of trials reported in major medical journals were funded by the pharmaceutical industry; biases in a way that trial results are interpreted and reported; lack of transparency that the MHRA was legally bound to abide by; lack of public profile and impact; lack of consumer input and serious adverse events in a Phase 1 Healthy Volunteer Clinical Trial conducted in the UK several years ago.

17.7 Comparison of Regional Drug Regulations and Clinical Trial Approval Process

In comparison of the Australian, British and American systems of clinical trials approval, the American system for clinical trials approval has some similarity to the Australian, but also some obvious exceptions (related to CTX/CTN schemes). Until the 1 May 2004 the UK system was basically the same as the one in Australia, with a two-tiered approach. One of the requirements of the EU Directive was that all drug trials are to be conducted according to Good Clinical Practice (GCP) and DDXs (Doctors and Dentists Exemptions) and other exemption schemes were not valid beyond 1 May 2004.

After implementation of EU Directive (Directive 2001/20/EC) in the UK, the regulatory system placed new responsibilities on the sponsor, investigator, and even

the regulatory body (such as MHRA). This has brought conduct of clinical trials more in line with the US model. In addition, it required the sponsoring organisation (sponsor) to be a recognised legal entity in the EU or to have an EU recognised legal representative. In terms of Investigational Medicinal Products (IMP), the new system requires production of IMP and the placebos in licenced facilities and in compliance with the Code of Good Manufacturing Practice (GMP). The Code requires that the manufacturer of IMPs hold a current Manufacturer's Authorisation, and a key requirement for that is that the holder of the authorisation is a Qualified Person (QP). The European Directive goes further and requires that QPs are suitably trained and registered by the appropriate regulatory agency in each EU member state. The QP is required to take considerable personal responsibility for the quality aspects of IMP.

Due to their differences in structure, functional units and scope there are significant differences between the EMA and the FDA. The differences in the review style may be summarised as follows:

- EMA is an administrative framework, and National Agencies are the scientific reviewers—differences in culture and medical practices, FDA reviewers are within the same Agency.
- EMA's organisational and review structure is top-down, while in the FDA is bottom-up.
- EMA reviewers look into overall benefit/risk projected on the entire data, while the FDA reviewers are more specific (i.e. requires adequate and well-controlled studies).
- Same data package to both authorities may not necessarily result in the same outcome.

In comparison between US/UK current requirements and the Australian two-tiered clinical trial model is presented by the CTN and CTX schemes. CTN is a notification scheme that enables all material relating to the proposed trial, including the clinical trial protocol to be submitted directly to the HREC. The HREC is responsible for assessing the scientific validity of the trial design, the safety and efficacy, the ethical acceptability of the trial process and approval of the trial protocol. CTN trials cannot commence until a notification letter is sent to the TGA. However, it can commence without any approval given by the TGA. In terms of CTX application (equivalent to IND) another difference is related to the Investigator Brochure (IB) that an IND requires with each clinical trial application, but the current TGA set up does not specifically require this. The IB is a compendium of scientific and clinical data and can be submitted with either the CTX or the CTN scheme, although is not an absolute requirement. The TGA requires the sponsor to be an Australian entity [individual (e.g. a medical practitioner), a body or organisation (i.e. a hospital, non-government organisation), or a company (i.e. a pharmaceutical company)]. In the American system, the FDA accepts foreign clinical studies (not conducted under an IND) if they comply with specific requirements (i.e. provided they are well designed, performed by qualified investigators, and conducted in accordance with ethical principles acceptable to the world community as stated

in “Declaration of Helsinki”). Generally speaking, the previous situation (prior to 1 May 2004) was quite attractive for pharmaceutical companies so they have moved a number of early phase clinical trials programs from the USA to Britain, Europe and Australia. In terms of already implemented changes in EU countries, the Commonwealth Department of Health has been carrying out a review of Australian clinical trials system. It is reasonable to expect further harmonisation and discontinuation of the existing differences.

17.8 Conclusions

Paradigms arising from drug regulatory frameworks and their inevitable harmonisation can be applied to biological therapeutics regulations. Use of a shared approach in early clinical trials might reduce time and resources for new drugs (including new cell drugs) to reach late-stage clinical trials. This may increase collaborative efforts across the globe and enhance chances of new biologic drugs reaching the market. To date, this has been a long and painful process, led by major industry players with significant funding.

Regulatory frameworks established by national agencies should provide stable, structured and reliable but not overly restrictive support. Advancing the regulatory framework’s harmonisation process will eventually increase the likelihood of biomedical discoveries for millions of neurological, cardiovascular, oncology, haematology, reconstructive surgery, paediatric oncology and other patients.

Phase I clinical trials are proven to be the most challenging step in this process due to limited funding, laborious and long manufacturing procedures and the need for a multi-disciplinary team with a unique skill set.

Manufacturing of human placenta-derived hpMSC for phase I clinical trials, expected to assess safety of product, is a complex process. Adherence to the Code of Good Manufacturing principles and Quality Management System principles has become mandatory from the regulator’s perspective and from the patient and staff safety perspective. Further trials of placenta-derived MSC are underway at our centre.

Thoroughly planned and safely conducted clinical trials in accordance with ethical principles and with adherence to rigorous regulatory requirements are needed to advance the field and provide valid clinical research data. The “open system” of manufacture is labour-intensive and to move to large multi-centre trials a large scale closed bioreactor system compliant with GMP will be required. We are currently pursuing this technology.

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Chapter 18
Mesenchymal Stem Cell Therapy for Peripheral Vascular Diseases

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Abstract Adult multipotent mesenchymal stem or stromal cells (MSCs) have been at the forefront of basic and clinical research for years and have unique biological and immunomodulatory properties making them an attractive cell source for tissue regeneration and repair. MSCs have been reported to home to and accumulate around the damaged tissue, and the primary mode of action is believed to be mediated through paracrine activity. The secretome profile of MSCs include among others various anti-inflammatory and angiogenic growth factors, suggesting that these cells are potentially useful for treating peripheral arterial diseases (PAD) like critical limb ischemia (CLI). In addition, because of their non-immunogenic nature and potent immunosuppressive properties, MSCs have the ability to survive in an allogeneic environment. Recent clinical trial data using stem cells with angiogenic properties have shown some success in the treatment of PAD. Since bone marrow-derived MSCs (BMMSCs) have been the most well-characterized population of MSC with high angiogenic potential, we have developed an investigational medicinal product (IMP) using BMMSCs from normal adult healthy volunteers and have performed clinical trials with CLI patients. This review broadly describes the advancements made during the last several years with MSCs and other types of stem cells for the treatment of PAD, with special reference to CLI.

Keywords Critical limb ischemia • Mesenchymal stem cells • Neoangiogenesis • Ankle brachial pressure index • Rest pain

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18.1 Introduction

Angiogenesis, the sprouting of new blood vessels from preexisting vasculature, is an essential feature in the growth and maintenance of tissues. It is a hallmark of wound healing, and angiogenesis also has an important physiological role in various ischemic and inflammatory diseases [1]. Angiogenesis is subject to a complex control system that involves both proangiogenic and antiangiogenic factors [1]. In adults, the process of angiogenesis is tightly controlled by "angiogenic balance." Therapeutic induction of neoangiogenesis is a potential treatment for chronic ischemia especially in peripheral arterial disease (PAD) and acute myocardial ischemia (AMI). Rapid revascularization of the injured ischemic tissue to prevent further deterioration is essential for the restoration of their biological function [2, 3].

PAD is an omnibus term encompassing diseases of the extremities due to mainly atherosclerosis, though many other conditions are also responsible. These include Thromboangitis obliterans (TAO) or Buerger's disease, various autoimmune disorders like SLE, and acute conditions like embolism [4]. It is estimated that there are eight million patients in the USA who are suffering from various types of PAD. Worldwide, the prevalence of PAD has been estimated to be in the range of 3–10% in the general population rising to about 15–20% in patients more than 70 years of age [4]. Various risk factors causally related to PAD include smoking, diabetes, hyperlipidemia, and hypertension. Critical limb ischemia (CLI) is a severe form of PAD which results from near complete occlusion of the peripheral arteries resulting in severe rest pain and non-healing ischemic skin lesions and finally gangrene of the extremity.

Diagnostic studies include measurement of hemodynamic changes by the Ankle Brachial Pressure index (ABPI), transcutaneous partial oxygen pressure ($TcPO_2$), exercise testing to elicit symptoms, segmental pressure monitoring, and Doppler examinations of the vascular system. Magnetic resonance imaging (MRI) and computed tomography (CT) angiography can also aid in diagnosis [5]. The management of CLI is based on risk factor management and surgical or endovascular revascularization. Despite claims that 90% limb salvage rate are possible with CLI patients, there is still major risk of amputation and a significant proportion of CLI patients are considered "no option" patients [6]. It has therefore become necessary to conceptualize and develop therapies that will induce revascularization and remodel the vascular system to prevent the complications of CLI.

18.2 Angiogenesis by Stem Cells

Cell-based "therapeutic angiogenesis" started with several new studies on neovascularization. Until the end of the 1990s, the differentiation of mesodermal cells into angioblasts and their subsequent endothelial differentiation were

believed to occur exclusively during embryonic development [7]. However, in a seminal paper, Asahara et al. demonstrated the role of peripheral blood-derived mononuclear cells (PBMNC) to differentiate into endothelial lineages [8], it changed the dogma on angiogenesis. Later, Kamihata et al. also demonstrated that bone marrow-derived mononuclear cells enhance angiogenesis into ischemic sites via harmonic supply of endothelial progenitor cells and angiogenic factors [9, 10]. Recently, mesenchymal stem cells (MSCs) due to their ease of isolation and large-scale culture expansion in vitro, along with their multipotential properties, have made these cells an attractive candidate for cell therapy. MSCs are reported to have been isolated from various adult tissues like bone marrow, cord blood and cord tissue, adipose, placenta, dental pulp, etc. [11, 12]. Among all the tissue sources, bone marrow-derived MSCs (BMMSCs) are most well characterized and established in various laboratories, and remains the principal source of MSCs for majority of preclinical and clinical studies [13–15]. BMMSCs are extensively used for treating patients with ischemic heart disease and postmyocardial infarction [16].

18.3 Role of Mesenchymal Stem Cells in Angiogenesis

The mechanism through which MSCs exert angiogenesis is mainly by secreting angiogenic cytokines and also through differentiation into endothelial cells. The angiogenic support provided by MSCs can be considered one more supportive effect, since the re-establishment of blood supply is fundamental for recovery of damaged tissues. The pro-angiogenic effect of MSCs has been demonstrated in several studies both in vitro and in vivo [16, 17]. MSCs have been shown to express and secrete stromal cell-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF); matrix metalloproteinases (MMPs), all of which are important for triggering and maintaining angiogenesis. VEGF has been identified as a key component in the development of blood vessels, and in conjunction with SDF1, VEGF-induced endothelial cell survival, vascular branching, and pericyte recruitment [18].

Some of the MSCs were found to express endothelial markers including CD31 and von Willebrand Factor (VWF), while others expressed smooth muscle markers including α-smooth muscle Actin and Desmin [19, 20]. Taken together these data suggest that MSCs may function as vascular progenitors. However, apart from their angiogenic activity, MSCs obtained from bone marrow and other tissues have also been shown to mediate anti-inflammatory, anti-apoptotic, anti-fibrotic, mitogenic, and wound healing properties [21]. The complex interplay of some of these biological mediators secreted by MSCs has been shown to be important in regulating regeneration of a variety of damaged or diseased organs of the body although complete clarity with respect to their tissue-specific function still requires extensive investigations [22, 23].

18.4 Immunomodulatory Properties of MSCs

One of the key characteristics of MSCs, regardless of the organs from which they are isolated, is that these cells are generally hypoimmunogenic and possess immunosuppressive activity. As a result, use of MSCs for allogeneic therapy does not require HLA matching [24]. Although allogeneic cell therapy often calls for using traditional immunosuppressive medications, this may not be the case for MSCs transplantation. The basis of their hypo- or non-immunogenic nature is that MSCs express low to intermediate levels of HLA class I antigens and negative for cell surface expression of HLA class II and other co-stimulatory molecules like CD40, CD80, and CD86 required for allospecific T cell stimulation [24]. Upon treatment with interferon (IFN)-γ, BMMSCs express HLA class II antigens on the surface; however, this expression was not found to alter the immunomodulatory activity of these cells [25, 26]. Since MSC's role in immune modulation is well documented through soluble factors and direct physical contact, affecting the innate and adaptive immune system, these cells would be the better candidates for treating vascular diseases, inflammatory and autoimmune disorders. It should be reiterated that these immunomodulatory properties may help the transplanted BMMSCs to stay in vivo for a period of time during which angiogenic factors secreted by these cells are able to initiate neoangiogenesis in allogeneic CLI patients.

18.5 Preclinical Studies

18.5.1 Safety Studies

Stempeutics Research has conducted preclinical safety studies using BMMSCs. The tests included acute toxicity studies (14-day single dose and repeat dose) by intravenous (IV) and intramuscular (IM) administration of BMMSCs, sub-chronic toxicity studies (90 days single dose) in two animal species (rats and rabbits) by two routes (IV and IM) of administration and by genotoxicity studies. The toxicity studies were conducted using human equivalent doses in respective animals to a maximum dose of 20×10^6 BMMSCs/kg body weight. In addition, tumorigenicity and teratogenicity studies were also conducted using BMMSCs. Preclinical results have shown that these cells are safe to administer, non-tumorigenic, non-teratogenic, and did not induce genotoxicity.

18.5.2 Preclinical Efficacy Model of CLI

Numerous animal models have proved that transplantation of MSCs or mononuclear cells (MNCs) from different sources augmented arteriogenesis in the ischemic

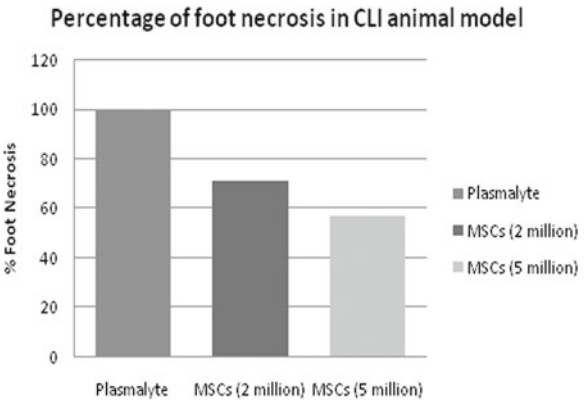

Fig. 18.1 Percentage of foot necrosis at 28 days after femoral vessel ligation with either PlasmaLyte (control group) or MSCs. 42.87% reduction in food necrosis ($p = 0.03321$) was observed in 5×10^6 MSCs dose group as compared to control group

limb of the animal. Kim et al. transplanted human umbilical cord blood (UCB)-derived MSCs in nude mice with hind limb ischemia by femoral artery ligation [27]. Immediately after the resection of one femoral artery, 1.3×10^6 culture expanded MSCs were injected (IM) into the ischemic position of the hind limb. Up to 60% of the hind limbs were salvaged in the femoral artery-ligated animals [27]. To confirm the homing and incorporation of administered MSCs to the site of the vessels, in situ hybridization for human-specific *Alu* sequence was performed. MSCs were detected in the arterial walls of the ischemic hind limb in the treated group. Iwase et al. compared the angiogenic potency between MSCs and MNCs in a rat model of hind limb ischemia [28]. Three weeks after cell transplantation, the laser Doppler perfusion index was found to be highest in animals that received MSCs, followed by the animals in the MNC group while the score was minimal in the control animals. Similarly, capillary density was also observed to be highest in the MSC animal group. It was concluded that MSC transplantation caused significantly greater improvement in hind limb ischemia than MNC transplantation.

Stempeutics Research developed a CLI animal model in BALB/c nude mice by femoral artery and vein ligation. Human BMMSCs were administered in two different doses (2×10^6 and 5×10^6), immediately after the ligation. In the control group, all animals developed foot necrosis whereas 2×10^6 and 5×10^6 MSC-treated animals showed 28.57% and 42.87% protection, respectively (Fig. 18.1). These findings suggest that allogeneic human BMMSCs have the therapeutic potential for treating CLI patients.

18.5.3 Clinical Trials in CLI

On searching clinicaltrials.gov using the keywords stem cells and CLI, we found 33 completed or ongoing clinical trials. Twenty-five of them were using mononuclear stem cells and eight were using mesenchymal stem cells.

TACT (Therapeutic Angiogenesis using Cell Transplantation) was the first large report on the use of bone marrow-derived mononuclear cells (BMMNCs) in the treatment of CLI [29]. In this pilot study, they investigated efficacy and safety of autologous implantation of BMMNCs in patients with ischemic limbs. It was concluded that autologous transplantation of BMMNCs is safe and effective for achievement of therapeutic angiogenesis, because of the natural ability of marrow cells to supply endothelial progenitor cells and to secrete various angiogenic factors or cytokines.

This was the beginning of the use of stem cells/BM stem cell therapy in peripheral ischemia in a number of studies across the globe. The studies published in literature in the last 5 years are given in Table 18.1. In these studies the degree of ischemia varied between Rutherford grade 2 and severe CLI (Rutherford grade 6). Some studies are hampered by small numbers of subjects, lack of control groups, and by differing outcome parameters. Despite these limitations, the majority of the trials showed positive outcome on perfusion parameters (ABPI, $TcPO_2$) and clinical outcome (ulcer healing, pain-free walking distance, rest pain, and amputation free survival). Collectively, these clinical trial data suggest that the MNCs derived from bone marrow contain one or more cell types that are able to induce angiogenesis in CLI patients.

Many clinical trials are using autologous BMMNCs for seeing the efficacy in PAD. However, there are significant limitations to the development of BMMNCs as a therapy for patients with CLI due to numerous reasons. First, the active cellular constituent of bone marrow that is the agent of repair is not well characterized. Second, it is widely accepted that therapeutically active bone marrow constituents likely represent only 1 in 10,000 bone marrow cells [42]. Third, aspirating bone marrow is an invasive process, and lastly, concerns exist that patients most likely to be affected by atherosclerosis are also likely to have impaired marrow function [43]. The use of allogeneic BM-MSCs has important advantages. They likely represent an enriched population of cells with therapeutic capacity. They are readily prepared from healthy donors and may be used as an allogeneic, "off-the-shelf" cryopreserved product [44]. They are easy to administer, as evidenced by the intramuscular approach used in many studies. The details of trials involving mesenchymal stem cells are given in Table 18.2.

18.5.4 Stempeutics Research Experience in Critical Limb Ischemia Clinical Trials

18.5.4.1 In India

A multicentric study was conducted by Stempeutics Research, Bangalore, using allogeneic BMMSCs in patients with CLI (unpublished data). This clinical trial was a 6-month long, prospective, double-blinded, randomized, placebo-controlled, single dose study. The protocol was approved by Drug Controller General of India

Table 18.1 Clinical trials conducted in CLI patients using stem cells

S. no.	Authors	# Patients	ABPI	$TcPO_2$	Pain	Amp	+/–
1	Miyamota et al. [30]	8, TAO, CLI	–	–	D	–	+
2	Kajiguchi et al. [31]	7, CLI, TAO	+/–	I	D	?	+/–
3	Huang et al. [32]	74, PAD, DM	I	I	D	–	+
4	Hermandez et al. [33]	12, PAD, DM	I	I	D	D	+
5	Gu et al. [34]	16, PAD/CLI	I	I	D	D	+
6	Chochola et al. [35]	28, CLI, PAD	I	I	D	D	+
7	Wester et al. [36]	8, CLI	–	–	D	D	+
8	Van Tongeren et al. [37]	27, PAD	I	I	D	?	+
9	De Vriese et al. [38]	16, PAD	+/–	I	D	?	+/–
10	Motukuru et al. [39]	38, TAO	I	I	D	D	+
11	Amann et al. [40]	51, CLI	I	I	D	D	+
12	Prohazka et al. [41]	37, CLI, DM	I	I	D	–	+
13	Kawamoto et al. [21]	17, PAD, TAO	I	I	D	–	+
14	Lu et al. [22]	41, CLI, DM	I	I	D	–	+
15	Walter et al. [24]	40, CLI	–	I	D	–	+
16	Idei et al. [25]	51, PAD, TAO	I	I	D	D	+

PAD peripheral artery disease, *CLI* critical limb ischemia, *TAO* thromboangitis obliterans, *ABPI* ankle brachial pressure index, *$TcPO_2$* transcutaneous partial oxygen pressure, *Amp* amputation, *I* increased, *D* decreased. Overall result positive (+) or negative (–) or equivocal (+/–)

(Indian Food and Drug Administration) and by the Institutional Ethical Committees (IEC) of the four participating hospitals in India. The study was registered in the NIH website (NCT 00883870). It included patients with established CLI as per Rutherford classification in category II-4, III-5, or III-6; who had Infra-inguinal arterial occlusive disease and were not eligible for or had failed traditional revascularization treatment (no-option patients) and had ABPI ≤ 0.6 or ankle pressure ≤ 70 mm Hg or $TcPO_2$ ≤ 60 mmHg in the foot. Patients were administered multiple intramuscular injection (40–60 injections per patient based on body weight) of BMMSCs at a dose of two million cells per kilogram or placebo (PlasmaLyte A). A total of 20 patients were recruited in the trial with 10 patients each in the cell and placebo arm.

The results showed that physical examination, vital signs, hematological and biochemical parameters did not vary from baseline between the two groups of patients during the 6-month follow-up. An improvement in the rest pain scores was observed in both the treatment arms ($p = 0.1099$) (Fig. 18.2a). An increase in mean ABPI was observed from baseline to the end of 6-month follow-up (visit 6) in the cell arm group, which was statistically significant compared to the placebo ($p = 0.0018$) (Fig. 18.2b). Decrease in the numbers of ulcers was observed in the treatment arm (11 ulcers became 2) as compared to the placebo arm (7 ulcers became 2) after 6-month follow-up. However, there was no difference in the incidence of amputations between the cell and placebo arms.

Table 18.2 Summary of clinical trials using mesenchymal stem cells in CLI patients

S. no.	Conditions	Interventions	Sponsor/ Collaborators	Ph	*N*	Duration of follow-up	Outcome measures	PMID/ Clinicaltrials.gov identifier
1	Diabetic critical limb ischemia and foot ulcer	Autologous BMMSC vs. BMMNC	Department of Endocrinology and Metabolism, Southwest Hospital, Third Military Medical University, Chongqing, China	P1	41	24 weeks	Ulcer healing rate of the BMMSC group was significantly higher than that of BMMNCs at 6 weeks Improvements painless walking time, ankle-brachial index (ABI), transcutaneous oxygen pressure (TcO_2), and magnetic resonance angiography (MRA) analysis induced by the BMMSCs transplantation were more significant than those by BMMNCs	21216483
2	Chronic non-healing ulcers (diabetic foot ulcers and Buerger disease)	Autologous BMMSC	Department of Biochemistry, S.C.B Medical College, Cuttack, Orissa, India	NA	24	12 weeks	The BMMSC group had significant improvement in pain-free walking distance and reduction in ulcer size as compared to those in the control group	19929258

3	Critical limb ischemia	Autologous adipose-derived mesenchymal stem cells 0.5, 1, 2 million cells/kg	Fundacion Progreso y Salud, Spain	P1|P2	36	12 months	Neovasculogenesis, major adverse event, ankle Brachial Index, University of Texas Classification at target limb	NCT01257776
4	Buerger's disease	Allogeneic mesenchymal stem cells	Stempeutics Research Pvt Ltd, Bangalore, India	P2	126	2 years	Relief of the rest pain, healing of ulcerations or reduction of ulcer area, pain-free walking distance, major amputation-free survival, ankle brachial pressure index (ABPI), increase in transcutaneous partial oxygen pressure ($TcPO_2$), quality of life by King's College VascuQOL questionnaire, angiogenesis, adverse events	NCT01484574
5	Critical limb ischemia	Arm 1: BMMSC+BMMNC Arm2: BMMNC alone	National University of Malaysia Cytopeutics Pte. Ltd	P2	50	12 months	Change in angiogenesis, change in blood supply, change in ulcer size, visual analog score, exercise treadmill test	NCT01456819

(continued)

Table 18.2 (continued)

S. no.	Conditions	Interventions	Sponsor/ Collaborators	Ph	*N*	Duration of follow-up	Outcome measures	PMID/ Clinicaltrials. gov identifier
6	Critical limb ischemia	Arm1: BMMSC Arm 2: Placebo	Stempeutics Research Pvt Ltd, Bangalore, India	P1|P2	20	2 years	AE and symptomatic relief, increase in transcutaneous partial oxygen pressure ($TcPO_2$) and Ankle brachial pressure index (ABPI)—measured by Doppler	NCT00883870
7	Diabetic patients with chronic critical limb ischemia	Autologous mesenchymal stem cells from adipose tissue. 0.5, 1, 2 million cells/kg	Fundacion Progreso y Salud, Spain	P1|P2	36	2 years	Safety assessment, angiography changes, magnetic resonance angiography changes. clinical outcome	NCT01079403
8	Buerger's disease	Autologous adipose tissue-derived MSCs transplantation	RNL Bio Company Ltd, Korea	P1|P2	18	24 weeks	Treadmill walking distance, VAS (Visual Analog Scale), toe-brachial pressure index (TBPI), transcutaneous oxygen pressure, ($TcPO_2$), arterial brachial pressure index (ABPI), pain-free walking distance (PFWD), angiography, Laser Doppler, use of a analgesic medicine, safety evaluation	NCT01302015

9	Critical limb ischemia	Arm1: PTA + Infusion of MSC_Apceth Arm2: PTA	Apceth GmbH & Co. KG, Germany	P1|P2	30	1 year	Collection of adverse events, Safety laboratory values, ECG findings, analysis of inflammation markers, comparison of course of hemodynamic and vascular processes	NCT01351610
10	Peripheral vascular diseases	Endometrial regenerative cells 25 million, 50 million, or 100 million ERC by intramuscular injection	Medistem Inc., USA	P1|P2	15	52 weeks	Adverse and serious events, improvements posttreatment in rest pain (VAS), toe pressure and ABI, transcutaneous oximetry and ulcer status (with picture)	NCT01558908

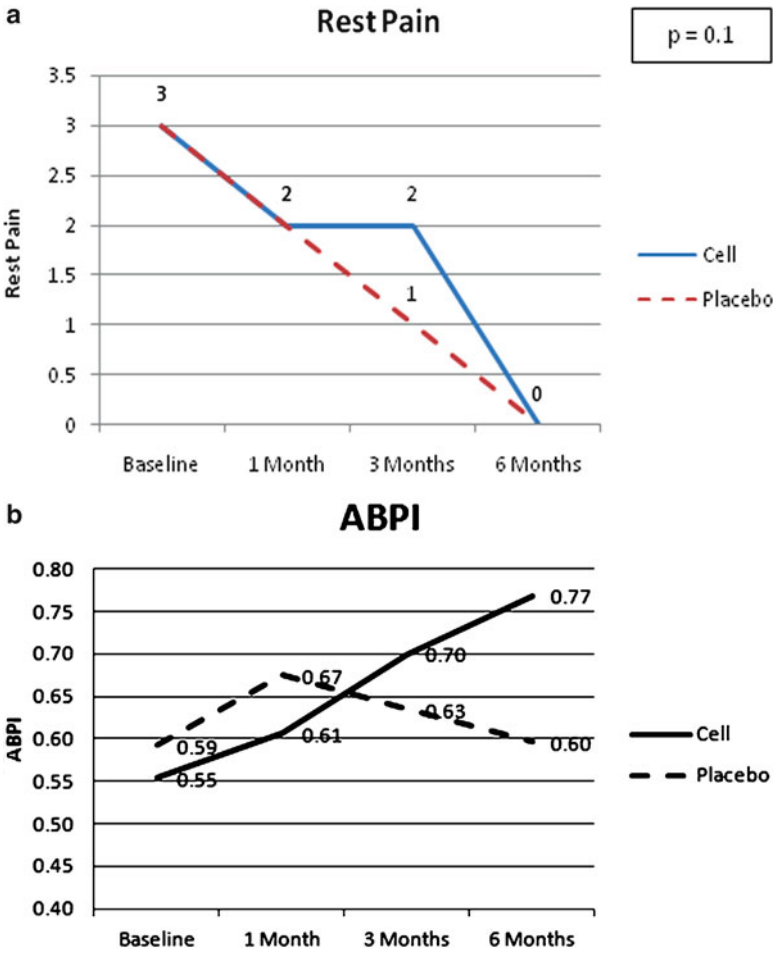

Fig. 18.2 Efficacy data in CLI patients following IM injection of allogeneic BMMSCs. (**a**) Changes in rest pain in patients with CLI at baseline (screening visit), 1-month, 3-month, and 6-month follow-up period for both cell and placebo arms are shown. Rest pain score decreased in both the arms from score of 3 to 0 at the time of 6 months follow-up ($p = 0.1$). (**b**) Changes in Ankle Brachial Pressure Index (ABPI) in patients with CLI at baseline (screening visit), 1-month, 3-month, and 6-month follow-up in cell arm and placebo arm are shown. ABPI increased from 0.55 to 0.77 in the cell arm at the time of 6-month follow-up ($p = 0.0018$)

Total adverse events (AEs) in the cell arm were 12 (22.64%) as compared to 41 (77.35%) in placebo arm. Five patients experienced nine serious adverse events (SAEs) during the course of the study (5 in cell arm and 4 in placebo arm). Seven SAEs were recorded because of hospitalization for disease process-related complications or for conducting amputations. Two SAEs were due to death. The data of the

fatal SAEs were reviewed by the Independent Data Safety Monitoring Board (DSMB), and it was opined that the events were unlikely to be caused by BMMSCs, placebo, or the procedure, and it was concluded that deaths occurred due to the underlying disease process.

Data generated from this clinical trial suggest that bone marrow-derived ex vivo cultured adult allogeneic MSCs are safe when injected IM at a dose of two million cells per kilogram body weight. Few efficacy parameters like ABPI, ankle pressure, and decrease in number of ulcers showed positive trend; however, there is a need to further evaluate the efficacy of allogeneic MSCs in a larger number of CLI patients in a phase II study.

18.5.4.2 In Malaysia

A single center investigator led study was conducted in Malaysia using a similar protocol except that the cells were delivered by intra-arterial injection. This study was approved by the University of Malaya Ethics Committee and recruited eight patients. As two of them had bilateral disease, a total of ten limbs were evaluated.

Seven limbs were classified as Rutherford Classification III-5/6 and three as III-4. Two patients died during the course of the study (due to myocardial events) and one patient had his study limb amputated, thus leaving six limbs to be evaluated at the 6-month time point of the study. The patients were evaluated for relief of rest pain, ulcer healing, and freedom from major amputation. The ABPI was performed at predetermined time points. Five of the six limbs had relief from pain and all had substantial ulcer healing. The rest pain improved significantly at the time of 6-month follow-up compared to the baseline values ($p<0.01$) (Fig. 18.3a). ABPI was also significantly increased at both 3 ($p<0.05$) and 6 months ($p<0.01$) follow-up after BMMSCs administration (Fig. 18.3b). The amputation rate was 14% (1/6) which is substantially better than historical controls. There were no adverse events related to the procedure or to the MSC injection.

This study has shown that intra-arterial injection of allogeneic MSCs is safe and well tolerated when injected intra-arterially in patients with CLI and some efficacy parameters showed significant improvement. Further studies are planned to conclusively prove the efficacy of mesenchymal stem cells in CLI by intra-arterial route of injection.

18.6 Future Perspectives

Bone marrow-derived MSCs, due to their potential for restoring organ function by engraftment and paracrine activity, and for their immunomodulatory properties, have become useful to treat vascular diseases. In addition, MSCs produce endothelial and epithelial growth factors that might promote tissue repair by angiogenesis. In spite of all these properties, there are many questions that still remained unresolved.

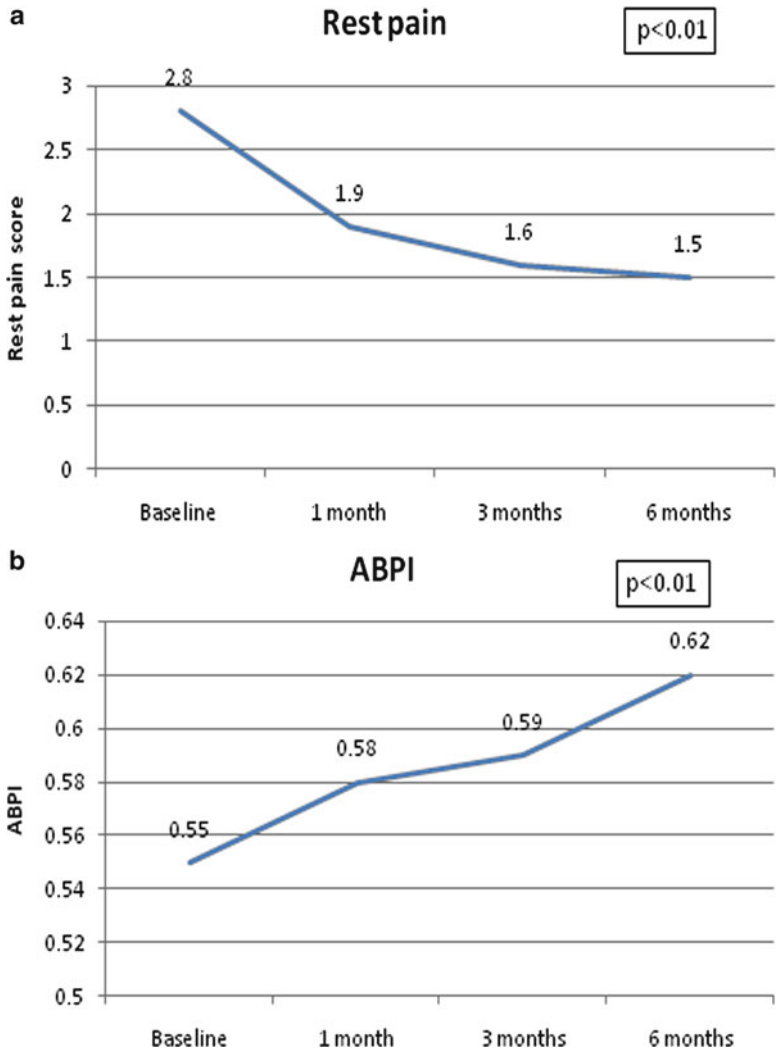

Fig. 18.3 Efficacy data in CLI patients following intra-arterial injection of allogeneic BMMSCs. (**a**) Changes in rest pain in patients with CLI at baseline (screening visit), 1-month, 3-month, and 6-month follow-up after intra-arterial administration of BMMSCs. Rest pain score decreased from 2.8 to 1.5 after 6-month follow-up ($p < 0.01$). (**b**) Changes in Ankle Brachial Pressure Index (ABPI) in patients with CLI at baseline (screening visit), 1-month, 3-month, and 6-month follow-up after intra-arterial administration of BMMSCs. ABPI increased from 0.55 to 0.62 after 6-month follow-up ($p < 0.01$)

First one is the safety of the stem cell therapy; it is essential to be sure that these novel methods of therapy are safe for the patient. Because of their capacity to release angiogenic growth factors, such as VEGF and bFGF, there was a concern that MSCs might favor the development or growth of tumors in patients by stimulating

angiogenesis. There has also been some apprehension that MSCs have the potential to give rise to neoplasia. Fortunately, both of these concerns have been unfounded in clinical trials conducted so far. Next important question which needs to be addressed is the type of cells for the treatment of vascular diseases. Presently BMMNCs, PBMNCs, MSCs, and several other cell types were used for this purpose although no thorough comparative studies have been conducted. Clinical trials comparing different types of cells are required to answer this question. Other points need to be addressed are optimal route of delivery and dosage of cells required for the treatment, and whether single or multiple doses are necessary for successful revascularization. Intramuscular or intra-arterial or a combination of both are the commonest route of injection used for the treatment of human PAD. Since many of the CLI patients at the time of treatment, present with ulcer, another option of cell administration could be to inject these cells around the ulcer. Clearly, all of these unresolved issues cannot be addressed in a single study. Carefully planned clinical trials using BMMSCs obtained from patients (autologous) and from normal healthy volunteers (allogeneic) may shed valuable insight into the curative properties of these cells in promoting angiogenesis in CLI patients. Undoubtedly, a great deal of progress is required at both basic and clinical research fronts before these cells can be routinely used in the clinic for treating various forms of PAD.

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Chapter 19
Mesenchymal Stromal Cells in the Clinic: What Do the Clinical Trials Say?

Vivek Tanavde and Mohan C. Vemuri

Abstract In the last 5 years, a large number of trials have been undertaken for the clinical application of cell-based therapy with human mesenchymal stromal cells (MSCs) for a variety of human autoimmune and degenerative diseases. In preclinical and clinical studies with ischemic injury, diabetes, wound healing, graft versus host disease, MSCs are emerging as promising candidates with therapeutic potential. MSC features such as homing efficiency to injured site, ability to produce several trophic factors in critical quantities needed for repair, immunomodulatory features to facilitate engraftment are expected to be the underlying mechanisms for therapeutic benefit in these disease states. Although early results are promising, much work is required as cellular therapies need careful isolation of cells, expansion, characterization, and proper delivery of injectable transplant ready cells that need to be prepared in good manufacturing practice (GMP) conditions to meet the safety and specification, reproducibility with no or minimal lot-to-lot variation, and efficacy following transplantation in to disease subjects. There have been 230 clinical trials as of April 2012 with MSCs that have been registered with Food and Drug Administration (FDA) site in various stages of investigation with autologus as well as allogenic sources of bone marrow-derived cells. This review summarizes the outcome of the completed trials and lays foundation for the expected outcome of the ongoing trials.

Keywords MSC • Clinical trials • Preclinical trials • Cardiac • Stroke • Spinal cord injury • Bone and cartilage • Diabetes • Critical limb ischemia • GVHD

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19.1 Introduction

Mesenchymal stromal cells (MSCs) are multipotent cells, found in a variety of tissues like bone marrow, adipose tissue, dental pulp, skin, etc. These cells can differentiate into tissues of mesodermal origin like bone, cartilage, fat, skeletal muscle, etc. In addition MSCs have also shown to be able to differentiate to tissues of ectodermal origin like neurons. This ability of MSCs to differentiate into a variety of tissues makes them attractive therapeutic agents to treat a variety of disorders. This review will focus on summarizing the ongoing and recently completed clinical trials using MSCs. This review does not attempt to summarize the status of MSCs in the clinic, as excellent reviews on that topic [1, 2] are recently published.

MSCs are easy to culture and unlike hematopoietic stem cells, can be expanded in culture without differentiation. These cells can also be grown in defined xeno-free media for therapeutic use. The cultured cells can be characterized by the use of surface markers such as those designated by the International Society for Cellular Therapy (ISCT) [3]. Although many clinical trials use undifferentiated MSCs, these cells can also be differentiated into specific lineages in vitro. Further these cells can also be grown on numerous scaffolds which could be used for tissue engineering.

MSCs are already being used in a number of clinical trials for treating variety of disorders. A search on the NIH clinical trials.gov database yielded over 230 trials utilizing MSCs for treating a variety of disorders (Fig. 19.1). Of these 40 trials were completed while an equal number had stopped recruiting, but were still active. Three trials were suspended, or withdrawn and one trial for heart failure was terminated. The remaining trials were still actively recruiting, highlighting the enormous interest in this area. Thirty-seven trials were phase I trials, 90 were intermediate between phase I and phase II, 53 were phase II and 11 were phase III studies (Fig. 19.2).

In these trials, patient enrolment numbers varied from 8 to 10 patients for small studies to 290 patients in a phase II study of osteoporotic fractures. Majority of these trials were interventional while only 9 trials were observational. Forty-five of these trials were funded purely by industry, 15 by a combination of industry and academic research institutions while the remaining were investigator lead. This shows that majority of trials involving MSCs are being led by investigators and very few are being lead by companies which desire to launch a product. One hundred and three of these trials were focused on or involved children. This highlights the importance of MSCs in early intervention of childhood disorders that can be manifested at a later age.

These trials involved the use of MSCs in a variety of disorders. Liver failure was the largest indication (19 studies), followed by graft versus host disease (14 studies). Osteoarthritis, critical limb ischemia, Crohn's disease, type 1 and type 2 diabetes were the other disorders involving the use of MSCs for clinical trials.

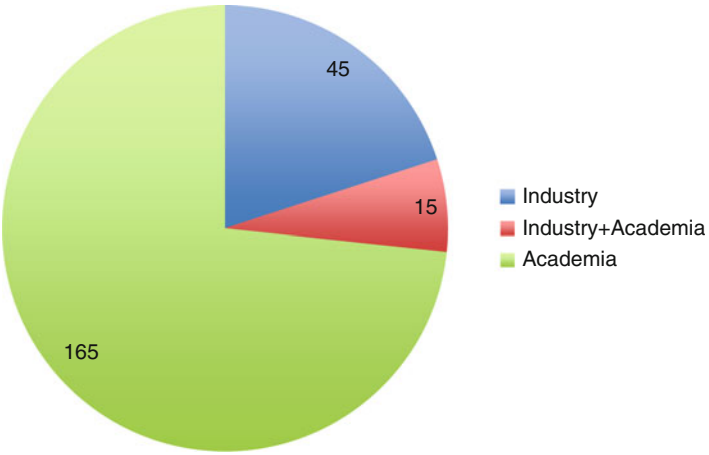

Fig. 19.1 Distribution of MSC trials funded by Academia and Industry. The pie chart indicates proportion of MSC trials funded by Industry vs. Academia. The figure shows that while majority of the trials are Academia funded, a significant proportion of clinical trials are funded by Industry. This highlights the commercial interest in the use of MSC as therapeutics. The *numbers* indicate number of trials

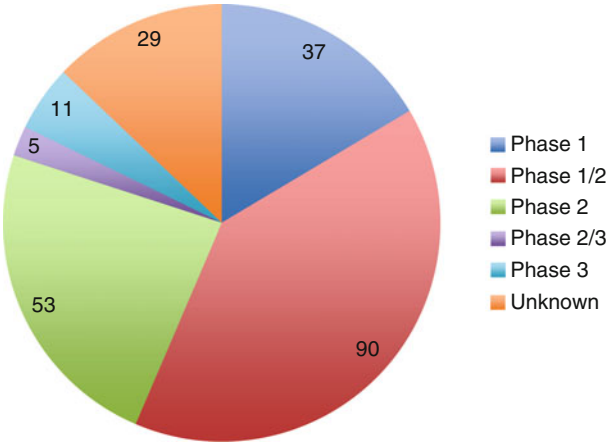

Fig. 19.2 Distribution of MSC trials dependent on their phase of study. The figure shows that majority of the trials are in phase I or intermediate between phase I and phase II. Very few trials have reached phase III. This is reflective of the nascent nature of this field since most trials are early trials to determine primarily safety and in some cases efficacy of transplanted MSCs in treating these disorders. The *numbers* on the pie chart indicate number of trials

19.2 MSCs for Cardiac Therapy

There was tremendous excitement in the field for use of MSCs to improve cardiac function. The initial trials like the SCOPE study were predominantly safety studies assessing the safety of bone marrow collection or MSC administration in patients with acute myocardial infarction or congestive heart failure. After the initial euphoria of using MSCs in acute myocardial infarction as well as congestive heart failure, it was clear that transplanting these cells had limited beneficial effect. Recent evidence indicates that MSCs do not form cardiomyocytes, but may have indirect beneficial effect on cardiac function [4, 5]. Although initial small studies reported benefits of MSC transplantation, these beneficial effects were not observed in larger controlled randomized trials. The clinicaltrials.gov database lists 11 current or completed trials involving the use of MSCs in treating cardiac disorders. Of these 3 trials were carried out for myocardial infarction, whereas one trial at the Rigshospitalet in Denmark is exploring the use of MSCs for treating congestive heart failure. All these trials involve the use of bone marrow MSCs. One trial in Mexico is using autologous adipose-derived stem cells to treat heart failure. Only one trial by US-based Capricor will be using allogenic trials for acute myocardial infarction. A recently completed STEMI trial in India also used allogenic MSCs for myocardial infarction (CTRI/2009/091/000176).

Many of the beneficial effects of MSCs can be attributed to indirect effects. Transplanted MSCs can contribute to neoangiogenesis in cardiac tissue. Exosomes secreted by MSCs have also been shown to improve cardiac function [6]. Lu et al. reported that macrophages in damaged myocardium phagocytose transplanted MSCs and secrete factors that stimulate stunned myocardium [7].

Thus it is possible that the beneficial effects of MSCs in treating cardiac disorders do not actually result from direct transplantation of MSCs. Therefore it may be possible to create MSC-based therapeutics for treating cardiac disorders. This may obviate the need for direct transplantation of MSCs since MSC-derived products like cytokines, exosomes or cellular fragments may be sufficient for these treatments. Thus the most important message from all these clinical trials is the possibility that the endogenous stem and progenitor cells have the potential to participate in repairing and restoring the diseased cardiac function. These insights although optimistic underline the critical need for better designed clinical trials with clear end point read outs and better prediction of efficacy of cell therapy.

19.3 MSCs in Neurological Disorders

Although MSCs can differentiate into neurons with low efficiency, this has not stopped the investigational use of MSCs for treating neurological disorders. The clinicaltrials.gov database lists 19 trials utilizing MSCs to treat neurological disorders. These trials cover a range of diseases from ALS to Alzheimer's disease to stroke.

The majority of these trials are in spinal cord injury and use autologous MSCs for treating these disorders. Also, most of the trials are in phase 1/2 mainly assessing the safety of these cells to treat neuronal disorders.

19.3.1 MSCs in Stroke

The clinical trials database lists 3 trials using MSCs for stroke. The trial by Stemedica Technolgies in San Diego uses autologous MSCs for treating ischemic stroke and is currently ongoing. A trial by Stempeutics Research Malaysia (NCT01461720) will use cultured allogenic MSCs in stroke patients and will start recruiting patients soon. A trial in China (NCT01461720) is utilizing allogenic MSCs in the treatment for stroke. A larger proportion of trials in stroke use allogenic MSCs compared to MSCs in other disorders. This might be due to the immune privileged environment in the brain.

19.3.2 MSCs in Spinal Cord Injury

There is lot of preclinical and limited clinical data showing the beneficial effects of MSCs in treating spinal cord injury [8–11]. Therefore most clinical trials involving use of MSCs are in the area of spinal cord injury. Of the 6 trials listed for spinal cord injury, 4 trials are using bone marrow-derived MSCs for treating spinal cord injuries, the trial from RNL Bio Korea uses adipose tissue MSCs while a phase II trial from China uses allogenic umbilical cord-derived MSCs.

19.4 MSC Trials in Bone and Cartilage Disorders

Since MSCs can differentiate into bone and cartilage, these disorders were one of the earliest targeted for MSC therapy. The NIH clinical trials registry lists 22 trials using MSCs for treating bone and cartilage disorders. Of these, most of the trials are for treating osteoarthritis of knee joints or hip. Bone marrow MSCs are also being investigated to treat osteogenesis imperfecta. Two studies are also using autologous MSCs for treating nonunion fractures that are difficult to heal otherwise. A trial at St. Judes Hospital is using allogenic MSCs to treat osteodysplasia. The use of Cartistem, a umbilical cord-derived MSC product [12] by Medipost of South Korea is being evaluated for the treatment of microfractures. MSCs were used as vehicles for treatment [13] or compounds regulating MSC differentiation like oxytocin [14] have been proposed for treating osteoarthritis. Stempeutics in India and Cytopeutics in Malaysia are investigating the use of

MSC transplantation in treating osteoporosis. MSCs have also been used in treating cartilage disorders particularly in injured cartilage or degenerative joint disease [15, 16].

19.4.1 Use of Scaffolds for Tissue Engineering of MSCs

Many of these trials involved growing MSCs on scaffolds that mimic the mechanical properties of the tissue being targeted. For bone this usually involves an inert hard surface that supports the growth of MSCs [17–20]. For cartilage materials like collagen [21], chitosan [22] or extracellular matrix-derived scaffolds [19] have been used to seed MSCs. A good summary of different scaffold materials used in tissue engineering can be found in reviews by Boo, Warren and Gigante [20, 21, 23].

19.5 MSCs as Immunosuppresants in Graft-Versus-Host Disease

Since MSCs do not express MHC Class II molecules on their surface and act as immunosuppresants, these have been used in the treatment of graft-versus-host disease (GVHD). The clinical trials database lists 15 trials involving the use of MSCs in treating GVHD. The drug Prochymal is an MSC product developed by Osiris Therapeutics for treating GVHD [24]. Its role has been investigated in treating GVHD especially in patients where the condition is steroid refractory. Apart from the Osiris studies, trials investigating the use of MSCs for treating GVHD are being carried out in Spain, China, Korea, India, and Belgium.

Apart from GVHD, MSCs are also being investigated in the treatment of other autoimmune disorders like lupus and Crohn's disease. Nine trials have investigated the use of MSCs in Crohn's disease, an immune linked disorder of the gastrointestinal system. Most of these trials are phase II/III indicating the promising result from phase I studies. Companies like Cytomed and Beike are also investigating the use of MSCs in treating lupus in phase II trials.

19.6 MSCs in Cancer Therapy

MSCs have been used in the management and treatment of cancers in two ways. The first is use of MSCs for increasing engraftment in HSC transplants in hematological malignancies. In addition to reducing GVHD [25, 26], MSCs also aid in the engraftment

of donor cells [27]. Co-transplantation of MSCs as facilitating cells also preserves the desirable graft v/s tumor effect [28].

19.7 MSCs for Treating Liver Disease

MSCs are being investigated extensively in treating liver disorders. The NIH database lists 18 trials investigating the use of MSCs in treating liver disorders. Most of these are liver failure due to cirrhosis or in some cases fibrosis. MSCs are used to augment the regenerative process in the liver or to directly replenish hepatocytes. Since MSCs can differentiate into hepatocytes in a number of preclinical animal models [29–32], the current trials investigate the safety and efficacy of this treatment in humans. Readers are recommended to the detailed reviews by Christ and Gilgenkranttz that summarize the impact of MSCs in hepatic disorders [33, 34].

19.8 Critical Limb Ischemia and Buerger's Disease

MSCs have been shown to play an important role in wound healing and therefore have been used in treating lower limb ischemias, foot ulcers as well in Buerger's disease [35]. The NIH clinical trials database lists six studies investigating the use of MSCs in treating critical limb ischemia or Buerger's disease. This is especially pertinent to countries like India, where it is common for people with these disorders to walk barefoot. Thus two such trials are being carried out by Stempeutics in India. Trials in Germany, Spain and Malaysia are also investigating bone marrow and adipose tissue-derived MSCs for treating critical limb ischemia.

19.9 MSCs in Diabetes

MSCs are used in directly treating insulin-dependent diabetes or diabetes-related limb ischemia [36] or foot ulcers. Trials at University of Sao Paulo and Qingdao University are investigating the use of MSCs in treating diabetes mellitus. Since MSCs are used in treatment of wounds and ulcers, the utility of these cells is also being examined in the treatment of such ulcers and wounds in diabetic patients in a number of trials. MSCs have been the front-runners relative to other stem cell types and are highly represented in clinical trials. Whether they could be effective in treating autoimmune disorders such as type 1 diabetes is still a question and remains to be established whether these cells can repair, replace, or restore the function of beta islet cells secreting insulin.

19.10 MSCs in Cell Therapy: Emerging Issues

19.10.1 Protocols

Cell therapy protocols require careful isolation of cells, expansion, differentiation, cryopreservation and preparation of transplant ready cells for delivery and meet the expected safety and efficacy, prior to their use in patients. Designing clinical trials and interpreting data from these trials is another challenge that cannot be underestimated. The first challenge is defining MSCs. Many trials use different ways to characterize and define MSCs making it difficult to compare data across trials. In 2006 the ISCT came up with a definition of MSCs and all trials using MSCs therapeutically are expected to define MSCs according to this definition. The other challenges are more generic to all cell-based therapies. It is difficult to prove safety and efficacy of these cells. As was evident with the use of MSCs in cardiac disorders, earlier promising results from small-scale studies may not hold up in larger randomized trials. Also some adverse effects of transplanted MSCs may be felt after many years (even decades) making long-term follow-up of these patients very important. Further it could take many years for transplanted MSCs to clear from the recipient. This is very different from traditional drugs where clearance is rapid and the amount of drug cleared from the recipient's system can be precisely measured. The other major challenge for use of MSCs in the clinic (especially in the development of large batches of MSCs for allogenic use) is the difficulty of establishing chemistry manufacturing controls for MSCs. These are standard controls for pharmaceuticals that characterize the drugs and ensure that the administered drug will behave in vivo as expected. Making such accurate predictions and such precise characterizations for MSCs is often very difficult or impossible. For example, even if we are able to characterize MSCs accurately, how do we predict exactly how transplanted MSCs will behave in vivo? This requires newer chemistry manufacturing controls defined for cell-based therapies including MSCs which would be different from such controls currently used for drugs.

19.10.2 Controlling Differentiation of Transplanted MSCs in Various Tissues

In a clinical study it is important to assess the safety and efficacy of the drug being administered. For MSCs, this means assessing the toxicity of transplanted MSCs and predicting the fate of these cells. It is important to achieve targeted differentiation of MSCs into the desired lineage only. It is extremely difficult to achieve such targeted differentiation of MSCs in vivo. Differentiation into undesired lineages may contribute to the toxicity of these cells. Differentiation of transplanted MSCs into fibroblasts in the heart is an example of such differentiation into an undesired lineage. This also applies to MSCs seeded on devices that may be transplanted. For

example, MSCs seeded onto 3D scaffolds for transplantation into cartilage may differentiate into bone cells which could cause toxicity of the implants. Understanding the precise cellular mechanisms governing MSC differentiation thus becomes very important [37].

19.10.3 Expanding MSCs in Large-Scale Cultures Under cGMP Conditions

Although MSCs can expand to a large extent without differentiating, their expansion capacity is often limited. MSCs have been expanded in bioreactors [38–40]. For therapeutic applications it is essential to expand MSCs in a closed system [41] under xeno-free conditions [42–44]. The development of serum-free media for expanding MSCs [45] led to the development of xeno-free protocols for MSC expansion. This is essential for the widespread adoption of MSC-based therapeutics. However these media and MSC isolation procedures are expensive and this remains the single most important hurdle in the mass adoption of MSC-based therapeutics.

19.11 Summary and Conclusion

In summary, MSCs are being used in a variety of disorders to treat multiple diseases. Many of these trials are ongoing and some are recently completed. Thus the findings are still being analyzed or are not publicly available. The long-term fallout of using MSCs as therapeutics still remains uncertain, while the early results are quite promising. A recent study by von Bahr [46] demonstrates that MSCs are cleared rapidly in recipients of MSC infusions undergoing hematopoietic stem cell transplants. Thus this study concludes that the long-term risk of MSC transplantation is limited since the cells are cleared rapidly in the recipients. Such studies of safety coupled with preliminary studies showing efficacy form the basis for future large randomized trials. Such studies will conclusively ascertain the benefits of MSCs in specific disorders. It is also possible that in many trials variables like cell dose, route and frequency of administration, stage of disease, etc. will have to be optimized before we can see the beneficial effects of MSCs.

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