Adult Stromal (Skeletal, Mesenchymal) Stem Cells: Advances Towards Clinical Applications

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Abstract Mesenchymal Stem Cells (MSC) are non-hematopoietic adult stromal cells that reside in a perivascular niche in close association with pericytes and endothelial cells and possess self-renewal and multi-lineage differentiation capacity. The origin, unique properties, and therapeutic benefits of MSC are under intensive investigation worldwide. Several challenges with regard to the proper source of clinical-grade MSC and the efficacy of MSC-based treatment strategies need to be addressed before MSC can be routinely used in the clinic. Here, we discuss three areas that can potentially facilitate the translation of MSC into clinic: Generation of MSC-like cells from human pluripotent stem cells, strategies to enhance homing of MSC to injured tissues, and targeting of MSC in vivo.

Keywords Mesenchymal stem cells • MSC-like cells • Pluripotent stem cells • Homing • In vivo targeting

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Abbreviations

AMD	Age-related macular degeneration
AMI	Acute myocardial infarction
Bzb	Bortezomib
CCR1	C-C Chemokine receptor type 1
CXCR4	C-X-C Chemokine receptor type 4
EB	Embryoid body
FAK	Focal adhesion kinase
GMP	Good manufacturing practice
hESC	Human embryonic stem cells
HLA	Human leukocyte antigen
hPSC	Human pluripotent stem cells
ICM	Inner cell mass
iPSC	Induced pluripotent stem cells
MHC	Major histocompatibility complex
miRNA	MicroRNA
MSC	Mesenchymal stem cells
Runx2	Runt-related transcription factor 2
SCID	Severe combined immunodeficiency
SDF-1α	Stromal cell-derived factor-1
siRNA	Small-interfering RNA

1 Introduction

Mesenchymal stem cells (MSC) are multipotent cells that were first identified by Friedenstein as bone marrow osteogenic stem cells [1]. The term "mesenchymal stem cell" was coined by Caplan to describe a population of cells that are involved in the formation of bone and cartilage during embryonic development, bone turnover, and repair throughout adulthood [2]. However, the term "mesenchymal" is contentious and not generally accepted [3]. Other names also exist for MSC including multipotent mesenchymal stromal cells, skeletal stem cells, adult stromal stem cells, and bone marrow stromal cells [4, 5]. MSC are defined as non-hematopoietic, plastic adherent multipotent stem cells that are present in the bone marrow stroma and can differentiate into cells of mesodermal lineage including osteoblasts, adipocytes, and chondrocytes. In ex vivo culture, MSC are positive for a number of CD markers: CD105, CD106, CD90, CD73, CD140b, CD166 and negative for CD31, CD45, CD34, CD14, CD133 and the major histocompatibility complex (MHC) class II markers [4, 6]. In addition to their presence in bone marrow, MSC-like cell populations have been isolated from the stromal compartment of adipose tissue, umbilical cord, dental pulp, skeletal muscle, synovium, and periodontal ligament [7–13]. While MSC-like cell populations share a common molecular signature with

bone marrow MSC, they exhibit differences in their molecular phenotype and differentiation potential characteristic for their tissue of origin [14]. A common in vivo location of MSC in the bone marrow and in other tissues is in a perivascular niche in close association with pericytes and endothelial cells [15].

2 Towards Clinical Use

MSC hold a great promise for clinical use in tissue regeneration in a large number of clinical conditions. 379 clinical trials, worldwide, are currently undergoing investigations into the therapeutic benefits of MSC (http://clinicaltrials.gov). These range from enhancing hematopoiesis following hematopoietic stem cell transplantation to enhancing tissue regeneration for cardiomyopathies, nerve tissue, bone and cartilage repair following injury and chronic disease.

Several factors limit the clinical use of MSC [16], including the inability to obtain the large number of MSC required for clinical transplantation due to in vitro replicative senescence [17], heterogeneity of ex vivo cultured MSC with respect to their differentiation capacity, and lack of specific markers that identify MSC prospectively and are predictive of their in vivo phenotype. In the current review, we will discuss progress in studies related to three areas that received a lot of attention due to their possible use to facilitate clinical use of MSC: (1) use of human pluripotent stem cells as a source for generation of an unlimited number of MSC, (2) development of approaches to enhance in vivo migration of MSC into injured tissues, and (3) novel strategies for targeting MSC in vivo with the aim of enhancing bone formation.

3 Generation of MSC-Like Cells from Human Pluripotent Stem Cells

Human pluripotent stem cells (hPSC) are a group of specialized cells that have the unique ability to differentiate into cells of the mesoderm, endoderm, and ectoderm lineages and are thus termed pluripotent. There are two major sources of hPSC: human embryonic stem cells (hESC) and induced pluripotent stem cells (iPSC). hESC are generated through isolation of the inner cell mass (ICM) from a 5- to 6-day-old human blastocyst [18]. Since the derivation of hESC by Thomson in 1998 [18] much effort has been focused to develop protocols for differentiation of hESC into lineage-specific cell types [19]. The creation of induced pluripotent stem cells (iPSC) from adult somatic cells [20–22] has added a new dimension to the field of regenerative medicine by offering the possibility of generating autologous pluripotent stem cells [23]. ESC and iPSC are similar in their expression of the self-renewal markers and ability to differentiate into the three basic cell lineages: ectoderm, endoderm, and mesoderm [20–22, 24, 25]. Pluripotent stem cells offer much promise

within the field of regenerative medicine due to their unlimited proliferation ability, scalability, and differentiation capacity.

A number of methods have been reported for derivation of functional MSC-like cells from PSC, using embryoid body formation (EB), monolayer differentiation, coculture, selective isolation of spontaneously differentiated cells, and cultures using biomaterials [23, 26–29].

EB Formation: Standard methods demonstrate that EBs can be formed spontaneously from small clumps of pluripotent cells that are passaged either mechanically or using enzymatic methods and cultured in suspension using low adhesion plastic vessels. This method allows spontaneous nondirected differentiation or directed differentiation of PSC, through addition of growth factors/morphogens/cytokines. EBs imitate the structure of the early embryo and recapitulate many of the early embryonic developmental events like gastrulation, polarization, and primitive streak formation [26, 30]. However, a disadvantage of the EB method is that it provides heterogeneous populations of MSC-like cells. In an attempt to reduce cellular heterogeneity, a number of alternative approaches have been developed including methods of synchronized growth and differentiation through forced aggregation by centrifugation [31], bioreactor cultures [32], and stirred suspension cultures [33] and recently Son et al. [34] published data demonstrating a simple method using periodic passaging of hEBs to maintain uniformity on size and proliferation whilst preserving their differentiation potential.

Monolayer Differentiation: 2-D PSC cultures have an advantage over EB-based differentiation as it is possible to visualize the progression of ex vivo differentiation. However, this technique fails to recapitulate the gastrulation-like processes apparent in EB formation. Development of MSC-like cells has been obtained by using induction media that allows synchronized differentiation, e.g. adding Rock inhibitor Y27632 [35], by selection by continuous subculture over a number of weeks to select for stromal (MSC-like) cells [36, 37], or by cell sorting based on specific surface markers, e.g., the selection of a $CD105^{+}/CD24^{-}$ cell population [38].

Coculture: A number of groups have used coculture of hESC with differentiated cells to induce differentiation into an MSC-like phenotype. Barberi et al. employed coculture with murine OP9 cells followed by sorting for $CD73^{+}$ MSC-like cells [39]. This method of induced differentiation presupposes that secreted factors from the differentiated cells can supply microenvironmental cues necessary for differentiation, but the nature of these factors is not known.

Spontaneous Differentiation: Spontaneous differentiation into MSC-like cells often occurs at the edges of the hPSC colonies, obtained when hPSC are cultured in a feeder-free system. In the “raclure” method, the cells at the edges of the colonies are manually scrapped [40, 41], or cells can be enriched through adherence to selective extracellular matrix components such as hyaluronic acid (HA)-coated plates [28],

or through forced differentiation through overgrowth of cultures [42]. In our laboratory, we found that selection of MSC-like cells based on selective adherence to HA-coated culture plates resulted in obtaining a morphologically homogeneous cell population with a similar phenotype to bone marrow-derived MSC [28]. In addition, Liu et al. demonstrated that hESC and iPSC differentiated into MSC-like cells through plating of single cells on a fibrillar type 1 collagen matrix [29].

The MSC-like cells derived from hESC or iPSC using the above-mentioned approaches exhibit a phenotypic profile comparable to MSC as defined by CD markers and differentiation ability into one or more of the osteoblastic, chondrocytic, or adipocytic lineages. While most of the reported differentiation capacities are based on in vitro data, a number of groups have demonstrated the ability of hPSC-derived MSC-like cells to form bone in vivo following osteogenic induction ex vivo or through direct implantation of the cells in osteoinductive scaffolds [28, 43, 44].

4 Concerns of Using hPSC-Derived MSC-Like Cells in Cellular Therapy

For clinical use, hPSC-derived MSC-like cells should be obtained from GMP (good manufacture practice) compliant hPSC lines. There have been an increasing number of reported hESC lines [45–48] and iPSC lines [49] derived under GMP standards. Additionally, clinical-grade derivation protocols for MSC-like cells have been reported [50]. The necessity for extensive ex vivo culture, which would be required for clinical therapy, has raised concerns about the possibility of genetic changes and the development of a transformed phenotype. A number of reports have highlighted the issue of karyotypic stability during routine maintenance of hESC cultured ex vivo where gains in chromosomes 12, 17, and X have been reported [51–55]. Of additional concern is the unintentional transplantation of undifferentiated hPSC in conjunction with their differentiated progeny that may lead to teratoma formation upon transplantation. As the purity of hPSC differentiated cultures is variable, attempts are being made to deplete undifferentiated hPSC within the cultures either by using cytotoxic agents, mechanically removing undifferentiated cells [56, 57], or separating out undifferentiated cells using fluorescent tags which identify undifferentiated cells [58, 59]. Thus, before hPSC-derived MSC are considered for cellular therapy safety criteria are needed to be instituted [60, 61].

MSC-like cells derived from iPS cells should be compatible with their recipient and thus will not elicit an immunological rejection reaction. Interestingly, differentiated cells derived from hESC may be hypoimmunegenic. Drukker et al. [62] demonstrated absence of the MHC class II molecules and the presence of low levels of class I molecules in hESC. Additionally, normal irradiated mice transplanted with bone marrow from immune compromised (SCID) mice were transfused with human peripheral blood mononuclear cells to test the possible immunological reaction or

rejection of transplanted hESC. Over the course of a month, transplanted hESC did not demonstrate significant rejection [62]. More recently Araki et al. demonstrated limited or no immune response in differentiated mouse ESC and iPSC [63].

It is envisaged that off-the-shelf MSC-like cells should be available in stem cell banks that contain hPSC lines that cover the majority of the Western European population tissue types. It has been estimate that 150 hESC cell lines in Europe [64] and 170 cell lines for the Japanese population [65] would be needed to obtain an acceptable degree of HLA matching which would only require a minimum of immune suppressor therapy.

iPSC were initially derived using a combination of four transcription factors (OCT4, SOX2, KLF4, and cMyc) to reprogram the somatic cells to their pluripotent status [20]. Whilst the success of reprogramming was a huge step forward towards generation of cells for therapy, standard methods used to generate iPSC may result in cells not suitable for therapy due to the use of viral vectors. New strategies are being developed to overcome these concerns using plasmids [66], recombinant proteins [67], or RNA molecules [68, 69].

5 Directing MSC to Injured Tissues

Current thinking vis-a-vis the clinical use of MSC in therapy is modeled on the hematopoietic stem cell transplantation model where HSC are infused intravenously and consequently home to the bone marrow where they establish hematopoiesis [70]. Although homing of MSC to sites of injury and their involvement in healing and/or regeneration of defected tissues is a natural repair mechanism, it was observed that this endogenous ability can be further enhanced by exogenously administered MSC [71, 72]. Systemic infusion of MSC for treatment of tissue injury represents a more attractive procedure for clinical applications. In addition, studies on MSC migration to injured tissues have been shaped by concepts related to leukocyte recruitment from the circulation to inflammation sites, through a coordinated multistep biological process termed “cell homing” that includes infused cell rolling/adhering onto sinusoidal endothelial cells followed by their firm adhesion preventing their back movement to circulation, resulting in transmigration to their destined tissues [73]. Employing this model for MSC has been supported by evidence for the presence of osteoprogenitors or MSC-like cells in the circulation that can home to bone marrow or inflammatory sites [74].

Following injury, damaged cells secrete a number of chemokines that act as attractants to cells participating in tissue repair [75]. However, one of the major challenges facing MSC-based cell therapy is the observed low and inefficient homing of systemically infused MSC to non-injured tissues [76]. Several groups have demonstrated successful but limited homing after systemic delivery to ischemic, irradiated, or otherwise injured skeletal tissues in which only a small fraction of transplanted MSC can be found in the target tissue [77–79].

5.1 Novel Approaches to Enhance Homing of MSC into Injured Tissues

It is well known that adhesion and integrin molecules are important key players in determining the potential of cellular homing [80]. For example, the CD44 antigen is a cell surface glycoprotein involved in cell adhesion and migration [81]. A specialized glycoform of CD44 called hematopoietic cell E-/L-selectin ligand (HCELL) is an E-selectin ligand expressed on human cells [82]. Using real-time confocal microscopy cell trafficking was monitored in immune-compromised mouse calvaria. These results indicated that overexpression of HCELL E-selectin on MSC caused, within hours, enhanced osteotropic migration to the bone marrow [83]. Recently, modification of MSC cell surface integrins to enhance homing of MSC to bone surfaces was achieved by attaching a synthetic ligand (LLP2A) against integrin α4β1 on the MSC surface to a bisphosphonate (alendronate, Ale). Upon administration in in vivo animal models the LLP2A-ALE-modified MSC showed enhanced homing to bone surfaces with improved bone formation at the endo-cortical, trabecular, and periosteal surfaces when compared to non-modified MSC [84]. In another study, cell adhesion molecules were chemically attached to the cell surface to improve rolling efficiency of MSC. This chemical approach involved introduction of biotin groups to the cell surface by treatment with sulfonated biotinyl-*N*-hydroxy-succinimide, the addition of streptavidin, and attachment of a biotinylated cell rolling ligand (sialyl Lewisx (SLeX)) found on the surface of leukocytes [85]. This approach can be used to potentially target P-selectin expressing endothelium in the bone marrow or at sites of inflammation [86].

Another hypothesized explanation for the poor homing capacity of MSC is their inadequate expression of homing-associated chemokines. For example, CXCR4, a homing signaling molecule known for its interaction with its cognate ligand Stromal cell-derived factor-1 (SDF-1α), is expressed at low levels on the MSC cell surface [87, 88]. In a number of in vivo studies, homing of transplanted MSC to tumors [89], myocardium [90], and bone marrow [78] has been improved by overexpression of CXCR4 on the MSC surface. For example, MSC overexpressing CXCR4 were infused intravenously 24 h after coronary occlusion in a rat model of AMI and were found to home to the infarcted myocardium resulting in better recovery of left ventricular function as compared to rats infused with control (low CXCR4 expressing) cells [87]. In another study, C3H10T1/2 cells, a multipotent mouse stem cell line, overexpressing CXCR4 were injected intravenously in immune-competent glucocorticoid-induced osteoporotic mice. These cells had enhanced homing efficiency to the bone marrow and increased bone mass in the osteoporotic mice [79]. Another member of the chemokine family is the C-C chemokine receptor type 1 (CCR1), known to be involved in the recruitment of immune cells to sites of inflammation, e.g., injured myocardium [91]. Mouse bone marrow MSC, overexpressing CCR1, were injected intra-myocardially in a mouse model of AMI. One hour post coronary artery ligation, MSC overexpressing CCR1 had accumulated in the

infarcted myocardium at significantly higher levels than control MSC. This led to significant reduction in infarct size, reduced cardio-myocyte apoptosis, increased capillary density, and restoration of cardiac function via enhancement of transplanted cells' viability and engraftment [92].

All these studies demonstrate work in progress towards developing a clinically relevant protocol for intravenous infusion of the MSC to patients in need of enhanced tissue regeneration.

6 Targeting of MSC In Vivo

Therapeutic strategies that employ ex vivo cultured cells are associated with some limitations such as the need for substantial number of cells requiring extensive ex vivo cell expansion, the need for GMP facilities, as well as development of robust methods for differentiation induction [16]. Targeting of the endogenous MSC populations, using small molecules, small-interfering RNA (siRNA), or MicroRNA (miRNA), is an attractive alternative and is suitable for treatment of diseases where the mature cell populations, which are to be targeted by the drug, are depleted or do not respond to standard treatment. An example of such a clinical setting is the use of osteoblast-targeting anabolic therapies for treatment of bone loss, in which osteoblasts are decreased in number and activity [93].

Small molecules are very attractive agents to be used in clinical applications, due to the opportunity of fine-tuning their chemical structure using traditional chemistry techniques, high stability, adaptability to large-scale production leading to substantial reduction of the treatment costs, and a potential for oral delivery [39, 94, 95]. Some examples of these approaches have been recently reported.

Bortezomib (Bzb) is a small molecule proteasome inhibitor that is used in the clinic for treatment of multiple myeloma [96]. It has been shown that Bzb targets bone marrow MSC in vivo and induces their differentiation toward the osteoblastic lineage through regulation of runt-related transcription factor 2 (Runx2), known as a master regulator of osteogenesis [97, 98]. Intraperitoneal (i.p.) administration of Bzb to mice for 3 weeks increased bone mass, trabecular bone connectivity, trabecular number, serum osteocalcin, as well as bone formation rate demonstrating enhanced in vivo osteoblastic bone formation activity. Moreover, it was shown that in contrast to MSC, osteoprogenitors and osteoclasts did not respond to Bzb treatment [98].

siRNA can specifically silence the synthesis of any desired protein by base paring to its mRNA sequence [99]. To date, more than 20 siRNA-based drugs are under clinical investigation for treatment of a variety of conditions including solid tumors, acute kidney injury, age-related macular degeneration (AMD), diabetic macular edema, hepatitis C, AIDS-associated lymphoma, and respiratory syncytial virus infection [100]. Administration of siRNA and silencing the synthesis of a gene of interest can be used to alter the differentiation fate of MSC in vivo [101]. However, the large therapeutic doses of systematically administered siRNA that is needed to exert the desired clinical outcome may lead to activation of immune response, as

well as adverse effects on other tissues. Thus, the development of novel systems that deliver siRNA specifically to the cell population of interest is highly desirable. Recently, a novel targeting system has been developed that delivers siRNA to the bone-forming surfaces enriched for MSC and osteoprogenitors [102]. This system involves dioleoyl trimethylammonium propane (DOTAP)-based cationic liposomes attached to six repetitive sequences of aspartate, serine, serine ((AspSerSer)6). This system has been used for in vivo systemic delivery of siRNA targeting Plekho1 (a negative regulator of bone formation) in rats and led to significant enhancement of bone formation, enhanced the bone micro-architecture, and increased the bone mass in both healthy and osteoporotic rats [102]. In addition to siRNA, miRNAs have potential use in therapy. miRNAs are endogenous, short, noncoding RNAs that regulate diverse biological processes mostly through translational repression of their target genes [103]. miRNAs can be employed to modulate the differentiation fate of MSC in vitro and in vivo [103]. Exogenous supplementation or ectopic expression of miRNAs as well as using anti-miRs to antagonize the effect of miRNAs are promising strategies to be employed for treatment of different clinical conditions [104]. In our group, we have demonstrated that miR-138 negatively regulates in vitro osteoblast differentiation and in vivo bone formation of MSC, by targeting focal adhesion kinase (FAK), a kinase playing a central role in promoting osteoblast differentiation [105]. Using a preclinical in vivo bone formation model, we showed that pharmacological inhibition of miR-138 by antimiR-138 increased ectopic bone formation and thus it is possible to develop antimiR-138 into a novel strategy for treatment of bone loss conditions [105].

7 Conclusions and Future Perspectives

Regenerative medicine holds promise to restore normal tissue functions in the body using stem cell transplantation or ex vivo grown tissues and organs generated through a combination of stem cells and biomaterials, i.e., tissue engineering approaches. The transition from the laboratory to the clinic has proven to be difficult and currently there is no standard stem cell-based therapy for non-cancer indications. Conversely, a large number of clinical trials testing the ability of different types of stem cells including MSC in a number of disease conditions are being conducted and include conditions such as nonunion fractures, ulcerative colitis, type 1 diabetes mellitus, liver cirrhosis, idiopathic dilated cardiomyopathy, multiple sclerosis, spinal cord injury, acute and chronic graft-versus-host disease, middle cerebral artery infarct, osteoarthritis, relapsed/refractory severe acquired aplastic anemia, chronic critical limb ischemia, Parkinson's disease, acute myocardial infarction, hematological malignancies, Crohn's disease, acute leukemia, lupus nephritis, and non-healing wounds (please see: http://clinicaltrials.gov). It is hoped that these trials will establish the efficacy of stem cells and MSC in therapy and their place among other current treatment modalities.

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