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Concise Review: Multifaceted Characterization of Human Mesenchymal Stem Cells for Use in Regenerative Medicine

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Key Words. Mesenchymal stem/stromal cells • Bone marrow • Characterization • Release criteria • Regenerative medicine

Abstract

Mesenchymal stem cells (MSC) hold great potential for regenerative medicine because of their ability for self-renewal and differentiation into tissue-specific cells such as osteoblasts, chondrocytes, and adipocytes. MSCs orchestrate tissue development, maintenance and repair, and are useful for musculoskeletal regenerative therapies to treat age-related orthopedic degenerative diseases and other clinical conditions. Importantly, MSCs produce secretory factors that play critical roles in tissue repair that support both engraftment and trophic functions (autocrine and paracrine). The development of uniform protocols for both preparation and characterization of MSCs, including standardized functional assays for evaluation of their biological potential, are critical factors contributing to their clinical utility. Quality control and release criteria for MSCs should include cell surface markers, differentiation potential, and other essential cell parameters. For example, cell surface marker profiles (surfactome), bone-forming capacities in ectopic and orthotopic models, as well as cell size and granularity, telomere length, senescence status, trophic factor secretion (secretome), and immunomodulation, should be thoroughly assessed to predict MSC utility for regenerative medicine. We propose that these and other functionalities of MSCs should be characterized prior to use in clinical applications as part of comprehensive and uniform guidelines and release criteria for their clinical-grade production to achieve predictably favorable treatment outcomes for stem cell therapy. Stem Cells Translational Medicine *2017;6:2173–2185*

Significance Statement

There is a pressing need for more wide-ranging characterization metrics for mesenchymal stem cells (MSCs) that better and more accurately predict treatment outcomes of MSC-based therapies. This Review provides a detailed account of what are currently thought to be defining characteristics of MSCs and further considers recent advances that may prove to be important criteria when considering clinical applications. The relationship between in vitro characteristics and in vivo potency and strategies to improve the efficacy of MSC therapy is also addressed.

Introduction

Mesenchymal stem cells (MSC) constitute a heterogeneous subset of stromal regenerative cells which can be harvested from several adult tissues. Other descriptive names for MSC populations in the literature include mesenchymal stromal cells, mesenchymal progenitor cells, multipotent mesenchymal stromal cells, bone marrow stromal cells, bone marrow-derived MSC, multipotent stromal cells, mesenchymal precursor cells, skeletal stem cells, as well as medicinal signaling cells. They are multipotent cells capable of differentiating into various types of specialized cells including osteoblasts, chondrocytes, and adipocytes [1]. Recent studies indicate that MSCs resemble pericytes and emerge from the peripheral stromal region surrounding blood vessels, thus clarifying their broad regenerative potential in adult tissues, although there are also other sources for MSCs [2–4]. Their relative ease of isolation, combined with their capacities for self-renewal [5] and multipotentiality make MSCs a promising treatment option for a variety of clinical conditions. Yet, administration of MSCs (either intravenously or by direct injection in tissue) has not yielded consistent clinical results, because injected cells exhibit limited survival in host tissue. The fact that clinical improvement may be seen even despite the apparent short survival times of MSCs has led to alternative ideas about trophic effects [6]. Several wide-ranging investigations have attempted

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Figure 1. Profiling of MSCs. The diagram depicts the key parameters for the characterization of adult stem cells from different sources. Three of these parameters are linked to cell growth, survival, quiescence and/or senescence (i.e., viability and growth, CFU-Fs, telomere length), two are associated with cell identity (i.e., multilineage differentiation and surface marker expression), and the remaining two refer to the ability of MSCs to communicate with their microenvironment (i.e., immunomodulation and paracrine effects of trophic factors). Immunomodulation is important for regulating macrophage function during tissue repair (e.g., M1 to M2 macrophage transition) and for anticipating graft rejection (e.g., mixed lymphocyte reaction). Collectively, these parameters should be considered for the development of release criteria that validate the quality of GMP-grade MSCs for stem cell therapy. Abbreviations: MSCs, mesenchymal stem cells; CFU-Fs, colony-forming units-fibroblastic.

to address this issue of unpredictable outcomes by seeking to establish standard practices for the isolation, characterization, and maintenance of cells in culture. In this Review, we discuss human adult bone marrow-derived MSCs, their various characterization methods, including an assessment of trophic factors secreted by isolated and culture-expanded cells. Our group has recently proposed benchmarks for MSC functionality that require an improvement in MSC selection criteria [7]. This Review considers several functional aspects of MSCs (Fig. 1) as they pertain to potency, and of the need to adopt multiple-parameter analyses for useful stem cell selection.

Identification and Tissue Sources of MSCs

The first identified multipotent stromal precursor cell populations from the bone marrow were described as nonphagocytic, fibroblast-like in appearance and able to form adherent colonies that were henceforth termed "colony-forming units-fibroblastic" (CFU-F) for this population [8, 9]. Other studies revealed that bone marrow-derived MSCs represent precursor cells for mesenchymal tissues. Some investigations have reported conversion of multipotent stem cells into cells from another lineage through a process termed trans-differentiation, although there are varying opinions on this phenomenon [10–14]. While epigenetic transcriptional mechanisms control neuronal versus mesenchymal cell fates [15], MSCs can be induced experimentally to express neural markers [16].

Although MSCs were initially identified in bone marrow, MSC-like populations have since been harvested from autologous and allogeneic sources, including adipose tissue [17], peripheral blood [18, 19], lung [20], marrow spaces of long bone [21], synovial fluids [22], periodontal ligament [23], and muscle [24]. In addition, MSCs are also obtained from placenta [25, 26], umbilical cord [27], and cord blood [28, 29] as well as dental pulp [30, 31]. Investigations into the lineage of these cells strongly suggest that progenitor cells of cultured MSCs arise from around the blood vessels (capillaries, arteries and veins) in vivo, and are thus of perivascular origin [3, 31, 32]. Notably, MSCs obtained from various sources differ in their biological characteristics [33–36]. A recent comprehensive report on the proteome and transcriptome profiles of MSCs revealed source specific markers [37]. In addition, differences that exist in CFU-F efficiency, surfactome profiles, multilineage differentiation as well as paracrine functions [35, 36, 38–41] may determine their different clinical applications.

Recent reports have indicated that MSCs from allogeneic sources are more commonly used in trials than autologous MSCs [42, 43], even though both sources of cells have demonstrated comparable clinical effects [42, 44–46]. Generally referred to as "universal donor cells" [44, 47], owing to their immune tolerance property, these cells possess several clinical advantages [48]. Nevertheless, as with any cell-based therapy, it is of utmost importance to fully evaluate the safety and efficacy of allogeneic strategies before clinical use [49, 50]. MSCs from bone marrow are the most commonly investigated candidates that are providing most of the cells being used to create functional clinical therapies. In this Review, the isolation and functional characteristics described pertain to human bone marrow-derived MSCs.

Isolation of MSCs

MSCs are obtained after bone marrow aspiration and then isolated by sieving for plastic adherence in vitro. They readily form colonies capable of clonal expansion and differentiation. Other isolation methods with different degrees of sophistication have been investigated, including density gradient cell separation [51], as well as fluorescence- or magnetic-activated cell sorting [52, 53]. The latter two flow cytometric methods rely on expression of cell surface markers displaying relatively high specificity for MSCs. Clearly, standard isolation procedures and generic molecular characterization of MSCs are vital for any consistent cell isolations [54, 55]. The key characteristics that have hitherto defined MSCs have been based on their capacity for colony formation, potential for self-renewal, expression of surface markers, and subsequent capacity for multilineage differentiation [56].

Multifaceted Characterization of MSCs

Colony Formation

In vitro, plastic-adherent clonogenic cells, denoted as CFU-Fs, can be obtained from bone marrow and give rise to colonies during their initial growth [31, 57]. CFU-Fs are thought to be mostly composed of primary bone marrow-derived MSCs that upon further proliferative expansion in culture, constitute mesenchymal stem/stromal cells. Colonies of MSCs display heterogeneous morphological characteristics ranging from fibroblastoid to spindle-shaped or from large-flattened to small-round cells. Passaged cells are usually seeded at 100 to 150 cells per 10-cm dish and allowed to adhere and form colonies over a period of 14 days that are visualized by staining with crystal violet or toluidine blue [58]. Evaluation of the CFU-F potential is usually being done by seeding bone marrow cells at densities of 0.5–3 million in 50–75 cm^2 culture vessels [7]. Bone marrow mononuclear cells are typically seeded at 50,000–200,000 cells per cm^2 to yield colonies of MSCs. Although this approach provides a relatively crude estimate of MSC titers in bone marrow cells [59], CFU-F efficiency remains a routinely used and accepted standard to identify and characterize MSCs [56].

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Surface Phenotype

To acquire a more complete understanding of MSC biology and to generate a reliable stem cell product for clinical trials and routine patient care in the future, it is necessary to isolate homogenous population of MSCs. The principal approach for improving homogeneity of MSC populations uses antibodies that target specific cell surface markers. This homogeneity is a relative term, because MSC populations have natural variation in the expression of cell surface markers around a common mean. The identification of MSCs in vivo is far from straightforward, owing to extremely low frequencies in tissues [59, 60]. Furthermore, isolation methods are impeded because MSCs are dynamic and exhibit phenotypic variation over time ("plasticity"). Also, there is only a limited number of useful MSC markers, but none of these is definitively specific for MSCs in a strict sense (i.e., absolutely required and sufficient to establish MSC identity). Nevertheless, it is well established that cultured colonies of MSCs express CD105, CD73, and CD90, but do not express CD45, CD34, CD14 or CD11b, CD19, and HLA-DR [56]. Some labeling strategies have also been used to successfully isolate MSCs enriched for markers such as STRO-1 [52, 61–64], CD146 [5], SSEA-4 [65], CD271 (NGFR) [66–68], and MSC antigen 1 (MSCA-1) [69, 70], although there is no absolute agreement yet on the markers that could prospectively assist in the isolation of MSCs from either fresh bone marrow or other tissues. Initial studies by Pittenger and coworkers have identified markers such as SH2 and SH3, which correspond to CD105 and CD73, respectively. These markers, together with CD90, have been considered by the International Society for Cellular Therapy (ICST) as the primary markers expressed on greater than 95% of MSCs in a given culture. However, it should be noted that the expression of CD105, CD90, and CD73 may not be absolutely specific to undifferentiated multipotent MSCs, as some of these markers are also expressed by vascular populations [71, 72], smooth muscle cells [73], and mature stromal cells such as fibroblasts [71, 74]. Consequently, there is a critical need to develop highly sensitive cell sorting and immunohistochemical assays and reagents to distinguish immature/undifferentiated MSCs from committed stromal cell populations.

Currently not included in the ISCT panel is STRO-1, a particularly important marker, with relatively high specificity for early-passage bone marrow-derived MSCs. STRO-1 facilitates the identification, isolation, and functional characterization of clonogenic stromal cell progenitors [52, 61–63, 75]. The absolute selectivity of STRO-1 for naïve MSCs is yet to be resolved and the presence of STRO-1 antigen on MSCs is progressively downregulated following culture expansion. Notwithstanding these limitations, the putative STRO-1 antigen remains very useful because STRO-1 positive cells have favorable stem cell properties for translational applications [76].

Other strategies using cell-sorting have exploited the expression of CD49a (integrin α1; ITGA1) [39], PDGFR-α/β (platelet-derived growth factor receptors PDGFA and PDGFB), EGF receptor (EGFR), insulin-like growth factor receptor (IGFR), and STRO-3 [52, 77] to enable isolation of MSC populations enriched for multilineage differentiation potential (see Table 1). Andersen and colleagues have isolated antibodies against Collagen VI (COL6A1), CD44 and HLA-DR, and that have proven useful for identifying subpopulations in MSC cultures [79]. CD146 (melanoma cell adhesion molecule, MCAM) has attracted major interest following reports on its expression being linked to pericytes [3, 5]. It has also been shown that expression of CD146 on MSCs expressing CD271 (nerve growth factor receptor, NGFR) is associated with their in situ localization [80, 81]. However, it was also shown, particularly for CD146, that its expression is variable during in vitro culture and its cell surface presence fluctuates depending on the type of culture media [4]. Bone marrow-derived CFU-Fs express surface markers such as STRO-1, CD271 [68], CD49a [39, 82], stage-specific embryonic antigen-4 (SSEA-4) [65], and CD146 [83]. CD271+ cells display multipotentiality and is considered a suitable marker of bone marrow-derived MSCs [84]. Other reports have also detailed the use of D7-FIB (a fibroblast or epithelial surface antigen) [85] and CD56 (neural cell adhesion molecule, NCAM) [86] for multipotent MSC isolation. Nestin, a neural stem cell marker, has characterized as a selective marker for bone marrow-derived MSCs [87, 88]. On a parallel note, the use of mouse models to study MSC biology has yielded novel information, highlighting the similarities in the expression of some of the surface markers, including CD140a [89, 90] and CD295 (leptin receptor) [91], between mouse and human MSCs [92]. However, species-specific heterogeneity in phenotype and in vivo residence of MSCs must be taken into account when extrapolating information derived from other species.

Lastly, it is important to understand that the innate levels of expression of a set of surface markers are not a guarantee of MSC homogeneity. Since labs around the world use different sets of antigens for characterization, comparisons reveal that there is no consistency in the use of cell surface antigens for the isolation of MSCs and there is no marker that uniquely identifies MSCs that could be used reliably for their isolation. In our previous study comparing in vitro and in vivo functions of bone marrow-derived MSCs from multiple donors, we showed that STRO-1 and PDGFRα (CD140a) were able to identify MSCs that were more potent at forming bone in vivo [7]. We showed that MSCs with high-growth capacity had higher levels of expression of these markers and promoted increased bone-formation compared with low-growth capacity cells, thus highlighting the possible utility of STRO-1 and PDGFRα markers to aid in selection of efficacious MSCs for bone regenerative applications. Also, it is important to note that MSCs from different tissue origins have different surface marker expression [93, 94]. Consequently, investigators are now performing characterization to determine the genomic and proteomic profiles of MSCs to establish mechanisms that mediate self-renewal and maintenance of homogenous cell populations. In keeping with the trophic functions of MSCs (see below), their secretory and exosomal profiles may reveal unique biomarkers that reflect their biological properties and could potentially aid in their selection.

Multi-Lineage Differentiation

While surface markers are easily assessable, a proper definition of what constitutes an MSC can be completed by their ability to differentiate into classic mesodermal lineages of bone, fat, and cartilage. When late passage cultures are left in maintenance media for longer periods (weeks) and cells become confluent, at least a subset is capable of spontaneously mineralizing, indicating that bone-marrow MSCs are predisposed to differentiation into the bone lineage [95]. This property is not exclusive of bone marrow MSCs and is also exhibited by human umbilical cord perivascular cells [96]. Factors such as ascorbic acid and dexamethasone, at defined concentrations, are able to direct the MSCs toward osteogenic differentiation. Similarly, BMPs, WNTs, FGFs and other heparan sulfate-sensitive morphogens, and growth factors are

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Table 1. Surface antigen expression on human bone marrow-derived mesenchymal stem cells (MSCs)

CD (cluster of differentiation)	Gene Symbol	Protein description	MSC specificity
CD11a	*ITGAL*	Integrin alpha L chain	–
CD11b	*ITGAM*	Integrin alpha M chain	–
CD13	*ANPEP*	Aminopeptidase N	–
CD14	*CD14*	Myeloid cell-specific leucine-rich glycoprotein	–
CD19	*CD19*	B-lymphocyte surface antigen B4	–
CD29	*ITGB1*	Integrin β1 chain	+
CD31	*PECAM1*	Platelet endothelial cell adhesion molecule	+
CD34	*CD34*	Hematopoietic progenitor cell antigen CD34, transmembrane phosphoglycoprotein	+/–
CD36#	*CD36*	Collagen Type I Receptor, Thrombospondin Receptor	+/–
CD44#	*CD44*	Hyaluronan receptor	+
CD45	*PTPRC*	Lymphocyte common antigen; protein tyrosine phosphatase, receptor type, C	–
CD49a	*ITGA1*	Integrin subunit alpha 1 chain	+
CD49b	*ITGA2*	Integrin subunit alpha 2 chain	+
CD49c	*ITGA3*	Integrin subunit alpha 3 chain	+
CD49d	*ITGA4*	Integrin subunit alpha 4 chain	+
CD49e	*ITGA5*	Integrin subunit alpha 5 chain	+
CD51	*ITGAV*	Integrin subunit alpha V chain	+
CD54	*ICAM1*	Intracellular adhesion molecule	+
CD58	*CD58*	Lymphocyte function-associated antigen	+
CD61	*ITGB3*	Integrin β3 chain	+
CD71	*TFRC*	Transferrin receptor	+
CD73*	*NT5E*	Ecto-5′-nucleotidase	+
CD90*	*THY1*	Thy-1	+
CD102	*ICAM2*	Intracellular adhesion molecule	+
CD104	*ITGB4*	Integrin β4 chain	+
CD105*	*ENG*	Endoglin, TGFβ R III	+
CD106	*VCAM1*	Vascular cell adhesion molecule	+
CD120a	*TNFRSF1A*	Tumor necrosis factor receptor 1A, TNF IR	+
CD120b	*TNFRSF1B*	Tumor necrosis factor receptor type II, TNF IIR	+
CD121a	*IL1R1*	Interleukin-1 receptor	+
CD124	*IL4R*	Interleukin-4 receptor	+
CD133	*PROM1*	Prominin-1 transmembrane glycoprotein	–
CD140a	*PDGFRA*	Platelet-derived growth factor receptor alpha	+
CD140b	*PDGFRB*	Platelet-derived growth factor receptor beta	+
CD146	*MCAM*	Melanoma cell adhesion molecule	+
CD166	*ALCAM*	Activated leukocyte cell adhesion molecule	+
CD200	*CD200*	OX-2 membrane glycoprotein	+
CD221	*IGF1R*	Insulin-like growth factor 1 receptor, IGF-R	+
CD271	*NGFR*	Nerve growth factor receptor, NGF-R	+
SSEA-4	*SSEA4*	Stage specific embryonic antigen-4	+
STRO-1	N.A.	Stromal antigen 1	+
W8-B2/MSCA-1	N.A.	MSC antigen 1	+

The signs (+) or (–) indicate the presence or absence of markers respectively. * refers to antigens that have been proposed by the International Society for Cellular Therapy (ISCT) to define human MSCs [56]; # refers to markers specifically retained by adipose stem cells, according to the recently revised ISCT and International Federation for Adipose Therapeutics and Science joint statement [78]. Abbreviation: N.A., not available.

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Figure 2. Dual functions of MSCs in tissue regeneration and repair. MSCs play a central role during regeneration and repair of musculoskeletal tissues (i.e., bone, cartilage, ligament, tendon, muscle and synovium). In addition, MSCs provide a microenvironment for hematopoietic stem cells, including cells of the myeloid and lymphoid lineages. Effects of MSCs on their microenvironment are mediated by secretion of trophic factors that have both autocrine and paracrine functions. Abbreviation: MSCs, mesenchymal stem cells.

able to stimulate osteogenic differentiation [97–102]. To stimulate adipogenesis, dexamethasone, indomethacin, insulin, and isobutylmethylxanthine are usually added to the cultures [103]. Ascorbate, insulin, transferrin, selenic acid, and TGF-β are well-established inducers of chondrogenesis [104–106]. It is broadly appreciated that differentiation protocols followed by laboratories around the world are not necessarily the same. Factors such as antibiotics and growth supplements such as serum and platelet lysate can influence the phenotypic properties of MSCs and their multi-lineage potential [107–109].

We note that when cultured under defined conditions with specific inducing factors, MSCs can be directed to differentiate into neural, myocyte, and epithelial cells, thereby demonstrating their endodermic and neuroectodermic differentiation potential [14, 95, 110, 111]. It is not clear whether this process represents culture-induced aberrant trans-differentiation or perhaps reflects the inherent natural ability of adult stromal cells to reprogram under specific conditions. Forced trans-differentiation in culture may perhaps be analogous to established developmental events (e.g., neural crest formation, epithelial-to-mesenchymal, and endothelial-to-mesenchymal transitions). In several studies using in vivo transdifferentiation models, MSCs have been reported to engraft and differentiate resulting in functional improvement of endogenous tissues [112–117], as well as integrate via cell fusion mechanisms [118–120].

Differences exist in MSC differentiation properties. Culture-expanded colonies display progressively limited differentiation potential. Reports from various groups have suggested that only a fraction of the total number of clones can differentiate into all the three lineages. The majority of the clones appear to be bi-potent and only able to commit to osteogenic and chondrogenic lineages. Clones that showed increased adipogenic potential had decreased chondrogenic potential, and vice versa. From our previous work, we observed a bimodal differentiation pattern in MSCs that alternate between osteogenesis and adipogenesis [7]. Furthermore, monopotent MSCs have also been reported [121–123]. Thus, tri-, bi-, and mono-potent colonies have been identified.

The reasons for heterogeneity in differentiation potential are not fully understood, but most likely reflect epigenetic adaptations that predispose cells to different cell fates depending on the source tissue. The latter concept remains conjecture at present, but is a logical implication from current concepts of the physiological micro-environment of stromal cells. For example, stromal cells surrounding blood vessels in fat tissue or the bone marrow cavity are exposed to different growth factors, morphogens, cytokines and chemokines. Ultimately, such extracellular signals are sensed by cell surface receptors and transduced to the nucleus to mediate epigenetic chromatin changes. The latter changes are more likely to ensure that stromal cells in fat tissue differentiate into pre-adipocytes, while analogous stromal cells in bone marrow may more easily convert into skeletal progenitor cells.

Considering the heterogeneity in lineage-predisposition of different MSC preparations, characterization of the lineage-differentiation potential is important, albeit that it is not prudent to base MSC selection solely on this biological property. Differentiation properties of MSCs are important for tissue maintenance and repair, as well as engineering strategies, and cell-based therapies that require engraftment and differentiation into host tissues. However, the clinical potential of the trophic functions of MSCs is recently gaining significant traction as the basis for new stem cell therapies.

Trophic Functions of MSCs

The trophic function of MSCs refers to their functional capacity to generate a reparative milieu through cell-to-cell contact concomitant with paracrine secretion of a broad array of bioactive macromolecules that promote immunomodulation of inflammatory cells that participate in tissue repair (e.g., T cells, macrophages, and mast cells) and differentiation of endogenous progenitor cells (e.g., osteo- and chondroprogenitors). The current catalogue of trophic factors includes growth factors, morphogens, chemokines, cytokines, extracellular vesicles ([EVs] e.g., exosomes), and glycosaminoglycans (GAGs) [6, 124–128] (Fig. 2). The immunomodulatory properties of MSCs support suppression of local immune responses and fibrotic tissue formation, while modulating angiogenesis, apoptosis, and cell proliferation. These properties collectively generate a microenvironment that enables injured tissues to mount a self-regulated regenerative response [129, 130].

Historically, MSCs originally attracted attention because of their "stemness" and potential use as therapeutic agents through engraftment to replace cells in damaged tissues. However, in many experimental settings, transplanted cells restore tissue functions with no detectable engraftment within host tissue or differentiation [131–133]. Reports on the trophic functions of MSCs date back to studies by Dexter and colleagues, that showed the ability of MSCs to support HSCs [134], to be able to suppress the local immune system by secretion of cytokines [131, 135, 136], to

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aid in regeneration of the meniscus [137], to promote neurotrophic and functional recovery after stroke [138], and promote cardiac repair [139]. The mechanisms governing these functions imply that MSCs facilitate normal tissue healing by cell-to-cell contact and/or secretion of bioactive factors.

Several published findings reinforce the proposition that persistent engraftment of the cells at a skeletal defect site is not mandatory for tissue healing or repair. Horwitz and colleagues showed that OI (osteogenesis imperfecta) in babies was improved with transplantation of allogenic bone marrow cells, and resulted in increased bone mineral density and reduced bone fractures, even though less than 2% of the donor MSCs were found to be engrafted [140]. Other studies have shown that few implanted MSCs survive 6 weeks post-implantation in a rat ectopic model [141] and 3 to 7 weeks in an orthotopic femoral defect model [100]. The observed bone regeneration is attributable to a burst of active trophic factors secreted by the implanted MSCs. Similarly, MSC transplants in other disease models have resulted in improved cardiac function, neurogenesis, pancreatic islet survival and functionality, as well as modulation of the immune system in graft-versus-host-disease [142]. These findings are generally consistent with the now prevalent idea that MSCs do not promote tissue repair only through engraftment, but also by delivery of bioactive factors.

Given the therapeutic potential of EVs secreted by MSCs [143–146], it would be useful to include characterization of EVs while assessing MSC potency. EVs have been shown to possess anti-inflammatory properties [147], rescue radiation damage to bone marrow MSCs [148], as well as mitigate airway hyperreactivity and lung inflammation in preclinical disease models [144]. Assaying for their reported immunomodulatory, cytoprotective, and regenerative properties may be important for advancing MSC-based EV-mediated therapies [147, 149, 150].

Despite the fact that MSCs isolated from separate donors show no major differences in their in vitro differentiation potential or in their surface markers expression [151], differences in their secretion profile may be the key to the observed variability in their in vivo healing capacity. The in vitro secretome of MSCs has been well documented and several secretory molecules relevant to MSC potency have been investigated [7]. Although recombinant bioactive factors are essential for the future of regenerative medicine, the use of most of them remains experimental, mainly due to difficulties in optimizing the clinical dose of the factors based on in vitro results and preclinical models. Understanding the secretory activity of MSCs, in conjunction with their in vivo behavior and paracrine effects, is thus of paramount importance for the exploitation of their clinical potential.

Immunomodulatory Properties of MSCs

The mechanisms underlying immunoregulation by MSCs are not fully understood, but involve cell-to-cell contact and secretory mechanisms. Typical in vitro modulatory functions of MSCs are inhibition of T cell [152] and B cell proliferation, as well as dendritic cell differentiation [153]. MSCs also regulate immune responses by upregulating the numbers of regulatory T cells (T_{regs}) which actively suppress effector T cell functions [154]. MSC-immune cell contact involves adhesion molecules [155]. In addition, factors including IL-10, indoleamine 2, 3-dioxygenase (IDO), VEGF, CCL-5 or RANTES, prostaglandin E2, and nitric oxide (NO) are secreted by MSCs (either constitutively or by interaction with target cells). Interleukin-6 (IL-6), TGFβ1, hepatocyte growth factor, CCL-1 or MCP-1 (monocyte chemoattractant protein), and leukemia inhibitory factor (LIF) are other

Figure 3. Interactions of MSC with immune cells. MSCs secrete soluble molecules, such as nitric oxide, PGE_2, IDO, IL-10 and TGFβ1. The secretion of these factors suppresses the proliferation and/or activity of a variety of immune cells, including T cells, B cells, Natural Killer cells, and dendritic cells, as well as activated T_{regs}. Abbreviations: IDO, indoleamine 2, 3-dioxygenase; IL-10, interleukin-10; MSCs, mesenchymal stem cells; PGE_2, prostaglandin; TGFβ1, transforming growth factor-beta 1; T_{regs}, regulatory T cells.

notable immunoregulatory factors secreted by MSCs [156, 157]. MSC-to-T cell contact induces IL-10 secretion, which attenuates T cell proliferation, and stimulates HLA-G5 secretion which in turn inhibits activated T cells and NK-cell cytotoxicity [158] (Fig. 3).

In vivo, systemic administration of MSCs facilitates immunosuppression in graft-versus-host-disease models [159], multiple sclerosis, inflammatory bowel disease, diabetes [160] as well as cardiomyopathies [161, 162]. Following successful outcomes from animal models, clinical trials for Crohn's disease (e.g., Mayo Clinic), acute graft-versus-host-disease (e.g., Osiris Therapeutics), and severe osteogenesis imperfecta by allogenic BMT (e.g., St. Jude Children's Research Hospital) have been conducted. Trials are ongoing for acute myocardial infarction, aplastic anemia, osteoarthritis, SLE, diabetes, and other conditions [142, 163]. Because MSC therapy appears to be promising for treating immunological disorders, characterization of MSC immunosuppressive functions will provide an important functional indicator for in vivo efficacy of MSCs, even though they may not be specific to multipotent MSCs, since stromal fibroblasts also exhibit immunosuppressive functions [164, 165]. Furthermore, it is also important to note that MSCs from different sources may differ in their mechanisms and capacities for immunomodulation [166].

Because of their trophic and immunomodulatory functions, MSCs are generally considered to possess greater advantages in cell-based regenerative medicine. However, it is important to note that MSCs can either support or suppress tumorigenesis (reviewed in [167, 168]). In contrast to their anti-apoptotic and anti-inflammatory functions, MSCs have been shown to interact with tumor cells via paracrine signaling and possibly increase the risk for metastasis by mediating epithelial-to-mesenchymal transition in addition to augmenting angiogenesis [169–172]. This less-desirable effect imparted by MSC immunomodulatory activities at tumor microenvironments warrants some caution in their use in circumstances of pre-existing tumor conditions.

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Figure 4. Standard operating procedures for isolating mesenchymal stem cells (MSCs). The diagram shows the basic steps for isolating and validating MSCs from bone marrow aspirates derived from either human donors or patients, including evaluation of key potency parameters of these cells before release to the clinic. Abbreviations: BM, bone marrow; cPD, cumulative population doubling; HSC, hematopoietic stem cells; ISCT, International Society for Cellular Therapy; MNC, mononuclear cells; PD, population doubling; PDGFR-α, platelet-derived growth factor receptor-alpha; STRO-1, stromal antigen-1. Asterisk (*) indicates proposition of additional criteria that could potentially facilitate better selection of MSCs.

Telomere Length Analysis

Cell preparations of MSCs have variable and limited proliferative potential. The variability depends on differences in sources and methods of isolation, as well as the age and health conditions of the donors [173, 174]. For clinical use, extensive subculturing is performed to attain the required cell numbers for therapy. As a result, cells rapidly reach a stage of growth arrest and replicative senescence as their telomeres progressively shorten with repeated cell replications in vitro. Obtaining both quality and quantity of MSCs for an efficacious therapy is a major bottleneck in translational medicine. Telomere maintenance is carried out via telomerase reverse transcriptase (hTERT) which functions to lengthen telomeres by adding repetitive TTAGGG sequences to chromosome termini. Overexpression of TERT in MSCs restores telomerase activity, preserves telomere length and increases MSC life span. The status of telomeres is a key parameter for MSC quality that should be routinely monitored; however, reports on telomerase functions in MSCs are incompatible [111, 175, 176]. Differences in results could be due to different sensitivities in measurement and the nonestablished reference levels of telomerase to define cells as either telomerase-positive or negative [177]. Therefore, assaying for telomere lengths, as well as overall telomere status in MSCs should assist in the benchmarking process and in quality control decisions required before MSC transplantation [7, 178].

Standardization of Strategies for Improving MSC Growth and Regenerative Efficacy

Uniform standards for MSC preparation are essential for fundamental characterization and clinical translation of MSCs. Standard operating procedures avoid variability in cell preparation that may arise for technical reasons. Yet, currently most laboratories use their own optimized protocols, and cell preparations between labs clearly vary. Therefore, it would be beneficial for laboratories to agree upon standard operating procedures and to improve comparison of results.

The illustration provided in Figure 4 is a schematic of a BM aspirate sample processing. The aspirate is layered over Ficoll-Paque to obtain mononuclear cell fractions by density gradient method, from which MSCs are isolated. Between 1 and 3 million MNCs are seeded in 50–75 cm^2 dishes or flasks to obtain colonies that provide an estimate of MSC numbers per 10^5 cells seeded. Flow cytometry is performed to determine the MSC percentages in the original sample before they are plated for isolation to get the baseline MSC levels in a given patient/donor sample. Also, growth capacities of cells (fast or slow growing) are assayed starting at P0 by taking a fraction of the MNCs and allowing MSC colonies to form and proliferate so that cumulative growth can be plotted. Cells that have undergone three or four passages are typically evaluated for matching the ISCT criteria [56]. In addition, other criteria such as population doubling time, amounts of growth factor/cytokine secretions, levels of STRO-1, PDGFR-α/β, and telomere length are some measures to assess efficacy.

Strategies to clear the hurdle of achieving clinically relevant numbers of MSCs include the use of growth media supplements like serum, platelet lysates, growth factors, and so forth. Importantly however, the use of such supplements is currently hindered by their cost, degradation in culture and thus their limited bioactivity. It is to be noted that the transfer of retained non-human antigens from serum may elicit an inappropriate immune response upon transplantation [179, 180], and therefore necessitates the use of human-derived components such as plasma or

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Figure 5. Mesenchymal stem cells (MSCs) in clinical trials and stem cell market forecast. **(A):** MSC-based clinical trials were charted by region based on search results sourced from https://ClinicalTrials.gov (retrieved July 1, 2017). **(B):** Scopus search results show the number of stem cell research articles published between 1995 and 2015, indicating the rising number of published studies on MSCs (retrieved July 1, 2017; https//:www.scopus.com).

serum platelet lysate as a suitable alternative [181, 182]. Sustaining the bioactivity of growth factors can be achieved by harnessing their interaction with GAGs such as heparan sulfate (HS) [100]. HS GAGs that bind to growth factors with high affinity can be purified using well-established chromatographic techniques [100, 183]. Introducing GAGs to the culture at the time of isolation, and preconditioning cells in HS appears to be a promising approach to improve MSC numbers while maintaining their characteristics. On the contrary, heparin has been shown to alter biological properties of MSCs and is not a recommended additive [184]. HS-GAGs could interact and protect growth factors from extracellular proteases, as well as from pH and thermal changes, so enhancing growth factor activity and downstream signaling, and ultimately stimulating MSCs to proliferate and be useful for tissue repair and regeneration. More recently, pre-conditioning strategies using BMP-2 and Wnt5a has proven useful for cartilage repair [185].

Economic Potential and Market Impact of MSC Research and Therapies

Stem cells are central components of regenerative medicine holding huge market potential that is projected to reach $170 billion by 2020, as per recent reports by Grand View Research, Inc. published in 2015. Several unmet medical needs drive the stem cell research economy. The consumers of this market are usually hospitals, clinical laboratories, stem cell banks, and academic institutes. Adult stem cells dominate the market as they do not raise the ethical controversy that surrounds embryonic stem cells, as well as due to relatively low production labor and maintenance costs, lower risk of tumors, and better immunocompatibility [186–189].

Human MSCs are currently administered for several clinical conditions, including bone, heart, neurodegenerative, and immunological disorders, and have reached phase I and II clinical trials [190]. We performed a search using the keyword "mesenchymal stem cell" in ClinicalTrials.gov in order to find the number of studies conducted worldwide. The potential of MSCs for clinical applications is supported by the fact that the clinical trials database currently lists nearly 650 clinical trials globally, excluding studies of unknown status (Fig. 5A) [Source: https://ClinicalTrials.gov]. Most of the trials in phase II are for conditions such as osteoarthritis, neurological diseases, pulmonary disorders, spinal cord injury, myocardial infarction, severe coronary ischemia, Crohn's disease, and diabetes mellitus. There is a strong correlation between global economic burden due to health disorders and the potential for stem cells to treat such ailments. We performed a Scopus search using strings "hematopoietic stem cells," "embryonic stem cells," "mesenchymal stem cells," "neuronal stem cells," "induced pluripotent stem cells," and "umbilical cord stem cells" to assess the number of research articles published between 1995 and 2015. Clearly, research trends keep pace with market trends alongside clinical trials (Fig. 5B), and it is anticipated that this industry will continue to open up, with products for cardiovascular, diabetes, and nerve repair becoming commercially available. To accelerate this, it is important that the new 3Rs (regulation, reimbursement, and realization of value) recently proposed by Caplan and colleagues are taken into consideration [191]. As MSC research continues to increase (Fig. 5B), the overall revenue for adult stem cell products is estimated at $10.9 billion by the end of this decade (Source: http://www.grandviewresearch.com).

In recent years, as cell preparations of MSCs become commercially available, several stem cell companies have formulated their own criteria for the selection of clinical grade cells: for example, the enrichment for STRO-1+ and STRO-3+ mesenchymal precursor cells by Mesoblast [64, 192], and the selection of MSCs secreting TNF-α receptor Type I at a minimum of 13 pg/10 million cells defined by Osiris Therapeutics [193]. Another important development was the identification of a subpopulation of MSCs by Smith and colleagues, which are characterized by their smaller size and rapid self-renewal potency. These cells are enriched for precursor cells that could be efficacious for therapy [194]. Stempeutics Research's specifications for their allogeneic BMMSC product, Stempeucel, includes parameters such as morphology (fibroblastic and spindle-shaped), cell counts of 180–220 million cells per bag, viability of $>$85%, ISCT-defined surface marker levels $>$80% along with CD166 $>$ 80% [71] and CD133 $<$ 5% as their release criteria for administration [195, 196]. As more strategies evolve and new criteria are published, the selection panel is continuously being developed. Therefore, it is essential to adopt broader characterization schemes if we seek to better understand MSC function and utility for commercial and clinical applications.

Conclusion

There is a compelling need to broaden the characterization landscape by identifying novel stable markers and refining selection

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criteria for establishing optimal classes of MSCs. Yet, current definitions of MSCs based on surface markers and/or differentiation parameters have so far been incomplete. Differences in the cellular phenotypes of MSCs can be attributed to the methods by which MSCs are isolated and expanded, ways of handling the cells, particularly seeding densities and media supplements, as well as other components of the culture conditions. The technical discrepancies in methods for defining MSC characteristics prevents general interpretations of results from stem cell laboratories or any beneficial effects of stem cell therapies observed in clinical trials with a range of stem cell preparations. Therefore, it is essential to obtain uniformity of methods for isolating and characterizing MSCs. To address ambiguities related to MSC identification and function, the ISCT criteria aimed to standardize isolation methods by serving as the basis for characterization of these cells, and to enable comparison of investigations among laboratories. This initiative is a key step in the right direction, but many more steps remain to be taken. Definition of novel biomarkers using genomic, epigenomic, transcriptomic, proteomic and metabolomic approaches, beyond the classical techniques that measure colony-forming ability, CD marker expression, telomere length and cellular morphology (among a myriad of other tests) may collectively provide for a new generation of highly sophisticated standardized tests as necessary quality control parameters for characterization of MSC preparations in clinical practice.

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Author Contributions

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Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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