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Concise Review: Bone Marrow-Derived Mesenchymal Stem Cells Change Phenotype Following In Vitro Culture: Implications for Basic Research and the Clinic

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ABSTRACT

Mesenchymal stem cells (MSCs) are increasingly being used in tissue engineering and cell-based therapies in all fields ranging from orthopedic to cardiovascular medicine. Despite years of research and numerous clinical trials, MSC therapies are still very much in development and not considered mainstream treatments. The majority of approaches rely on an in vitro cell expansion phase in monolayer to produce large cell numbers prior to implantation. It is clear from the literature that this in vitro expansion phase causes dramatic changes in MSC phenotype which has very significant implications for the development of effective therapies. Previous reviews have sought to better characterize these cells in their native and in vitro environments, described known stem cell interactions within the bone marrow, and discussed the use of innovative culture systems aiming to model the bone marrow stem cell niche. The purpose of this review is to provide an update on our knowledge of MSCs in their native environment, focusing on bone marrow-derived MSCs. We provide a detailed description of the differences between naive cells and those that have been cultured in vitro and examine the effect of isolation and culture parameters on these phenotypic changes. We explore the concept of "one step" MSC therapy and discuss the potential cellular and clinical benefits. Finally, we describe recent work attempting to model the MSC bone marrow niche, with focus on both basic research and clinical applications and consider the challenges associated with these new generation culture systems. STEM CELLS *2014;32:1713–1723*

INTRODUCTION

The discovery of plastic adherent, colony-forming cell populations derived from bone marrow, which were later shown to demonstrate trilineage differentiation potential, initiated the field of mesenchymal stem cell (MSC) research [1, 2]. In the context of cell therapy, MSCs offer several advantages over other candidate cell types such as embryonic or induced pluripotent stem cells, in that they are more readily available, can be used autologously, do not require extensive in vitro manipulation, and are generally associated with a lower risk of tumorigenicity. Previous reviews on MSCs have highlighted how microenvironmental changes are able to influence cellular phenotype [3–5], described known stem cell interactions in the bone marrow milieu [6–8], and explored the immunomodulatory properties of these cells from a clinical perspective [9]. Here, we bring together current knowledge of MSCs in their bone marrow niche environment and describe the dynamic nature of their phenotype in vitro. We compare the use of mononuclear cells (MNCs) versus MSCs in preclinical in vivo studies and in the clinic. Lastly, we discuss the need and potential translational benefits associated with the development of novel bone marrow niche culture systems.

MSC NICHES IN BONE MARROW

MSCs are thought to occupy anatomically distinct locations within the marrow; endosteal, stromal, and perivascular, with perivascular niches in both endosteal and stromal locations [4, 6, 10]. The phenotypical similarities of MSCs within their respective niches are currently unknown. Moreover, it is not apparent whether these discrete MSC populations are isolated and essentially self-replenishing, whether they are able to migrate from one niche to another in response to physiological cues or whether they exhibit functional differences. MSCs share their niche environments with many other cell types including osteoblasts and hematopoietic

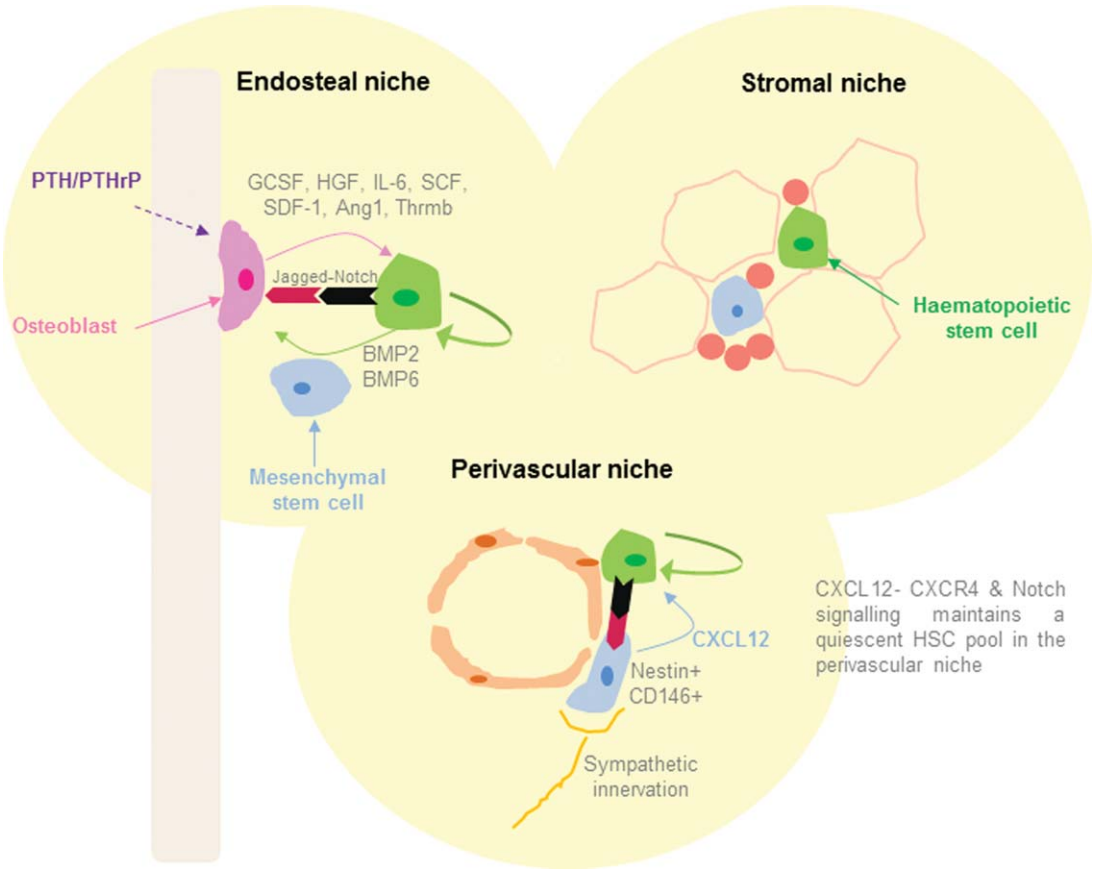

Figure 1. Schematic representation of the endosteal, stromal and perivascular mesenchymal stem cell niches in bone marrow. Within the endosteal niche, and under the control of parathyroid hormone/parathyroid related peptide (PTH/PTHrP), osteoblasts maintain hematopoetic stem cell renewal through Notch signaling and by the release of growth factors GCSF, HGF, IL-6, SCF, SDF-1, Ang1, and Thrm1. Hematopoietic cells stimulate the differentiation of mesenchymal progenitors into osteoblasts by BMP2 and BMP6 signaling. CXCL12-CXCR4 and Notch signaling between mesenchymal and hematopoietic stem cells maintains a quiescent hematopoietic pool in the perivascular niche. Interactions between mesenchymal and hematopoietic stem cells specifically at the stromal niche are less defined. Abbreviations: Ang1, angiopoietin-1; GCSF, granulocyte colony-stimulating factor; HGF, hepatocyte growth factor; IL-6, interleukin 6; SCF, stem cell factor; SDF-1, stromal cell-derived factor-1; Thrm1, thrombospondin-1.

stem cells (HSCs). These three cell types demonstrate crosstalk and appear to reciprocally regulate cell behavior and lineage commitment (Fig. 1).

Endosteal niche MSCs line the bone surface where they are physically associated with both osteoblasts and HSCs [6, 11]. Here, they provide a source of osteoprogenitors, and are also believed to contribute indirectly to osteogenesis by the secretion of growth factors and cytokines [12]. Osteoblasts secrete a plethora of hematopoietic growth factors including granulocyte colony-stimulating factor and hepatocyte growth factor [13, 14], angiopoietin [15], thrombopoietin [16, 17], Il-6, CXCL12 (also known as stromal-derived factor 1 [SDF-1]), and stem cell factor [11]. Under the control of parathyroid hormone/parathyroid related peptide, Notch signaling between osteoblasts and HSCs functions to expand the HSC pool while maintaining a primitive population of stem cells [11]. In turn, HSCs are capable of inducing the osteogenic differentiation of MSCs, which appears to be dependent upon BMP2 and BMP6 signaling [18]. Additional studies implicating monocytes [19], and specific osteoblast and mesenchymal progenitor subsets on HSC maintenance [20], highlight the complexity of cellular interactions in the bone marrow.

The vascular niche hypothesis for MSCs arose from in vitro observations demonstrating phenotypic similarity between pericytes and MSCs [21, 22]. This was later confirmed by the in vivo localization of a population of self-renewing, multipotent progenitor cells at perivascular sites in bone marrow and other tissues [23, 24]. CD146+ osteoprogenitors, termed adventitial reticular cells, were identified in the endothelium of marrow sinusoids and following ectopic transplantation into mice, were shown to induce the formation of bone and an associated hematopoietic marrow component [23]. A breakthrough study by Méndez-Ferrer in 2010 revealed that perivascular MSCs play a critical role in maintaining a quiescent HSC pool in bone marrow [6]. Nestin+ MSCs were found to colocalize with HSC at perivascular locations in the endosteum and stroma, express HSC maintenance genes, including CXCL12, and were associated with sympathetic nerve fibers which have previously been shown to regulate HSC trafficking into the bloodstream [25–27]. Depletion of Nestin+ MSCs reduced both the number of endogenous HSCs and their homing ability following transplantation, to the marrow in a lethally irradiated mouse model [6]. The importance of the CXCL12-CXCR4 signaling pathway with

regard to HSC mobilization has been known for over a decade [28]. CXCL12 was first discovered to be expressed by osteoblasts and endothelial cells [29], and later by stromal cells lining the endosteal surface and surrounding stromal sinusoids, termed CXCL12-abundant reticular cells (CAR cells) [30]. CAR and Nestin+ MSCs occupy similar locations and produce CXCL12, but whether these cells denote equivalent MSC populations or whether Nestin+ MSCs represent a more naive stem cell population is still unclear. A recent multiple knockout mouse study whereby CXCL12 expression was selectively deleted from (a) endothelial cells and mature osteoblasts, (b) osteoprogenitors and CAR cells, or (c) all mesodermal-derived cells revealed niche cell specific functions of this signaling pathway on HSC maintenance [31]. Results of this study suggest that osteoprogenitors and/or CAR cells serve to maintain the HSC pool and support B-lymphoid progenitor survival. CXCL12 knockout from mesodermal-derived cells (including MSCs) resulted in increased HSC cycling, indicating a role for maintaining HSC quiescence. In addition to the CXCL12-CXCR4 axis, Notch signaling has also shown to play a fundamental role in the ex vivo expansion and maintenance of HSCs by CD146+ Nestin+ MSCs [32].

Pericytes and MSCs

Functioning to maintain vessel maturation and stability, pericytes are typically identified by the cell surface marker expression of NG2 [33], platelet-derived growth factor (PDGF) receptor-beta [34], and CD146 [35]. In vitro cultured pericytes exhibit a marker profile and multipotent differentiation potential similar to that of MSCs, making discrimination between the two cell types difficult [21, 22, 24]. It has been proposed that pericytes represent the primitive ancestor cell of MSCs in vivo [24]. Two alternative hypotheses exist, that pericytes are simply MSCs in a perivascular locale or that they represent a distinct MSC subset population. A study comparing both primary and commercially available pericytes to heterogeneous MSC populations in endothelial coculture angiogenesis assays has indicated functional differences between these two cell types in vitro [36]. CD146+ MSC maintained endothelial tube-like formation on Matrigel™ and supported endothelial spouting, whereas CD146− MSC did not. Interestingly, initially CD146− MSC acquired CD146 expression following culture, making them distinguishable from pericytes only on the basis of angiogenic function. This work provides evidence that pericytes represent a distinct cell population in their own right. However, in the absence of comprehensive cell lineage tracing studies, whether pericytes indeed represent the common ancestral cell of all or discrete MSC subpopulations has yet to be determined. What is clear is that bone marrow contains a heterogeneous population of multipotent stromal stem cells. Attempts to define and discriminate between these cells have resulted in an ever growing repertoire of nomenclature; MSCs, mesenchymal progenitor cells, adventitial reticular cells, CAR cells, and pericytes, leading to a certain degree of ambiguity and confusion within the field. Are these cell populations distinct or are they simply the same cell in a different location that has adopted a phenotype in response to a change in microenvironment? A greater understanding of the developmental origin and differentiation pathways of MSCs, in vivo behavior, and the development of robust cellular identification methods may allow us to better understand the biology of these heterogeneous cell populations and fully appreciate their clinical relevance.

Isolation of MNCs and MSCs

In the absence of standardized isolation and culture expansion protocols for MSCs, the way in which these cells are cultured in vitro varies considerably between research groups. MSC isolation procedures typically use density centrifugation (with Ficoll™, Lymphoprep™, or Percoll™ density mediums) to separate the MNC fraction from the other marrow constituents (i.e., red blood cells, plasma, and lipids). This MNC fraction contains an enriched population of T cells, B cells, monocytes, HSCs, endothelial progenitor cells, and MSCs. Following plating onto tissue culture flasks, MSCs, which represent the adherent cell population, form colonies. It is generally assumed that MSCs adhere within a few days after initial seeding and that the cell culture is rid of contaminating, non-adherent hematopoietic cells following serial media changes.

There is concern that the use of different MNC isolation protocols between laboratories may result in phenotypic differences of both MNCs and MSCs that could subsequently affect functional outcome following their use in cell-based therapies. Differences in how marrow aspirates are extracted and processed, choice of density medium, wash and centrifugation steps, duration of cell attachment, and media/serum type may each play a role. This has been demonstrated by discrepancies in clinical outcome between two similar, randomized, placebo-based controlled trials investigating MNC therapy for the prevention of cardiac failure postmyocardial infarction. The REPAIR-AMI trial [37] reported improved left ventricular ejection fraction following MNC therapy versus placebo, compared to the ASTAMI trial which showed no improvement versus placebo [38]. A comprehensive study by Seeger et al. [39] demonstrated that the combined differences in MNC isolation protocols between these trials likely accounted for the disparity in clinical outcome. The trials used different density media, centrifugation speeds, wash steps, and storage conditions. Seeger et al. [39] found that the protocol used in the REPAIR-AMI trial resulted in improved MNC yield, CFU (colony-forming unit) efficiency, chemotactic response of MNC-derived MSC to SDF-1, and outcome of vascular repair in a mouse model of hind limb ischemia, which was concomitant with the reported improvement in clinical outcome. Despite this, another comparative study reported improved MNC yield, yet no significant difference in CFU-efficiency when comparing protocols used in the REPAIR-AMI versus ASTAMI trials [40]. Furthermore, other factors such as the proportion of apoptotic cells [41] and degree of red blood cell contamination [42] have been linked with the clinical efficacy of MNCs.

The density centrifugation process itself reduces the total yield of MNCs from bone marrow [40, 43, 44]. Interestingly, plating whole bone marrow directly has been reported not only to increase CFU-efficiency but also result in a population of MSC demonstrating longer telemore length versus isolation protocols using either Ficoll™ or Percoll™ [43]. This suggests

that a proportion of more naive MSCs may be lost as a result of density centrifugation. This has led others to use magnetic bead separation techniques which deplete the polymorphonuclear cell population on the basis of granularity or expression of CD15. MNC recovery from both rat and human bone marrow was reported to be highest after magnetic-activated cell sorting separation, followed by Percoll™ and then Ficoll™ density centrifugation (25.6±5.8%, 51.5±2.3% and 72.3±6.7% cell recovery, respectively) [44]. Conversely, other studies directly comparing the effects of Ficoll™ to Lymphoprep™ [40] or Percoll™ [43] showed no significant difference in MNC yield.

Importantly, there is an increasing body of evidence to suggest that an MSC population is present in the initial, nonadherent MNC fraction [45–48]. This initially nonadherent MSC population is capable of forming CFU upon serial plating [48]. Importantly, the survival and subsequent expansion of these cells were shown to be critically dependent upon FGF_2 signaling. These MSCs demonstrated greater proliferation and trilineage differentiation potential in vitro and improved osteogenesis in vivo compared to traditionally isolated cells. Together, these studies suggest the existence of heterogeneous MSC populations in the MNC fraction, and that those in the initially nonadherent fraction may represent a more naive stem cell population. Because of the apparent selection of an MSC population from the original marrow aspirate, we would propose that the term monolayer selection is adopted when discussing the isolation and culture of MSC using the above described techniques.

Expansion of MSCs in Monolayer

At p0, morphologically distinct MSC populations are present; small, spindle-shaped, proliferative cells, and larger, flattened cells which replicate at a comparably slower rate [49, 50]. Cell populations tend to become more homogenous in terms of appearance with subsequent in vitro expansion. It is widely acknowledged that, following extended in vitro culture, MSCs undergo replicative senescence [49–51]. The onset of growth arrest is subject to significant donor variation, and as a result reports range between 10 and 38 population doublings [49–52]. Many groups describe MSC growth kinetics in terms of passage number rather than population doublings which makes data interpretation and comparison between studies difficult. Although some groups have attempted to correlate donor age to proliferation rate [52, 53], donor age alone is not a reliable predictor of senescence. Rather, differences in growth rate are likely attributed to sampling variation during aspiration and the number of highly proliferative cells that are originally isolated [54]. Such sampling variation has been observed between MSC cultures acquired from two separate aspirates taken from the same donor [55]. Growth arrest of MSCs is associated with telomere shortening [52, 56]; however, epigenetic modifications such as DNA methylation may also play a role [55, 57]. Comprehensive profiling studies reveal that changes in gene expression occur immediately after isolation and are continuously acquired during culture [58]. Specifically, genes associated with the cell cycle, DNA replication, and repair become downregulated in senescent cultures [58]. After prolonged in vitro culture, MSCs appear to lose multipotency and display a propensity toward osteogenic differentiation [51, 56, 58]. Osteogenic lineage commitment appears to coincide with a reduction in proliferation and gene expression changes [56, 58]. Other studies report donor-dependant differential gene expression of integrins [59], extracellular matrix molecules, growth factors, and cytokines, including CXCL12 [60] throughout culture.

Seeding Density

Typical MSC seeding densities range between 2,000 and 5,000/cm^2; however, there is evidence to suggest that lower seeding densities enhance proliferation, which is thought to be attributed to a reduction in contact inhibition [59, 61–63]. Importantly, lowering seeding density does not appear to affect CD (cluster of differentiation) marker profile or in vivo osteogenic capability [61] or trilineage differentiation in vitro [63]. Similar improvement in proliferation was demonstrated following low density culture of MSCs supplemented with platelet lysate (PL) under GMP (good manufacturing practice)-compliant conditions without affecting CD marker profile or multipotency [59]. These findings have important translational implications for the optimization of large-scale clinical-grade cell expansion of MSCs for cell-based therapies.

Media Supplementation

MSCs are most commonly expanded in a basal media such as Dulbecco's modified Eagle's medium (DMEM)/DMEM F-12 or alpha-MEM with 10% fetal bovine serum (FBS). Widespread batch-batch variability in FBS constitutes the need for serum testing in order to provide optimal growth conditions. Many laboratories avoid the practice of serum-testing using commercially available MSC-qualified serum, at considerable cost. Alternatively, MSCs may be cultured using autologous serum (AS), PL, platelet-rich plasma (PrP), or under serum-free conditions using growth factor supplementation (for a detailed review see Tonti et al. [64]). MSCs expanded in FBS are reportedly less proliferative and subject to extensive gene expression changes compared to MSC expanded in AS [65]. FBS-supplemented MSCs upregulated expression of genes associated with cell-cycle inhibition and trilineage differentiation, whereas the transcriptome of MSC cultured with AS was comparably stable, suggesting AS may maintain MSCs in a more primitive state [65]. To our knowledge, there is one study in the literature directly comparing FBS to MSC-qualified serum upon primary human MSCs which reported no difference in growth rate, cell-surface marker expression, or adipogenic/osteogenic differentiation [66]. Notably, in the aforementioned study, MSCs were isolated from cancellous bone chips and additionally supplemented with epidermal growth factor, basic fibroblast growth factor, PDGF-BB, and dexamethasone.

Compared to FBS, PrP appears to improve the MSC proliferation rate [67–69]; however, the associated increase in expression of *runx2*, *sox9*, and *aggrecan* suggests that it may encourage early lineage commitment [68]. Moreover, PrP supplementation reduces both SDF-1 levels and the migratory effects of MSC secretome on HSCs [69]. When compared with either FBS or AS, PL has been reported to improve the proliferation rate without significantly affecting CD marker profile or in vitro differentiation potential [70–73]. The stimulatory effects of PL on MSC proliferation have been attributed to the presence of heat-denaturable factors, but comparable

growth rates using growth factor cocktails under serum/PL-free conditions have not yet been achieved [73]. Notably, PL supplementation is also associated with changes in MSC gene expression [71] and secretome profile [72]. Interestingly, both proliferation rate and osteogenic differentiation appear to be enhanced by PL from younger (<35 years) versus older (>45 years) donors; however, this was not attributed to individual or combinatorial levels of growth factors; PDGF-AB, TGF-b1, FGF, IGF-1, or hormones; estradiol, parathormone, leptin or 1,25 vitamin D_3 [74]. Tarte et al. [75] reported superior MSC growth with a combination of FBS and FGF_2 compared to PL using commercially available MSC lines. FGF_2 is a potent mitogen of MSCs, reported to maintain multipotency [48] and enhance chondrogenic differentiation in vitro [76–78]. Expansion of MSCs with FGF_2 supplementation has been shown to reduce CD146 expression in both primary cells [79] and commercially available cell lines [80], although the resulting phenotypic significance of this remains unclear. For most applications, the use of FGF_2 in routine MSC culture appears justified; however, in the absence of standardization, concentrations ranging between 0 and 10 ng/ml are reported, making comparisons between research studies difficult.

It is evident that the monolayer expansion phase of MSCs results in loss of multipotency, the ability to self-renew, and promotes a tendency toward osteogenesis. Thus, monolayer expanded cells are not truly representative of naive MSCs in the bone marrow which should be borne in mind for both basic research and possible ensuing clinical translation. Culture media type and the use of supplements clearly affect MSC genotype and phenotype and should be considered when selecting optimal culture conditions for clinical scale-up. Whether differences between in vitro culture protocols and subsequent modification of cellular phenotype are likely to affect functional outcomes following the in vivo transplantation of MSCs still remains unclear and warrants further investigation.

Cell Surface Marker Expression

In the absence of a universal, MSC-specific marker, MSCs are currently identified by a repertoire of proteins expressed on the cell surface. CD markers such as CD73 [2, 81] and CD105 [2, 82] were established as positive MSC markers over a decade ago. Proposed by the International Society for Cellular Therapy in 2006, a recommended panel of positive and negative cell surface markers is now commonly used to characterize MSCs [83]. In line with their report, MSCs are defined as being >95% positive for: CD105, CD73, and CD90 and >95% negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR. There are also a number of other positive cell surface markers that are expressed by MSCs: CD44 [2], CD166 [84, 85], Stro-1 [86], CD106 [2, 87], and CD146 [85, 87]. However, the function and significance of these markers in both a biological and therapeutic context are poorly understood. Moreover, reports of identical CD marker profiles between donor matched MSC and fibroblasts following monolayer expansion bring their specificity into question [88].

It is becoming increasingly apparent that cell surface marker expression profiles of in vitro expanded MSCs differ compared to both freshly isolated cells and those residing in their bone marrow niche environment. Table 1 provides a summary of such changes in marker expression, with an emphasis on primary human bone marrow-derived MSCs. Whereas CD73 and CD105 appear to be constitutively expressed regardless of environment [60, 85], the expression of CD44, CD271, CD146, and CD106 appears to change as a result of in vitro culture [59, 60, 85, 89]. Using a combination of multicolor flow cytometry analysis and CFU assays, an enriched population of MSCs has recently been identified in the CD44− subset of freshly isolated bone marrow MNCs from both mice and humans [60]. The authors demonstrated that CD44 expression was acquired early on in the in vitro expansion phase (both human and mouse). Additionally, DNA microarray revealed CD73 and CD146 were upregulated while CD271 and VCAM were downregulated following monolayer culture [60]. The observed upregulation of CD146 expression is in accordance with Blocki et al. [36] who demonstrated that initially CD146− MSC populations acquired expression of this putative pericyte marker during in vitro expansion [36]. Conversely, a reduction in CD106 and CD146 following monolayer culture has also been reported [89]. Discrepancies between such studies may be due to a combination of different culture conditions, donor variation, and differences in methodology such as antibody clone, immunostaining protocol, and gating/analysis of flow cytometry data. A recent immunohistochemical study has revealed different MSC marker expression profiles by distinct MSC niche populations in bone marrow [10]. Coexpression of neural ganglioside GD2 by endosteal and perivascular CD73+ MSC confirmed previous work implicating this neural protein as a novel MSC marker [90]. Interestingly, stromal cells identified at endosteal or stromal niches but not from perivascular locations expressed Oct4, Nanog, and SSEA-4, suggesting endosteal and stromal niche MSCs may represent a more primitive stem-cell population. Whether alterations to cell surface marker expression are indicative of phenotypic changes such as loss of multipotency has yet to be determined. A better understanding of putative MSC markers and their functions may allow us to use CD marker expression profiles as a predictive tool for cellular behavior both in vitro and in the clinic.

MNCs Versus In Vitro Expanded MSCs

Culture expanded MSCs have been used clinically for the treatment of various conditions including bone [92–94] and cartilage defects [95–97], acute myocardial infarction [98–100], and spinal cord injury [101–103]; however, complete, permanent functional recovery has yet to be demonstrated. The pronounced phenotypic changes associated with monolayer expanded MSCs have turned attention toward using unprocessed bone marrow or freshly isolated MNCs (Fig. 2). This approach has been supported by the development of bone marrow concentrating devices that can be used in the operating room [104]. On a cellular level, this may preserve MSC multipotency, their ability to self-renew, and homing capacity. This may not only strengthen therapeutic potency but also avoid treatment delay and extensive costs associated with the in vitro expansion phase. Currently recognized as an advanced therapy medicinal product by the European Medicines Agency, and as a human cell, tissue, and cellular and tissue-based product by the Federal Drug Agency, clinical grade MSCs must

Table 1. A comparison of cell surface marker expression profiles of naive, freshly isolated, and monolayer expanded mesenchymal stem cells

Marker	In vivo	Freshly isolated	Post monolayer expansion	Notes
CD44	?	− Qian et al. [60]	+ Qian et al. [60]	Mouse and human
			+ Halfon et al. [89]	Human
CD73	+ Rasini et al. [10]			Human, immunohistochemistry
		+ Jones et al. [85]	+ Jones et al. [85]	Human, previously freeze thawed
		+ Qian et al. [60]	+ Qian et al. [60]	Mouse and human.↑ Gene expression with time in culture
			+ Fekete et al. [59]	Human
CD105		+ Jones et al. [85]	+ Jones et al. [85]	Human, previously freeze thawed
	?	+ mouse/ low human, Qian et al. [60]	+ Fekete et al. [59]	Human, ↑gene expression with time in culture
				Human
CD90	?	?	+ Fekete et al. [59]	Human
CD166	?	?	+ Fekete et al. [59]	Human
CD271	+/− Rasini et al. [10]	–		Human, niche location dependant, immunohistochemistry
		+ Qian et al. [60]	− Fekete et al. [59]	Human
		+ Cox et al., 2012 [136]		Human
				Human, ↓ cell surface marker labeling with time in culture
CD146	+/− Rasini et al. [10]			Human, niche location dependant, immunohistochemistry
		+ Qian et al. [60]	+ Jones et al. [85]	Human, ↑gene expression during culture
		+ (variable) Jones et al. [85]	+ Halfon et al. [89]	Human, freeze thawed
		+/− Blocki et al. [36]	+ Blocki et al. [36]	↓ With time in culture
				Initially negative cells acquired expression during culture
CD106	?	+ (variable) Jones et al. [85]	+ Jones et al. [85]	Human, previously freeze thawed
		+ Qian et al. [60]	+ Halfon et al. [89]	Mouse and human
				↓ Cell surface marker labeling with time in culture
GD2	+ Rasini et al. [10]			Human, niche-dependent, immunohistochemistry
		+ Martinez et al. [90]	+ Martinez et al. [90]	Human
SCA1		+ Qian et al. [60]		Mouse
	+ Morikawa et al. [91]	+ Morikawa et al. [91]	?	Mouse
	+ Nakamura et al. [20]	+ Nakamura et al. [20]		Mouse
Stro-1		+ Qian et al. [60]		Human
	?		+ Simmons et al. [86]	Human
Nestin		+ Qian et al. [60]	?	Mouse
	+ Méndez-Ferrer et al. [6]	+ Méndez-Ferrer et al. [6]		Mouse
PDGFr	?	+ Qian et al. [60]	?	Mouse

Positive (+) and (−) cell surface antigen expression detailed accordingly.
Note: Cell surface antigen expression ascertained by flow cytometry unless otherwise stated.
Abbreviations: CD, cluster of differentiation; GD2, neural ganglioside 2; PDGFr, platelet-derived growth factor-1 receptor; SCA1, stem cell antigen-1; Stro-1, stromal-derived factor-1.

be cultured under defined GMP compliant conditions in Europe and U.S., respectively. As our knowledge of these cells increases, so does the regulatory framework governing their use which has and will undoubtedly continue to increase the cost of their use in cell-based therapies. With this in mind, the development of GMP compliant one-step MSC treatments may offer both clinical and economic advantages compared to using monolayer expanded cells. One limitation of a one step intra-operative approach could be the reduced number of MSCs delivered to the patient compared with current MSC-based therapies, where cell doses ranging between 1 and 100 million cells are described. Nevertheless, there is little supporting evidence in the literature to correlate cell number with clinical efficacy [9]. Our lack of knowledge and understanding regarding the clinical implications of cell dose represents a major unknown in the field which is constraining development of translational therapies and is thus deserving of our full attention.

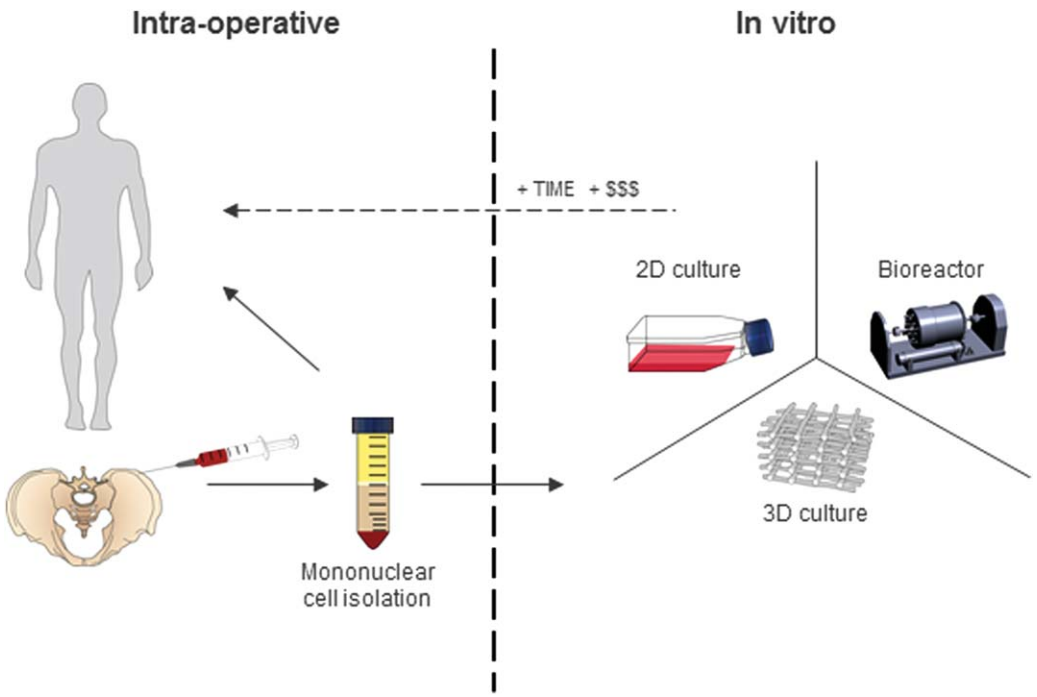

Figure 2. Intraoperative versus in vitro approaches to mesenchymal stem cell therapy and tissue engineering.

Bone marrow transplantation has been used effectively for the treatment of osteonecrosis [105] and fracture nonunions [106–109]. The therapeutic effects of bone marrow are thought to be attributed to its constitutive MNC population. In turn, MNCs are postulated to promote tissue regeneration via several mechanisms (a) by providing a source of progenitor cells (including MSCs) that contribute directly to tissue formation, (b) by producing growth factors which promote repair actions of native cells, and (c) through modulation of resident cell behavior by direct cell-cell signaling. MNCs may also provide vascular cell types and release angiogenic factors which promote angiogenesis, further aiding tissue regeneration. The regenerative capacity of MNCs has been reported in spinal fusion [110] and in animal models of bone [111] and cartilage repair [112, 113]. Conversely, clinical trials investigating intracoronary [114–116], intramyocardial [117], and transendocardial [118] MNC injection post myocardial infarction have demonstrated safety but little or no improvement in clinical outcome. The few existing comparative studies of MSC versus MNC tissue repair in the literature are inconclusive as to the superiority of one cell source over the other. Limb perfusion and capillary density were greater following MSC versus MNC transplantation in a rat model of hind limb ischemia [119]. This was corroborated by improved healing and clinical outcome using MSCs compared to MNCs in a pilot cell therapy study for the treatment of diabetic critical limb ischemia [120]. Conversely, in the case of bone repair, improved bone growth was observed following MNC versus MSC transplantation in patients with osteogenesis imperfecta [121]. Interestingly, MNCs were shown to contribute directly to bone formation whereas MSCs elicited a healing response via a paracrine mechanism [121]. Both MNCs and in vitro expanded MSCs have demonstrated safety and clinical efficacy in various preclinical in vivo models and in clinical cases of tissue regeneration; however, whether one cell source is superior to the other has yet to be elucidated. The answer may be tissue specific and is likely to be dependent upon other factors such as cell isolation procedure and route of delivery. A better understanding of the mechanisms responsible for their regenerative capacity is required in order to optimize their use in cell-based therapies.

Modeling the MSC NICHE in Bone Marrow

The development of one-step MSC and MNC therapies requires a greater understanding of the biology of these cells, which has prompted the need to develop new three-dimensional (3D) in vitro culture systems [122]. As reviewed by Sart et al. [123], several groups have demonstrated the advantages of postmonolayer expanded spheroid/aggregate culture systems with regard to maintaining MSC multipotency and their secretion of trophic factors [124–129]. A 3D culture system for freshly isolated MNCs would allow one to better characterize naive MSCs and determine how they could be manipulated in the clinic biochemically/genetically to enhance their therapeutic efficacy. Moreover, development of a MSC bone marrow niche model would improve our understanding of MSCs in their native environment; allow us to study their interactions with other bone marrow cells and how aspects of their behavior such as proliferation, differentiation, and migratory capacity are regulated. Ultimately, an improved knowledge of the MSC bone marrow niche may enable us to develop targeted pharmacological approaches that exploit the innate homing ability of these cells facilitating noninvasive tissue repair. Furthermore, such 3D culture systems may offer a

platform for drug screening, model to study bone marrow disorders such as leukemia, and possibly an improved method of in vitro expansion of MSCs and HSCs for clinical application.

One group have previously developed a perfusion culture system whereby MNCs were cultured in ceramic-hydroxyapatite scaffolds [130, 131]. This system generated constructs containing viable CD105+ and CD45+ cell populations which subsequently ossified ectopically following in vivo implantation into nude mice [131]. More recently, Claros et al. recently described a MNC culture system using type I collagen gels. The proportions of cells expressing typical MSC markers and CD45 changed during the culture period; however, it was not entirely clear whether this was attributed to cell loss (either due to poor retention/cell death) or differential proliferation rates between cell populations [132]. Notably, Oct4 and Nanog expressions were detected at day 14 but absent from MSC cultures expanded in monolayer. Whether the presence of these multipotent stem cell markers was representative of naive MSC or HSC populations is unclear. Another study reported the culture of unprocessed bone marrow into calcium phosphate scaffolds [133]. Immunohistochemical detection of CD105 and CD45 suggested this culture system supported MSC expansion but was not as supportive of the hematopoietic fraction. Increasing cell yield, while maintaining stemness, represents a significant challenge for the in vitro expansion of clinical grade HSCs. With this in mind, others have investigated the role of MSCs on HSC proliferation using 3D coculture systems. MSCs derived from Wharton's jelly were shown to stimulate the proliferation of cord blood-derived HSCs in fibrin-polycaprolactone scaffolds [134]. The same group went on to demonstrate the mitogenic and chemotactic effects of both Wharton's jelly-derived and bone marrow-derived MSCs on HSCs in collagen gels [135]. In summary, the development of culture systems able to support bone marrow niche cells, including naive MSCs, has yet to be achieved. Parameters such as media type, seeding density, nutrient/oxygen gradients, and scaffold characteristics represent important considerations when designing such models.

Conclusions

It is becoming increasingly apparent that monolayer expansion is not only selective, but leads to distinct changes in MSC phenotype, leading us to question what aspects of observed behavior are representative of in vivo behavior and which are artifact. This issue is potentiated by the absence of standardized isolation and culture techniques which has potentially serious implications for the development of effective MSC-based therapies. We suggest that the global standardization of culture parameters, although practically a challenge, would improve consistency between research groups and ultimately enhance the quality and impact of MSC research. For the development of both one-step and intrinsically targeted MSC therapies, there is a real need to develop a more suitable in vitro culture system that maintains MSCs in a naive, multipotent state as they are in bone marrow. The development of such a culture system poses significant challenges both conceptually and practically. As our knowledge of the bone marrow MSC niche grows, so does the complexity of the culture system required in order to accurately model it. In summary, a successful bone marrow MSC niche model would not only allow us to better understand the intrinsic function and reparative properties of these cells but may also allow us to develop more economical and clinically effective cell therapies.

Author Contributions

J.B.: concept, research of literature, and manuscript writing; M.S.: concept, manuscript writing, revision, and final approval; M.A.: concept, revision, and final approval; G.R.: manuscript writing, revision, and final approval.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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