Review

Human bone marrow mesenchymal stem cells in vivo

E. Jones and D. McGonagle

Great confusion still exists amongst cell biologists, musculoskeletal and other specialists interested in regenerative medicine regarding the in vivo identity of human bone marrow (BM) mesenchymal stem cells (MSCs). Contrary to views held in some quarters, methods for the robust identification and purification of BM MSCs are now well established. Human BM MSCs represent a phenotypically homogeneous cell population that share an identical phenotype with marrow adventitial reticular cells (ARCs), which are stromal cells similar in nature to pericytes. When an extensive panel of markers is used to characterize BM MSCs, it appears that the diverse MSC markers described in different laboratories are expressed on the same cell population. Rare cell phenotypical analysis and in vivo MSCs including CD73 and CD105 and the development of robust CFU-F assays produce no compelling evidence that BM MSCs circulate in healthy man. Furthermore, although investigators speak of a number of specific MSC markers, a true marker of MSC ‘stemness’ and multipotentiality has not yet been defined since culture-expanded MSCs may lose some of these markers, but remain multipotential. This knowledge provides a platform for understanding MSCs in vivo leading to novel approaches for therapy development, including in situ tissue engineering.

KEY WORDS: Mesenchymal stem cells, Surface markers, In vivo topography, MSCs in circulation.

Background

The concept of a mesenchymal stem cell (MSC) arose from the work of Friedenstein and colleagues four decades ago [1]. They noted that upon plastic adherence of bone marrow (BM) cells, a rare cell population developed into colony forming units that were fibroblastic (CFU-F) [2]. Following in vitro culture expansion, clonal cultures derived from individual CFU-Fs could be introduced into diffusion chambers in experimental models where the formation of bone, cartilage and stromal elements was observed. The frequency of these marrow CFU-Fs was extremely low, ranging from 1/10 000 to 1/100 000 BM mononuclear cells (MNCs) [3]. This is considerably lower than the frequency of CD34+ haematopoietic progenitor/stem cells (HSCs) that comprise about 1% of the MNC fraction [4]. Therefore, it was long recognized that the study of BM MSCs in vivo would represent a much tougher challenge than the study of HSCs. The interest in MSCs increased greatly almost a decade ago with the reporting of novel markers for culture-expanded MSCs including CD73 and CD105 and the development of robust in vitro assays of MSC tripotentiality [5]. Some investigators suggested that these findings were erroneously celebrated by the scientific community and media as the happy outcome of an extraordinary hunt for MSCs [6]. Indeed, the studies in question described the same culture-expanded CFU-F population that went back as far as Friedenstein’s work, and the identity of the unknown ancestral cell remained enigmatic. The only firm clue to the in vivo identity of BM MSCs came from the work of Simmon’s group [7] who showed that an antibody Stro-1 could be used to enrich CFU-Fs approximately 100-fold; however, their purification was still not feasible.

The ongoing confusion in the MSC field has been contributed to by the assumption that any marker expressed on culture-expanded MSCs was also likely to be present in vivo. Consequently, independent laboratories have begun to use different markers of expanded MSCs to search for MSCs in vivo [8, 9]. This has resulted in the perception that these in vivo MSCs were a heterogeneous cell population, and could be distinct from Stro-1+ stromal cells and progenitors. Indeed, the confusion to the in vivo identity of the BM MSC has lead to difficulty with terminology whereby the MSC acronym continues to signify both MSCs and marrow stromal stem cells [5, 6, 10]. Based on our studies of in vivo MSCs and related literature, the purpose of this article is to reconcile these apparent contradictions and to discuss their implications for regenerative medicine development.

Markers for the in vivo identification of MSCs

There is still a widely held perception that BM MSCs represent a phenotypically heterogeneous population of cells. There are a number of reasons for this. First, as far back as the pioneering work of Friedenstein et al. [11, 12], it has been recognized that not all CFU-Fs were highly proliferative and multipotential. Second, many different groups have used a limited number of diverse phenotypic markers to identify in vivo MSCs/CFU-Fs using magnetic enrichment or flow cytometry [8, 9, 13–17]. Taking a synthesis of these functional and phenotypic data to a logical (but not necessarily correct conclusion) has led to the impression that MSCs were both functionally and phenotypically heterogeneous.

To clarify this, we used multiparameter flow cytometry and cross-tested different MSC markers and purification methods, including plastic adherence for their selectivity and specificity for in vivo BM MSCs [14, 18]. We found that all these methods identified a phenotypically identical rare cell population that was distinct from BM haematopoietic cells by their very low CD45 expression and a larger cell size. The Stro-1 marker was uniformly expressed on these cells [14], but its cross-reaction with other BM populations somewhat limited its utility for BM MSC identification. Another disadvantage of the Stro-1 antibody related to the fact that its target antigen has not thus far been defined, despite the fact it has been over 15 yrs since its original discovery.

With this in mind, we next aimed to find the best positive marker for BM MSCs based on the criterion of the highest expression on MSCs and the lowest expression on all other marrow cell populations. As a result, it was demonstrated that the low-affinity nerve growth factor receptor (LNGFR), now clustered as CD271, was the most differentially expressed marker [14, 19]. Importantly, high-level expression of CD271 on BM MSCs was reported in at least four other independent studies [13, 20–22]. These observations have lead to the recent commercialization of CD271 as a preferred marker for the purification of...
a homogeneous population of cells that contains all the BM MSC activity [23]. Notably, another neural molecule (ganglioside molecule GD2) has been recently shown to be expressed on BM MSCs, both in vivo and following expansion in culture [24].

In the past, we were also able to show that both CD73 and CD105 were uniformly present on BM CD45<sup>−</sup>CD271<sup>+</sup> cells [18], as were many other putative MSC markers such as CD90 [14]. With the exception of CD146/Muc18, which showed a wide spectrum of positivity from low to brightly positive cells, every other marker tested in our studies has demonstrated no apparent heterogeneity and was either uniformly positive or uniformly negative on MSCs. Of course, with the passage of time and increased availability of good-quality commercial antibodies, further phenotypic heterogeneity within BM CD45<sup>low</sup>CD271<sup>+</sup> MSCs may be found, parallel to past discoveries in the HSC field, when CD34<sup>+</sup> population was later subdivided into CD34<sup>+</sup>CD38<sup>+</sup> and CD34<sup>+</sup>CD38<sup>−</sup> populations [25]. Nevertheless contrary to some currently propagated views [26, 27], a phenotypically distinct, in vivo BM MSC population has now been identified. Importantly, a striking consensus regarding the morphology of fresh MSCs is emerging, regardless of the method of isolation used. They appear as large cells that have prominent nucleoli and bleb-like projections, which extend further as MSCs adhere—this is different from the spindle-shaped morphology of typical cultured MSCs [14, 15, 21].

Based on functional assays, the presence of MSCs in extra skeletal locations including synovium, fat and even placental tissue and umbilical cord has been firmly established [28-31]. Identifying the MSC population from the much larger stromal fraction will be a more formidable challenge compared with MSC identification in the marrow. There has been a common opinion that CD73, CD105, CD90 and CD44 are highly specific for MSCs, and hence can discriminate multipotent cells from the more mundane tissue resident fibroblasts. More recently, however, several studies showed that these markers were ubiquitously expressed on stromal cells from many locations as well as on skin fibroblasts [32-34], and at best they only inform an investigator that the phenotyped cells are non-haematopoietic and stromal in origin. Of note, we originally utilized a fibroblast marker termed D7-FIB to purify MSCs from the BM and showed that these cells expressed other fibroblastic markers such as CD10 and CD13 [14]. In the BM, where the overwhelming majority of cells are haematopoietic, these markers may indeed be useful, but in connective tissues, where most of the cells are fibroblastic, their utility for the isolation of resident MSCs will be limited and a search for new, more specific markers, if they indeed exist, is needed. For the isolation of MSCs from post-partum tissues, such as placenta, an embryonic stem cell marker SSEA-4 was found to be useful [30] and, more recently, it was successfully applied for the isolation of MSCs from adult BM [35]. It remains to be investigated whether SSEA-4 identifies all, or only a subpopulation, of CD45<sup>−</sup>CD271<sup>+</sup> cells.

Another important issue to bear in mind is the stability of putative MSC markers in culture. Despite the loss of certain markers following passaging [7, 14] and the gain of others [18], MSC cultures remain multipotent, indicating that these markers are unlikely to be reflective of the MSC’s true ‘stem cell’ nature or its multipotentiality. More likely, many markers present on MSCs in vivo may be induced by the BM microenvironment or be reflective of some other MSC function in vivo that is lost upon plastic adherence and exposure to culture media. At this stage, it would appear that the heterogeneity in the MSC proliferative and differentiation capacities, first noted by Friedenstein et al. [1] cannot be explained on the basis of known surface markers alone.

**Marrow topography of MSCs**

Having defined major surface markers specific for MSCs in vivo, many investigators used immunofluorescence or immunohistochemical techniques to identify their possible in vivo niches [18, 36, 37]. It is well established that HSCs occupy a specific niche in the BM close to trabeculae [38] and that HSCs are regulated by several local cues including oxygen and calcium gradients and other molecules. Our phenotypical and morphological data together with data from other laboratories [6, 37] indicated that BM MSCs could be virtually identical to BM stromal supportive cells termed adventitial reticular cells (ARCs). In this respect, it is not surprising that many investigators still refer to MSCs as multipotent mesenchymal stromal cells [39] or marrow stromal stem cells [6, 15]. The morphological and phenotypical similarities between MSCs and ARCs are listed in the Table 1.

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<th>Table 1. Shared features of mesenchymal stem cells (MSCs) and bone marrow adventitial reticular cells (ARCs)</th>
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<td><strong>Morphology</strong></td>
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<td>Reticular with long processes</td>
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<td>LGFR/CD271</td>
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<td>Alkaline phosphatase</td>
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Another investigators have suggested that the topography of MSCs in the BM is that of a vascular pericyte, the cell lining of the outer surfaces of blood vessels [36]. In one view, ARCs themselves ‘can be seen as bona fide specialized pericytes of venous sinusoids in the marrow’ [37] linking both cells together. Indeed, MSCs sorted based on Stro-1<sup>+</sup>CD106<sup>−</sup> or Stro-1<sup>+</sup>CD146<sup>−</sup>phenotype, expressed α smooth muscle actin or were positive for 3G5 antigen, which is said to be specific for pericytes [15, 16]. These findings have led to a broader proposition that MSCs are generally distributed throughout the post-natal organism as vascular pericytes [47]. At the same time, cells that meet the criteria for MSCs, have been isolated from articular cartilage [48], which is avascular, arguing that MSCs do not have to be exclusively located in a perivascular niche. Indeed, bone lining cells (BLCs) as potential reservoirs of MSC activity in the BM cannot be overlooked. The presence of MSC activity in BLCs would explain why bone explants enzymatically pre-treated to remove all soft marrow tissues could generate fully competent MSC cultures in many published studies [49, 50]. Common to both ARCs and pericytes, BLCs have been shown to be negative for CD45 and positive for α smooth muscle actin [51].

In fact, all three non-haematopoietic BM cell types can possess some degree of the MSC activity. As shown on Fig. 1, all of them are positive for CD10, a marker uniformly expressed on BM CD45<sup>low</sup>CD271<sup>+</sup> MSCs. Other studies showed similar distribution of BM α smooth muscle actin-positive cells [52]. In general, a close lineage commonality between ARCs and pericytes [37, 40] or BLCs and ARCs [53] has been previously reported. Altogether, these findings suggest that compared with HSCs, BM MSCs may have a less-defined niche. In fact, the non-haematopoietic, non-endothelial marrow compartment might form a close network of phenotypically linked but topographically diverse cells, which could all have some degree of the MSC activity.

**The concept of MSC as a circulating cell**

It is firmly established that HSCs can be mobilized from their marrow niches and circulate following growth factor and/or chemotherapy administration. Furthermore, HSCs can home back to these marrow niches following systemic reinfusion and this forms the basis for peripheral blood autologous stem cell...
transplantation. Indeed the concept that MSCs may also circulate is not a new one, and as noted by Prockop, was suggested exactly 140 years ago—well before the advent of the molecular or even cellular biology eras [10]. Consequently, based on this and many subsequent studies, there is a widespread perception that MSCs circulate. But what is the recent evidence for MSCs circulating and homing to sites of injury in man? Actually, most of the evidence comes from experiments with rodent models where MSCs were systemically infused and appeared to be present in greater numbers at sites of injury [54, 55]. In our view, this scenario might simply represent the natural and anticipated consequence of a biophysical process whereby cells artificially introduced to the circulation get trapped at sites of injury. The criteria used to define circulating MSCs have not been sufficiently stringent. In many situations, single marker-based methods were used [56, 57], which may not be necessarily sufficient for rare cell detection. On the other hand, CFU-F assays could fail to detect MSCs considering the fact that MSCs could have lost their adherence properties following entry into circulation as suggested by Khosla and Eghbali-Fatourechi [57]. Using the latter assays, however, the presence of rare circulating MSC in many animal species and, occasionally, in man has been demonstrated [58], but much controversy still exists in this field.

Armed with the knowledge of the in vivo phenotype of BM MSCs, we have investigated the presence of MSCs in normal peripheral blood. We used four different pre-enrichment methods followed by multiparameter flow cytometry, which in the BM have demonstrated an excellent sensitivity for the MSC detection (1 MSC for 100,000 haemopoietic cells). With a starting population of between 300 and 500 million peripheral blood MNCs (equivalent to 300–500 ml of blood), a distinct MSC population could not be detected, regardless of a pre-enrichment method used (Fig. 2A). We did detect adherent spindle-shaped cells reminiscent of fibroblasts but these did not proliferate. These cells were actively phagocytic indicating their monocyte/macrophage lineage nature (Fig. 2B). Importantly, no fibroblastic colonies were grown from peripheral blood of all 16 donors studied. These data confirmed previous studies showing...
different pre-enrichment methods. (CD271) Phagocytic monocytic lineage cells (original magnification 400) from the BM contain both non-phagocytic MSCs (indicated by arrows) and phagocytic monocyto/macrophage (rather than MSC) lineage nature. (C) Positive control BM MSC detection following D7-FIB-based pre-enrichment. CD45–D7-FIB–CD271– population of BM MSCs is encircled. (D) Adherent cells from the BM contain both non-phagocytic MSCs (indicated by arrows) and phagocytic monocytic lineage cells (original magnification 400×).

that circulating MSCs were exceptionally rare in man [58]. It remains a possibility, however, that MSCs may be mobilized from the marrow by growth factors [59] or following acute injury [60]. Although the issue of MSC circulation remains controversial, it is possible to suggest some explanations to a few unresolved issues. First, MSC have been unequivocally shown to circulate during the first trimester of fetal development but scarcely thereafter [61]. This could be related to the completion of the process of vasculogenesis during early development, consistent with the idea of most MSCs taking a role of pericytes stabilizing blood vessels. Although MSCs are present in the cord blood [62], higher numbers of these cells were recently found in soft tissues with the idea of most MSCs taking a role of pericytes stabilizing blood vessels. Although MSCs are present in the cord blood [62], higher numbers of these cells were recently found in soft tissues within the cord itself [63, 31], consistent with this idea. Second, if MSCs circulate in adults but are virtually undetectable, then their physiological role is questionable. Third, we and others have previously shown a very close spatial association between MSCs and fat cells in the marrow [18, 64]. It is well known that fat globules can be released into blood following major orthopaedic trauma (termed fat embolization) [65]. Far from being a specific feature of MSCs to egress from the BM in response to injury, this latter example illustrates that MSC circulation could merely represent a transient phenomenon and the unavoidable consequence of skeletal trauma. In our view, the data showing MSC circulation in man, outside the setting of skeletal trauma, remains controversial; however, the potential circulation of BM MSCs following fracture may have a simple biophysical explanation.

The concept of MSC homing
A logical extension of the belief that MSCs circulate must be that, like HSCs, they possess the ability to egress from the marrow and home to other sites. There would be a strong conceptual argument for MSC trafficking from the marrow to sites of injury, if like HSCs, the marrow was their predominant topographic location. Given the recent demonstration that MSCs are fairly ubiquitously distributed throughout the skeleton [47], the question remains—why do they need to circulate and migrate for long distances at all? Nevertheless, if this question is to be pursued further, attempts to fully characterize tissue-specific MSCs and then track their movements following an infusion would represent a first step.

There is plenty of reported evidence that MSCs show some homing potential, which is influenced by chemokines and other molecules [66, 67]. This does not imply an ability to circulate systemically—in fact, it is equally plausible that MSC homing can occur over short distances, within tissues following some local injury. Phrased in a different way, the wide distribution of MSCs means that if homing occurs it may be over short distances and not be dependent on the systemic circulation. The proposed location of solid tissue MSCs in a perivascular niche [47] could greatly facilitate such local homing without the prior need to enter the systemic circulation.

Finally, a common sense approach to the therapy of cartilage or bone defects involves the direct injection or placement of cells into the defect of interest [68, 69]. At the practical level, those working in the front line of cellular therapy development for musculoskeletal diseases are not pursuing ‘systemic infusion strategies’, which appear to be more academically driven and derived from the HSC stem cell paradigm. It must be acknowledged that interest in MSC homing for the cellular therapy of congenital myopathies has been considered and there is evidence that MSCs participate in muscle regeneration [70]. But again, this is counterbalanced by the fact that the vast majority of infused cells seem to end up in the reticuloendothelial system [71]. The accumulation of MSCs in the muscle may be secondary to the associated vascular damage that accompanies any muscle injury.

Regardless of whether steady-state MSC circulation represents a natural physiological process or not, such cells may still have an important therapeutic value when infused systemically after limited culture-expansion. Nowhere is this clearer than in respect to the use of MSCs in graft-vs-host disease [72], as immunomodulatory cells [73] or in breast cancer trials [74], where MSCs were shown to augment haematopoietic cell recovery. These scenarios illustrate that MSCs could be therapeutically used in settings outside tissue regeneration and in these settings their mode of action could be different (via autocrine, paracrine and even endocrine actions) [75].

Implications
The proposed aim of regenerative medicine strategies based on MSCs is tissue reconstruction using, singly or in combination, MSCs, growth factors and scaffolds [76, 77]. The potential for MSC-based gene therapy has been added to this mélange [78, 79]. However, the concept that MSCs are functioning stromal cells makes it likely that following prolonged expansion in culture that they will exhibit cell senescence, loss of potency, genetic instability and even transformation. Indeed all of these concerns have been borne out by experimental work [80–83]. Given the known drawbacks of gene therapy in the malignancy field [84], it would not seem prudent to pursue such a strategy in cells that may already be rendered genetically unstable by prolonged in vitro culture.

There has been many proof-of-principle studies showing that culture-expanded MSCs can repair bone in the experimental setting [77, 85], but this has not lead, in our view, to many
ground-breaking translational developments thus far. Indeed, orthopaedic surgeons have not favoured such a cumbersome process as BM MSC expansion and re-administration, at least in the areas of orthopaedic trauma or fracture repair, but have used a more pragmatic approach utilizing whole BM injections into fracture sites, where excellent outcomes have been reported [69, 86, 87]. As few as 50,000 uncultured MSCs were found to provide effective bone repair in one such study [88]. This may be because BM MSCs in vivo may have much higher osteogenic differentiation potency compared with expanded MSCs as documented in one report [89].

In the Hennigou et al., study, CFU-F numbers in the implanted material were retrospectively established based on standard 14-day CFU-F assays [88]. However, the realization that uncultured MSCs can now be enumerated by flow cytometry could lead to the development of new strategies for fracture repair (and possibly, for other bone repair applications) whereby the number of fresh MSCs in the implanted cellular fractions could be accurately determined. Additionally, new technologies for repairing bone based on semi-purified, uncultured BM MSCs are now being actively developed [90]. However, in certain situations, mode of action may be more important than phenotypical characterization of the cellular product. For instance, in bone repair indications, the number of injected MSCs may be sufficient, but in applications based on immunomodulatory properties of MSCs, respective quality control procedures are likely to be more complicated.

Finally, a better appreciation of the in vivo biology of MSCs could lead to the emergence of in situ tissue engineering. This refers to the scenario whereby the manipulation of MSCs in vivo could be used for tissue repair without necessarily adding further stem cells to the joint. Indeed, ligamentization of artificial scaffolds placed in the correct orientation represented the first example of in situ tissue engineering in man [91]. From which joint structures MSCs actually traffic to such scaffolds and how they differentiate into ligaments has yet to be defined. Another examples of in situ tissue engineering are the administration of recombinant human bone morphogenetic proteins 2 or 7 for fracture repair or recombinant parathyroid hormone (PTH) for osteoporosis therapy (reviewed in [92]). All of these agents either locally or systemically increase bone mass and are likely to act on resident MSCs without the need for exogenous stem cells. Understanding the cellular basis for these processes has important implications for musculoskeletal stem cell therapy development.

In vivo identification of MSCs in the BM opens-up new avenues of research for understanding the nature and identity of MSCs at other sites including the synovium, periosteum [28, 93] and fat [29]. The formal identification of BM MSCs in these tissues would herald an exciting new era of research, not geared to expanding these cells indefinitely in culture, but rather understanding their in vivo biology and then devising therapeutic strategies to augment their function where defective and suppress it where exaggerated.

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