

Review

Non-hematopoietic bone marrow stem cells: Molecular control of expansion and differentiation

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Abstract

The first non-hematopoietic mesenchymal stem cells (MSCs) were discovered by Friedenstein in 1976, who described clonal, plastic adherent cells from bone marrow capable of differentiating into osteoblasts, adipocytes, and chondrocytes. More recently, investigators have now demonstrated that multi-potent MSCs can be recovered from a variety of other adult tissues and differentiate into numerous tissue lineages including myoblasts, hepatocytes and possibly even neural tissue. Because MSCs are multipotent and easily expanded in culture, there has been much interest in their clinical potential for tissue repair and gene therapy and as a result, numerous studies have been carried out demonstrating the migration and multi-organ engraftment potential of MSCs in animal models and in human clinical trials. This review describes the recent advances in the understanding of MSC biology.

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Mesenchymal stem cells

The non-hematopoietic mesenchymal stem cells (MSCs) of bone marrow were discovered by Friedenstein [1], who

described clonal, plastic adherent cells from bone marrow capable of differentiating into osteoblasts, adipocytes, and chondrocytes [2–5]. These cells are also stromal cells, structural components of the bone marrow that support *ex vivo* culture of hematopoiesis by providing extracellular matrix components, cytokines, and growth factors [1,6–10]. Numerous investigators have now demonstrated that multi-

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potent MSCs can be recovered from a variety of adult tissues and differentiate into a variety of tissue lineages including myoblasts, hepatocytes, and possibly even neural tissue [11–14].

MSCs from bone marrow

The protocols for isolating MSCs from different species vary, but human MSCs are typically isolated from the mononuclear layer of the bone marrow after separation by discontinuous density gradient centrifugation [1,3,15,16]. In some cases, the MSCs are further purified based in the expression of the primitive MSC marker, STRO-1 [17]. The mononuclear layer is simply cultured and the MSCs adhere to the culture plastic. Over time in culture, the non-adherent hematopoietic cells are washed away, resulting in small, adherent fibroblast-like cells. When cultured in α -minimal Eagle's medium containing fetal calf serum or fresh human serum [18], after an initial lag phase [10], the cells divide rapidly, with a donor-dependent average initial doubling time of 12 to 24 h, which is dependent on initial plating density [18]. As the cultures approach high density, the MSCs enter a stationary phase and transform from a spindle-like morphology to a larger, flatter phenotype [18]. Typically, the MSCs recovered from a 2-mL bone marrow aspirate can be expanded 500-fold over about 3 weeks resulting in theoretical yield of 12.5 to 35.5 billion cells [18]. The cells generally retain their multipotentiality [18] for at least 6–10 more passages. Therefore, the cells seem to provide attractive opportunities for cell-based therapy for a variety of diseases.

The non-hematopoietic compartment of bone marrow consists of a variety of heterogeneous populations of cells. As a result, there has been much effort directed towards separating the different cellular populations and evaluating their therapeutic potential. Most noteworthy are primitive MSCs that are isolated by anti-CD133 (prominin, AC133) or anti-low-affinity nerve growth factor receptor (p75, LNGFR) which have showed the most promise in recent studies.

MSC-like cells isolated from bone marrow by anti-CD133

CD133 (AC133, prominin) is a marker for human hematopoietic stem and progenitor cells [19,20]. This surface epitope is also expressed on the apical surfaces of neuro-epithelial cells, embryonic epithelia, adult kidney proximal tubules [21], and adult neural stem cells [22]. The membrane surfaces of bone marrow endothelial progenitor cells are also positive for CD133 [23]. The CD133 protein is targeted to membrane protrusions on stem cells, although its function is currently unknown [24]. Adherent human stem cells can be isolated by magnetic-activated cell (MACS) sorting of adult bone marrow using antibodies against AC133. Interestingly, these primitive adherent cells give rise to non-adherent CD34-negative cells that can repopulate the entire hematopoietic system of lethally-irradiated mice [25]. Furthermore, we and others have found that adherent CD133-

negative transitory amplifying cells can be generated from either adult bone marrow or umbilical cord blood CD133-positive adherent stem cells, and these cells are MSC-like in that they can produce osteoblasts, adipocytes, myoblasts, endothelial cells [26–28], and chondrocytes [28] *ex vivo*. When injected into immunodeficient NIH III mice, human CD133⁺ cells can contribute to multiple non-hematopoietic tissues including muscle [29], pulmonary, intestinal, and liver epithelia, and blood vessels [28]. Based on its expression on a variety of adult stem cells, and that its appearance precedes CD34 expression on hematopoietic stem cells [25], CD133 may be the earliest marker for all primitive stem cells in the adult mammal.

MSC-like cells isolated from bone marrow by anti-p75LNGFR

Nerve growth factor receptor antibodies were initially shown to stain the stromal compartment of bone marrow [30]. NGF antibodies were later used to purify 100% of the colony forming units-fibroblast (cfu-f) from the STRO-1⁺ fraction of human bone marrow cells [31]. Primitive human mesenchymal stem cells have now been isolated directly from the bone marrow by the p75LNGF receptor [32]. This fraction of the bone marrow was shown to be highly clonogenic and to possess a marked potential for the production of osteoblasts and adipocytes. Interestingly, the freshly isolated p75LNGFR-fraction was approximately 50% positive for CD133, which was down-regulated to 0–1% after culture [32]. In the laboratory, we have found that progenitor cells isolated from human bone marrow mononuclear cells by anti-p75LNGFR MACS express multiple neurotrophic factors such as NGF both in culture and after implantation into the dentate gyrus (hippocampus) of immunodeficient mouse brain [33].

MSC-like cells from other tissues

MSC-like cells can also be recovered from a rapidly expanding list of tissues. Cells with very similar properties to MSCs can also be recovered from human lipoaspirates [34], cryo-preserved human umbilical cord blood [35], placental tissue [36,37], and even human exfoliated deciduous teeth [38].

In vitro differentiation of MSCs and MSC-like cells

Because multipotent MSCs are easily expanded in culture and differentiate into multiple tissue lineages, there has been much interest in their clinical potential for tissue repair and gene therapy [39]. Numerous laboratories have now demonstrated that MSCs and MSC-like cells recovered from a variety of adult tissues differentiate into various tissue lineages *in vitro*. In particular, Verfaillie and colleagues, report that a specific type of murine MSC isolated from bone

marrow, muscle, or brain, termed multipotential adult progenitor cells (MAPCs), differentiates into a variety of tissue lineages including myoblasts, hepatocytes, and even neural tissue [11–14]. These processes are frequently complex and in most cases require the simplicity of an *in vitro* system to begin to elucidate the molecular cues required to induce differentiation. Nevertheless, the mechanism of differentiation to various tissue types is poorly understood, even *in vitro*. Much progress has been made, however, elucidating the processes that lead to the differentiation of MSCs to osteoblasts, chondrocytes, and adipose tissue.

Regulation of osteogenic differentiation by MSCs in vitro

MSCs are widely regarded as the stem cell for osteoblasts, the cells that synthesize bone matrix. *In vitro* differentiation of MSCs to osteoblasts has classically involved incubating a confluent monolayer of MSCs with ascorbic acid, β -glycerophosphate, and dexamethasone for 2–3 weeks [1–3,15]. Although these conditions are efficient, they are unlikely to reflect the physiological signals MSCs receive that induce osteogenesis *in vivo*. In more recent studies, the role of bone morphogenic proteins (BMPs) on osteogenesis has been investigated, resulting in numerous contrasting reports on the efficacy of BMPs in promoting bone growth in humans and animal models. Some of the confusion arises from extensive species-specific differences in the effect of BMPs *in vitro*. For example, BMP2 induces a profound increase in the induction of osteogenic markers in murine [39] and rat multipotent progenitor cells [40] but does not reproducibly induce osteogenesis in human MSCs from bone marrow [41]. In fact, BMP2 at concentrations as high as 100 ng mL⁻¹ fails to induce alkaline phosphatase or calcium deposition ([41]; Gregory and Gunn, unpublished observations) and results in increased expression of *Msx-2*, a transcription factor that has been reported to inhibit differentiation of osteoprogenitor cells [41]. It seems that even subtle variations in the method of MSC recovery can affect the outcome of *in vitro* differentiation experiments. For example, positive wingless (Wnt) signaling has been shown to be an inhibitor of osteogenic differentiation in MSCs recovered from bone marrow and trabecular bone [42] but an inducer under alternative conditions [43]. Furthermore, the Wnt inhibitor Dickkopf-1 (*Dkk-1*) also inhibits osteogenic differentiation [43,44]. Given that there are 19 currently identified Wnt ligands operating through at least 4 mechanisms and at least 10 isoforms of the Wnt co-receptor, Frizzled, it seems that subtle changes in cellular phenotype and culture conditions affect the Wnt signal transduction capabilities of MSCs profoundly.

Chondrogenic differentiation by MSCs in vitro

In vitro chondrogenesis by MSCs is typically carried out in micro-mass cultures in the presence of transforming

growth factor [45]. TGF β appears to induce chondrogenesis via protein kinases, including extracellular signal-regulated kinase 1, p38, protein kinase A, protein kinase C, and Jun kinase (reviewed in [46,47]). The TGF β -mediated kinase activation also induces Wnt expression which in turn, up-regulates the expression of the adhesion molecule N-cadherin [47]. N-cadherin appears necessary for cell adhesion complexes required for the early stages of mesenchymal condensation seen during the early stages of chondrogenesis *in vitro* and in the developing embryo [47]. TGF β also seems to synergize with BMP6, enhancing chondrogenesis by MSCs [48].

Adipogenic differentiation by MSCs in vitro

In vitro, adipogenic differentiation is classically induced by incubation of monolayers in a hormonal cocktail containing dexamethasone, isobutyl methyl xanthine (IBMX), and indomethacin. IBMX is a phosphodiesterase inhibitor, which blocks the conversion of cAMP to 5'AMP resulting in an up-regulation of protein kinase A. The protein kinase A activity results in the up-regulation of hormone sensitive lipase (HSL) through the action of the regulatory molecule, perilipin. HSL converts triacylglycerides to glycerol and free fatty acids. Indomethacin is a known ligand for the peroxisome proliferator activated receptor (PPAR) α/γ , a key early transcription factor in adipogenesis [49]. Suppression of Wnt signaling is required for the cells to undergo adipogenesis and this is achieved by PPAR γ through accelerating the degradation of β -catenin by the proteasome [50]. The necessary inhibition of canonical Wnt signaling for the progression of adipogenesis provides an interesting insight into the regulation of osteogenic versus adipogenic commitment by MSCs, since PPAR γ activation can inhibit osteogenesis [51]. This suggests that a fine balance between activated PPAR γ and canonical Wnt signaling controls the differentiation potential of MSCs to either bone or adipose.

In vivo differentiation of MSCs

In the 1970s, pioneering work by Friedenstein et al. demonstrated that MSCs could be grown *ex vivo* and maintain their differentiation capacity *in vivo* upon re-implantation [1]. Following transplantation into irradiated animals, MSCs were later shown to durably engraft in the bone, cartilage, and lungs of mice [52], and to produce fibroblasts or fibroblast-like cells that could be re-isolated and cultured from the lungs, calvaria, cartilage, long bones, tails, and skin [52]. Human MSCs have been transplanted *in utero* in sheep, and were demonstrated to contribute in a site-specific manner to chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma [53]. The human cells were found to persist in multiple tissues for as long as 13 months, despite the

development of immunological competence in the sheep, thus providing some of the first evidence that MSCs might possess unique immunological properties in addition to multiplicity for differentiation [53]. While the growing list of reports of MSCs contributing to tissue repair *in vivo* is beyond the scope of this review, notable recent examples include the repair of kidney [54], lung [55], and heart [56].

The future of MSCs in the clinic

Because MSCs and MSC-like cells are multipotent and easily expanded in culture, there has been much interest in their clinical potential for tissue repair and gene therapy [57] and as a result, clinical trials are currently underway demonstrating efficacy of MSCs in humans for a range of disorders. In particular, promising results have been obtained using hMSCs in clinical trials for Osteogenesis Imperfecta [58–60], metachromatic leukodystrophy, and Hurler syndrome [61]. Allogenic MSC infusions and host compatibility have been the subject of much debate in recent years. One remarkable aspect of MSC physiology is that the cells may actually inhibit inflammation and immunological responses in the host. *In vitro*, MSCs fail to induce allogenic responses in mixed lymphocyte reaction assays and they escape lysis by cytotoxic T-cells and natural killer-cells. The immuno-modulatory properties of MSCs are probably explained by their lack of an HLA type II receptor and the secretion of cytokines [62–64]. Indeed, allogenic MSC implants do not appear immunogenic in non-human primate [65] and human recipients receiving MSCs from sibling donors [58–61]. However, it is not certain that allogenic MSCs can survive *in vivo* after they differentiate.

It is becoming clear that adult stem cells, and particularly hMSCs, will be powerful tools for regenerative medicine and gene therapy. With a better understanding of their fascinating properties, MSCs may provide a novel family of therapeutic strategies for a variety of disorders in the future.

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