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Skeletal Stem/Osteoprogenitor Cells: Current Concepts, Alternate Hypotheses, and Relationship to the Bone Remodeling Compartment

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Abstract Plastic adherent bone marrow stromal cells have become synonymous with skeletal stem cells, and perhaps rightfully so, as these cells have been extremely well characterized over the past four decades, since their original description by Friedenstein. However, although this cell population is useful as an experimental model of precursors for osteoblasts and other mesenchymal lineages, the precise role of bone marrow stromal cells in bone remodeling, fracture repair, or repair of non-skeletal tissues remains unclear. Moreover, there is a conceptual problem in terms of postulating that these cells are osteoblast precursors at sites of bone remodeling on trabecular surfaces adjacent to red marrow and yet having to posit potentially entirely different mechanisms for the origins of osteoblasts at sites of cortical bone remodeling distant from red marrow. Thus, the identification and characterization in recent years of non-adherent stem and osteoprogenitor cells in the bone marrow, of similar cells in the peripheral circulation, and of stem/osteoprogenitor cells arising either from the perivascular compartment (pericytes) or within the developing vascular wall itself, has suggested alternative candidate cell populations that may help to resolve the problem of postulating different mechanisms of remodeling in trabecular versus cortical bone. When coupled with our evolving understanding of the bone remodeling compartment (BRC), a closed cavity penetrated by capillaries which appears to be the site of remodeling in both trabecular and cortical bone, it is likely that our conceptual understanding of the fundamental mechanisms of bone remodeling will need to be modified. J. Cell. Biochem. 103: 393–400, 2008. © 2007 Wiley-Liss, Inc.

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Studies by Friedenstein almost four decades ago established that the bone marrow stroma contains plastic adherent cells (colony forming unit-fibroblast, CFU-F) that can give rise to a broad spectrum of fully differentiated connective tissues, including bone, cartilage, adipose tissue, fibrous tissue, and myelosupportive stroma [Friedenstein et al., 1968]. These studies have led to considerable work aimed at the characterization of marrow stromal cells in rodent and in human systems. In addition to their ability to undergo in vitro differentiation into bone and other tissues, plastic adherent bone marrow stromal cells, when combined with a hydroxyappatite matrix and transplanted into immunodeficient mice, give rise to bone of donor origin (albeit with a relatively low frequency of ~10% of transplanted stromal cell clones), as well as support hematopoiesis by host cells [Bianco et al., 2006]. These plastic adherent stromal cells have attracted considerable attention as a potential source of multi-potent cells for tissue repair, although the clinical utility of these cells remains to be realized. Nonetheless, isolation of stromal cells from murine or human bone marrow based on adherence to plastic has become synonymous with isolating "stem cells," although the relationship of the cell populations isolated using these in vitro methods to true stem cells in vivo remains unclear.

While there is no doubt that isolation of bone marrow stromal cells on the basis of plastic adherence clearly leads to the identification of a population of cells with multi-lineage potential in vitro, over the past several decades there has been a growing body of work suggesting that there likely are other populations of cells in bone marrow and other tissues, as well as in

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peripheral blood, that also have characteristics of stem and/or osteoprogenitor cells. Since the area of traditional bone marrow stromal cells has been extensively reviewed elsewhere [Bianco et al., 2001], the current discussion focuses on these other, less well characterized cell populations which may nonetheless be of tremendous biological and therapeutic importance. Moreover, these alternative candidate stem/osteoprogenitor cells may allow for a better understanding of and integration with our evolving concepts of the mechanisms of bone remodeling in trabecular as well as cortical bone.

BONE MARROW NON-ADHERENT OSTEOPROGENITOR/STEM CELLS

Concurrent with the extensive body of work noted above on plastic adherent bone marrow stromal cells, Long et al. identified, in the 1990s, a non-adherent population of cells in bone marrow with osteogenic potential [Long et al., 1990, 1995, 1999]. The primary method used to isolate these cells was cell sorting using antibodies to bone-related proteins, including osteocalcin or alkaline phosphatase. Alkaline phosphatase is an established cell surface marker for the identification of osteoprogenitor cells; however, the fact that osteocalcin is a secreted protein that has typically been associated with a mature osteoblast phenotype (at least in rodent systems) led to some perhaps understandable skepticism regarding these initial findings. Nonetheless, the empirical observation that sorting with osteocalcin did isolate an osteoprogenitor population seems fairly convincing. Specifically, when osteocalcin positive cells from human bone marrow, which were small and had low granularity (determined by the forward and side scatter characteristics by flow cytometry), were cultured in the presence of TGF-β and accessory bone marrow cells (the identity of which is still unclear), these cells proliferated and differentiated into mature osteoblastic cells expressing bone-related genes and capable of in vitro mineralization. In retrospect, it is possible that cell sorting for osteocalcin was feasible due to the fact that osteocalcin possesses Gla-residues, which are known to bind to cell membranes [Huang et al., 2003] and may anchor osteocalcin to the cell upon being secreted, at least transiently as the cells are sorted. Alternatively, there is increasing evidence for a cell surface receptor that can bind osteocalcin [Bodine and Komm, 1999; Pi et al., 2005], and it is possible that the cells producing osteocalcin also express this receptor, allowing for binding of the secreted protein to the cell surface. Finally, in contrast to rodent systems, even relatively undifferentiated human osteoprogenitor cells appear to express at least low levels of osteocalcin mRNA [Gronthos et al., 1999], thus making the identification of human osteoprogenitor cells using sorting with an osteocalcin antibody more plausible.

Further evidence supporting the presence of osteoprogenitor or stem cells in the bone marrow non-adherent population was provided by Falla et al. [1993]. These investigators reasoned that since hematopoietic stem cells are relatively quiescent and can be enriched from mouse bone marrow by treating the animals with 5-fluorouracil (5-FU) (which depletes the marrow of all proliferating cells), perhaps the same method could be used to enrich for osteoprogenitor cells. Using a similar approach and 5-FU treatment, they obtained a 12-fold enrichment for murine osteoprogenitor cells from bone marrow. Interestingly, while 5-FU eliminated all adherent bone marrow stromal cells, non-adherent low density osteoprogenitor cells (likely a similar population to that isolated by Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000] from human bone marrow) were enriched. Collectively, the findings of Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000] and Falla et al. [1993] suggested that the non-adherent cells may represent a more primitive, non-cycling osteoprogenitor population that becomes adherent and begins to proliferate as the cells differentiate.

Despite these findings, the potential functional relevance of these non-adherent osteogenic bone marrow cells remained largely unexplored until more recent studies by Dominici et al. [2004], who compared hematopoietic versus mesenchymal reconstitution of irradiated mice using either bone marrow stromal adherent or non-adherent fractions. Thus, they obtained plastic-adherent bone marrow stromal cells from FVB/N mice and labeled them with a GFP marker using a retroviral vector. These cells were then infused into lethally irradiated host mice. As expected, the labeled stromal cells

did not contribute to the hematopoietic reconstitution of the host mice, and 0–2% (median, 1.5%) of osteoblasts or osteocytes in the host were GFP positive, indicating limited engraftment of infused bone marrow stromal cells into the host. By contrast, when the identical experiment was repeated using bone marrow non-adherent cells, >90% of blood leukocytes, erythrocytes, and platelets were GFP positive. Even more importantly, up to 50% (median, 18%) of osteoblasts and osteocytes in the host were now GFP positive. Moreover, molecular analysis demonstrated a common retroviral integration site in clonogenic hematopoietic cells and osteoprogenitors from each of seven animals studied, establishing a shared clonal origin for these cell types. These findings thus provided considerable credence to the previous work of Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000] and established that, at least in the experimental paradigm used by Dominici et al. [2004], non-adherent bone marrow cells have a >10-fold more robust bone-repopulating activity than do adherent bone marrow stromal cells. Moreover, the findings were also consistent with the previous work of Olmsted-Davis et al. [2003] suggesting the presence of a unique progenitor cell with both hematopoietic and osteoblastic differentiation potential in the non-adherent subset of bone marrow cells. Indeed, the functional utility of the bone marrow non-adherent cells was recently also demonstrated by work from the Karsenty group in which transplantation of bone marrow non-adherent cells from wild type mice into irradiated β-adrenergic receptor2 deficient (or homozygous null) mice as per the protocol of Dominici et al. [2004] reversed the high bone mass phenotype of the β-adrenergic receptor2 knock out mice [Elefteriou et al., 2005]. In addition, cultured osteoblasts from the host mice were positive for β-adrenergic receptor2 expression, consistent with the transplanted cells contributing to osteoblast formation in the host mice.

STEM/OSTEOPROGENITOR CELLS IN THE CIRCULATION

A potential conceptual problem with the notion that bone marrow stromal cells are the exclusive, or even major, source of osteoblasts in vivo is that the majority of osteoblasts, at least in the human skeleton, are found not on trabecular surfaces in contact with red marrow, but rather on endocortical surfaces or in intracortical remodeling sites distant from red marrow. How osteoblasts get to these cortical sites has been a source of considerable discussion and speculation; since it is known that osteoclasts at these sites likely arrive as monocytic precursors via the circulation [Fujikawa et al., 1996], a logical hypothesis is that osteoblasts may also take a similar route. However, this notion has been largely dismissed over the years since studies by Gothlin and Ericsson in the 1970s [Gothlin and Ericsson, 1976] arguably provided conclusive evidence against this possibility. Thus, they used a parabiotic rat model in which a femoral fracture was induced in both rats. One of the rats was subsequently shielded, while the other rat received radiation to destroy at least hematopoietic and possibly mesenchymal precursors (the latter are more radio-resistant). The shielded rat was then given a dose of ^{3}H-thymidine during a 20 min arrest of cross-circulation, and at various subsequent time points, the fracture sites in both rats were examined for the presence of labeled cells. Since the rat infused with the ^{3}H-thymidine was found to have label in monocytes, macrophages, endothelial cells, fibroblasts, chondroblasts, osteoblasts, and osteoclasts in the fracture callus, but the contra-lateral, parabiosed rat was found to have label only in monocytes, macrophages, and osteoclasts, the inevitable conclusion was that osteoblasts did not circulate. However, while this study has been accepted as conclusive evidence against circulating osteoblast precursors, the subsequent work of Falla et al. [1993] described earlier provides a potential explanation for the apparently negative findings of this study. Thus, if the circulating osteogenic cells came from the bone marrow non-adherent pool of pre-osteoblastic cells (which seems more likely than originating from plastic adherent bone marrow stromal cells), these cells would not have incorporated the pulse of ^{3}H-thymidine administered as the label (since they are relative quiescent as reflected by their resistance to 5-FU). As such, this study may not be as definitive as generally assumed in terms of providing conclusive evidence against circulating osteoprogenitor cells.

Since the original parabiosis study described above, there has been accumulating evidence for the presence of mesenchymal/osteoblast

precursor cells in peripheral blood, although the precise concentration of these cells has been the subject of some debate. Thus, Huss et al. [2000] identified cells in the peripheral circulation of a dog that, when cultured in vitro in the presence of IL-6, formed fibroblastic colonies which also expressed osteocalcin. These investigators created immortalized cell lines from these peripheral blood cells which were tagged with a green fluorescent protein (GFP) construct, and following injection of one of these lines into an irradiated dog, were able to demonstrate GFP-positive cells in the marrow cavity which appeared predominantly as "bone lining cells." Of note, these cells were also able to reconstitute the hematopoietic elements of the marrow, consistent with previous work from this group [Huss et al., 1995] as well as the studies by Olmsted-Davis et al. [2003] noted earlier demonstrating the existence of stem cells that can differentiate down both hematopoietic and mesenchymal lineages.

In subsequent studies, Zvaifler et al. [2000] used elutriation to isolate small, round mononuclear cells from human peripheral blood that, upon in vitro culturing, were replaced by fibroblast-like cells and large, round stromal cells. The concentration of these cells was approximately 0.3–0.7% of peripheral blood mononuclear cells, and they were capable of at least in vitro differentiation into osteoblastic and adipocytic cells. Subsequently, Kuznetsov et al. [2001] provided further evidence in support of circulating osteoprogenitor cells. These investigators obtained blood from a number of species and demonstrated the presence of plastic adherent colony-forming cells with osteogenic potential in the circulation of mice, rabbits, guinea pigs, and humans. However, the frequency of these plastic adherent circulating stromal cells was extremely rare, on the order of 1 in 10^6 peripheral blood mononuclear cells in mice and guinea pigs, 1 in 10^7 in rabbits, and 1 in 10^8 (or less) in humans. These cells did not express hematopoietic (CD34, CD45, CD14) or endothelial (endoglin, CD34, Factor VIII-related antigen, Muc-18, PAL-E, EN4) markers, but did express osteogenic markers (osteopontin, bone sialoprotein, type I collagen, osteonectin) as well as the β1 integrin subunit. Interestingly, the cells were negative for Stro-1, a commonly used marker for bone marrow stromal cells, and perhaps surprisingly, did not express AP, which is expressed in marrow stromal cells from a variety of species. The osteogenic potential of these cells was proven by an in vivo transplantation assay in which either polyclonal or single colony-derived cells were transplanted into the subcutis of immunocompromised mice, and the donor origin of the resulting fully differentiated bone cells was proven using species-specific probes. Moreover, these circulating stromal cells were at least bipotential, since they could also be converted into adipocytes in vitro in the presence of 20% rabbit serum.

Reasoning that requiring plastic adherence may well have underestimated the concentration of osteoprogenitor cells in peripheral blood, our group subsequently used methods very similar to those used by Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000] to identify circulating osteoblast lineage cells. Specifically, we used flow cytometry following staining with osteocalcin or alkaline phosphatase antibodies to demonstrate that osteocalcin or alkaline phosphatase positive cells were indeed present in peripheral blood in humans. Osteocalcin positive cells constituted 0.5–0.8% of circulating mononuclear cells [Eghbali-Fatourechi et al., 2005], which is very similar to the concentration of circulating mesenchymal precursor cells noted by Zvaifler et al. [2000] using elutriation; moreover, the concentration of osteocalcin positive cells was markedly increased (~5-fold) in peripheral blood of adolescent males going through the pubertal growth spurt and possibly in adults who had sustained fractures. Circulating osteocalcin positive cells also expressed bone related genes (osteocalcin, alkaline phosphatase, and collagen I) and formed mineral deposits in vitro and bone in vivo in immunodeficient mice [Eghbali-Fatourechi et al., 2005]. Interestingly, the circulating osteocalcin positive cells were predominantly small, round cells (<10 μm in diameter) [Eghbali-Fatourechi et al., 2007], phenotypically very similar to the cells originally isolated from the non-adherent bone marrow population by Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000].

Consistent with these studies in humans, Otsuru et al. [2007] have recently provided evidence in support of bone marrow-derived osteoblast progenitor cells in the peripheral circulation in mice. These investigators examined how bone marrow cells contributed, via the

circulation, to osteogenesis in a BMP-induced model of ectopic bone formation. To do so, they implanted a BMP-2-containing collagen pellet into muscle in mice which had received lethal dose-irradiation and subsequent GFP-transgenic bone marrow cell transplantation. Three weeks later, a significant number of GFP-positive osteoblastic cells were present in the newly generated ectopic bone; these cells could only have come from the bone marrow via the circulation. Further, they also demonstrated that peripheral blood mononuclear cells from the BMP-2-implanted mouse contained cells which could differentiate into osteoblasts in vitro. In addition, passive transfer of the peripheral blood mononuclear cells isolated from a BMP-2-implanted GFP-mouse to a second (non-GFP) BMP-2-implanted mouse led to GFP-positive osteoblast accumulation in the ectopic bone, consistent with GFP-positive circulating osteoblastic cells from the GFP-mouse homing to and participating in ectopic bone formation in the non-GFP-mouse.

Collectively, there is now thus fairly convincing evidence that osteoblast progenitors can be isolated from human and mouse peripheral blood using several different methods and experimental approaches. However, the concentration of these cells does appear to be dependent on the method(s) used to identify the cells, and the potential physiological role of these cells in normal bone remodeling or fracture repair remains to be fully defined.

BONE AND THE VASCULATURE: A COMMON PROGENITOR?

While it has traditionally been believed that endothelial and osteoblastic cells are derived from distinct progenitor populations, there is increasing evidence for overlap between these lineages. In fact, the Stro-1 antibody, which is widely used to identify mesenchymal stem cells in bone marrow and other tissues, was originally derived by immunizing mice with purified human cells expressing the hematopoietic/endothelial progenitor marker, CD34 [Simmons and Torok-Storb, 1991]. Moreover, human bone marrow CD34+ cells can differentiate into osteoblastic cells capable of forming mineralized nodules in vitro [Chen et al., 1997], and Tondreau et al. [2005] have found that populations of cells expressing another endothelial progenitor marker, CD133, from human bone marrow, umbilical cord blood, or peripheral blood from G-CSF-treated donors are enriched for mesenchymal stem cells capable of differentiating into osteoblasts. In recent studies, we examined whether circulating osteocalcin or alkaline phosphatase positive cells also co-stained with a CD34 antibody, and found that approximately 40% of osteocalcin positive and 50% of alkaline phosphatase positive cells from obtained from random blood donors co-expressed CD34; conversely, ~30% of circulating CD34+ cells co-stained with the osteocalcin antibody [Eghbali-Fatourechi et al., 2007]. The latter finding was independently confirmed by a study using single cell reverse-transcriptase polymerase chain reaction, which found that ~20% of human peripheral blood CD34+ cells expressed the mRNA for osteocalcin [Matsumoto et al., 2006]. In addition, when infused into immunocompromised rats following femur fractures, these CD34+ cells were found to localize to the fracture site and to differentiate into endothelial cells as well as osteoblasts and enhance fracture healing [Matsumoto et al., 2006].

The overlap between endothelial and osteoblastic lineages is of particular interest given the increasing recognition of the link between the vasculature and bone [Brandi and Collin-Osdoby, 2006]. Thus, a number of cross-sectional and prospective studies have demonstrated independent associations between bone mineral density (BMD) and vascular calcification [Barengolts et al., 1998; Hak et al., 2000; Kiel et al., 2001; Tanko et al., 2003; Pennisi et al., 2004; Tanko et al., 2005], which appears to be predictive of atherosclerosis and related cardiovascular risk [Sangiorgi et al., 1998; Iribarren et al., 2000]. In addition, low BMD has also been associated in prospective studies with an increase in the risk for cardiovascular events [Tanko et al., 2005]. The potential mechanism(s) for the link between osteoporosis and vascular disease have remained unclear, but of interest are recent data from Sambrook et al. [2006] demonstrating that high bone turnover itself is also associated with increased cardiovascular mortality in elderly subjects, independent of age, sex, overall health, serum parathyroid hormone levels, and hip fracture status.

A further connection between bone and the vasculature comes from increasing evidence that vascular pericytes are also capable of

forming osteoblasts [Doherty et al., 1998]. In addition, evidence that the adult rat aorta contains a CD34+ precursor that, under appropriate culture conditions, loses CD34 expression and acquires the characteristics of a pericyte (including α-smooth muscle actin expression) [Howson et al., 2005] raises the intriguing possibility that the vasculature may provide not only the blood supply, but perhaps all of the cells needed for normal bone remodeling and/or fracture repair.

THE BONE REMODELING COMPARTMENT: LINKING BONE AND BLOOD VESSELS AS WELL AS REMODELING IN TRABECULAR AND CORTICAL BONE

As noted earlier, the concept that adherent bone marrow stromal cells are the exclusive or major source of osteoblasts is difficult to reconcile with the fact that cortical bone remodeling occurs at sites distant from red marrow, which is the predominant site of resident bone marrow stromal cells. Thus, it has been necessary to postulate two distinct mechanisms for bone remodeling: one in trabecular bone next to red marrow, where the osteoprogenitor cells traveled from the marrow directly to bone surfaces, and a second process within cortical bone, where the osteoprogenitor cells arrived via alternate, as yet unclear mechanisms. However, recent evidence that bone remodeling in both cortical and trabecular bone largely occurs in highly vascular bone remodeling compartments (BRCs) that are covered by a layer of bone lining cells which are penetrated by capillaries suggests a potential resolution to this problem (Fig. 1) [Hauge et al., 2001; Eriksen et al., 2007]. In this model, cells destined to become osteoblasts on bone surfaces likely enter the BRC not directly from the bone marrow (which would not be possible for BRCs in cortical bone distant from the marrow), but rather via the capillaries that penetrate the BRCs. Based on the above discussion, there are several possible candidates for the precursor cells that ultimately give rise to osteoblasts. Thus, in addition to classical bone marrow stromal cells, it is possible that bone marrow non-adherent cells, as originally identified by Long et al. [Long et al., 1990, 1995, 1999; Eipers et al., 2000; Kale and Long, 2000], access the BRC via this mechanism, or alternatively, that circulating osteoblastic cells contribute to the pool of osteoblastic cells entering the BRC. On the other hand, given the potential overlap between osteoblastic and endothelial cells noted above, the evidence that vascular pericytes can differentiate into osteoblasts [Doherty et al., 1998], and recent work demonstrating that pericytes themselves may arise from a CD34+ progenitor in the vessel wall [Howson et al., 2005], it is also possible that

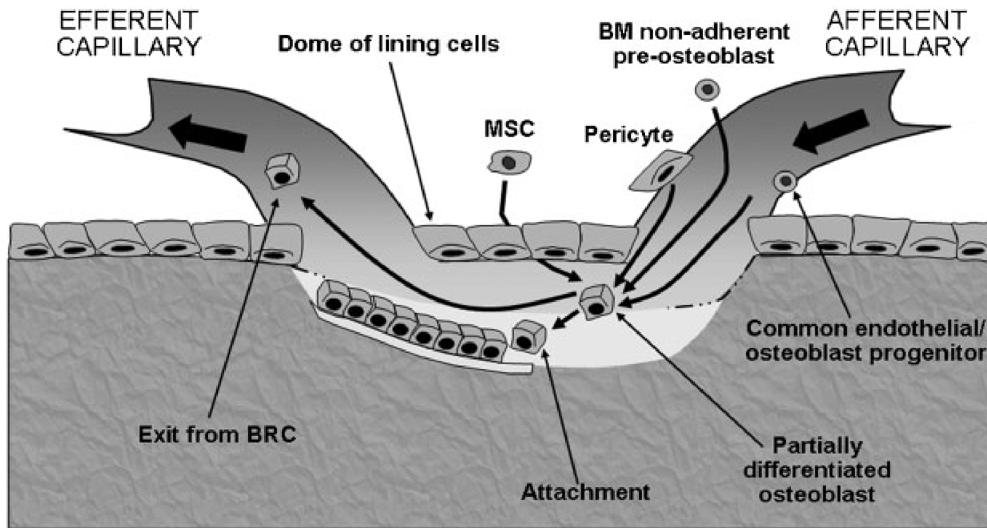

Fig. 1. Model of the bone remodeling compartment (BRC) as described by Hauge et al. [2001]. In this model, osteoblastic cells enter the BRC principally via the afferent capillary and could include non-adherent (or adherent) bone marrow (BM) cells, circulating cells, or osteoblastic cells originating from precursor cells in the vasculature. MSC, marrow stromal cell. Reproduced from Eghbali-Fatourechi et al. [2007] with permission.

precursor cells in the vasculature (i.e., within the capillary wall penetrating the BRC) may give rise to osteoblastic progenitors. Fundamentally, however, the same process can now be postulated to occur in BRCs in trabecular and in cortical bone, obviating the need to postulate distinct mechanisms for bone remodeling in these two compartments.

SUMMARY AND CONCLUSIONS

While it is clear that the study of plastic adherent bone marrow stromal cells has yielded important insights into the differentiation pathways of osteoblasts and other mesenchymal lineages, there are conceptual limitations to the notion that these cells are the exclusive, or perhaps even major, sources of osteoblasts on bone surfaces in vivo. The description of the BRC opens up new ways of thinking about bone remodeling, including the parsimonious unification of this process in trabecular and cortical bone. However, the concept of the BRC also highlights why our current understanding of osteoprogenitor cells, and perhaps, stem cells in general, needs to broaden beyond that of the bone marrow stromal cells described by Friedenstein almost 40 years ago. Thus, further characterization of not only adherent bone marrow stromal cells, but rather also the additional candidates discussed in this perspective, including (but not limited to) non-adherent bone marrow cells, cells present in the peripheral circulation, and progenitor cells either surrounding blood vessels (pericytes) or those derived from the developing blood vessels is essential both for a more complete understanding of skeletal stem cell biology and for harnessing the potential of these sources of progenitor cells for therapy of skeletal and perhaps nonskeletal disorders.

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