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Skeletal Stem Cells: Origins, Functions and Uncertainties

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Abstract

The development and maintenance of the skeleton requires a steady source of skeletal progenitors to provide the osteoblasts and chondrocytes necessary for bone and cartilage growth and development. The current model for skeletal stem cells (SSCs) posits that SSC/progenitor cells are present in bone marrow (BM) and other osteogenic sites such as cranial sutures where they undergo self-renewal and differentiation to give rise to the main skeletal tissues. SSCs hold great promise for understanding skeletal biology and genetic diseases of bone as well as for the advancement of bone tissue engineering and regenerative medicine strategies. In the past few years, a considerable effort has been devoted to identifying and purifying skeletal stem cells and determining their contribution to bone formation and homeostasis. Here, we review recent progress in this area with particular emphasis on the discovery of specific SSC markers, their use in tracking the progression of cell populations along specific lineages and the regulation of SSCs in both the appendicular and cranial skeleton.

Keywords

Skeletal stem cells; (Non)-Perivascular niches; Markers; Fate determination; Bone marrow; Suture mesenchyme

Introduction

Skeletal stem cells (SSCs), also known as mesenchymal stem cells or stromal stem cells, are the postnatal multipotent progenitors of skeletal tissues capable of generating all the major non-hematopoietic cell types associated with bone (i.e. osteoblasts, chondrocytes, marrow adipocytes, fibroblasts, and bone marrow stromal cells) [1]. SSCs are slow-cycling cells that maintain themselves through long-term self-renewal. In the presence of a differentiation signal, SSCs undergo asymmetric cell divisions such that daughter cells become committed

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Conflict of Interest

Fatma F. Mohamed and Renny T. Franceschi each declare no potential conflicts of interest.

Human and Animal Rights and Informed Consent

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to one or more of the skeletal lineages to eventually provide new cells necessary for bone growth and remodeling.

In this review, we will explore current knowledge in SSC biology, highlighting the identification of candidate markers, their use for prospective SSC isolation and lineage tracing as well as advances in our understanding of SSC regulation in the appendicular and cranial skeletons.

Discovery of clonogenic cells in BM with SSC-like properties

Any stem cell discussion must begin with work from the hematology field where the first stem cell populations were definitively isolated and characterized. Hematopoietic stem cells (HSCs) were isolated by flow cytometry using specific cell surface markers that defined a population of bone marrow (BM) cells capable of generating all the blood cell types. In what is considered to be the definitive experiment for proving the existence of a stem cell, single HSC were shown to give rise to all types of donor-derived blood cells upon transplantation into sub-lethally irradiated recipient mice[2, 3]. The ability of a single HSC to repopulate the whole hematopoietic system reflects its self-renewal and multilineage differentiation potential.

While transplantation and single cell analysis is still considered to be the “gold standard” for investigating functional HSCs in vivo, this approach is not feasible for defining multipotent SSCs, due to their low abundance in BM, slow cycling time, resistance to ablation with irradiation, and the possible requirement for a supportive niche for SSC growth [4]. As a surrogate, ex vivo expansion of single clonogenic BM cells followed by in vivo ectopic transplantation has been used to functionally evaluate the ability of putative SSC populations to generate the major skeletal tissues [5]. This approach originated with the seminal work of Friedenstein and colleagues in the late 1960s who provided the first evidence for the existence of osteogenic progenitor cells in bone marrow stroma [6]. Adherent fibroblast-like cells were identified in whole bone marrow cultures having SSC properties. Specifically, single fibroblast-like cells were shown to initiate growth independent of cell density to form colonies of at least 50 cells, subsequently referred to as colony forming units-fibroblasts (CFU-Fs)[7]. Individual CFU-Fs had the capacity to differentiate ex vivo into the principal skeletal lineages (osteogenic, adipogenic, and chondrogenic) and, upon subcutaneous transplantation, form heterotopic bone organs, which subsequently filled with host-derived hematopoietic cells[6, 8–10]. These findings indicate that single clonogenic cells identified ex vivo are multipotent. CFU-F formation remains as an important criterion for characterizing purified SSC populations. Based on these studies, it was hypothesized that a common progenitor or stem cell retained in the postnatal BM can give rise a range of skeletal tissues [10].

While this is an attractive model, a key question raised is whether ex vivo expanded clonogenic cells are equivalent to their in vivo counterparts or are an artifact of cell culture. Only recently has it been possible to relate ex vivo studies with SSC populations in vivo. Advances in this field have come from a variety of quarters including studies on non-HSC BM cells that support hematopoiesis as components of an HSC niche, analysis of

perivascular stromal cell populations from multiple tissues as well as detailed lineage tracing experiments using a variety of genetic markers.

SSCs and the hematopoietic niche

Interestingly, important advances in SSC biology came as the byproduct of studies whose primary goal was to identify a cell population in BM capable of supporting HSC-mediated hematopoiesis. The observation that hematopoiesis is restricted to bone once the skeleton is formed and that ossicles formed by implanted bone marrow stroma can support hematopoiesis suggested that certain non-hematopoietic lineage cells in BM are necessary for hematopoietic maintenance [8, 11]. Initial studies by Taichman and colleagues showed that osteoblasts isolated from human trabecular bone could produce regulatory molecules capable of maintaining human hematopoietic progenitor cells *ex vivo* [12–14]. In addition, localization studies showed that HSCs were often found in close proximity to endosteal bone surfaces, which was again indicative of a supporting role for endosteal osteoblasts in hematopoiesis [15, 16]. These early studies are significant because they paved the way for subsequent experiments designed to characterize the hematopoietic niche *in vivo* [17, 18]. Although the concept that osteoblasts are a major component of the niche was subsequently brought into question (e.g. *in vivo* osteoblast-selective knockout of hematopoietic niche factors failed to inhibit hematopoiesis [19]), the general paradigm that cells within the skeletal lineage including SSCs make up the HSC niche continues to be supported by more recent studies as described below.

As the quest to define the hematopoietic niche continued, a number of BM stroma components associated with the perivascular region such as endothelial cells, fibroblast and CXCL12-abundant reticular (CAR) cells were all proposed to support hematopoietic stem cells (HSCs) [20–22]. These reports reflected the complexity and uncertainty surrounding the identity of the niche [23]. Although the overlap between these cells and skeletal progenitors was not initially apparent, more detailed analysis uncovered some interesting associations. For example, BM perivascular cells were identified that, in addition to expressing hematopoiesis-supporting factors, such as CXC chemokine ligand 12 (CXCL12), stem cell factor (SCF) and angiopoietin-1 (Ang1), were also capable of undergoing osteoblast and adipocyte differentiation (reviewed in [24, 25]). Consistent with the concept that this cell population contains SSCs, ablation of CXCL12-expressing stromal cells, including CAR cells, was shown to not only deplete HSCs from BM *in vivo*, but also to severely reduce the osteoblast and adipocyte differentiation capacity of BM stromal cells [26]. Also, conditional knockout of CXCL12 using Prx1-Cre, which targets early skeletal progenitors, but not cells of the hematopoietic lineage, leads to significant reductions in BM cellularity and HSC frequency [27]. While these and other lines of evidence indicate that skeletal progenitor/stem cells are contained within the HSC niche and support hematopoiesis [28–30], these studies also provoked a number of investigations into the role of this cell population in skeletal development and maintenance.

Perivascular identification of skeletal stem/progenitor cells

In a separate, but related line of investigation, studies focused on the possible association of stem cells including SSC with blood vessels. Perivascular cells or pericytes were initially purified from human BM by FACS using the marker, melanoma cell adhesion molecule (MCAM/CD146) [30], a previously characterized endothelial cell antigen[31]. Purified MCAM/CD146+ cells accounted for most of the CFU-Fs in BM. Furthermore, clonogenic MCAM/CD146+ cells formed bone ossicles with an intact marrow cavity after ectopic transplantation and gave rise to a small subset of MCAM/CD146+ stromal cells including adventitial reticular cells around blood vessels, which contributed to organization of the hematopoietic niche [30]. Subsequent work showed that MCAM/CD146+ cells could be isolated from multiple human tissues including BM, skeletal muscle, pancreas, adipose tissue, and placenta[32]. However, these cells had different properties depending on the tissue of origin. Thus, MCAM/CD146+ cells from BM exhibited a tri-lineage differentiation potential ex vivo and, after transplantation, could form osteoblasts and stromal cells while MCAM/CD146+ cells from skeletal muscle were inherently myogenic, but not osteogenic[33]. These findings suggest that SSCs in BM differ from their counterparts in the perivascular niche of other tissues. Of note, findings from these studies were largely based on the use of ectopic transplantation assays and may be affected by ex vivo manipulation and scaffold microenvironment[33].

Demonstration of a perivascular origin for human SSCs provided new insights into our understanding of stem cell biology, and stimulated parallel studies in mice [28], which were able to exploit numerous genetic models to accelerate progress. In the course of these studies, a number of SSC markers were identified. These include, nestin (*Nes*), the leptin receptor (*Lepr*), osterix (*Osx*), myxovirus resistance-1 (*Mx1*), platelet-derived growth factor receptor α (*Pdgfra*), paired-related homeobox gene-1 (*Prx1*) and homeobox gene a11 (*Hoxa11*), which will be individually discussed.

Nestin was first identified as an intermediate filament protein and neural stem cell marker [34]. Although it is unlikely that the endogenous *Nes* gene is expressed in SSCs [35–37], a transgenic Nestin-GFP mouse where GFP expression is controlled by a 1.8 kb enhancer from the second intron of the Nestin gene has proven to be a very useful tool for identifying and purifying SCCs. FACS purification of GFP+ cells identified a relatively rare perivascular stromal cell population with SSC activity. These cells were enriched in CFU-F activity, had the capacity for multi-lineage differentiation ex vivo as well as self-renewal upon serial transplantation[28]. Investigations into the in vivo contribution of this cell population to skeletal development showed that Nes-GFP+ cells first appear at embryonic day 10.5 (E10.5) in the perichondrium around cartilaginous rudiments where they colocalize with the endothelial marker, CD31[36]. As endochondral bone formation proceeds, a non-endothelial Nes-GFP+ subpopulation emerges beginning at E13.5 that becomes associated with vasculature [36]. Lineage tracing experiments suggest that this population of Nes-GFP+ cells are derived from type II collagen-expressing chondrocytes via a Runx2- and Indian hedgehog (*Ihh*)-dependent mechanism. *Ihh*- and Runx2-deficient mice have a significantly reduced number of endothelial Nes-GFP+ cells and a complete loss of non-endothelial Nes-GFP+ cells in the perichondrium[36].

PDGFR α , an early mesodermal marker [38], is commonly used alone or in combination with standard stem cell markers to isolate stromal cells enriched in SSCs activity. PDGFR α combined with Sca-1 (stem cell antigen-1) identifies two distinct populations: PaS+ cells (defined as PDGFR α +, Sca-1+, CD45-, TER119- and representing 0.03% of BM cells) located around arterioles [39] and PaS- cells (defined as PDGFR α +, Sca-1-, CD45-, TER119- representing 0.22% of BM cells), which primarily reside around sinusoids[40]. Differences between PaS+ and PaS- cells are also seen when HSC niche factors are examined; PaS+ cells express high levels of Ang-1[39], whereas PaS- cells express high levels of CXCL12, thereby representing a subgroup of CAR cells [40]. PaS+ cells can differentiate to osteoblasts, adipocytes, reticular cells and endothelial cells upon systemic transplantation in vivo [39]. PDGFR α is also used in combination with CD51 (α V integrin) to further define and enriched SSC populations. PDGFR α + CD51+ BM stromal cells (defined as PDGFR α +CD51+ CD45- Ter119- CD31-) were shown to recapitulate the SSCs activity of Nes-GFP+ cells in BM [41]. Endogenous Nestin expression, as detected by real-time PCR, was also enriched in stromal PDGFR α + CD51+ cells compared with single-positive or negative stromal cells[42]. The use of PDGFR α as a mesenchymal stem cell marker and its role in skeletal and non-skeletal tissues during development was recently reviewed [42].

The leptin receptor (LepR) was shown to be an important SSC marker specifically in adult mice. LepR+ cells represent 0.3% of total BM and localize with perivascular stromal cells around sinusoids and arterioles [40, 43]. These cells account for most of the CFU-Fs in adult BM, express MSCs markers such as PDGFR α , PDGFR β , Prx1-Cre, CD51, and CD105 [29, 40, 41, 44, 45] and overlap with Nes-GFP+ cells^{dim} and with CAR cells [26]. Clonogenic LepR+ cells were shown to have tri-lineage differentiation potential ex vivo, and when transplanted subcutaneously, give rise to bone, fat and stroma[40]. In addition to SSC activity, LepR+ cells strongly express HSCs niche factors such as CXCL12 and SCF [46]. Lineage tracing of LepR+ marrow cells demonstrate that these cells mainly arise postnatally and serve as precursors for osteoblasts and adipocytes. LepR+ cells are thought to be necessary for BM homeostasis and tissue repair after fracture and ablation/irradiation injury [40, 45]. LepR+ cells; however, are rare in prenatal BM (first appearing at E17.5 [45]) and showed no or little contribution to bone or cartilage during fetal development [40], although they may contribute to cartilage during injury repair [40, 45].

Osterix (Osx), a transcription factor essential for osteoblast differentiation, is expressed in immature osteogenic cells [47]. In studies using an inducible Osx-CreERT mouse, Osx was shown to mark at least three distinct mesenchymal precursor cells in the fetal, perinatal, and adult bone marrow[45]. First, Osx+ precursor cells appear in embryonic perichondrium and co-invade with blood vessels to transiently contribute to nascent bone tissues and primitive BM stroma in the developing primary spongiosa[45]. During the perinatal period, a second group of Osx+ precursor cells emerge, which contribute to osteolineages and to vasculature-associated, long-lived stromal progenitors that replace prenatal stromal cells in adult BM [45, 47, 48]. Adult Osx+ cells; however, only contribute to osteoblasts, not to BM stroma [45]. These results show a stage-specific contribution of Osx+ cells to skeletal development, and also suggest that Osx+ cells could be recruited to BM vasculature and become skeletal progenitors [45]. In line with these findings, tracing perinatal Osx+ cells over time reveals

overlap with *Nes*-GFP⁺ cells, both of which are immunostained with *LepR* [36, 45], and suggest that perinatal *Osx*⁺ cells are precursors of *Lepr*⁺ SSCs in the adult BM. However, whether *Osx*⁺ cells give rise to *Nes*-GFP⁺ cells or are derived from *Nes*-GFP⁺ cells is less certain[45].

Mx1-Cre (myxovirus resistance-1-Cre) was shown to label osteolineage-restricted progenitor cells in vivo having SSC properties [44]. *Mx1*⁺ cells are clonogenic, have tri-lineage differentiation potential ex vivo, and robustly contribute to the osteolineage, but not to other cell types, during homeostasis and regeneration. *Mx1*⁺ cells partly overlap with *Nestin*-GFP⁺ cells. Adult *Mx1*⁺ cells are often perivascular, on the endosteal bone surface and in calvarial suture mesenchyme where cranial SSCs are thought to reside[44]. In addition to its expression in BM stromal progenitors, *Mx1* is also expressed in other cell types including hematopoietic and vascular cells [40, 44], which limit its specificity to selectively enrich for skeletal progenitor cells.

Paired-related homeobox gene-1 (*Prx1*) a transcription co-activator expressed in the early limb bud and craniofacial mesenchyme, is critical for normal skeletal and craniofacial development [49–51]. *Prx1*⁺ cells are multipotent progenitor cells enriched in CFU-Fs [40] that overlap with the *PaS*⁺ cell population described above [52]. *Prx1* is uniformly expressed in marrow *LepR*⁺ cells [40]. Surprisingly, no endogenous expression of *Nestin*, *MCAM*/*CD146*, or *LepR* is detected in *Prx1*-Cre labeled cells, which may either reflect the specificity of *Prx1* to embryonic SSC or inefficiency of this Cre to label all targeted cells [52].

The homeobox gene, *Hoxa11*, along with other *Hox11* genes is essential for the embryonic development of the middle limb elements or zeugopod (radius and ulna; tibia and fibula). Interestingly, *Hoxa11* is expressed with the same regional specificity in adult mice and is associated with SSCs [53]. A GFP-knockin genetic approach was used to show that *Hoxa11*⁺ cells appear early in the developing limb bud in the outer perichondrium around cartilaginous rudiments. As the zeugopod anlage develops, *Hoxa11* expression is restricted to the outer perichondrial stromal cells outside the osteoblast layer that surrounds the developing elements [54, 55]. *Hox11* persists in the periosteum and also within the BM throughout adulthood [53]. Marrow *Hoxa11*⁺ cells are detected within a non-hematopoietic, non-endothelial stromal cell population that is positive for *PDGFRα*, *CD51*, and *LepR* and capable of tri-lineage differentiation and self-renewal ex vivo. When transplanted into a fracture callus, these cells contribute to cartilage and bone while maintaining their regional specificity[53]. When *Hox11* genes were mutated (*Hoxa*, *b* and *c11*), fracture healing in these zeugopod-derived elements was defective while healing the humerus was normal. In addition, SSCs from mutants were unable to undergo proper differentiation to give rise cell types essential for injury repair, and exhibited skewed adipogenic differentiation ex vivo [53]. Additionally, BM cells positive for *Hoxa11*EGFP, *PDGFRα*, and *CD51* showed a 3-fold enrichment of clonogenic potential compared with *PDGFRα*⁺ *CD51*⁺ cells. Interestingly, the skeletal element-restricted expression seen for *Hoxa11* is also seen for other *Hox* genes (stylopod; *Hox9*, *Hox10*; autopod, *Hox12*, *Hox13*; sternum, *Hoxa5*, *Hoxb6*, *Hoxc6*). The demonstration of regional specificity of SSCs based on the *Hox* code represents a paradigm shift in the field and adds a new layer of complexity to their biological identity

[53] that may have important implications for tissue regeneration. However, whether adult Hoxa11+ cells originate from embryonic Hoxa11-expressing cells and how much they contribute to postnatal skeletal growth and homeostasis is less well-understood.

Identification of Non-perivascular Skeletal Stem/Progenitor Cells

In addition to being present in perivascular regions of BM, SSC-like populations have been identified in metaphyseal regions of BM, in growth plate cartilage and in cranial sutures.

Gremlin1 (Grem1), a bone morphogenetic protein antagonist, marks a distinct SSC population in the primary spongiosa of the metaphysis immediately adjacent to the growth plate that have been referred to as osteochondroreticular (OCR) stem cells [35]. Using a lineage-tracing approach, Grem1+ cells were found to represent a rare and exclusively mesenchymal population (0.0025% of all live, mono-nucleated BM cells after collagenase digestion). Grem1+ cells first appear at E9.5 in hindlimb and contribute to most cells within primitive mesenchyme and the primary spongiosa by E21 [35]. They give rise to osteoblasts, chondrocytes, and stromal cells within the metaphysis and epiphysis, but not to adipocytes or perisinusoidal cells. In support of their function during development, ablation of Grem1 cells using DTA toxin leads to a low-bone mass phenotype. Grem1+ cells from adult BM are also enriched for stem cell activity including efficient colony formation (CFU-F) ex vivo, self-renewal, and osteogenic and chondrogenic differentiation during regeneration, and may be useful for treating skeletal diseases such as osteoarthritis, osteoporosis, and fracture. Grem1+OCR stem cells are distinct from perisinusoidal Nes-GFP+ cells in that they are more clonogenic and preferentially active during prenatal and perinatal development versus perisinusoidal SSCs that are more active during later adulthood.

Similar findings concerning the specification of bone, cartilage and stromal tissues from cartilage-associated stem/progenitor cells were obtained using a Rainbow mouse model [4]. Using this approach, individual cartilage-associated cells and their progeny were shown to form bone, cartilage and stromal tissue, but not hematopoietic cells, marrow fat or muscle. Immuno-phenotyping was then used to separate growth plate-associated cells into eight subgroups with varied ability to form different skeletal tissues after serial ectopic transplantation. Results of this analysis indicated that skeletogenesis may proceed through a developmental hierarchy of lineage-restricted progenitors starting with multipotential SSCs capable of self-renewal and differentiation into multiple lineages being at the top of a hierarchical tree followed by more lineage-restricted progenitors that form cartilage, bone or marrow stroma [4]. Consistent with these findings, other groups showed that chondrocytes and their precursors could contribute to early skeletal progenitors in growing bone. This was achieved in fate-mapping studies using cartilage-specific inducible Cre's such as collagen II (Col2-CreER), Sox9, a transcription factor expressed in primitive mesenchyme (Sox9-CreER), and aggrecan (Acan-CreER). For example, it was shown that Col2+ cells could give rise to chondrocytes, osteoblasts, CXC12-expressing stromal cells and BM skeletal progenitor cells in adult mice. Col2+ cells also contributed to transient prenatal Osx-CreER-labelled stromal cells that were replaced by perinatal Osx-CreER-marked stromal cells (discussed earlier). These cartilage-derived progenitor cells were proposed to support the explosive growth that occurs uniquely in growing mammals[56].

Cranial sutures are active growth sites for intramembranous bone formation in the cranial vault [57] and are the location of an additional, non-perivascular SSC population that contributes to craniofacial bone growth and injury repair [58]. Cells in cranial sutures have different fates depending on their position within the suture [59]; suture mesenchymal cells adjacent to the osteogenic front of the calvaria bones differentiate into osteoblasts and become incorporated into the growing bones, while mid-suture mesenchymal cells remained undifferentiated[59]. On the basis of lineage tracing studies described below, these mid-suture mesenchymal cells have been proposed to be calvaria mesenchymal stem. For example, glioma-associated oncogene 1 (Gli1), an intermediate in hedgehog signaling, was shown to mark undifferentiated cells in the suture mesenchyme of adult mouse craniofacial bones. Gli1+ cells are broadly expressed in neonatal calvaria, but become restricted to suture mesenchyme as mice age [58]. Fate mapping of Gli1+ cells shows that they contribute to craniofacial bones during growth and injury repair. Findings also suggest that Gli1+ cells are required for normal calvaria development because depletion of this cell population leads to craniofacial malformations, such as craniosynostosis, the premature closure of cranial sutures[58]. In culture, Gli1+ cells exhibit osteogenic and chondrogenic differentiation potential, but have a lower tendency to differentiate into adipocytes compared with cells from bone marrow[58]. Together, these findings support the use of Gli1 as a marker to identify SSCs in adult calvaria and raise the possibility of using this specific population to treat skull deformations caused by craniosynostosis.

Similar suture mesenchymal cells expressing high levels of the Wnt pathway intermediate, Axin2, were also shown to possess characteristics of SSCs[60]. Axin2+ cells reside solely in suture midline, and may represent a subset of Gli1+cells. Axin2+ cells are capable of long-term self-renewal and differentiation to osteoblasts during development, remodeling and regeneration. Interestingly, LepR (a previous identified marker for perivascular BM SSCs) is highly enriched in Axin2+ cells; however, data suggest that Axin2 is not expressed in SSCs of long bones[60].

In addition to being an early mesenchymal cell marker and critical factor for formation of the appendicular skeleton (as described above), Prx1 is expressed in a subpopulation of the adult Gli1+ suture cells [51]. The craniofacial expression of Prx1 is mainly in the posterior frontal, coronal, sagittal and lambdoid sutures, but not in other craniofacial sutures or in calvarial bone marrow of adult mice. Prx1+ cells are abundant during development, but gradually decline in adulthood. Calvarial Prx1+ cells express high levels of PDGFR α and MCAM/CD146 (previous defined markers for SSCs), as well as Dkk1 and Sost, two inhibitors of the Wnt signaling pathway that were suggested to contribute to the maintenance of the undifferentiated quiescent status of Prx1+ cells[51]. Studies where global depletion of Prx1+ cells was achieved using diphtheria toxin A (DTA) demonstrated that these cells are necessary for development of most calvaria bones and sutures. In the adult, suture-derived Prx1+ cells were shown to be necessary for calvarial bone regeneration [51].

Regulation of SSC differentiation – Osteoblasts versus Adipocytes

Cell fate decisions controlling whether SSCs will form bone or other tissues are critical for maintenance of bone mass. While SSCs can differentiate into multiple cell types

(osteoblasts, chondrocytes, marrow stromal cells, bone lining cells, marrow adipocytes); the reciprocal relationship between osteogenic and adipogenic differentiation is of particular interest because of the association of marrow adiposity with pathological conditions including age-related bone loss/osteoporosis, immobilization-related osteopenia and diabetes. Here we review some important factors affecting SSC lineage commitment including physical and mechanical characteristics of the SSC microenvironment and hormone/growth factor signaling. In the first category are a number of studies showing that SSC fate decisions are affected by the extracellular matrix (ECM) environment including topography [61], elasticity [62], and geometry [63, 64]. For example, manipulation of cell shape by growth on adhesive surfaces of different sizes is able to affect lineage commitment of SSCs *ex vivo*, with round and unspread cells preferentially differentiating to adipocytes, and flattened, spread cells differentiating to osteoblasts[64]. Because changes in cell morphology are related to changes in cytoskeletal tension, these studies establish a link between mechanical signaling and SSC differentiation (reviewed in [65]). The mechanical interaction between SSCs and their ECM is largely mediated by integrins, the trans-membrane receptors that integrate extracellular signaling to activate downstream pathways, including focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK) and Rho/Rho-associated protein kinase (ROCK). Integrins interact with the collagen-rich ECM, and, upon mechanical stimulation, trigger FAK-activation of the MAPK pathway, which subsequently activates RUNX2 and inhibits PPAR γ , resulting in a loading-dependent switch in lineage commitment of SSCs from adipocytes to osteoblasts [66, 67]. At least part of this switch is explained by MAPK-dependent phosphorylation of RUNX2 and PPAR γ transcription factors, which stimulates RUNX2-dependent induction of osteoblast gene expression and inhibits PPAR γ -dependent adipogenesis [68]. Mechanical and cytoskeletal signals also direct cell fate by controlling the activity of YAP/TAZ (Yes-associated protein/transcriptional co-activator with PDZ-binding motif), the two downstream transcriptional regulators of the Hippo signaling pathway (reviewed in[69]). Once active, YAP/TAZ translocate from cytoplasm into nucleus, where they interact with other transcription factors. Recent findings indicate that YAP/TAZ in cooperation with two transcriptional repressors, Snail and Slug, regulate self-renewal and differentiation of SSCs [70, 71]. In fact, Snail/Slug are required for normal SSCs function since conditional knockout of both factors severely compromised SSC self-renewal, differentiation and bone formation. These studies suggest that Snail/Slug interacts with YAP/TAZ to activate the expression of YAP/TAZ targeted genes including RUNX2, thereby controlling SSC function. Mechanical activation of YAP/TAZ via contractile activity of α -smooth muscle actin (α -SMA) controls differentiation of these cells to osteoblasts[72]. α -SMA+ SSCs exhibited high contractile activity, low clonogenicity, and a differentiation potential restricted to osteogenesis, while knockdown of α -SMA was sufficient to restore clonogenicity and adipogenesis[72].

Extracellular signaling mediated by non-integrin receptors may also control SSC lineage allocation. For instance, discoidin domain receptor 2 (DDR2), a non-integrin receptor activated by fibrillar collagens, including type I collagen [73], is required for normal bone development[74, 75]. As was recently shown, type I collagen activation of DDR2 stimulates MAPK-dependent RUNX2 and PPAR γ phosphorylation to promote osteoblast differentiation and inhibit adipogenesis [75]. In addition to extracellular factors, the ability

of SSCs to remodel their own collagenous ECM is also critical for determining cell fate. Matrix metalloproteinase 14 (MMP14), also known as MT1-MMP, controls SSC commitment by remodeling the ECM niche and altering cell shape, which then triggers activation of β 1-integrin and downstream signaling through YAP/TAZ [76]. Targeted inactivation of MT1-MMP redirects SSC differentiation from osteoblasts to adipocytes and chondrocytes [76].

Fate decision of SSCs can also be controlled by hormonal factors such as leptin, an adipokine secreted by adipocytes[77], parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP)[78]. Leptin signaling regulates bone mass systemically by acting on Leptin receptor (LepR)-expressing neurons in the hypothalamus[79] and locally by directly binding LepRs on SSCs, osteoblasts and osteoclasts (reviewed in[80]). Conditional knockout of LepR in SSC-enriched stromal cells impaired adipogenesis and promoted osteogenesis, suggesting that LepR promotes adipogenesis (and inhibits osteogenesis) by acting on BM SSCs [43]. Interestingly, Morrison and colleagues showed that SSCs in adult BM could be regulated by nutritional status as a consequence of local leptin/LepR signaling. LepR+ SSCs were shown to act locally to mediate diet-induced leptin signaling to promote adipogenesis and bone loss in BM, likely through Jak2/Stat3 signaling [43]. Intermittent administration of PTH stimulates bone formation by increasing osteoblast activity [81] and recruiting bone marrow stromal cells to the osteoblast lineage[82]. Recent studies revealed that PTH could also regulate the SSC fate since conditional knockout of PTH receptor-1 (PTH1R) in skeletal progenitor/stem cells resulted in increased adipogenesis accompanied by high bone resorption[78].

Conclusions and Future Directions

Over the past 50 years, much progress has been made in our understanding the nature and function of SSCs beginning with the initial identification of clonogenic multipotent cells in ex vivo marrow cultures followed by the discovery of the relationship between skeletal progenitors and the hematopoietic niche, identification of specific SSC cell surface markers and, most recently, lineage tracing studies that relate specific progenitor populations to differentiated osteoblasts, chondrocytes, marrow adipocytes and marrow stromal cells (summarized in Table 1 and Figure 1). Of equal importance is the expansion of knowledge related to the control of SSC differentiation to specific lineages. Current knowledge reveals a unique spatial distribution of SSCs in BM, cartilage and suture mesenchyme as well as temporal and lineage-specific contributions of specific cell populations during skeletal development and adult homeostasis. It has been proposed that stem cell pools from cartilage and perivascular niches have complementary functions with SSCs associated with growth plate cartilage being dominant and active during development and early postnatal life, while perivascular SSCs increase their contribution to skeletal homeostasis in adulthood [35]. Despite these advances, much remains to be learned about SSC biology due to inherent biases associated with experimental assays and limitations of the genetic tools being used to define stem cell populations. Future studies will require the identification more reliable SSC markers (or combinations of markers), more specific Cre alleles for lineage tracing and increased understanding of signals controlling SSC differentiation. These new tools will allow further refinement of our understanding of distinct SSC populations, their

interrelationships and contribution to skeletal functions during prenatal and postnatal life. Knowledge gained will be critical for understanding how the skeleton develops, matures and ages as well as for the development of stem cell based therapies to treat bone diseases and facilitate skeletal tissue engineering.

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Figure 1. Markers for specific skeletal stem cell populations

(A) In long bones, SSCs labeled with various markers suggest a unique distribution in growth plate cartilage and in perivascular regions around blood vessels, where some SSC subpopulations show an overlapping distribution. The differentiation potentials of cells labeled with specific markers are shown in parentheses, **O**: Osteoblasts, **A**: Adipocytes, **C**: chondrocytes, **S**: stromal cells. (B) Distinct postnatal calvarial SSCs contributing to craniofacial bones are shown to reside within the suture mesenchyme. Left panel: Color

code is used to show cells marked by specific Cre's that reside either in central or peripheral regions of the suture. Right panel shows major cranial sutures.

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Table 1

Putative skeletal stem cell markers

Markers	Localization/method used	Cells labeled	References
Growth plate cartilage-associated SSCs			
α V integrin (CD51) & endoglin (CD105)	FACS followed with ectopic transplantation	Osteoblasts, chondrocytes, stromal cells	[4]
Grem1-CreER	Endogenous expression confirmed with qRT-PCR analysis/Lineage tracing	Chondrocytes, Col1(2.3)-GFP+ osteoblasts & reticular marrow stromal cells	[35]
Col2-CreER	Lineage tracing	Chondrocytes, perichondrial cells in the growth plate, Col1(2.3)-GFP+ osteoblasts, osteocytes & CXCL12+stromal cells	[56]
Sox9-CreER/Acan-Cre-ER	Lineage tracing	Sox9-expressing Chondrocytes, perichondrial cells, & Col1(2.3)-GFP+ osteoblasts (also Ocn-positive)	[56]
Perivascular-associated SSCs			
MCAM/CD146	FACS/Ectopic transplantation	Osteoblasts, stromal cells	[30]
Nes-GFP	FACS & lineage tracing	Perivascular stromal cells endothelial cells, Osx-expressing cells, osteoblasts, osteocytes,	[28,36]
PDGFR α Sca-1	FACS isolation of GFP-labeled cells/BM transplantation	Ocn-expressing osteoblasts, reticular cells, endothelial cells & perilipin+ adipocytes (in adipose tissue)	[39]
PDGFR α CD51	FACS & immunostaining (IF)	Nes-GFP & Nes-expressing cells	[41]
LepR-Cre	Endogenous LepR expression confirmed with immunostaining (IF)/Irradiation, tibia fracture & immunostaining (IF)	Col1(2.3)-GFP+ osteoblasts, perilipin+ adipocytes & aggrecan+ chondrocytes (fracture only)	[40]
Mx1-Cre	FACS/Immunostaining (IF)	Osx-expressing cells & Ocn-expressing osteoblasts	[44]
Prx1-Cre	FACS	PDGFR α + Sca-1+ mesenchymal progenitor cells	[52]
Hoxa11-GFP	FACS, immunostaining (IF) & Hoxa11-GFP labeled with LepR-Cre injected into the fracture callus	Sox9-expressing chondrocytes & Osx-expressing osteoblasts	[53]
OSX-CreER	Lineage tracing	Osteoblasts, stromal cells & Nes-GFP+ cells	[45]
Cranial suture SSCs			
Gli1-CreER	Lineage tracing & calvaria defects	Osteolineages in osteogenic fronts, periosteum, dura & osteocytes	[58]
Axin2-CreER	B-gal staining, lineage tracing & calvaria defects/immunostaining (IF)	Osx-expressing osteoblasts & osteocytes	[60]
Prx1-Cre & Prx1-CreER	Endogenous expression was confirmed by in situ hybridization/Lineage tracing, calvaria defects & suture transplant	Osteolineages in osteogenic fronts, periosteum, dura & osteocytes	[51]