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Skeletal Stem Cells for Bone Development and Repair: Diversity Matters

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Abstract

Purpose of Review—Skeletal stem cells (SSCs) are considered to play important roles in bone development and repair. These cells have been historically defined by their in vitro potential for self-renewal and differentiation into “trilineage” cells; however, little is known about their in vivo identity. Here, we discuss recent progress on SSCs and how they potentially contribute to bone development and repair.

Recent Findings—Bone is composed of diverse tissues, which include cartilage and its perichondrium, cortical bone and its periosteum, and bone marrow and its trabecular bone and stromal compartment. We are now at the initial stage of understanding the precise identity of SSCs in each bone tissue. The emerging concept is that functionally dedicated SSCs are encased by their own unique cellular and extracellular matrix microenvironment, and locally support its own compartment.

Summary—Diverse groups of SSCs are likely to work in concert to achieve development and repair of the highly functional skeletal organ.

Keywords

Skeletal stem cells (SSCs); Mesenchymal stem cells (MSCs); Bone regeneration; Bone development; In vivo lineage-tracing experiments; Single-cell RNA-seq

Introduction

Somatic stem cells, also known as tissue-specific stem cells, play important roles in tissue growth and homeostasis throughout postnatal life, based on their two hallmark capabilities of (1) self-renewal, which is the ability to continue to reproduce themselves, and (2) multipotency, which is the ability to give rise to multiple types of differentiated cells. There are a variety of somatic stem cells in the body, such as in the blood, intestine, central nervous system, skin, hair follicle, and bone [1–8]. Skeletal stem cells (SSCs), a type of somatic stem cells dedicated to bones, are considered to play important roles in

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development, homeostasis, and regeneration of bone tissues. They are generally defined as self-renewing cells with the “trilineage” potential to differentiate into chondrocytes, osteoblasts, marrow stromal cells, or adipocytes in vitro. However, the in vivo identity of SSCs remains largely elusive.

A variety of SSCs are generated during the course of bone development. Formation of mesenchymal condensations within the limb bud is the initial step for endochondral bone development, by which most of bones are formed. Undifferentiated mesenchymal cells in these condensations become chondrocytes, which form and expand the cartilage template through rounds of cell proliferation and differentiation. At the same time, the perichondrium is formed around the cartilage template as layers of fibroblastic cells. When chondrocytes within the cartilage template undergo hypertrophy, the perichondrium becomes differentiated and forms the osteogenic perichondrium that is located in the bilateral sides of the hypertrophic zone. These chondrocytes and perichondrial cells eventually generate most of skeletal compartments, including articular cartilage, growth plate, perichondrium, periosteum, cortical bone, trabecular bone, and bone marrow, indicating that these two types of cells provide sources of SSCs. SSCs develop within these diverse types of skeletal tissues, primarily during the postnatal stage.

A well-accepted model is that somatic stem cells follow through a hierarchy model, in which these stem cells stand at the pinnacle of the cell lineage and are responsible for generating most of differentiated cells. For example, hematopoietic stem cells are the only cells capable of reconstituting the entire blood system upon transplantation, producing all types of blood cells [1–5]. In addition, somatic stem cells in epithelial tissues, such as intestinal [6, 7] and hair follicle stem cells [8], continue to repopulate their own compartment by continuously generating differentiated cells throughout life. This hierarchical model established in these showcase stem cell tissues has been considered to be unanimously applicable to SSCs. However, recent lines of evidence suggest that SSCs might follow a model that is dissimilar to other stem cells; that is, multiple types of SSCs, instead of a single master stem cell, work concomitantly to support major biological processes. In this review, we discuss three distinct types of SSCs, which are growth plate stem cells, periosteal stem cells, and bone marrow stem cells, and how they can possibly regulate bone development and repair.

Techniques to Identify Skeletal Stem Cells

SSCs have been isolated using several types of approaches. An in vitro colony-forming unit-fibroblast (CFU-F) assay, which is adapted from an assay to define hematopoietic progenitor cells in classical studies [9–11], and a subsequent transplantation assay have long been used as a gold standard for defining SSCs. These cells can rapidly expand on cell culture dishes and can make ossicles with bone and bone marrow after transplantation [10, 11]. In addition, human and mouse SSCs have been isolated by a panel of cell surface markers for fluorescence-activated cell sorting (FACS). In brief, human SSCs are defined by PDPN⁺CD146⁺CD73⁺CD164⁺ nonhematopoietic mesenchymal cells [12], whereas mouse SSCs are defined by CD51⁺CD200⁺CD90⁺CD105⁺ nonhematopoietic mesenchymal cells [13]. Human and mouse SSCs characterized by Chan et al. do not share the same cell surface markers for unknown reasons. Other combinations of cell surface markers used to define

SSCs include $\text{PDGFR}\alpha^+\text{Sca1}^+\text{CD45}^-\text{TER119}^-$ [14], $\text{CD73}^+\text{CD31}^-$ [15, 16], $\text{CD271}^+\text{CD45}^-$ [17–20], and CD106^+ [21].

However, it is not clear how these SSCs, defined by these in vitro and transplantation assays, are formed during normal bone development. *Prrx1*, which is expressed in the lateral plate mesoderm of the early limb bud, marks precursors of all skeletal cells of the appendicular skeleton, as *Prrx1-cre* marks all skeletal cells when combined with a reporter strain [22–24]. Following the expression of *Prrx1*, *Sox9* becomes expressed by a large subset of condensing mesenchymal cells. These Sox9^+ cells in condensations function as osteo-chondro progenitors and provide precursors for all chondrocytes and osteoblasts in subsequent stages, thus playing an essential role for endochondral bone development [25, 26]. *Sox9*, as well as its immediate target matrix genes such as type II collagen alpha 1 chain (*Col2a1*) and aggrecan (*Acan*), are expressed by cells in the cartilage template that contribute to a majority of skeletal cells, as demonstrated by in vivo lineage-tracing experiments based on a tamoxifen-inducible *creER* recombinase system [27, 28]. Therefore, chondrocytes and their precursors within the cartilage template and the growth plate provide a major source of skeletal cells in development.

Bones develop progressively from initially simple condensations to more functionally compartmentalized mineralized tissues. The current evidence indicates that functionally dedicated types of SSCs develop in a site-specific manner during bone development. Recent technological advances, including clonal lineage-tracing approaches using an inducible *creER* recombinase system, which allows specific spatial and temporal labeling of cells, and single-cell sequencing analyses, which unveils cellular diversity and distinct molecular signature of individual cells, provide new insight into multiple types of SSCs, including growth plate stem cells, periosteal stem cells (PSCs), and bone marrow skeletal stem cells, each of which playing distinct functions in their own compartment [10, 29•, 30, 31•, 32•]. How these SSCs are related to one another remains clarified. Here we discuss the characteristics of each type of SSCs.

Growth Plate Stem Cells

The growth plate cartilage is a central piece of endochondral bones, which plays an important role for bone growth. The growth plate develops relatively early from undifferentiated mesenchymal condensations during fetal bone development. Cells in the condensation become chondrocytes and continue to proliferate and differentiate into round, flat, and hypertrophic chondrocytes. These chondrocytes organize themselves in an avascular structure termed the cartilage template. Primary ossification center with nascent bone marrow space is formed as a result of vascular invasion into the hypertrophic zone of the cartilage template. Hypertrophic chondrocytes send cues to direct the formation of mineralized matrix within the template. As the primary ossification center continues to expand, chondrocytes are organized at the both sides of long bones as the fetal growth plate, composed of round, flat, and hypertrophic layers [33]. This “tri-layer” structure of the growth plate develops further during postnatal bone development, into three distinct layers of resting, proliferating, and hypertrophic chondrocytes [34].

Early skeletal progenitor cells are formed among cells in the growth plate cartilage that are marked by typical chondrocyte markers such as Col2a1, Sox9, and Acan. These cells continue to become osteoblasts and bone marrow stromal cells (BMSCs) in growing bone marrow for a long period, and support explosive bone growth uniquely occurring during the early postnatal period [27]. Cells marked by a typical hypertrophic chondrocyte marker Col10a1 are on their path to become cells of the osteoblast lineage [35]. These series of lineage-tracing studies have provided definite evidence that chondrocytes and their precursors in the growth plate cartilage can differentiate into additional skeletal cell types including osteoblasts and marrow stromal cells, which may include SSCs or other populations of skeletal progenitor cells.

SSCs with robust transplantability can be isolated from developing growth plates using a panel of cell surface markers and cell transplantation experiments, both in humans and mice [12, 13]. Human SSCs are defined as PDPN⁺CD146⁻CD73⁺CD164⁺ non-hematopoietic mesenchymal cells, which are isolated by mechanical and collagenase digestion from the fetal femoral head cartilage that includes proliferating, pre-hypertrophic, and hypertrophic zone. Similarly, mouse SSCs are defined as CD51⁺CD200⁺CD90⁻CD105⁻ non-hematopoietic mesenchymal cells, which are isolated from the neonatal femoral growth plate by collagenase and mechanical dissociation.

The important question here is where these transplantable SSCs are housed within the growth plate. It has long been proposed that the resting zone contains stem-like cells, based on an elegant rabbit transplantation model [36]. More recently, skeletal stem cells have been identified within the resting zone of the postnatal growth plate, which is formed within the epiphysis in conjunction with the formation of the secondary ossification center, a highly vascularized structure formed during postnatal bone development. Particularly, a specific subset of chondrocytes expressing parathyroid hormone-related protein (PTHrP) in the resting zone can behave as a unique class of skeletal stem cells, as demonstrated by in vivo lineage-tracing experiments in mice [29••, 30]. PTHrP released from the resting zone maintains the growth plate by interacting with Indian hedgehog (Ihh) released from the hypertrophic zone, through the PTHrP–Ihh negative feedback loop. PTHrP⁺ chondrocytes marked by a *Pthrp-mCherry* knock-in allele or a *Pthrp-creER* line are specifically located in the resting zone. PTHrP⁺ chondrocytes express a panel of mouse SSC markers defined by Chan et al. mentioned above, and demonstrate SSC properties in cultured conditions. Lineage-tracing experiments using *Pthrp-creER* demonstrate that these PTHrP⁺ cells continue to self-renew within resting zone through asymmetric divisions and provide a source of columnar chondrocytes for a long time. Some of their progeny become osteoblasts and bone marrow stromal cells beneath the growth plate. Therefore, a special type of PTHrP⁺ chondrocytes in the resting zone can behave as SSCs with robust self-renewability and multipotency at the postnatal stage and simultaneously express a panel of markers for transplantable SSCs.

Stem cells generally undergo either asymmetric division, which produces a new stem cell and a daughter cell that eventually differentiates, or symmetric division, which reproduces two new stem cells or two differentiating daughter cells. Growth plate stem cells have an important function in keeping the balance of these cell divisions in physiological conditions,

in order to maintain the growth plate structure. The current evidence supports that this process is regulated by some of the important signaling pathways. Newton et al. demonstrate that chondroprogenitors deplete themselves due to a consumption program in fetal and neonatal stages, but they acquire self-renewability when the secondary ossification center (SOC) is formed within the epiphysis, which results in the formation of large and stable monoclonal columns of chondrocytes (a renewal program). Activation of mTORC1 signaling in chondrocytes disorganizes the resting zone, associated with an increase in the number and thickness of multi-columnar clones. This is due to the shift from asymmetric to symmetric cell division induced by mTORC1 signaling [30]. In addition, hedgehog (Hh) signaling appears to play an essential role for regulating SSCs in the growth plate. Newton et al. administered Hh antagonist vismodegib or agonist SAG to the mice around 4–5 weeks of age and dissected them shortly thereafter. Hh antagonist reduces the clone size within the resting zone, causing the premature fusion of the growth plate, whereas Hh agonist increases proliferation within the resting zone. In contrast, Mizuhashi et al. administered Hh antagonist LDE225 or agonist SAG to the mice around 2 weeks of age and dissected these mice thereafter. Both Hh antagonist and agonist induce a significant reduction in the number of PTHrP⁺ SSCs and their descendants. Together, an appropriate level of Hh signaling appears to be essential to maintaining the proper cell fates of PTHrP⁺ SSCs residing in the resting zone through both cell autonomous and non-cell autonomous mechanisms.

Future Directions for Growth Plate Stem Cells

SSCs in the resting zone of the growth plate, particularly PTHrP⁺ SSCs, have the potential to self-renew and differentiate into multiple types of skeletal cells in vivo. However, further details remain to be clarified. First, PTHrP⁺ SSCs contribute to an only small number of osteoblasts and stromal cells in the marrow space, indicating that most of descendants of these stem cells may undergo apoptosis without arriving at the marrow space. The mechanism regulating their cell fate choice between survival and death during their transition to the marrow remains undefined. In addition, growth plate chondrocytes can arrive at the marrow space through multiple pathways. For example, borderline chondrocytes located at the periphery of the growth plate adjoining the perichondrium represent an alternative route for chondrocytes to become osteoblasts and marrow stromal cells [29••]. Second, how PTHrP⁺ SSCs maintain their stemness and asymmetric divisions within the resting zone for a long time remains unknown, although mTORC1 signaling is now identified as one of the important pathways. It is possible that these important properties are maintained as a result of dynamic cellular interactions between PTHrP⁺ SSCs and PTHrP⁻ chondrocytes, as well as between PTHrP⁺ SSCs and their adjoining “niche” cells in the SOC. Third, the origin of SSCs in the resting zone remains largely ambiguous. These SSCs might be derived from Wnt-responsive chondroprogenitors in the outermost layer of the growth plate [37] or perichondrial cells in the groove of Ranvier that might migrate into the growth plate [38]. Unraveling cellular identities and functions of distinct subsets of growth plate chondrocytes will aid us to better define growth plate stem cells.

Perichondrial/Periosteal Stem Cells

The perichondrium and the periosteum are fibrous tissues surrounding the growth plate cartilage and the cortical bone, respectively, which play important roles in bone development and repair. The perichondrium initially develops at the periphery of mesenchymal condensations in a highly vascularized environment as a multi-layer fibrous tissue [39]. The perichondrium has essential roles in supporting bone development, by providing a source of skeletal progenitor cells as well as cues for adjacent chondrocytes. For example, deletion of *Fgf9* and *Fgf18*, which are abundantly expressed in the perichondrium, decreases chondrocyte proliferation, largely due to lack of FGF9 and FGF18 binding to FGFR3 expressed by chondrocytes [40]. Additionally, Notch effector genes *Notch2* and *Hes1* are expressed in perichondrial cells surrounding condensations. *Hes1* suppresses chondrogenic differentiation of mesenchymal progenitor cells in cultured conditions [41]. After the formation of the primary ossification center, the perichondrium remains outside of the growth plate; fetal perichondrial cells in the osteogenic perichondrium expressing *Osx* can translocate into the nascent marrow space [42]. In the postnatal stage, the perichondrium remains surrounding the growth plate. This includes the groove of Ranvier, which has been suggested as the reservoir of skeletal progenitor cells that can migrate to the surface of the articular cartilage and growth plate cartilage [38], although direct evidence based on in vivo lineage-tracing experiments is still lacking.

The periosteum is a fibrous membranous tissue covering the outer surface of the cortical bone. The periosteum is mainly composed of two layers, the outer fibrous layer and the inner cambium layer. The cambium layer is considered to house osteoblasts and their precursors, as well as SSCs [43, 44]. The current prevailing notion is that periosteal cells and osteoblasts in the bone collar are derived from the fetal perichondrium, as demonstrated by a tissue transplantation study [45, 46].

Periosteal stem cells (PSCs), residing in the periosteum of long bones in mice, are characterized with robust self-renewability and multipotency through both endochondral and intramembranous pathways during bone fracture healing and upon transplantation, respectively. Duchamp et al. show that these properties of PSCs are more robust than those of BMSCs, based on a transplantation model [47]. PSCs can be identified as a subset of periosteal cells marked by *Cathepsin K* (*Ctsk*)-*cre*, which is also a well-known marker for osteoclasts. *Ctsk*-*cre*-marked cells appear in the perichondrium shortly after E14.5 and continue to be present in the periosteum and the groove of Ranvier during the following fetal and postnatal stages [31•, 48]. Interestingly, *Ctsk*-*cre*-marked cells do not appear to contribute to the bone marrow stromal and trabecular compartment. Periosteal cells marked by *Ctsk*-*cre* are heterogeneous as demonstrated by a single-cell RNA-seq analysis, and subcluster into mouse SSCs and their immediately downstream progenitor populations that Chan et al. defined previously. *Ctsk*-*cre*-marked PSCs can differentiate into only osteoblasts after transplantation to the kidney capsule, while they can differentiate into trilineage cells in vitro under cultured conditions. Moreover, *Ctsk*-*cre*-marked PSCs do not contribute to cortical bone osteoblasts in vivo.

PSCs can robustly contribute to bone regeneration via endochondral pathways. *α SMA-creER*-marked cells [49, 50], *Ctsk-cre*-marked cells, and pIpC-inducible Mx1-cre-marked *α SMA*⁺ cells [51] can differentiate into fracture callus chondrocytes and osteoblasts during long bone fracture healing [31•]. Studies using transplantation of GFP-labeled periosteal cells and BMSCs into the wild-type fracture site demonstrate that periosteal cells have higher regenerative capacity than BMSCs [47], demonstrating the usefulness of PSCs for bone fracture healing and regeneration.

Future Directions for Perichondrial/Periosteal Stem Cells

The properties of PSCs, which are particularly advantageous in the context of bone fracture healing and transplantation, have now been unraveled. However, there are fundamental questions that need to be addressed in future studies. First, the relationship between fetal perichondrial cells and periosteal cells at the later stage has not been unambiguously defined; thus, the origin of PSCs remains unclear. The current evidence shows that fetal *Osx*⁺ perichondrial cells are not the source of periosteal cells in the later stage, as demonstrated by a lineage-tracing study by *Osx-creER* [42]. In fact, descendants of fetal *Osx*⁺ perichondrial cells are transient and eventually disappear from the bone collar, without contributing to any periosteal cells or perichondrial cells in the groove of Ranvier in the following stage [27, 42, 52]. An earlier population of perichondrial cells that provide a source of *Osx*⁺ fetal perichondrial cells needs to be identified to clarify the cell lineage, particularly to define whether these unidentified early cell populations can contribute to periosteal cells at later stages. Second, how significantly PSCs can contribute to bone fracture healing needs to be defined through more rigorous analyses. For example, *α SMA-creER* can mark periosteal cells as well as cells in primary spongiosa [49], and constitutively active *Ctsk-cre* lacks temporal control of recombination. A transplantation model cannot evaluate the regenerative capabilities of these periosteal cells in unperturbed conditions. The function of SSCs is likely to be regulated by their native environment, particularly by their adjacent cells. Considering that bone marrow stromal cells can concomitantly contribute to bone fracture healing [32•, 52, 53], an inducible *creER* line specific to periosteal cells is highly desirable to interrogate the function of periosteal cells under both physiological and regenerative conditions.

Bone Marrow Skeletal Stem Cells (a.k.a. Mesenchymal Stem Cells)

Mesenchymal stem cells (MSCs), or those later redefined as SSCs, have been isolated from bone marrow over five decades [9, 54] and applied widely to regenerative medicine [55]. These cells are preferentially located around bone marrow sinusoids, particularly among CD146⁺ perisinusoidal stromal cells in humans [56]. These perisinusoidal stromal cells demonstrate a characteristic morphology as reticular cells and express high levels of alkaline phosphatase [57]. However, the *in vivo* identity of SSCs or MSCs is still controversial primarily due to lack of SSC-specific markers or genetic tools that can reliably identify these cells *in vivo*.

SSCs have been isolated from bone marrow using a panel of cell surface markers such as *PDGFR α* ⁺*Sca1*⁺ cells [14], CD51⁺CD200⁺CD90⁻CD105⁻ [13], and CD73⁺ stromal cells

[15] in mice. Bone marrow SSCs represent a subset of CFU-Fs [10] identified in cultured conditions, therefore constituting a small subset of bone marrow stromal cells in their native environment. Bone marrow stromal cells assume perivascular locations surrounding sinusoids (open-pore capillaries) or arterioles (distal arteries), and universally express cytokines such as C-X-C motif chemokine 12 (CXCL12, also known as stromal cell-derived factor 1 (SDF1)) [58] and stem cell factor (SCF, also known as KIT ligand) [59] to attract and retain hematopoietic progenitor cells in the marrow space. Leptin receptor (LepR) is expressed in bone marrow stromal cells that largely overlap with CXCL12-abundant reticular (CAR) cells. These *LepR-cre*-marked and *Ebf3-creER*-marked bone marrow stromal cells, which almost completely overlap with CAR cells, can differentiate into osteoblasts in the trabecular and cortical osteoblast compartment in physiological and regenerative conditions, as well as into marrow adipocytes [53, 60].

Although these bone marrow stromal cells or CFU-Fs are generally considered to be uniform cell populations, recent single-cell RNA-sequencing studies have revealed the diversity of these cell populations [32•, 61–63]. In fact, CAR cells subcluster into several distinct groups, composed of two major groups of pre-adipocyte-like reticular cells and pre-osteoblast-like cells. A *Cxcl12-creER* line can specifically mark a quiescent subset of CAR cells that correspond to pre-adipocyte-like reticular cells. *Cxcl12-creER*⁺ cells are located preferentially in the central marrow space and do not contribute to cortical osteoblasts even after a long chase in physiological conditions. Interestingly, *Cxcl12-creER*⁺ cells remain spatially restricted to a far proximal portion of the femoral marrow space after active long bone growth. In fact, these quiescent *Cxcl12-creER*⁺ cells with a terminally differentiated state are the product of other mesenchymal precursor cells during bone growth; these precursor cells may include *Col2a1*⁺ cells that reside in proximity to the growth plate, *Gli1*⁺ cells at the chondroosseous junction immediately beneath the growth plate, *Osx*⁺ cells in the primary spongiosa, and/or *PTHrP*⁺ resting chondrocytes in the resting zone [27, 29•, 52, 64].

Cxcl12-creER⁺ and *LepR-cre*⁺ cells robustly contribute to osteoblasts and chondrocytes during bone fracture healing. These cells can also differentiate into marrow adipocytes spontaneously or under adipogenic conditions based on high-fat diet or radiation [32•, 53]. Therefore, CXCL12⁺LepR⁺ bone marrow stromal cells as a population have diverse differentiation potentials that meet the criteria of SSCs in the native condition. It is important to note, however, that the way that *Cxcl12-creER*⁺ cells become osteoblasts does not fit into a classical model of stem cell differentiation. Single-cell RNA-sequence analysis of cells lineage-marked by *Cxcl12-creER* shows that pre-adipocyte-like reticular cells can rapidly convert their identity into SSC-like cells in response to injury, then further differentiate into mature osteoblasts via the activation of the canonical Wnt signaling pathway. Therefore, *Cxcl12-creER*⁺ stromal cells contribute to bone regeneration through (1) dedifferentiation into stem cells and (2) further differentiation into mature osteoblasts. Other types of cells, including *Osx*⁺ osteoblasts and *Dlx5*⁺ osteoblast precursor cells, may also contribute to bone regeneration concomitantly. Therefore, diverse groups of bone marrow stromal cells, osteoblast precursor cells, and even more mature osteoblasts, rather than a small number of SSCs alone, can concertedly achieve bone regeneration, particularly through the process termed cellular plasticity [65].

Future Directions for Bone Marrow Skeletal Stem Cells

Quiescent bone marrow stromal cells abundantly expressing *CXCL12*, which are preferentially marked by *Cxcl12-creER*, are located in the central marrow space and do not produce new marrow stromal cells or differentiate into cortical bone osteoblasts in physiological conditions. At this moment, the upstream precursors for these cells remain unknown. Candidate precursors include *Gli1*⁺ or *Osx*⁺ cells, but these cells are transient and disappear from the original position after a long chase [52, 64]. *Col2a1*⁺ cells in proximity to the growth plate and *PTHrP*⁺ SSCs in the resting zone can continue to provide bone marrow stromal cells, but their contribution is limited. The future endeavor should be devoted to discover bona fide SSCs in bone marrow, which possibly reside in the metaphyseal bone marrow space right beneath the growth plate. An alternative hypothesis would be that SSCs do not exist in the marrow space in the first place. For example, under regenerative conditions, *Cxcl12-creER*⁺ bone marrow stromal cells can be reverted into stem cell-like cells that are characterized with an intermediate state between mature osteoblasts and CAR cells. Therefore, we may be able to discover bona fide bone marrow SSCs by further scrutinizing the identity of these intermediate stem cell-like cells. This will bring this field a step closer to redefining these elusive stem cells with more precise criteria.

Conclusions

SSCs have been characterized mainly by cell sorting with a combination of surface markers or only retrospectively after a prolonged period of clonal cell culture. The limitation of these methods is that spatial information of SSCs is permanently lost upon cell isolation. Identifying the location of these cells within highly complex bone tissues will be essential to understand their roles in bone development and regeneration. Murine lineage-tracing studies using *cre*, or ideally inducible *creER* recombinase systems, which can mark a specific cell population in time and space, will continue to be an important approach to unravel their behaviors under native conditions (Table 1). A single-cell RNA-sequencing analysis can be effectively combined with in vivo lineage-tracing tools to reveal the identity of distinct groups of cells constituting a given cell population. These two technologies have a strong potential to improve our current knowledge on SSCs. The diversity of SSCs in the native conditions is now becoming evident. Multiple types of distinct SSCs, rather than a single master SSC, work in concordance to achieve skeletal development and regeneration; in this process, each SSC might make only moderate contribution. One example is *PTHrP*⁺ skeletal stem cells in the resting zone of the growth plate, which continue to produce columnar chondrocytes throughout postnatal life, but their descendants only moderately contribute to osteoblasts or stromal cells in the marrow space. Taken together, SSCs are sequestered into their own compartment and regulated by their own cellular and matrix environment, and acquire their stemness at the specific stages of skeletal development. This gives SSCs a uniquely local role in regulating development and maintaining homeostasis.

In contrast, SSCs may coordinate bone regeneration in a different way from they regulate development and homeostasis (Table 2). The current evidence indicates that SSCs can acquire a new potential under regenerative conditions. For example, *PTHrP*⁺ SSCs directly differentiate into osteoblasts in response to micro-ablation injury, while *Ctsk*⁺ PSCs

contribute to endochondral bone formation in bone fracture healing. Diverse groups of bone marrow stromal cells as a group contribute to bone regeneration, including highly quiescent CAR cells marked by *Cxcl12-creER*, *Osx*⁺, and *Dlx5*⁺ osteoblast precursors. Importantly, functionally mature bone marrow stromal cells can dedifferentiate into skeletal stem cell-like cells through cellular plasticity, before redifferentiating into osteoblasts. In contrast, the identity of bona fide SSCs in bone marrow is still largely unknown (Fig. 1).

Thanks to recent technological progress, including an increasing repertoire of novel cell type-specific inducible *creER* lines and single-cell RNA-sequencing approaches, it is now possible to redefine the relationship among multiple types of SSCs and their relationships with more mature differentiated cells. Further research is needed to elucidate the fundamental characteristics of SSCs and their roles in skeletal development and regeneration.

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References

Papers of particular interest, published recently, have been highlighted as:

• Of importance

•• Of major importance

1. Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med.* 2014;20(8): 833–46. [PubMed: 25100529]
2. Méndez-Ferrer S, Michurina TV, Ferraro F, Mazloom AR, Macarthur BD, Lira SA, et al. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature.* 2010;466(7308):829–34. [PubMed: 20703299]
3. Wei Q, Frenette PS. Niches for hematopoietic stem cells and their progeny. *Immunity.* 2018;48(4):632–48. [PubMed: 29669248]
4. Notta F, Zandi S, Takayama N, Dobson S, Gan OI, Wilson G, et al. Distinct routes of lineage development reshape the human blood hierarchy across ontogeny. *Science.* 2016;351(6269):aab2116. [PubMed: 26541609]
5. Metcalf D On hematopoietic stem cell fate. *Immunity.* 2007;26(6): 669–73. [PubMed: 17582339]
6. Santos AJM, Lo YH, Mah AT, Kuo CJ. The intestinal stem cell niche: homeostasis and adaptations. *Trends Cell Biol.* 2018;28(12): 1062–78. [PubMed: 30195922]
7. Gehart H, Clevers H. Tales from the crypt: new insights into intestinal stem cells. *Nat Rev Gastroenterol Hepatol.* 2019;16(1):19–34. [PubMed: 30429586]
8. Gonzales KAU, Fuchs E. Skin and its regenerative powers: an alliance between stem cells and their niche. *Dev Cell.* 2017;43(4): 387–401. [PubMed: 29161590]
9. Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morpholog.* 1966;16(3):381–90.
10. Bianco P, Cao X, Frenette PS, Mao JJ, Robey PG, Simmons PJ, et al. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med.* 2013;19(1):35–42. [PubMed: 23296015]
11. Bianco P “Mesenchymal” stem cells. *Annu Rev Cell Dev Biol.* 2014;30:677–704. [PubMed: 25150008]

12. Chan CKF, Gulati GS, Sinha R, Tompkins JV, Lopez M, Carter AC, et al. Identification of the human skeletal stem cell. *Cell*. 2018;175(1):43–56.e21. [PubMed: 30241615]
13. Chan CK, Seo EY, Chen JY, Lo D, McArdle A, Sinha R, et al. Identification and specification of the mouse skeletal stem cell. *Cell*. 2015;160(1–2):285–98. [PubMed: 25594184]
14. Morikawa S, Mabuchi Y, Kubota Y, Nagai Y, Niibe K, Hiratsu E, et al. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J Exp Med*. 2009;206(11):2483–96. [PubMed: 19841085]
15. Breitbach M, Kimura K, Luis TC, Fuegeman CJ, Woll PS, Hesse M, et al. In vivo labeling by CD73 marks multipotent stromal cells and highlights endothelial heterogeneity in the bone marrow niche. *Cell Stem Cell*. 2018;22(2):262–76.e7. [PubMed: 29451855]
16. Cuthbert R, Boxall SA, Tan HB, Giannoudis PV, McGonagle D, Jones E. Single-platform quality control assay to quantify multipotential stromal cells in bone marrow aspirates prior to bulk manufacture or direct therapeutic use. *Cytotherapy*. 2012;14(4):431–40. [PubMed: 22268519]
17. Álvarez-Viejo M, Menéndez-Menéndez Y, Otero-Hernández J. CD271 as a marker to identify mesenchymal stem cells from diverse sources before culture. *World J Stem Cells*. 2015;7(2):470–6. [PubMed: 25815130]
18. Das B, Kashino SS, Pulu I, Kalita D, Swami V, Yeger H, et al. CD271(+) bone marrow mesenchymal stem cells may provide a niche for dormant *Mycobacterium tuberculosis*. *Sci Transl Med*. 2013;5(170):170ra13.
19. Boxall SA, Jones E. Markers for characterization of bone marrow multipotential stromal cells. *Stem Cells Int*. 2012;2012:975871. [PubMed: 22666272]
20. Coutu DL, Kokkalis KD, Kunz L, Schroeder T. Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. *Nat Biotechnol*. 2017;35(12):1202–10. [PubMed: 29131149]
21. Yang ZX, Han ZB, Ji YR, Wang YW, Liang L, Chi Y, et al. CD106 identifies a subpopulation of mesenchymal stem cells with unique immunomodulatory properties. *PLoS One*. 2013;8(3):e59354. [PubMed: 23555021]
22. Martin JF, Olson EN. Identification of a prx1 limb enhancer. *Genesis*. 2000;26(4):225–9. [PubMed: 10748458]
23. Logan M, Martin JF, Nagy A, Lobe C, Olson EN, Tabin CJ. Expression of Cre recombinase in the developing mouse limb bud driven by a Prx1 enhancer. *Genesis*. 2002;33(2):77–80. [PubMed: 12112875]
24. Greenbaum A, Hsu YM, Day RB, Schuettelpelz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*. 2013;495(7440):227–30. [PubMed: 23434756]
25. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrughe B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev*. 2002;16(21):2813–28. [PubMed: 12414734]
26. Akiyama H, Kim JE, Nakashima K, Balmes G, Iwai N, Deng JM, et al. Osteo-chondroprogenitor cells are derived from Sox9 expressing precursors. *Proc Natl Acad Sci USA*. 2005;102(41):14665–70. [PubMed: 16203988]
27. Ono N, Ono W, Nagasawa T, Kronenberg HM. A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. *Nat Cell Biol*. 2014;16(12):1157–67. [PubMed: 25419849]
28. Ono N, Kronenberg HM. Mesenchymal progenitor cells for the osteogenic lineage. *Curr Mol Biol Rep*. 2015;1(3):95–100. [PubMed: 26526380]
- 29••. Mizuhashi K, Ono W, Matsushita Y, Sakagami N, Takahashi A, Saunders TL, et al. Resting zone of the growth plate houses a unique class of skeletal stem cells. *Nature*. 2018;563(7730):254–8 [PubMed: 30401834] This research reveals that the resting zone of the growth plate houses PTHrP+ chondrocytes that behave as a unique type of SSCs.
30. Newton PT, Li L, Zhou B, Schweingruber C, Hovorakova M, Xie M, et al. A radical switch in clonality reveals a stem cell niche in the epiphyseal growth plate. *Nature*. 2019;567(7747):234–8. [PubMed: 30814736]

- 31•. Debnath S, Yallowitz AR, McCormick J, Lalani S, Zhang T, Xu R, et al. Discovery of a periosteal stem cell mediating intramembranous bone formation. *Nature*. 2018;562(7725):133–9 [PubMed: 30250253] This research unveils the role of periosteal SSCs in physiological and regenerative conditions.
- 32••. Matsushita Y, Nagata M, Kozloff KM, Welch JD, Mizuhashi K, Tokavanich N, et al. A Wnt-mediated conversion of the bone marrow stromal cell identity supports regeneration. *Nat Commun* 2020;11(1):332. [PubMed: 31949165] This research reveals that quiescent mature bone marrow stromal cells (BMSCs) lining sinusoidal blood vessels can convert their identity into skeletal stem cells (SSCs) and coordinate bone regeneration.
33. Kronenberg HM. Developmental regulation of the growth plate. *Nature*. 2003;423(6937):332–6. [PubMed: 12748651]
34. Ono N, Kronenberg HM. Bone repair and stem cells. *Curr Opin Genet Dev*. 2016;40:103–7. [PubMed: 27399886]
35. Yang L, Tsang KY, Tang HC, Chan D, Cheah KS. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. *Proc Natl Acad Sci USA*. 2014;111(33): 12097–102. [PubMed: 25092332]
36. Abad V, Meyers JL, Weise M, Gafni RI, Barnes KM, Nilsson O, et al. The role of the resting zone in growth plate chondrogenesis. *Endocrinology*. 2002;143(5):1851–7. [PubMed: 11956168]
37. Usami Y, Gunawardena AT, Francois NB, Otsuru S, Takano H, Hirose K, et al. Possible contribution of Wnt-responsive chondroprogenitors to the postnatal murine growth plate. *J Bone Miner Res*. 2019;34:964–74. [PubMed: 30602070]
38. Karlsson C, Thormemo M, Henriksson HB, Lindahl A. Identification of a stem cell niche in the zone of Ranvier within the knee joint. *J Anat*. 2009;215(3):355–63. [PubMed: 19563472]
39. Long F, Chung UI, Ohba S, McMahon J, Kronenberg HM, McMahon AP. Ihh signaling is directly required for the osteoblast lineage in the endochondral skeleton. *Development*. 2004;131(6): 1309–18. [PubMed: 14973297]
40. Ornitz DM, Marie PJ. Fibroblast growth factor signaling in skeletal development and disease. *Genes Dev*. 2015;29(14):1463–86. [PubMed: 26220993]
41. Dong Y, Jesse AM, Kohn A, Gunnell LM, Honjo T, Zuscik MJ, et al. RBPjkappa-dependent notch signaling regulates mesenchymal progenitor cell proliferation and differentiation during skeletal development. *Development*. 2010;137(9):1461–71. [PubMed: 20335360]
42. Maes C, Kobayashi T, Selig MK, Torrekens S, Roth SI, Mackem S, et al. Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Dev Cell*. 2010;19(2):329–44. [PubMed: 20708594]
43. Chang H, Knothe Tate ML. Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. *Stem Cells Transl Med*. 2012;1(6):480–91. [PubMed: 23197852]
44. Duchamp de Lageneste O, Colnot C. Periostin in bone regeneration. *Adv Exp Med Biol*. 2019;1132:49–61. [PubMed: 31037624]
45. Colnot C, Lu C, Hu D, Helms JA. Distinguishing the contributions of the perichondrium, cartilage, and vascular endothelium to skeletal development. *Dev Biol*. 2004;269(1):55–69. [PubMed: 15081357]
46. Colnot C, Zhang X, Knothe Tate ML. Current insights on the regenerative potential of the periosteum: molecular, cellular, and endogenous engineering approaches. *J Orthop Res*. 2012;30(12): 1869–78. [PubMed: 22778049]
47. Duchamp de Lageneste O, Julien A, Abou-Khalil R, Frangi G, Carvalho C, Cagnard N, et al. Periosteum contains skeletal stem cells with high bone regenerative potential controlled by Periostin. *Nat Commun*. 2018;9(1):773. [PubMed: 29472541]
48. Yang W, Wang J, Moore DC, Liang H, Dooner M, Wu Q, et al. Ptpn11 deletion in a novel progenitor causes metachondromatosis by inducing hedgehog signalling. *Nature*. 2013;499(7459):491–5. [PubMed: 23863940]
49. Grcevic D, Pejda S, Matthews BG, Repic D, Wang L, Li H, et al. In vivo fate mapping identifies mesenchymal progenitor cells. *Stem Cells*. 2012;30(2):187–96. [PubMed: 22083974]

50. Matthews BG, Grcevic D, Wang L, Hagiwara Y, Roguljic H, Joshi P, et al. Analysis of α SMA-labeled progenitor cell commitment identifies notch signaling as an important pathway in fracture healing. *J Bone Miner Res.* 2014;29(5):1283–94. [PubMed: 24190076]
51. Ortinau LC, Wang H, Lei K, Deveza L, Jeong Y, Hara Y, et al. Identification of functionally distinct Mx1+ α SMA+ periosteal skeletal stem cells. *Cell Stem Cell.* 2019;25(6):784–96.e5. [PubMed: 31809737]
52. Mizoguchi T, Pinho S, Ahmed J, Kunisaki Y, Hanoun M, Mendelson A, et al. Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev Cell.* 2014;29(3):340–9. [PubMed: 24823377]
53. Zhou BO, Yue R, Murphy MM, Peyer JG, Morrison SJ. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell.* 2014;15(2):154–68. [PubMed: 24953181]
54. Tavassoli M, Crosby WH. Transplantation of marrow to extramedullary sites. *Science.* 1968;161(3836):54–6. [PubMed: 4871792]
55. Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM. Concise review: multifaceted characterization of human mesenchymal stem cells for use in regenerative medicine. *Stem Cells Transl Med.* 2017;6(12):2173–85. [PubMed: 29076267]
56. Sacchetti B, Funari A, Michienzi S, Di Cesare S, Piersanti S, Saggio I, et al. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell.* 2007;131(2):324–36. [PubMed: 17956733]
57. Krebsbach PH, Kuznetsov SA, Bianco P, Robey PG. Bone marrow stromal cells: characterization and clinical application. *Crit Rev Oral Biol Med.* 1999;10(2):165–81. [PubMed: 10759420]
58. Ara T, Tokoyoda K, Sugiyama T, Egawa T, Kawabata K, Nagasawa T. Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity.* 2003;19(2):257–67. [PubMed: 12932359]
59. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature.* 2012;481(7382):457–62. [PubMed: 22281595]
60. Seike M, Omatsu Y, Watanabe H, Kondoh G, Nagasawa T. Stem cell niche-specific Ebf3 maintains the bone marrow cavity. *Genes Dev.* 2018;32(5–6):359–72. [PubMed: 29563184]
61. Tikhonova AN, Dolgalev I, Hu H, Sivaraj KK, Hoxha E, Cuesta-Domínguez Á, et al. The bone marrow microenvironment at single-cell resolution. *Nature.* 2019;569(7755):222–8. [PubMed: 30971824]
62. Baryawno N, Przybylski D, Kowalczyk MS, Kfoury Y, Severe N, Gustafsson K, et al. A cellular taxonomy of the bone marrow stroma in homeostasis and leukemia. *Cell.* 2019;177(7):1915–32.e16. [PubMed: 31130381]
63. Zhao M, Tao F, Venkatraman A, Li Z, Smith SE, Unruh J, et al. NCadherin-expressing bone and marrow stromal progenitor cells maintain reserve hematopoietic stem cells. *Cell Rep.* 2019;26(3):652–69.e6. [PubMed: 30650358]
64. Shi Y, He G, Lee WC, McKenzie JA, Silva MJ, Long F. Gli1 identifies osteogenic progenitors for bone formation and fracture repair. *Nat Commun.* 2017;8(1):2043. [PubMed: 29230039]
65. Merrell AJ, Stanger BZ. Adult cell plasticity in vivo: dedifferentiation and transdifferentiation are back in style. *Nat Rev Mol Cell Biol.* 2016;17(7):413–25. [PubMed: 26979497]

Fig. 1.

During bone development, SSCs are sequestered into their own compartment and regulated by their own cellular and matrix environment, and acquire their stemness at the specific stages of skeletal development. During bone regeneration, each SSC can acquire a new potential and robustly contributes to regenerated bone

Table 1

Cell lineage of potential SSCs in physiological conditions

Gene	Constitutively active cre or inducible creER	Tamoxifen injection	Chase	Cell lineage
<i>Prrx</i> ^{23, 24}	Cre	–	E16.5 Adult	All cells derived from limb bud mesoderm All skeletal cells in the limbs, including SSCs
<i>Col2a1</i> ²⁷	Cre CreER	– E13.5	P3 E14.5 P21 P5	Chondrocytes, perichondrial cells, osteoblasts, and bone marrow stromal cells Chondrocytes and perichondrial cells Cells in the growth plate, perichondrium, bone, and bone marrow Columnar chondrocytes and borderline chondrocytes in the growth plate, a small number of cells in the perichondrium and the metaphyseal spongiosa
<i>Plhrp</i> ²⁹	CreER	P6	1Y P12 1Y	Chondrocytes, osteoblasts, stromal cells, and adipocytes in the metaphyseal bone A subset of the resting chondrocytes (growth plates SSCs)
<i>Cathepsin K</i> ³¹	Cre	–	E14.5 P10 E14.5	Columnar chondrocytes in the growth plate, osteoblasts, and bone marrow stromal cells Cells in the perichondrium
<i>Osterix</i> ^{42, 52}	CreER	E13.5	E14.5 E16.5 13W P6 24W	Cells in the periosteal mesenchyme and the endosteal marrow component, as osteoclasts Perichondrial cells in the osteogenic perichondrium, and proliferating chondrocytes in the cartilage template Cells in the trabecular bone and bone marrow stromal cells, not in the periosteum Cells decreased in the metaphyseal marrow space
<i>αSMA</i> ⁴⁹	CreER	4–5W	2D chase 17D chase	Cells in the primary spongiosa and around the cortical bone Osteoblasts, osteocytes, and bone marrow stromal cells in the metaphyseal marrow space Cells in the primary spongiosa and within the periosteum
<i>Lept</i> ⁵³	Cre	–	P0.5 2M 14M	Some osteoblasts and osteocytes in the marrow space Bone marrow stromal cells mostly in metaphysis Bone marrow stromal cells in metaphysis and diaphysis
<i>Ebf3</i> ⁶⁰	CreER	10W(<4)	2D chase 13M chase	Bone marrow stromal cells, osteoblasts, osteocytes, and adipocytes throughout the marrow space Bone marrow stromal cells exclusive of osteoblasts
<i>Cxcl12</i> ³²	CreER	P3 8W	P5 1Y 2D chase 1Y	Bone marrow stromal cells, osteoblasts, and adipocytes Bone marrow stromal cells in the central marrow space Bone marrow stromal cells and some trabecular osteoblasts in far proximal portion of the marrow space Bone marrow stromal cells in the central marrow space
				Bone marrow stromal cells and some trabecular osteoblasts in the central marrow space

Table 2

Cell lineage of potential SSCs in regenerative conditions

Gene	Constitutively active cre or inducible creER	Tamoxifen injection	Surgery	Chase	Cell lineage
Phrp ²⁹	CreER	P6	Chondrocyte ablation	P21 Day 7	Osteoblasts
Cathepsin K ³¹	Cre	–	Complete fracture	6W Days 6, 9, and 15	Chondrocytes, osteoblasts and periosteal cells
^{49, 50} _{aSMA}	CreER	Day0, –1	Complete fracture	3–5M Days 2, 6, 7, and 14	Chondrocytes and osteoblasts
Osterix ⁵²	CreER	P5	Complete fracture	32W Day 8	Chondrocytes and osteoblasts
Lepr ⁵³	Cre	–	Complete fracture	2M Days 14 and 56	Chondrocytes and osteoblasts
Cxcl12 ³²	CreER	Day – 7	Complete fracture	6–10W Days 2, 7, 14, and 56	Chondrocytes and osteoblasts
			Bone marrow ablation		Chondrocytes and osteoblasts
			Drill-hole injury		Osteoblasts