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Stem and progenitor cells in skeletal development

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Abstract

Accumulating evidence supports the idea that stem and progenitor cells play important roles in skeletal development. Over the last decade, the definition of skeletal stem and progenitor cells has evolved from cells simply defined by their in vitro behaviors to cells fully defined by a combination of sophisticated approaches, including serial transplantation assays and in vivo lineage-tracing experiments. These approaches have led to better identification of the characteristics of skeletal stem cells residing in multiple sites, including the perichondrium of the fetal bone, the resting zone of the postnatal growth plate, the bone marrow space and the periosteum in adulthood. These diverse groups of skeletal stem cells appear to closely collaborate and achieve a number of important biological functions of bones, including not only bone development and growth, but also bone maintenance and repair. Although these are important findings, we are only beginning to understand the diversity and the nature of skeletal stem and progenitor cells, and how they actually behave in vivo.

1. Introduction

Deliberate coordination of cell differentiation is essential to skeletal development. The skeletal system is comprised of closely connected but functionally distinct tissues such as bones, cartilages and tendons that connect the former two with muscles. Bones, as a central component of the skeletal system, are characterized by strong and rigid structures owing to mineralized matrix, but their functions are not limited to protection of vital organs or levering effects allowing body movement. Bones host and nurture hematopoietic cells within their marrow space; at the same time, they secrete hormones that regulate carbohydrate and mineral ion metabolism, provide large stores of calcium and phosphate available for regulation of systemic mineral ion homeostasis, as well as fertility and brain function. Bones, therefore, have many functions, which are achieved by the coexistence of multiple distinct types of highly active differentiated cells within their structure.

The currently prevailing view is that stem and progenitor cells stand at the pinnacle of the skeletal lineage and provide a significant source of these differentiated cells. Stem cells are characterized by two important functions: self-renewal, which is the ability to replicate themselves while maintaining their properties, and multipotency, which is the ability to give

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rise to multiple types of differentiated cell types. Progenitor cells are their downstream offspring with similar but potentially more limited capabilities. Bones undergo a number of biologically important steps throughout their life cycle, such as morphogenesis and development, explosive growth and functional maturation, maintenance and repair of proper architecture and function. There is a constant demand for differentiated cells at each step so that bones can become bigger and stronger, while maintaining their strength and functions throughout life. Thus, the significance of stem and progenitor cells playing major roles in these processes has been emphasized.

Stem and progenitor cells play distinctive roles in supporting growth and repair of bones in stage-specific and tissue-specific manners. In skeletal development, bones start as relatively simple primordial structures termed mesenchymal condensations, which then increase their complexity over time and differentiate into each component of the skeletal system. While stem cells in mesenchymal condensations act as multipotent stem cells that can give rise to the entire spectrum of the skeletal cell lineage, tissue-specific stem cells with more dedicated functions at later stages might be even more important to achieve proper tissue growth and regeneration. How stem and progenitor cells alter their properties over various stages of skeletal development are not well understood. This is largely due to the technical and conceptual limitation that these particular cell types cannot be easily identified within each skeletal tissue, since they are embedded within highly complex three-dimensional structures. In addition, complexity and plasticity of the skeletal cell lineage and lack of stage-specific markers contribute to hampering our understanding of these important cell populations. The notion that one or a few types of omnipotent skeletal stem cells can orchestrate the entire process of skeletal development and regeneration might be too simplistic. The current notion rather supports the hypothesis that multiple distinct types of skeletal stem and progenitor cells collaborate and cooperatively establish the network of the skeletal system. In the first chapter, we discuss recent advances in the concept of stem and progenitor cells in skeletal development.

2. Colony-forming unit fibroblasts (CFU-Fs) and mesenchymal/skeletal stem cells (MSCs/SSCs): A traditional definition for skeletal stem and progenitor cells

Most of the work on stem and progenitor cells in skeletal tissues has been strongly motivated by the goal of regenerative medicine, which is to identify cells capable of restoring functions to human bones. The bulk of existing knowledge on stem and progenitor cells of the skeletal lineage has been built on experiments based on human and rodent bone marrow cells. Traditionally, culture of bone marrow cells and subsequent heterotopic transplantation of in vitro expanded cells into immunodeficient mice has been used as the gold standard to identify these putative stem cells (Bianco, 2014). The first discovery that bone marrow may include stem cells capable of making bones was almost serendipitously made in 1960s. When whole human bone marrow cells were subcutaneously transplanted into immunodeficient mice, they formed ossicles that included blood cells inside (Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). Later, colony-forming unit fibroblasts (CFU-Fs), which are defined as cells capable of adhering to a plastic culture dish and establishing

colonies, were identified as cells responsible for heterotopic ossicle formation (Castro-Malaspina et al., 1980). These hybrid ossicles contained osteoblasts and stromal cells of the donor origin and blood cells of the recipient origin. Bone marrow cells from rodents showed similar properties. Therefore, CFU-Fs residing in human and rodent bone marrow are capable of reconstituting bone marrow in a new environment.

CFU-Fs are highly heterogeneous in gene expressions and functions, despite universally possessing similar colony-forming capabilities in cultured conditions. According to the current definition, mesenchymal stem cells (MSCs), or more accurately "skeletal" stem cells, represent a subset of CFU-Fs (Bianco et al., 2013). In fact, only a subset of CFU-Fs possesses self-renewability and multipotency, as not all individual CFU-Fs have the capability to give rise to so-called trilineage cells, i.e., osteoblasts, chondrocytes and adipocytes in vitro, or to form ossicles upon transplantation (Sacchetti et al., 2007). Interestingly, individually-cloned CFU-Fs demonstrate much reduced efficiency for ossicle formation upon transplantation (Sacchetti et al., 2007), suggesting that heterotopic interactions among multiple and heterogeneous CFU-Fs might facilitate reconstituting a hematopoietic microenvironment. Locations and properties of CFU-Fs and MSCs cannot be easily clarified in the native environment because these cells can be identified only retrospectively after exposure to culture conditions.

In human bone marrow, use of cell surface markers and cell sorting technologies led to the hypothesis that these putative stem and progenitor cells reside in a perivascular location and assume the morphology closely resembling adventitial reticular cells in bone marrow sinusoids (Bianco, 2014). Initially, the STRO-1 antibody was used to identify and enrich clonal human bone marrow mesenchymal cells (Shi & Gronthos, 2003). Further studies showed that all CFU-Fs were recovered in the $CD146^+$ fraction of human bone marrow cells. $CD146^+$ cells meet the criteria of "skeletal stem cells (SSCs)," as they can be serially transplanted and generate $CD146^+$ cells on secondary transplantation. These $CD146^+$ cells correspond to adventitial reticular cells lining bone marrow sinusoids *in vivo*. Further studies revealed that CD51 (αV integrin)$^+$ platelet-derived growth factor receptor-α (PDGFRα)$^+$ cells represent a small subset of $CD146^+$ cells with even more enriched colony-forming activities (Pinho et al., 2013). Therefore, these studies established the idea that cell surface markers that are typically expressed by perivascular stromal cells can be used to enrich human CFU-Fs in bone marrow.

Genetically modified mice, especially those engineered to express easily assayable proteins, such as green fluorescent protein (GFP), have been particularly useful in identifying putative stem and progenitor cell populations, when combined with cell surface markers and cell sorting technologies. Many of these markers are expressed in proximity to bone marrow vasculature, such as PDGFRα, stem cell antigen-1 (Sca1), chemokine (C-X-C motif) ligand 12 (CXCL12), nestin and α-smooth muscle actin (αSMA).PDGFRα^+Sca1$^+$ nonhematopoietic cells (PαS cells) reside in a perivascular space *in vivo* and are enriched for CFU-Fs (Morikawa et al., 2009). These cells, if uncultured and cotransplanted with HSCs, can engraft into irradiated recipients and become osteoblasts, stromal cells, adipocytes and, more importantly, PαS cells themselves, suggesting their self-renewal capability *in vivo*, while cultured PαS cells do not have such capability. Nestin is an

intermediate filament protein and a marker for neural stem cells. *Nestin*-GFP is highly expressed in pericytes of bone marrow arterioles (small arteries), and *Nestin*-GFP$^+$ cells in bone marrow include all CFU-F activities and form self-renewable "mesenspheres" that can go through serial heterotopic transplantations (Méndez-Ferrer et al., 2010). The CD51$^+$PDGFRα$^+$ fraction of *Nestin*-GFP$^+$ cells is further enriched for CFU-F activities (Pinho et al., 2013). Alpha-smooth muscle actin (αSMA) is a marker for pericytes of bone marrow arteries. Pericytes associated with vasculature in bone marrow are marked by αSMA-GFP/mCherry transgenic expression and exhibit trilineage differentiation potential *in vitro* (Grcevic et al., 2012). Further, connective tissue growth factor (CTGF) is expressed by peri-trabecular stromal cells, and CTGF-GFP$^+$ cells appear to give rise to clonal cells in vitro that are further transplantable (Wang et al., 2015). It is increasingly evident that bone marrow is not the sole location from which CFU-Fs and MSCs can be isolated. Other important locations in bones, such as the periosteum and the growth plate, also house clonogenic cell populations that may have distinct functions *in vivo* (we will mention these points later in the chapter). Therefore, comprehensive approaches combining cell surface and transgenic markers have proven to be particularly useful in gaining insight into CFU-Fs and MSCs.

3. How relevant are CFU-Fs and MSCs/SSCs to skeletal development?

As described above, analyses focusing on CFU-Fs and MSCs have produced a wealth of insight into stem and progenitor cells in skeletal tissues. However, the potential limitation of these studies is that they require multiple maneuvers to evaluate their properties, i.e., cell isolation from their native environment, cell culture in exogenous conditions and transplantation into recipient mice. It is tempting to think that this knowledge accumulated from transplantation assays can be extrapolated into cell lineage development of native skeletal tissues. An appealing hypothesis would be that these skeletal stem and progenitor cells defined by transplantation assays could actively participate in bone formation in their native environment. Yet, whether these cells (CFU-Fs and MSCs) represent precursors of osteoblasts in normal development or during bone remodeling, or perhaps represent cells that respond to injury, is an important question that needs to be addressed through more rigorous analyses.

The emerging idea is that stem cells defined by transplantation assays may have limited relevance to physiological conditions. In the field of hematopoietic stem cell research, transplantation assays have been used as the gold standard to evaluate stem cell properties. However, an elegant study utilizing a cellular barcoding system based on a Sleeping Beauty transposase reveals that these traditionally defined hematopoietic stem cells have minimal contribution to native hematopoiesis; rather lineage-restricted progenitors are the main drivers for blood cell production during most of adulthood (Sun et al., 2014). Whether the same principle applies to MSCs is unknown, current and further lineage-tracing experiments are needed to fully clarify the possible role of bone marrow MSCs in normal skeletal homeostasis and repair.

4. In vivo lineage-tracing experiments in mice: An unambiguous approach to reveal cell fates

The gold standard for investigating fates of particular cells *in vivo* in their native environment is lineage-tracing experiments using transgenic mice. This approach is important for studying the process of development because cell lineages can be investigated without any perturbation. Typically, lineage-tracing strategies utilize the *cre-loxP* technology to permanently mark cells of interest using a bigenic system. *Cre* recombinase is expressed in a promoter/enhancer-specified manner in the first transgene, and acts on the reporter locus in the second transgene. In the reporter locus, multiple sequences containing polyA sequences, as well as translation termination codons in all three reading frames ("STOP" sequences) prevent continued transcription and translation of reporter genes. These "STOP" sequences are flanked by *loxP* sites; when *cre* recombinase acts on the *Rosa*26 locus and removes these sequences, the reporter gene becomes expressed under the direction of a ubiquitously active promoter. In a modified version, the *cre* recombinase is covalently bound to the ligand-binding domain of the estrogen receptor (*creERt*) that has been mutated so that 4-hydroxytamoxifen (4-OHT), but not estradiol, can bind and alter its tertiary structure. Translocation of the *creERt* complex to the nucleus depends on the presence of 4-OHT, which is an active form of tamoxifen produced after being metabolized in the liver. Therefore, in the *creERt* system, administration of tamoxifen can temporarily activate *cre-loxP* recombination only for 24–48h until 4-OHT is cleared away from cells. Recombination in the reporter locus is irreversible. Therefore, the reporter gene is continually expressed in the targeted cells and their descendants as long as they survive, even after the promoter that drove expression of *cre* recombinase is no longer active.

Several different versions of the modified *Rosa26* reporter allele have been developed, including *R26R*-LacZ (encoding β-galactosidase), *R26R*-YFP (encoding yellow fluorescent protein), *R26R*-tdTomato (encoding tandem dimer of red fluorescent protein, DsRed), and *R26R*-Confetti. The last allele encodes four different fluorescent proteins (nuclear GFP, YFP, tdTomato and CFP [cyan fluorescent protein]), in which one of them becomes stochastically expressed after *cre-loxP* recombination. It should be noted that each version has its own advantages and disadvantages. For example, although the *R26R*-Confetti allele provides four different colors that may be instrumental for *in vivo* clonal analysis, this allele is relatively insensitive to *cre-loxP* recombination because of the complexity of the transgene design requiring both excision and inversion of *loxP*-flanked sequences (N. Ono, Unpublished Observations).

The lineage-tracing technology has been extensively applied to investigate progenitor-descendant relationships in an unperturbed native environment. To achieve this purpose, it is important to ensure that promoters driving the transgene expression are active in a narrow array of desirable cell types. It is also highly desirable that given promoters have minimal "off-site" activities, especially in their putative descendant cells. Although this genetic approach has successfully uncovered important information on stem and progenitor cells in skeletal tissues, heterogeneity of cell populations of interest marked by a promoter-based approach may complicate overall interpretation of presented data. This is particularly true in

studies using a “constitutively active” version of *cre* recombinase. The difference between “constitutively active” *cre* and “inducible” *creERt* is significantly impactful. Because *cre* lacks a temporal factor and induces recombination whenever the given promoter becomes active, the possible relationships between different cell types cannot be easily ascertained. More sophisticated inducible genetic tools that can specifically mark putative stem and progenitor cells in particular skeletal tissues are highly desirable to better characterize these important cell populations.

5. Endochondral bone development/Phase 1: Formation of the growth plate

To understand potential roles of stem and progenitor cells for skeletal development, it is essential to understand the fundamental processes of skeletal development. Here, we review these processes in conjunction with currently available findings from *in vivo* lineage-tracing or fate-mapping studies. Bones are formed through two common mechanisms: intramembranous and endochondral bone formation. The former intramembranous bone formation is a relatively simple process in which immature mesenchymal cells directly differentiate into osteoblasts that lay down the mineralized matrix. The latter endochondral bone formation is a complex process in which initial cartilaginous templates are replaced later by bones. In both mechanisms, a primordial structure called mesenchymal condensations set the frame for the future domain of bones. In this process, mesenchymal cells in a specific domain of the embryonic tissue align together to form cell clusters that exclude blood vessels.

Most bones in mammals are formed through endochondral bone formation. In this process, mesenchymal cells in condensations further differentiate into two distinct but closely related cell types, chondrocytes and perichondrial cells. Chondrocytes develop in the vasculature-free central portion of condensations, whereas perichondrial cells develop in the highly vascularized outer layer of condensations. This process is initiated when condensing mesenchymal cells express the transcription factor Sox9, a master regulator of chondrogenesis. Indeed, Sox9 is absolutely required for these mesenchymal cells to remain within condensations. Sox9 can directly bind to regulatory elements of major cartilage matrix genes, including those encoding collagens (such as type II, IX and XI collagen) and proteoglycans (e.g., aggrecan). As a result, cartilaginous extracellular matrix is established, and mesenchymal cells in condensations become programmed as chondrocytes or perichondrial cells in their surrounding region.

Subsequently, chondrocytes form the fetal growth plate. This is triggered when chondrocytes in the center of the cartilage stop proliferation, drastically change their cell morphology and differentiate into hypertrophic chondrocytes. Simultaneously, chondrocytes in the flanking region of the cartilage also differentiate and establish the highly hierarchical structure of the growth plate (Kozhemyakina, Lassar, & Zelzer, 2015). The fetal growth plate is primarily composed of three distinct types of chondrocytes: round, flat and hypertrophic chondrocytes. Especially, flat chondrocytes are stacked up on each other like pancakes, and continue to proliferate to generate columnar chondrocytes. These chondrocytes eventually stop

proliferating, change their cell morphology again, and differentiate into prehypertrophic and hypertrophic chondrocytes.

Lines of evidence clearly indicate that stem and progenitor cells during these early stages are established locally within mesenchymal condensations and their subsequent structures. In the limb, bones originate from the lateral plate mesoderm during development. The transcription factor Prrx1 is expressed in these mesodermal cells. In fact, *Prrx1-cre*, in which cre recombinase is expressed under the direction of a 2.4kb *Prrx1* promoter, marks essentially all limb mesenchymal cells at later stages, including chondrocytes, perichondrial cells, osteoblasts and stromal cells, but not muscle cells (Logan et al., 2002). As mentioned earlier, Sox9 is expressed in condensing mesenchyme and potently stimulates expression of type II collagen (Col2). Both *Sox9-cre* and *Col2-cre* mark essentially all chondrocytes, perichondrial cells and osteoblasts in a similar way to *Prrx1-cre* (Akiyama et al., 2005). For the technical reasons mentioned in the preceding section, it remains as the subject of future studies if, when, and where early stem and progenitor cells are established during skeletal development.

6. Endochondral bone development/Phase 2: Formation of the perichondrium and osteoblast precursors

Hypertrophic chondrocytes are the master regulators of endochondral bone formation. These cells abundantly express type X collagen (Col10), whose expression is also tightly regulated by Sox9 and its other transcription factors (Dy et al., 2012; He, Ohba, Hojo, & McMahon, 2016). More importantly, hypertrophic chondrocytes secrete paracrine factors, including vascular endothelial growth factor (VEGF) that induce invasion of blood vessels from the perichondrium, and Indian hedgehog (Ihh) that regulates proliferation and differentiation of neighboring chondrocytes and perichondrial cells. Ihh executes a number of important functions through its interactions with its receptor, patched 1 (Ptch1). Ptch1 action blocks the activation of a G protein coupled receptor, smoothened (Smo), and thereby blocks the action of the transcription factors of the Gli family. This inhibitory action of Ptch1 is canceled when Ihh binds to Ptch1. Ihh facilitates formation of columnar chondrocytes by inducing differentiation of these cells from round chondrocytes (Kobayashi et al., 2005). Ihh also promotes proliferation of flat chondrocytes and their differentiation into hypertrophic chondrocytes (Mak, Kronenberg, Chuang, Mackem, & Yang, 2008). Ihh also acts further on periarticular chondrocytes at the end of the cartilage and promotes production of parathyroid hormone-related peptide (PTHrP) (St-Jacques, Hammerschmidt, & McMahon, 1999). PTHrP maintains flat chondrocytes in the proliferating pool and delays their differentiation into hypertrophic chondrocytes through its receptor, the PTH/PTHrP receptor, therefore indirectly delaying Ihh production (Schipani et al., 1997). These series of interactions establish the PTHrP-Ihh feedback loop that is essential to maintaining the growth plate structure (Lanske et al., 1996; Vortkamp et al., 1996).

Cells in the perichondrium adjacent to hypertrophic chondrocytes, especially those on the innermost layer of the perichondrium, become committed to the osteoblast lineage largely due to the actions of Ihh released from these cells (Ono, Ono, Mizoguchi, et al., 2014). This

portion of the perichondrium is described as the osteogenic perichondrium, and is the first location in which osteoblast precursors appear in endochondral bone formation. These cells enter the program regulated by transcription factors Runx2 and osterix (Osx), in which Osx acts at a level genetically downstream of Runx2 (Nakashima et al., 2002). These cells further differentiate into mature osteoblasts and abundantly produce bone matrix proteins including type I collagen (Col1), osteopontin, and osteocalcin to make the bone collar, which replaces a layer of the perichondrium. The bone collar eventually becomes the cortical bone, while other perichondrial cells become cells in the periosteum composed of multiple layers of mesenchymal cells with diverse functions (we will mention the periosteum in later sections).

7. Endochondral bone development/Phase 3: Formation of the primary ossification center and the bone marrow cavity

The nascent bone marrow cavity is formed within the cartilage template by attracting blood vessels from the perichondrium. Hypertrophic chondrocytes orchestrate this process by producing VEGF, one of the most potent mediators of angiogenesis (Maes, 2017). At the same time, mesenchymal cells coinvade into the nascent bone marrow cavity and give rise to the mesenchymal stromal compartment of the marrow space. There are at least three routes for these incoming mesenchymal cells.

The first route is through the adjacent perichondrium. Cells committed to the osteoblast lineage on the innermost layer of the osteogenic perichondrium translocate into the nascent bone marrow cavity in a pericyte-like fashion before they become mature osteoblasts. This was demonstrated by lineage-tracing experiments using an *Osx-creERt* line and a *Col1(3.2kb)-creERt* line (Maes et al., 2010); the former labels precursors for osteoblasts, while the latter labels mature matrix-producing osteoblasts in the fetal perichondrium. Importantly, only Osx^+ cells can translocate into the nascent marrow cavity; therefore, such migrating capability is unique to relatively undifferentiated populations of osteoblasts. Interestingly, Osx^+ cells from the fetal perichondrium can proliferate only for a limited period, and eventually disappear from the marrow space (Mizoguchi et al., 2014; Ono, Ono, Mizoguchi, et al., 2014). Thus, the osteogenic perichondrium in the fetal growth plate appears to represent only a transient source of the marrow stromal compartment.

The second route is through the hypertrophic layer of the growth plate. Some hypertrophic chondrocytes do not die from apoptosis but transform into cells that eventually become osteoblasts in an area right beneath the hypertrophic layer (Yang, Tsang, Tang, Chan, & Cheah, 2014). This idea that hypertrophic chondrocytes can behave as precursors of osteoblasts has a growing body of evidence based on lineage-tracing experiments. Cells marked by *Col10-cre* or *Col10-creERt* can differentiate into osteoblasts (Yang et al., 2014; Zhou, von der Mark, et al., 2014). How cells with a large size can turn into very compact mesenchymal cells in the marrow space, and whether this pathway represents a major source of osteoblasts need to be addressed by further experimentation. In addition, heterogeneity of cell populations marked by a *Col10* promoter/enhancer needs to be carefully dissected. The hypertrophic layer represents another transient source of the marrow stromal compartment.

The third route is through the “borderland” between the growth plate and the perichondrium. Borderline chondrocytes are a special group of chondrocytes immediate beneath the perichondrium, which line up parallel to perichondrial cells but perpendicular to other growth plate chondrocytes (Bianco, Cancedda, Riminucci, & Cancedda, 1998). Our lineage-tracing experiments using *PTHrP-creERt* (which will be mentioned in more detail later) demonstrate that borderline chondrocytes in the neonatal stage can translocate into the marrow space (N.O., Unpublished Data). $PTHrP^{+}$ borderline chondrocytes can proliferate only for a limited period, and eventually disappear from the marrow space. Therefore, the borderland represents another transient source of the marrow stromal compartment.

These mesenchymal cells of heterogeneous origins contribute to osteoblasts and stromal cells within a highly vascularized environment called the primary spongiosa. Whether different cellular origins denote functional differences of these mesenchymal cells is unknown. Interestingly, *Runx2* and *Osx* are also expressed by prehypertrophic and hypertrophic chondrocytes. The partly overlapping gene programs of hypertrophic chondrocytes and osteoblastic cells suggest that mesenchymal cells arriving at the marrow cavity might already have some epigenetic signatures tuned to the osteoblast lineage. Supporting this notion, perivascular marrow stromal cells possess different transcriptome signatures depending on different developmental origins (Sacchetti et al., 2016), suggesting that mesenchymal cells may retain part of their original identities in their perivascular destinations.

As the growth plate continues to grow further, the marrow cavity and the primary ossification center continue to enlarge. In this process, osteoblasts and stromal cells need to be replenished constantly. This requires beyond three transient routes for migrating mesenchymal cells that we described above. Stem and progenitor cells of particular skeletal components are likely to provide the source of continuous generation of these mesenchymal cells. There are two possible mechanisms.

The first mechanism is that growth plate chondrocytes and perichondrial cells continue to feed into the growing marrow cavity. This is likely to happen because continuous mitotic activities in the flat proliferating layer of the growth plate give rise to an ample number of hypertrophic and borderline chondrocytes in the end, some of which may transform into these mesenchymal cells. Moreover, vigorous cell proliferation in the perichondrium provides a sufficient number of cells that can eventually feed into the marrow space. Evidence for this mechanism is supported by lineage-tracing experiments using multiple transgenes active in chondrocytes and their precursors. Cells within and in proximity of the growth plate marked by *Col2-creERt*, *Sox9-creERt* and *Acan-creERt* continue to generate growth plate chondrocytes, osteoblasts and stromal cells (Ono, Ono, Nagasawa, & Kronenberg, 2014), unambiguously demonstrating that these transgenes can mark the self-renewing population that may represent stem and progenitor cells of skeletal development. Also, cells marked by a Notch-responsive transgene, *Hes1-creERt*, can mark early cells prior to the commitment to the osteoblast lineage in the fetal perichondrium, and these cells can generate osteoblasts and stromal cells for a longer period and to a greater extent than fetal perichondrial *Osx-creERt* osteoblast precursors do (N.O., Unpublished Data), suggesting that this transgene is expressed in putative stem and progenitor cells of the perichondrium.

The second mechanism is that precursors for osteoblasts and stromal cells are re-established within the marrow space and replicate themselves for an extended period. This is also likely to happen because a group of bone marrow stromal cells is believed to behave as stem and progenitor cells with the important properties of self-renewability and multilineage differentiation potential, including those called “mesenchymal stem cells (MSCs).” Bone marrow stromal cells are generally positioned at a perivascular location surrounding arterioles (small arteries) and sinusoids (capillary vessels), and universally express CXCL12 (also known as stromal cell-derived factor 1, SDF-1) (Ara et al., 2003) and stem cell factor (SCF, also known as KIT ligand) (Keller, Ortiz, & Ruscetti, 1995) to attract and retain hematopoietic progenitor cells in the marrow cavity. It is generally believed that bone marrow mesenchymal precursors are located in proximity to blood vessels (we will discuss their potential cell fates in the next section). It is possible that aforementioned transgenes (*Col2-creERt*, *Sox9-creERt* and *Acan-creERt*) are also active in these newly established marrow precursor cells or mark precursors of these marrow stem cells, although more details need to be clarified. When *Sox9-creERt* is used to mark skeletal precursors shortly after birth (Ono, Ono, Nagasawa, et al., 2014) or when mice are 6 weeks old (Balani, Ono, & Kronenberg, 2017), a chase over time shows that these cells slowly and relatively modestly become CXCL12-expressing cells, which are likely to include the perivascular cells described above, in addition to rapidly becoming type I collagen (Col1a1)-expressing osteoblasts.

8. Endochondral bone development/Phase 4: Formation of the postnatal growth plate and continued growth of the marrow space

The postnatal growth plate continues to be active well into early adulthood, playing roles as an important driver for active bone growth. The formation of this structure is closely linked with the formation of the secondary ossification center within the fetal growth plate, which differentiate the existing round layer into two tissues with distinct functions: the postnatal growth plate and the articular cartilage, a transient and a permanent cartilage, respectively. The postnatal growth plate is formed as a disk-like tissue between the primary and secondary ossification centers with characteristic columnar chondrocytes. It is similar to the fetal growth plate, but there are certain differences. Most notably, chondrocytes at the top of the postnatal growth plate are slowly dividing, termed as resting or reserve chondrocytes. As described in the preceding section, PTHrP is expressed in the periarticular layer in the fetal growth plate. In the postnatal growth plate, PTHrP is also expressed in the resting zone. This resting zone has been suggested to contain stem-like cells that give rise to clones of proliferating chondrocytes and produce cytokines that orient columns parallel to the long axis of the bone, as demonstrated by surgical autograft tissue transplantation experiments in rabbits (Abad et al., 2002). Our lineage-tracing experiments using a *PTHrP-creERt* line confirmed and expanded this finding further: $PTHrP^{+}$ resting chondrocytes continue to form columnar chondrocytes long term, which undergo hypertrophy and became osteoblasts and marrow stromal cells beneath the growth plate (Mizuhashi et al., 2018); interestingly, these cells do not become marrow adipocytes (we will discuss more about this point later). $PTHrP^{+}$ chondrocytes possess colony-forming abilities, behave as “skeletal stem cells (SSCs)” and overlap with transplantable “mouse SSCs (mSSCs)” identified by a combination of cell

surface markers, including αV integrin (CD51), Thy (CD90), endoglin (CD105) and OX2 (CD200) that are found in the growth plate (Chan et al., 2015). Similar stem cells have been also found in the human growth plate, termed “human SSCs (hSSCs)” identified by expression of $PDPN^{+}CD146^{-}$ $CD73^{+}CD146^{+}$ (Chan et al., 2018). These studies present an emerging idea that the resting zone of the postnatal growth plate is one of the niches in which stem and progenitor cells for bone growth are housed. How these resting chondrocytes maintain themselves long term within the postnatal growth plate is largely unknown; however, actions of Ihh released from the hypertrophic layer appear to be important in maintaining stem cells and their niche.

The postnatal growth plate is flanked by a highly vascularized marrow space enriched with trabecular bones in the primary and secondary ossification centers. The perivascular space of these vasculature-rich epiphyseal and metaphyseal regions is considered to contain abundant stem and progenitor cells pertinent to active bone growth. Gremlin 1 (Grem1), a bone morphogenetic protein (BMP) antagonist, is expressed in marrow stromal cells of the perivascular space. Lineage-tracing experiments using a *Grem1-creERt* demonstrate that these cells can become osteoblasts, chondrocytes and reticular stromal cells, but not marrow adipocytes, and are therefore identified as osteo-chondro-reticular (OCR) stem cells (Worthley et al., 2015). These vasculature-rich regions are adjacent to the prehypertrophic/hypertrophic layer that abundantly releases Ihh; therefore, these stem and progenitor cells might be highly responsive to Hedgehog signaling. Transcription factor Gli1 is an authentic Hedgehog-responsive gene that mediates a number of biological actions of Hedgehog signaling. Gli1 marks “metaphyseal mesenchymal progenitors (MMPs),” as lineage-tracing experiments using a *Gli1-creERt* demonstrate that these cells, unlike *Grem1-creERt*, contribute to osteoblasts, adipocytes and stromal cells (Shi et al., 2017). Interestingly, a group of these stem and progenitor cells might be transiently committed to the osteoblast lineage that can be defined by Osx expression. Lineage-tracing experiments using an *Osx-creERt* line demonstrate that these cells in the perinatal stage can contribute to osteoblasts and stromal cells that persist in the marrow long term (Mizoguchi et al., 2014); interestingly, Osx^{+} cells in adulthood do not possess such capabilities. As mentioned earlier, pericytes associated with bone marrow arteries show specific elongated cell morphology and express α-smooth muscle actin (α-SMA). Lineage-tracing experiments using an *αSMA-creERt* demonstrate that these pericytes rather represent transient precursors for osteoblasts and, possibly, stromal cells (Grcevic et al., 2012). These lines of studies illustrate the variable fates of cells capable of generating multiple mesenchymal lineages. The studies also support the notion that distinct types of stem and progenitor cells for bone growth exist to support explosive growth of bone and its marrow space in early life.

9. Endochondral bone development/Phase 5: Establishment and maintenance of the adult bone marrow stroma

Bones need constant maintenance to sustain their structures and function throughout the lifespan even after bone growth slows and stops. As the growth plate activity slows down toward adulthood, the contribution of chondrocytes and perichondrial cells to the marrow space is likely to become negligible. It is probably at this post-growth stage that bone

marrow stromal cells make a significant contribution to diverse skeletal cell types. Because matrix-producing osteoblasts are relatively short-lived, a continuous supply of new osteoblasts is necessary to maintain the bone structure. The current notion is that bone marrow stromal cells can differentiate into osteoblasts during normal adult skeletal homeostasis.

The first endeavor to mark and trace the fate of bone marrow stromal cells was made in 2010 using a *Nestin-creERt* line (Méndez-Ferrer et al., 2010). Although these cells certainly become osteoblasts and chondrocytes, this transgene simultaneously marks a large number of endothelial cells (Ono, Ono, Mizoguchi, et al., 2014), complicating the interpretation of overall outcomes. Leptin receptor (LepR) is expressed by bone marrow stromal cells around sinusoids and arterioles (Zhou, Yue, Murphy, Peyer, & Morrison, 2014). Fate-mapping studies using a *LepR-cre* knock-in allele demonstrate that these cells contribute to a large fraction of CFU-Fs, osteoblasts and marrow adipocytes in adult bones, particularly only after 2 months of age in mice (Zhou, Yue, et al., 2014). CXCL12 is abundantly expressed by marrow stromal cells surrounding sinusoids, which are termed CXCL12-abundant reticular (CAR) cells. The work from Nagasawa's group, using *Ebf3-creERt* to mark CAR cells in a tamoxifen-dependent manner, shows that CAR cells can become osteoblasts in adulthood (Seike, Omatsu, Watanabe, Kondoh, & Nagasawa, 2018). Our lineage-tracing experiments using a *Cxcl12-creERt* line demonstrate that these cells remain dormant without generating new reticular cells during growth of the marrow space, and become trabecular osteoblasts, but not cortical osteoblasts (N.O., Unpublished Data). The question whether there is any particular stem and progenitor cell populations maintaining the adult bone marrow stroma has not been completely answered. It is also possible that more differentiated cells (such as "bone-lining cells") play more important roles.

10. Periosteum and craniofacial sutures

Outside the marrow space encased by the cortical bone, the periosteum is formed as a result of activities of perichondrial cells. This tissue also contains clonogenic cell populations (CFU-Fs) and possesses highly regenerative capabilities. Lineage-tracing experiments demonstrate that perichondrial cells marked by *Prrx1-creERt* and *αSMA-creERt* lines can respond to injury and robustly generate chondrocytes and osteoblasts during fracture healing (Duchamp de Lageneste et al., 2018; Kawanami, Matsushita, Chan, & Murakami, 2009; Matthews et al., 2014). The suture of craniofacial bones is somewhat similar to the perichondrium of endochondral bones, and considered as the niche where stem cells reside. Lineage-tracing experiments demonstrate that suture mesenchymal cells marked by *Gli1-creERt* and *Prrx1-creERt* can contribute to normal turnover and regeneration of calvarial bones (Wilk et al., 2017; Zhao et al., 2015). The significance of these cells populations to normal skeletal development needs to be clarified by further experimentation. Recent work from Greenblatt's group confirms that the periosteum is the source of stem cells that can form osteoblasts and contribute heavily to callus formation after fracture, but do not support hematopoiesis (Debnath et al., 2018).

11. Sox9+ osteoblast precursors

Earlier, we mentioned that *Sox9-creERt* can be "chased" into reticular cells expressing high levels of CXCL12, first described by Nagasawa's group, that strongly support hematopoiesis. At early times after administration of tamoxifen to mice shortly after birth or 6 weeks later, *Sox9-creERt* marks, in addition to growth plate chondrocytes, three groups of cells in bone. The largest group are metaphyseal cells immediately adjacent to the growth plate, separating the growth plate from the marrow contents; a second group marks cells in the perichondrium, probably those noted in (Debnath et al., 2018); a third group is found immediately below the osteoblast/lining cells layer at the surface of cortical and trabecular bone. Cells from these three locations can be chased into groups of osteoblasts lining trabecular bone, and osteoblasts at the endosteal and periosteal surfaces. It is potentially relevant to stem cells found in many other tissues expressing Sox9, such as hair follicle stem cells (Nowak, Polak, Pasolli, & Fuchs, 2008), breast (Guo et al., 2012), liver (Furuyama et al., 2011), intestine (Formeister et al., 2009) and pancreatic stem cells (Kawaguchi, 2013).

12. Parathyroid hormone (PTH) action on skeletal precursors

Parathyroid hormone (PTH) is a major regulator of mammalian calcium homeostasis that acts on bone (Hock, 2001). When teriparatide (hPTH (1–34)) is administered by daily subcutaneous injection to humans or rodents, bone mass increases as a result of an increase in the number of bone-forming osteoblasts and an increase in bone formation rate (Esbrit & Alcaraz, 2013). The cause of this teriparatide-mediated increase in the number of mature osteoblasts has been extensively studied. The *Sox9-creERt* lineage tracing experiment showed that, besides acting on the post-mitotic mature osteoblasts and osteocytes, teriparatide-mediated increase in bone mass also involves action on very early cells of the osteoblast lineage. Teriparatide suppresses apoptosis of early skeletal precursors, thus contributes to the overall increase in the number of osteoblast precursors seen in the first 7 days after the start of teriparatide administration (Balani et al., 2017). The early cells of the osteoblast lineage express *Pth1r* (*PTH/PTHrP receptor*) mRNA. When the PTH receptor is knocked out specifically from *Sox9-creERt*-expressing cells, teriparatide failed to increase the number of osteoblast precursors, suggesting that the actions of teriparatide on early skeletal precursors require direct signaling via PTH/PTHrP receptors (Balani et al., 2017). When daily subcutaneous administration of PTH was continued for a longer duration, the number of reporter-marked *Sox9-creERt* cells continued to increase and differentiated into osteoblasts with a higher rate of differentiation compared to controls. In control mice, a portion of *Sox9-creERt* descendant cells are adipocytes, in addition to stromal cells and osteoblasts. However, no adipocytes that descended from *Sox9-creERt* cells are observed at the end of 4 weeks of teriparatide administration. As expected, when teriparatide administration is then halted and mice are followed for another 4 weeks, most of the increase in bone mass caused by teriparatide administration is reversed. Strikingly, however, the bone marrow of the teriparatide-withdrawn mice shows a dramatic increase in adipocytes descended from the *Sox9-creERt*+ cells, unlike what happens in the controls. This observation is consistent with previous work done by other groups showing that PTH suppresses adipocytic differentiation of human stromal cells *in vitro* (Yu et al., 2012). However, this experimental design cannot determine whether Sox9-expressing cells or,

instead, one or more groups of descendant cells were the source of the new marrow adipocytes. Strikingly, when an analogous experiment is performed using *Ocn-creERt*, reporter mice that primarily label mature osteoblasts and their descendants, several adipocytes seen after teriparatide withdrawal are descendants of *Ocn-creERt*+ cells (Balani et al., 2017). The conversion of *Sox9-creERt*+ or *Ocn-creERt*+ cells to adipocytes was not seen as long as teriparatide was administered once daily, suggesting that the molecular cues that direct adipocytic differentiation of early precursors is suppressed as long as PTH is administered.

In the marrow adipocyte pool, the overall contribution of cells that differentiated from *Sox9-creERt* or *Ocn-creERt* cells ranged between 10% and 30%. In these studies, tamoxifen was administered to 6-week-old mice, at the same time as the initiation of teriparatide treatment. Interestingly, when the withdrawal experiment was performed in *Sox9-creERt* mice, in which Tomato+ cells were labeled soon after birth at day P3 and teriparatide administration began at 6-weeks of age, Tomato+ cells contributed >98% of the marrow adipocytes formed after PTH-withdrawal in vivo. These findings suggest that a larger fraction of the adipocyte precursors express Sox9 at P3 than at 6 weeks of age, perhaps because Sox9 descendants, no longer expressing Sox9, accumulate with age and can serve as adipocyte precursors.

13. Wnt/β-catenin signaling and cell fate decision

Wnt/β-catenin signaling plays a crucial role in controlling osteoblast and adipocyte differentiation. Removal of β-catenin from bone marrow stromal cells (BMSCs) *in vitro* blocks osteoblastic differentiation and causes these cells to more readily differentiate into the adipocytic lineage (Christodoulides, Lagathu, Sethi, & Vidal-Puig, 2009). Wnt protein supplementation to culture media increases osteoblast differentiation and suppresses adipogenesis in the bone marrow-derived ST2 stromal cell line. β-Catenin activation plays a key role in the canonical Wnt pathway and regulates Wnt target gene transcription. When this pathway is activated by any of the variety of Wnt ligands (Wnt10b, Wnt1, Wnt3, for example), activated β-catenin is transported into the nucleus and initiates targeted gene transcription (MacDonald, Tamai, & He, 2009). Stabilization of β-catenin in BMSCs promotes osteoblast differentiation and inhibits their adipogenic differentiation. In contrast, overexpression of β-catenin in pre-adipocytes suppresses their differentiation into mature adipocytes (Ross et al., 2000). Conditional deletion of β-catenin *in vivo* from osterix-expressing preosteoblasts leads to a substantial fraction of these cells differentiating into adipocytes (Song et al., 2012). Teriparatide administration, both *in vitro* and *in vivo*, increases Wnt/β-catenin signaling (Lee & Partridge, 2009). Teriparatide administration also suppresses Wnt inhibitors such as DKK-1 (Guo et al., 2010) and sclerostin (Keller & Kneissel, 2005); this, in turn, leads to an increase in Wnt/β-catenin signaling. Our report (Balani et al., 2017) suggests that teriparatide withdrawal causes a sudden decrease in Wnt/β-catenin signaling in skeletal stem cells, based on measurements of the non-phosphorylated (active) form of β-catenin accumulated in vivo. Teriparatide-withdrawn mice showed a dramatic decrease in the active β-catenin in *Sox9-creERt*+ cells compared to the levels in control mice subjected to continued one daily PTH administration. The molecular mechanism of this decrease in activation of β-catenin is not known, but we hypothesize that there must be mechanisms of skeletal homeostasis that regulate the increase and decrease of

bone mass to optimize the stresses on individual osteoblasts/osteocytes under normal conditions. We further speculate that the increase in bone mass in response to one daily teriparatide administration leads to a higher bone mass than the mouse otherwise "needs," and that the bones sense this high-bone mass state. That is, sudden cessation of teriparatide administration may activate homeostatic mechanisms that decrease bone mass until bone mass falls and reaches its basal level. When the number of osteoblasts/osteocytes is artificially high after teriparatide administration, they may each receive insufficient stress signals from gravity and muscle pull to activate canonical Wnt signaling. This could lead to a decrease in activation of β-catenin, and then to lower bone mass and an increase in adipocyte generation from accumulated precursors.

14. Conclusions and perspectives

Bone cells have multiple missions throughout life. Early in life, growth is relatively explosive, while in adulthood, bones must maintain multiple cellular configurations, from the rapidly turning over trabecular bone to the more slowly remodeling cortical bone and repair the bone when cracks or fractures occur. The missions during adulthood include regulation of mineral ion homeostasis and hematopoiesis, and provision of levers for muscle-generated movement and protection of inner organs. Considering all these tasks, it is not surprising that multiple sites are used to generate skeletal stem cells, from the bone marrow and perichondrium of fetal bone, the resting zone of the postnatal growth plate, the marrow cavity in adulthood, and the periosteum. These stem cells have distinct properties and missions that are regulated both by local factors and by hormones. Here we have delineated how both the tools of regenerative medicine and of lineage tracing can be used to identify skeletal stem cells. Although we are off to a good start, we are only beginning to understand the relationships between the different skeletal stem cells. Very importantly, it is likely that most stem cell groups identified *in vivo* are quite heterogeneous, since they are primarily identified by the expression of one particular promoter driving *cre* expression. Finding ways to effectively combine the powerful cell sorting tools of regenerative medicine with the cell lineage tools needed to determine what actually happens *in vivo* in normal or injured bone remains an enormous challenge for the future.

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