Skeletal Stem and Progenitor Cells in Bone Development and Repair

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ABSTRACT

Bone development, growth, and repair are complex processes involving various cell types and interactions, with central roles played by skeletal stem and progenitor cells. Recent research brought new insights into the skeletal precursor populations that mediate intramembranous and endochondral bone development. Later in life, many of the cellular and molecular mechanisms determining development are reactivated upon fracture, with powerful trauma-induced signaling cues triggering a variety of postnatal skeletal stem/progenitor cells (SSPCs) residing near the bone defect. Interestingly, in this injury context, the current evidence suggests that the fates of both SSPCs and differentiated skeletal cells can be considerably flexible and dynamic, and that multiple cell sources can be activated to operate as functional progenitors generating chondrocytes and/or osteoblasts. The combined implementation of *in vivo* lineage tracing, cell surface marker-based selection, single-cell molecular analyses, and high-resolution *in situ* imaging has strongly improved our insights into the diversity and roles of developmental and reparative stem/progenitor subsets, while also unveiling the complexity of their dynamics, hierarchies, and relationships. Albeit incompletely understood at present, findings supporting lineage flexibility and possibly plasticity among sources of osteogenic cells challenge the classical dogma of a single primitive, self-renewing, multipotent stem cell driving bone tissue formation and regeneration from the apex of a hierarchical and strictly unidirectional

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differentiation tree. We here review the state of the field and the newest discoveries in the origin, identity, and fates of skeletal progenitor cells during bone development and growth, discuss the contributions of adult SSPC populations to fracture repair, and reflect on the dynamism and relationships among skeletal precursors and differentiated cell lineages. Further research directed at unraveling the heterogeneity and capacities of SSPCs, as well as the regulatory cues determining their fate and functioning, will offer vital new options for clinical translation toward compromised fracture healing and bone regenerative medicine.

Lay summary

Skeletal progenitor cells are crucial for bone development and growth, as they provide the cellular building blocks (chondrocytes and osteoblasts) that form the cartilage and bone tissues that the skeleton is composed of. In adult life, the occurrence of a bone fracture reactivates similar tissue-forming mechanisms, starting with the trauma triggering various postnatal skeletal stem/progenitor cells (SSPCs) residing near the bone defect to divide and migrate. These cells subsequently generate functional fracture-repairing cells by differentiating into mature chondrocytes and/or osteoblasts. In recent years, the combined use of various advanced research approaches and new techniques has strongly improved our insights into the origin, identity, fates, and roles of developmental and reparative skeletal stem cells and progenitor subsets. Concomitantly, this research also unveiled considerable complexity in their dynamics, diversity, hierarchies, and relationships, which is incompletely understood at present. In this review, we discuss the state of the field and the newest discoveries in the identity and roles of skeletal stem and progenitor cells mediating bone development, growth, and repair. Further research on these cell populations, including determining their exact nature, fate, and functioning, and how they can be harvested and regulated, is critical to develop new treatments for non-healing fractures.

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INTRODUCTION AND KEY APPROACHES FOR IDENTIFYING AND STUDYING ENDOGENOUS SKELETAL STEM AND PROGENITOR CELLS IN VIVO

Skeletal stem and progenitor cell populations are critical players in bone development and regeneration. Various populations of precursor cells contribute to the formation of transient pools of chondrocytes and osteoblasts; these are herein collectively termed 'skeletal progenitors' for fetal and neonatal periods and 'skeletal stem/progenitor cells' (SSPCs) in adult/adolescent bones to align with the general notion in stem cell biology that during development, the origin progenitor cells are not stable and unlikely to be self-renewing in exact ways as seen for adult tissue stem cells.[1] The highly specified term 'skeletal stem cells' (SSCs) is reserved for cells in the post-adolescent organism proven to display the two characteristic hallmarks of stem cells *in vivo*, namely multipotency and self-renewal. Multipotency of SSCs most commonly implies that the cells have the potential to give rise to multiple skeletal lineages, through differentiating into mature osteoblasts, chondrocytes, and/or marrow adipocytes (referred to as "trilineage differentiation potential" especially when tested *in vitro*). Multipotency *in vivo* can be shown through cartilage pellet and bony ossicle formation assays in which the selected populations of cells are transplanted ectopically into a recipient mouse (e.g., subcutaneously or beneath the kidney capsule), or via lineage mapping/tracing in the endogenous skeletal environment.[2-5] We define lineage/fate mapping as a method based on a mouse in which Cre recombinase expression is driven by a specific gene promoter, that is crossed to a reporter strain; in the resultant double transgenic mouse, recombination of the

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reporter (at the DNA level) occurs in cells expressing the Cre protein, and is transmitted into their progeny. Recording or visualizing the reporter signal thus allows for revealing all the cells with a history of expression of the Cre-driving promoter, including current as well as formerly expressing cells and their descendants. Lineage tracing is similar to lineage mapping but uses an inducible CreERt line, adding a time-restricted pulse-chase component whereby cells of a given (promotor-determined) genetic lineage are labelled at a given moment only, and subsequently followed over time in their tracks, fates, and behavior in the native *in vivo* environment.[5,6] Self-renewal is defined as the capacity to maintain the stem cell pool by generating very similar daughter stem cells while at the same time generating committed progenitors. Self-renewal is a key stem cell feature that is however relatively challenging to demonstrate *in vivo*, as this principally requires serial transplantation experiments.[2,4] Despite the impressive recent progress in the exciting and fast-moving field of skeletal stem cell research, it is important to note that most precursor cell populations currently studied, whether identified by a panel of cell surface markers or by a single genetic lineage driver, are still considerably heterogeneous and do not comprise purely of SSCs. Therefore, the use of the more broadly defined term 'SSPCs' is currently more appropriate to denote any of the bone tissue-residing cell populations that can be assumed to at least include bona fide SSCs, even though usually along with less primitive (committed or uncommitted) progenitors with proliferative and skeletogenic differentiation potential.

In recent years, a lot of work has been performed using advanced methodologies and new technologies, to enhance the knowledge on the identity, location, characteristics, and fates of developmental skeletal progenitors and postnatal SSPCs. Much of the progress in dissecting the hierarchy chart of stem and progenitor cell commitment and differentiation has built on two main approaches: fluorescence-activated cell sorting (FACS)-based identification and isolation of cell subsets followed by ectopic implantation, and genetic lineage tracing combined with high-resolution imaging to track cell fates *in vivo*, both used along with single-cell omics for

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138 further disentangling the cellular heterogeneity. Both approaches are associated with distinct
139 advantages and limitations.
140 Groundbreaking work was performed in identifying SSPC subsets by FACS based on a
141 combined panel of cell surface markers in both human and mouse bones.[7,8] These cells could
142 be FACS-isolated and studied following transplantation in ectopic ossicles, delineating the
143 differentiation potentials and hierarchies among specified cell subsets.[7,8] A major strength of
144 this approach is the outlook on applicability for cell therapy purposes; on the other hand, a
145 disadvantage is the limited information on the precise *in situ* localization and endogenous
146 properties of the cells. These spatial aspects and mechanistic insights into the roles of SSPCs
147 in various physiological and pathophysiological settings can be particularly well addressed
148 using *in vivo* lineage tracing and other genetic strategies in mice. While the use of a single
149 genetic marker to characterize the SSPCs under study in most or all cases leads to relatively
150 broad targeting of a lineage that encompasses a heterogeneous subset of SSPCs (with different
151 markers/gene promotors labeling partially overlapping and partially distinct cell populations),
152 these approaches have enabled researchers to label and trace various cell populations and reveal
153 their fate over time, their differentiation trajectories in the spatial skeletal environment, and
154 their importance and functional roles in physiological/pathological settings (by applying
155 cellular ablation, mutagenesis, and molecular profiling of traced populations). These advances
156 have been expedited by the increasing availability of transgenic mice carrying constitutively
157 active Cre or tamoxifen-inducible CreER^T recombinase constructs under the control of specific
158 gene promoters, in combination with the development of sensitive fluorescent reporter mice,
159 which can be single-colored such as the Ai9/tdTomato (tdTom) strain[9] or multicolored (e.g.,
160 R26R-confetti)[10]. An important consideration to adopt in these approaches is that there might
161 potentially be some transfer of Cre mRNA, or perhaps Cre protein or reporter signal, to
162 neighbouring cells by inter-cellular transfer through matrix vesicles, which could possibly
163 result in false-positive in terms of lineage descendance.[11-13] In all lineage tracing experiments,
164 defining the specificity of a given CreER^T line using a rigorous short-chase analysis is also

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crucial before making assumptions on the cell fate. Given that no single genetic driver can exclusively target SSCs in homogeneous ways, the researcher's interpretation needs to consider that the reporter marks a heterogeneous SSPC population and all its progeny. Of note, recent developments include dual reporter systems (e.g., combined Cre-loxP and Dre-Rox or Flp-Frt-based mouse models) that allow for greater precision and narrowing of the targeted and traced cell populations.[14,15] Furthermore, the upcoming implementation of single-cell clonal lineage tracing, using cell barcoding strategies, will enable researchers to trace individual cells and interrogate cell states, lineage histories, and plasticity; for a review of these new approaches and their technical considerations and limitations, see[6,16]. Overall, using *in vivo* animal models, dedicated cell subsets and their roles can be analyzed in various sophisticated ways, by advanced *in situ* imaging methodologies, flow cytometry marker panels, high-resolution bulk and single-cell RNA-sequencing (scRNA-seq), and spatial transcriptomics as well as emerging proteomics, epigenomics, and metabolomics platforms. Although many outstanding questions remain, these technologies are rapidly delivering new insights into skeletal biology and the cell populations constituting sources of bone-forming osteoblasts during development, growth, homeostasis, and repair.

In this review we will focus on developmental and reparative skeletal precursor populations. These populations give rise to all the chondrocytes and osteoblasts that represent the principal mediators of skeletal development, growth, and repair, as they form the cartilage and bone matrix. The engagement of fetal/neonatal skeletal progenitors is critical for the initial shaping of bones during embryonic development and for building up bone mass during juvenile growth. Later in life, adult SSPCs are essential for the repair of bone defects upon tissue injury or fracture. A deeper understanding of the microenvironment-derived signals and the intrinsic pathways regulating precursor cell activation and differentiation into functional chondrocytes and osteoblasts, is needed to develop novel approaches for intervening therapeutically when the healing of bone and cartilage defects is compromised. Currently, few osteo-anabolic treatments

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are available, and no effective treatment exists for curing non-union fractures, underscoring the clinical need. Skeletal progenitors and SSPCs, and analogous populations derived from induced pluripotent stem cells (iPSCs), bear great promise to enhance bone healing in delayed-unions and to repair non-unions, for instance, by pharmaceutically activating endogenous SSPCs or efficiently delivering potent exogenous SSPCs via cell therapy.[17-19] To achieve this, a major area of insufficiently met research needs in the field is the in-depth characterization of the human SSPC landscape, and the effective translation of that knowledge into clinical and applied settings. Although great efforts are being done to map the human skeletal system at the single-cell level through large omics initiatives[20,21], at present, much of the cellular-molecular knowledge is still derived from murine models. Hence, these will be the focus of this review, but the emerging insights into human biology will be indicated.

TRANSIENT SKELETAL PROGENITORS STEER BONE FORMATION DURING DEVELOPMENT AND PERINATAL GROWTH

The skeleton is composed of bones of different shapes and sizes, most of which are derived from the mesoderm, except for some skull and facial bones that are derived from the cranial neural crest, an ectodermal mesenchyme derivative. Following condensation of immature mesenchymal progenitor cells at the proper sites, bone can be formed via two distinct processes, namely intramembranous or endochondral ossification. During intramembranous ossification, mesenchymal progenitors directly differentiate into osteoblasts. In contrast, in bones that form via endochondral ossification, osteochondral progenitor cells first form a cartilage template that is subsequently transformed into bone. Increasing insights are being obtained into the origin of the skeletal progenitors that give rise to bone-forming osteoblasts in both modes of ossification.

Early fetal skeletal progenitor sources for the initiation of skeletogenesis

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Bones in the cranial, axial, and appendicular skeleton are derived from spatially specified mesodermal populations: the cranium, vertebrae, ribs, and sternum are derived from the paraxial mesoderm, more specifically the sclerotome parts of the somites, which are blocks of mesodermal cells located on either side of the notochord, whereas most bones in the limbs and limb girdles (shoulder, hip) are derived from the lateral plate mesoderm. While the early fetal origin of cells establishing the skeleton is thus relatively diverse, the cells initiating bone formation follow largely similar molecular programs, governed by critical transcription factors, and regulated by selected morphogenic signaling pathways. Firstly, the cells localize to the site of bone initiation and then aggregate to form mesenchymal cell condensations, which outline the future skeletal elements. The locations of the condensations and patterning of the skeleton is controlled by strictly coordinated, region-specific patterning factors, which include morphogens and homeobox transcription factors encoded by the Hox genes.[22-25] In bones that form via intramembranous ossification (i.e., the flat bones, such as the cranial bones (calvaria), parts of the scapula, and clavicle), the cells of the mesenchymal condensation undergo direct osteoblast lineage specification and differentiation, controlled by the transcription factors *Runx2* (Runt-related transcription factor 2) and osterix (Osx, encoded by the *SP7* gene). Mature osteoblasts secrete unossified bone matrix (osteoid), which is subsequently mineralized (**Figure 1**). Within condensations forming long bones through endochondral ossification, progenitors differentiate into the chondrogenic lineage, driven by the transcription factors *Sox9, Sox5 and Sox6* (members of the sex-determining region Y (SRY)-related high-mobility-group (HMG)-box (Sox) family). The chondrocytes form an initial cartilage template, by secreting an extracellular matrix (ECM) rich in collagen type II (Col2) and proteoglycans such as aggrecan, which will progressively be replaced with bone (**Figure 2**). The central transcriptional control and differentiation path of mesenchymal skeletal progenitor cells into chondrocytes and/or osteoblasts is regulated by many cues, including various locally secreted ligands such as bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), Wnts, and hedgehog proteins, and Notch family members.

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Osteogenic skeletal progenitors in intramembranous bone development

The flat bones – most typically represented by the cranial bones – develop and grow through proliferation and differentiation of osteogenic skeletal progenitors and bone deposition at the osteogenic fronts, which are the active sites of bone formation throughout development and growth[26] (**Figure 1**). Particularly, the sutures, the unossified connective tissue regions that separate the growing bones of the skull, are seen as the local stem cell niches that harbor the undifferentiated cell pools.[26,27] Glioma-associated oncogene family zinc finger 1 (Gli1), an essential transcription factor of the hedgehog signaling pathway, has been identified as a marker for a stem cell population within the suture mesenchyme, giving rise to the osteogenic fronts, the periosteum overlaying the outer surface of the calvaria, and the endosteum of the craniofacial bones.[28] These cells were not found to be associated with vasculature but exhibited stem cell marker expression, clonal expansion, and trilineage differentiation potential *in vitro*. Ablation of Gli1-expressing ($Gli1^+$) cells during development led to craniosynostosis, skull growth arrest, osteoporosis, and compromised injury repair.[28] Very similarly, Axin2-expressing cells were identified as quiescent suture stem cells with self-renewal, clonal expansion, and multilineage differentiation abilities during calvarial development and defect repair[29], and their maintenance was found to require expression of BMP receptor 1A (BMPR1A).[30] Skeletal progenitor cells expressing paired-related homeobox gene-1 (Prrx1) also contribute to calvarial bone development and repair.[31] Moreover, cranial suture stem/progenitor cells in mice have also been identified by a panel of surface markers defining '$CD51^+CD200^+$ cells'[32], and by expression of Cathepsin K (Ctsk), a protease mainly known for its bone-resorbing function in osteoclasts, combined with cell surface markers (selecting for the lineage-negative $(Lin)^-$,$Thy1.2^-6C3^-CD200^+CD105^-CD51^+$ cells).[33] Ctsk-lineage cells are specialized for intramembranous bone formation and contribute to calvarial mineralization.[33] Interestingly, very recent studies unveiled another stem cell population present in the skull sutures that is distinct from $Ctsk^+$ SSPCs despite both populations

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expressing Gli1. These cells are marked by expression of the discoidin domain receptor 2 gene (*DDR2*), which encodes a non-integrin collagen receptor that mediates interactions between cells and the ECM.[34]

Bok *et al* show that DDR2$^+$ calvarial cells exhibit stem cell characteristics both *in vitro* and *in vivo*, by using serial transplantation, lineage tracing, label retention, colony formation and clonal multipotency experiments, among others.[34] Interestingly, both Ctsk-Cre driven conditional knockout of *Twist1* (the gene in which loss-of-function mutations are responsible for Saethre-Chotzen syndrome, causing autosomal dominant craniosynostosis in humans), as well as diphtheria toxin-mediated ablation of Ctsk$^+$ SSPCs, led to their replacement by DDR2$^+$ SSPCs. This was accompanied with a shift from intramembranous towards pathologic endochondral ossification (normally not seen in most sutures), driving suture fusion as well as hypomineralization.[34] Of note, whereas endochondral bone formation is typically coupled with the establishment of a hematopoiesis-supporting niche environment, the endochondral ossification mediated by calvarial DDR2$^+$ SSPCs was found to be devoid of hematopoiesis, suggesting that a distinct form of endochondral ossification without hematopoiesis exists alongside the traditional endochondral bone formation process.[34]

The study by Bok *et al* thus identified two distinct stem cell populations within the sutures (Ctsk$^+$ cells and DDR2$^+$ cells), the interaction of which is needed for proper formation of the skull bones. The fact that both populations have complementary functions under physiological conditions, but work in opposing directions under conditions that cause disorders as craniosynostosis, highlights the complexity of skeletal stem cell biology and provides an interesting example of stem cell cooperation.[35] Interestingly, besides marking DDR2-expressing stem cells within the calvarial sutures and in the resting zone cartilage of the cranial base synchondrosis, the collagen receptor DDR2 also plays a functional role in these cells.[36] Global DDR2 deficiency impairs skull development in mice, and DDR2 mutations cause human craniofacial abnormalities in the genetic disease spondylo-meta-epiphyseal dysplasia (SMED). Now, the phenotype of mice globally deficient for DDR2 was found to be recapitulated by an

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inducible Gli1-CreER^{T}-driven deletion of DDR2, supporting that interactions between stem/progenitor cells and the collagen-rich ECM are a fundamental requirement for the development of the craniofacial skeleton.[36]

Together, these findings provide an example of how multiple stem cell populations can co-exist and interact, and postulate that disruptions in stem cell–stem cell and stem cell-matrix interactions could underly skeletal diseases.[34,36] Clearly, much remains to be investigated in this important area of research. In fact, our knowledge of the molecular mechanisms regulating the maintenance and differentiation of suture stem cells, and their interaction with each other and with the ECM, is still very limited, although balanced activities of the hedgehog and BMP pathways, of FGF and Wnt/β-catenin signaling, and of insulin-like growth factor (IGF)-1, appear to be needed.[27,32,34,37,38] Detailed further coverage of the recent discoveries in cranial suture biology can be found in several dedicated reviews.[26,27,37,38]

Of note, cortical bone formation to expand the shaft of the long bones is increasingly often seen as a type of intramembranous ossification. Recent observations show that skeletal progenitor cells marked by Prrx1-expression[31] or by Ctsk-expression[33] within the perichondrium (the connecting tissue encircling the growth cartilage) differentiate directly into the postnatal periosteal osteoblasts of the long bones, without chondrocyte intermediate. Thus, these cells can be seen as unipotent stem cells under physiological conditions, that can acquire multipotency upon tissue injury.

Osteochondral skeletal progenitors in endochondral bone development

The skeletal progenitor cells giving rise to the endochondral bones emerge as condensations of immature mesenchymal cells that will undergo chondrogenic differentiation. Focusing on the long bones of the appendicular skeleton in tetrapods, which include mice and humans, the process starts with the development of limb buds, which are small protrusions that emerge from the body wall of the embryo (around embryonic day (E) 9.5 in the mouse) (**Figure 2**). The outgrowing limb bud patterns along (1) the proximal-distal (P/D) axis (running from the base

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of the limb to the tip, e.g., shoulder to digit tip), (2) the anterior-posterior (A/P) axis (parallel with the body axis and from the thumb (anterior) to the little finger (posterior)), and (3) the dorsal-ventral (D/V) axis (from the back of the hand (dorsal) to the palm (ventral)). This patterned development is specified by polarizing signaling centers in the outgrowing bud, giving cells in different axial positions distinct developmental potential.[22,39,40] As such, the P/D axis is primarily regulated by the declining gradient of retinoid acid from the main body and the FGF signals (primarily FGF8) secreted from the apical ectodermal ridge (AER), a thickened ectodermal structure formed at the distal extreme of the limb bud. The A/P axis is controlled by Sonic hedgehog (Shh) morphogen diffusing from the zone of polarizing activity (ZPA), a group of mesenchymal cells located at the posterior distal limb margin immediately adjacent to the AER (**Figure 2**). The D/V polarity of the limb is regulated by the non-AER ectoderm covering the limb bud, through Wnt and BMP signaling (particularly Wnt7a).[22,39,41-43]

The developmental origin of skeletal progenitors traces back to these early fetal limb buds, where the condensing mesenchymal progenitor cells express the transcription factor *Prrx1*. Lineage mapping studies that used a mouse in which Cre-expression was driven by the *Prrx1* gene promoter and reported by the original Rosa26-locus-based β-galactosidase transgene (*Prrx1*-Cre;R26R-gal mice), showed Cre recombinase activity in the entire early limb bud mesenchyme.[44] These early Prrx1-positive cells consequently give rise to all the mesenchymal cell populations in the appendicular skeletal elements: chondrocytes, perichondrium, periosteum, and osteoblasts, as well as the fibroblasts in connective tissues of the limbs, tendon cells, and synovial cells.[45,46]

At condensation sites within the limb bud mesenchyme, osteochondral progenitors will start to express Sox9, which, along with its family members Sox5 and Sox6, is a master transcriptional regulator of chondrogenesis and directly activates the expression of chondrocyte-specific matrix proteins such as Col2, Col11, and aggrecan (encoded by the *Acan* gene).[47] Cell fate mapping using Sox9-Cre knock-in mice crossed to the R26R reporter strain, and lineage tracing

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by Sox9-CreER^{T} mice, evidenced that Sox9-lineage cells give rise to Col2-expressing cells.[45,48] Furthermore, Sox9-expressing cells are also located in the perichondrium, which later generates Osx-expressing osteoprogenitors and osteoblasts.[48,49] As such, Prrx1-expressing skeletal progenitor cells are at the top of the hierarchy, immediately followed by Sox9^{+} cells, as a source of the chondrocytes and osteolineage cells during early limb development[45] (**Figure 2**).

As the fetal chondrocytes proliferate and deposit abundant matrix, the cartilage anlagen grows. Subsequently, chondrocytes in the center stop dividing and mature into Indian hedgehog (Ihh)-expressing pre-hypertrophic chondrocytes, and further to hypertrophic chondrocytes that express Col10 and ultimately calcify some of the matrix.[50,51] Cartilage is an inherently avascular tissue, but hypertrophic chondrocytes start producing vascular endothelial growth factor (VEGF), promoting blood vessel invasion.[52,53] This initiates the formation of the primary ossification center (POC), the central bone cavity that, along with the blood vessel ingrowth, quickly becomes populated with hematopoietic cells, cartilage-resorbing osteoclasts, stromal/osteoprogenitor cells, and osteoblasts depositing bone on the remaining calcified cartilage spicules.[50,53] (**Figure 2**).

The precise sources for osteoblasts that give rise to trabecular bone have already for a long time been an area of intense investigation. Initial cell lineage tracing studies employed mice expressing a CreER^{T} construct controlled by the Osx (*Sp7*) gene promotor, pulsed with tamoxifen in early embryogenesis, focusing on E12.5 and E13.5.[49] This strategy marked the earliest Osx-expressing cells in the perichondrium, but also partially hypertrophic chondrocytes. Subsequent cell fate analysis at tight progressive time intervals documented that Osx^{+} perichondrial cells enter the developing POC by moving along with blood vessels during the initial vascular invasion of the fetal cartilage anlagen, and subsequently contribute to the trabecular osteoblast pool[49] (**Figure 2**). Ensuing work further detailed how cells with a history of Osx-expression, originally located both in the perichondrium and the growth plate, constitute

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a primary source of bone marrow stromal cells (BMSCs) and osteoblasts as well as adipocytes in the bone marrow.[54-57]

It is now well-established that both the perichondrium and growth plate cartilage are sources of osteolineage cells during developmental endochondral ossification. Recent lineage tracing work using selective cell-specifying markers, succeeded in distinguishing the fate of fetal perichondrial cells (marked by Dlx5-CreER^T activity at E12.5) and the fate of fetal growth plate chondrocytes (traced as FGFR3-lineage cells).[56] By comparing the fates of traced Dlx5$^+$ and FGFR3$^+$ cells, it was shown that perichondrial cells labeled at E12.5 form the cortical bone and constitute a source of adipocyte-biased postnatal BMSCs in the diaphysis, whereas the early chondrocytes mostly give rise to BMSCs with primarily an osteogenic predilection and to osteoblasts in the developing metaphysis.[56] Thus, while confirming the entry of perichondrial progenitors into the nascent POC, this study additionally helped to distinguish the contribution of two sources of osteoprogenitors, one from the perichondrium and another from the fetal growth plate chondrocytes (see next section), to the later SSPC pools.

Interestingly, the observation that already at early fetal stages, fractions of Osx$^+$-labeled cells associate tightly with the endothelium in a pericyte-like manner[49,58] may suggest that developmental osteoprogenitors, like many postnatal SSPC populations, might depend on the perivascular microenvironment. During embryonic and early postnatal life, these Osx$^+$ cell-covered skeletal blood vessels were described to be formed by a specialized endothelial cell subtype, termed ‘type E’ (embryonic), presenting abundantly within the bone marrow and bearing osteogenic supportive characteristics.[59] Type E endothelial cells are characterized by high expression of CD31, endomucin (EMCN), and apelin (encoded by the *Apln* gene) and appear to give rise to ‘type H’ endothelium peri-/postnatally (still highly expressing CD31, EMCN, and apelin), which on its turn can convert later to the ‘L-type’ endothelium (low in CD31 and EMCN) that constitutes the sinusoidal vascular network in adult bone marrow, as was shown by *in vivo* lineage tracing using Apln-CreER knock-in mice.[59] During

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endochondral bone formation, type H capillaries are located primarily in the metaphysis, where they are laden with pericytic SSPCs expressing platelet-derived growth factor receptor (PDGFR)β, Osx, C-X-C motif chemokine 12 (CXCL12), nestin, and/or neural/glial antigen 2 (NG2), especially in juvenile, fast-growing mice.[60,61] These type H-associated SSPCs may further orchestrate endochondral ossification by supporting or even driving cartilage turnover, as PDGFRβ-lineage SSPCs were shown to give rise to a population of fatty acid-binding protein 5-expressing ($FABP5^+$) cells at the chondro-osseous border termed septoclasts, which are cartilage-resorbing mesenchymal-lineage cells that strongly express matrix metalloproteinases such as Mmp9, Mmp13, Mmp14, and Mmp11.[62] Tight stromal-vascular alliances involving both Osx^+ skeletal progenitor cells and fetal/perinatal skeletal blood vessels also play an additional hematopoiesis-supporting role during development, by providing the proper local microenvironment or niche enabling the initial seeding and settlement of hematopoietic stem cells (HSCs) into developing bones.[58,63] This process whereby HSCs originating from the fetal liver start to home towards bone closely follows the invasion of the bone rudiment by blood vessels and osteogenic progenitors, presumably steered by chemo-attractive signals such as CXCL12 (also known as stromal cell-derived factor-1α (SDF-1α)), and stem cell factor (SCF, also known as kit ligand or steel factor) produced by skeletal progenitors and/or other niche cells[64]. These events thus initiate the establishment of bone marrow hematopoiesis inside the cavity, around E16.5 in mouse tibias, coinciding with the formation of the POC.[58,63,65]

Differentiation and fate of growth plate chondrocytes: chondrocytes as a source of osteoblasts

The growth plate is highly organized, with distinct morphologically discernable layers of progressive cellular maturation of chondrocytes from the extremity toward the POC (**Figures 2 and 3**). The upper 'resting' zone of the growth plate harbors cells characterized by slow cell cycle progression. Descendants of these cells are transit-amplifying cells forming the so-called 'proliferative' zone, which consists of stacks of flattened, highly proliferative cells organized

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in longitudinally oriented columns. As mentioned, after the proliferative phase, post-mitotic chondrocytes further differentiate towards a pre-hypertrophic phase and thereafter into hypertrophic chondrocytes, the process associated with a dramatic increase in cell volume (up to 15 times).[66] The growth plate drives the elongation of the long bones, requiring tight regulation of the pace of chondrocyte development, which is coordinated by a negative feedback signaling loop governed by parathyroid hormone-related protein (PTHrP, encoded by the *Pthlh* gene) and Ihh.[50] Chondrocytes in the upper resting zone secrete PTHrP, which binds to its receptor (the shared PTHrP and parathyroid hormone (PTH) receptor, PTH1R) expressed by the proliferating chondrocytes, thereby suppressing their differentiation into hypertrophic chondrocytes, and keeping the cells in the proliferative state. As the cells' distance from the resting zone increases, the impact of PTHrP fades, and the cells can escape the differentiation block. As soon as they become pre-hypertrophic, the chondrocytes start producing Ihh, which diffuses back to the upper layers and increases the expression of PTHrP by the cells in the resting zone. Ihh also increases the rate of chondrocyte proliferation and triggers osteoblast differentiation in the adjacent perichondrium by inducing Runx2 expression.[50,51]

Over the last forty-fifty years the common view was that all the terminally differentiated chondrocytes undergo apoptosis when they reach the chondro-osseous border, with the remaining cartilage matrix serving as a template on which trabeculae are formed by osteoblasts coming from other sources. However, this dogma has been profoundly challenged over the last decade. Lineage fate mapping and tracing experiments in mice have shown that at least a fraction of the hypertrophic chondrocytes in neonatal/juvenile murine growth plates can evolve into stromal cells populating the adjacent metaphysis and give rise themselves to trabecular osteoblasts for prolonged periods of time (**Figure 3**). Initially reported by 4 independent laboratories in 2014[48,67-69], this phenomenon of trans-differentiation of hypertrophic chondrocytes has subsequently been confirmed by several other laboratories employing various lineage tracing strategies, suggesting it to include a spatial component. Specifically,

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chondrocytes located at the periphery of the growth plate, facing the perichondrium/periosteum – originally described by Paolo Bianco as 'borderline chondrocytes'[70] – are a source of endosteal osteoblasts lining the inside of the cortical bone[49,71] (**Figures 2 and 3**). More central located cells in fetal/neonatal growth plates (derived from e.g., PTHrP$^+$ cells[72], Col2$^+$ cells (labeled after the formation of the POC using inducible Col2-CreER^T mice pulsed with tamoxifen to exclude the perichondrium)[48,71,73], Acan$^+$ cells[48,74], or Col10$^+$ cells[67-69,75-78]), can behave as precursors for osteolineage cells, and give rise to a subset of trabecular osteoblasts in the metaphyseal region below the growth plate. Interestingly, this transition process may, in fact, entangle an initial conversion of hypertrophic chondrocytes into SSCs in the metaphysis, located just underneath the growth plate and important for trabecular bone formation, and (perhaps further downstream) into some of the reticular cells in the bone marrow that are characterized by high expression of CXCL12.[71,77,78] These BMSCs, called CXCL12-abundant reticular (CAR) cells, are themselves SSPCs that can subsequently give rise to osteolineage cells and marrow adipocytes[71,77] (**Figure 3**).

At present, however, it is not fully clear yet what determines the fate of a chondrocyte, to what extent it concerns apoptosis versus de- or trans-differentiation, and which factors control these events and their multipotent differentiation ability *in vivo.* Contributions have been reported for Ihh signaling[56,73,79], β-catenin[80], the transcription factors IRX3 and IRX5[76], and a tyrosine phosphatase called SHP2.[81] Furthermore, recent work indicated that MMP14 activity in chondrocyte-derived osteoblast lineage cells is involved in modulating the anabolic response to PTH signaling through PTH1R cleavage.[82]

Adolescence and the appearance of bona fide cartilage stem cells in the murine growth plate

After the period of morphogenesis, development, and intense juvenile growth, bone lengthening ceases around late adolescence in humans while gradually decreasing over the lifetime in mice. Accordingly, the growth plates disappear ('close') in humans, although mice remain to have growth plates open until very late in life. Recent research using lineage tracing has revealed the

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existence of post-adolescent epiphyseal SSPCs in the growth plates of mice[72,83-85] (**Figures 2 and 3**). Such postnatal, long-term SSPCs have been labelled *in vivo* as FoxA2-expressing ($FoxA2^+$)[84], $Axin2^+$[85], $PTHrP^+$[72], and CD73-expressing Col2-lineage cells[83], and showed the two stemness criteria, namely self-renewing capacity, and multipotency.

Growth plate SSPCs reside in the upper resting chondrocyte zone of the growth plate, a stem cell-rich region adjacent to the emerging secondary ossification center (SOC) or epiphysis (**Figures 2 and 3**). The fact that the appearance of these distinct cartilage SSPCs in the post-development period coincides with the development of the SOC, may possibly denote the need for this structure and its associated signals and mechanical environment as stem cell niche.[86,87] Epiphyseal cartilage SSPCs are slow-cycling cells that serve as precursors for growth plate chondrocytes and might additionally support the maintenance of endochondral bones during adult life in mice, as these cells and their hypertrophic chondrocyte descendants contribute to the generation of BMSCs and osteoblasts as described above.[88] It is at present unclear, however, if these SSPCs exist in the human growth plate, and how important they are for human growth or bone maintenance, given that the growth plates in humans close and growth ceases at late puberty.

In mice, the evidence indicates that the maintenance and functioning of growth plate SSPCs critically rely on delicately balanced signalling via the hedgehog, Wnt/β-catenin, and mammalian target of rapamycin (mTOR) pathways.[72,83,85,89] For instance, firstly, pharmacological lowering or elevating the levels of hedgehog signaling caused loss of cartilage SSPCs, indicating that proper hedgehog levels are required to maintain their self-renewal.[72,83] Recent genetic activation of hedgehog signaling in $PTHrP^+$ cells (by crossing tamoxifen-inducible PTHrP-CreER mice with Patched-1 floxed and tdTom reporter alleles) showed that hedgehog-activated $PTHrP^+$ chondrocytes in the resting zone showed increased clonal expansion, associated with hyperplasia of the growth plate, suggesting that hedgehog activation can drive cartilage SSPCs into transit-amplifying states as proliferating chondrocytes.[79,90] Moreover, transient pharmacological activation of hedgehog signaling was found to increase

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the number of cartilage stem cells, leading to leg lengthening[90] As described above, pre-hypertrophic chondrocytes create a gradient of Ihh, which influences cells within the resting zone, but a recent publication showed that hedgehog signaling streamed from the periosteum is also crucial for the regulation and maintenance of SSPCs in postnatal life.[91] Secondly, cells responding to canonical Wnt (Axin2-expressing cells), localized at the periphery of the growth plate facing the groove of Ranvier, were shown to contribute to lateral growth of the growth plate and to stem/progenitor cell functioning in the growth plate, which was inhibited by β-catenin ablation.[85] On the other hand, slow-cycling PTHrP-lineage cartilage SSPCs need to be maintained in a Wnt-inhibitory environment, as activation of β-catenin in these cells decreases their ability to generate progeny.[89] This aligns with the observation that activation of β-catenin in the growth plate causes its abrupt fusion.[92] Additionally, albeit speculatively, mechano-sensing signaling pathways might be involved in the maintenance and function of cartilage SSPCs and their niche, as the formation of the SOC dramatically changes the environment and mechanical load and stress that the upper zone of the growth plate, containing the SSPCs, is exposed to[93]; yet, the mechanisms of mechano-sensing in and its influence on SSPCs in the growth plate remain to be determined. Clearly, the specific constituents fulfilling cartilage SSPC-supportive niche functions have not yet been identified to date.

Emergence of postnatal SSPC populations in the bone marrow

Studies increasingly suggest that the developmental progenitor cells controlling bone formation during embryogenesis and perinatal growth are transient, and that they get replaced after adolescence by a second wave of distinct, postnatal stem cells.[55,74] A recent publication reported that osteoblasts for bone modelling in prenatal life and until adolescence were largely derived from chondrocytes (marked by the Acan-CreER^T transgene), whereas new osteoblasts in the post-adolescent and adult stages were mainly descending from cells traced with a leptin receptor (Lepr)-CreER^T line.[74] Interestingly, based on a mutually exclusive, dual-recombinase

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fate-mapping strategy, it was proposed that the Col2 lineage is hierarchically upstream of LepR$^+$ BMSCs and osteoblasts.[74] It is unclear at this moment, though, whether the Col2$^+$ cells that give rise to LepR$^+$ BMSCs would be chondrocytes or perichondrial cells or others, given the general ability of Col2 to label a broad set of skeletal progenitors, chondrocytes, and SSPCs, particularly if a non-inducible Cre is utilized.[48,49] Neonatal LepR$^+$ cells do not contribute much to osteoblastogenesis, whereas these cells are the major source of osteoblasts in adult bones.[74] Recently, the endosteum was identified in young mice as a source of Fgfr3$^+$ SSPCs with the ability to form chondro-osteal cells, while they, too, are later replaced by Lepr$^+$ cells.[94]

The post-adolescent, adult LepR$^+$ SSPCs reside as abundant perivascular stromal cells of reticular appearance in the bone marrow, and support the maintenance of bone mass during adult bone remodeling by providing lifelong sources for osteoblasts.[95]. LepR$^+$ cells and CAR cells in the bone marrow largely overlap, and both comprise SSPC subpopulations primed towards either osteogenesis or adipogenesis (termed 'Osteo-CAR' and 'Adipo-CAR' subsets).[96-98] Osteogenesis-primed SSPCs preferentially associate with arterioles ('H type' vessels), whereas adipogenesis-directed SSPCs are mostly peri-sinusoidal (i.e., located around the diaphyseal 'L type' blood vessels) or non-vascular.[61,96,98,99] The present knowledge of the identity, fates, functions, and relationships of SSPC populations in adult bone physiology, ageing, and disease, is reviewed and discussed in detail elsewhere (pending publication by the same author group).

Thus, it seems that short-term developmental skeletal progenitors are followed by long-term adult SSPCs, and that distinct cellular sources contribute to the osteoblast pool before and after adolescence. Not everything is clear at this moment, though. For instance, whether transient progenitors are replaced during adolescence by adult SSPCs with self-renewing capacities, or whether a portion of the cells acquires self-renewing capacity and gives rise to adult SSPCs, is not very clear. Also, while some long-term lineage tracing studies indicated that primary, early fetal Osx$^+$ cells (labeled at E14.5) give rise to long-lived BMSCs[54] and are still able to deliver progeny contributing to fracture repair following bone trauma in adult life[57], others reported

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that the fetal Osx^+ progenitor pool is transient, with a secondary wave of Osx^+ cells originating in early postnatal life (labeled at P5 or later) containing the adult reparative potential.[55] Further research will be needed to fully clarify the hierarchy between developmental and adult SSPCs.

BONE REGENERATION DURING FRACTURE HEALING IS MEDIATED BY MULTIPLE SOURCES OF REPARATIVE SSPCs

Bone has the extraordinary capacity to heal without scar tissue formation, thus representing one of few adult tissues able to regenerate. Generally, bone repair after fracture is a rapid and efficient process, which largely recapitulates the processes and signaling pathways regulating embryonic bone development.[100-102] Like developmental bone formation, fracture repair also occurs in close interplay between SSPCs and the skeletal vascular network.[53] Many of the genes that control fetal bone development show increased expression during fracture healing.[103] Ultimately, the "re-generation" process at the site of the defect normally results in newly formed bone that is fundamentally indistinguishable from the original bone prior to the injury.

Microfractures and stabilized fractures principally heal through intramembranous ossification, with SSPCs differentiating directly into osteoblasts, without chondrogenesis. Semi- or non-stabilized fractures largely heal through endochondral ossification, involving the formation of a soft fibro-cartilaginous callus intermediate at the fracture site, followed by its subsequent substitution by bone tissue in a process resembling endochondral bone formation (**Figure 4**). In both types of fracture healing, the initial inflammatory reaction and hematoma formation, probably along with tissue hypoxia resulting from the vascular disruption, lead to the release of a myriad of growth factors and cytokines.[104] These include several proangiogenic factors, such as VEGF[105,106], placental growth factor (PlGF)[107], platelet-derived growth factor (PDGF)[57,108], and thrombospondin-4 (TSP-4)[109], chemotactic factors such as CCL5[110], proteolytic enzymes as MMP-9 and plasminogen activators[111-113], and various other signals

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including transforming growth factor-β (TGFβ), IGFs, BMPs, tumor necrosis factor α (TNF-α), Wnts, Notch family members, FGFs, and hedgehogs[114-120], which are altogether indispensable for proper fracture healing, because they initiate the activation, proliferation, and recruitment of the necessary SSPCs to mediate the subsequent repair (**Figure 5)**.[101,102,121] PDGF-B, for instance, is a well-known, potent mitogen for skeletal precursor cells[122,123], and conditional loss-of-function of its main signaling receptor PDGFRβ in mice showed that PDGF-PDGFRβ signaling is critical during early fracture healing for inducing the proliferation, trafficking, and angiotropism of PDGFRβ-expressing SSPCs, thereby mediating callus formation.[57,108]

Dynamic responses of various subsets of SSPCs to acute tissue damage facilitate the effective repair and regeneration of injured bone, determining its native regeneration capacity. The prime sources of fracture-repairing cells are the periosteum and the bone marrow stroma, as discussed below. Yet, additional direct and/or indirect contributions by muscle-derived progenitor cells seem to be eminent.[124,125] For instance, paired box protein 7 (Pax7)-positive satellite cells from muscle adjacent to bone injury sites were reported to indirectly support fracture healing, by secreting chondrogenic and osteogenic growth factors such as BMPs, IGF-1 and FGF-2.[124] Possible contributions by circulating osteogenic cells are still a topic of debate given the low frequency of circulating SSPCs, especially in humans.[126-128]

The adult periosteum as a source of reparative SSPCs

The periosteum is a thin layer of connective tissue that overlays the outer bone shaft. It consists of an outer strongly vascularized and fibroelastic layer, and a highly cellular inner cambium layer, believed to contain most SSPCs, committed osteogenic precursors, osteoblasts, and fibroblasts. Although the relative contributions of the fibrous and cambium layers have not been fully characterized, the periosteum provides a rich niche for multipotent osteo-chondroprogenitor cells that exhibit a remarkable regenerative capacity (**Figures 4 and 5**). Bone injury first activates periosteal SSPCs to proliferate, resulting in an early thickening of

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622 the periosteal tissue layer adjacent to the fracture site, and subsequently triggers their migration
623 and differentiation. Experiments disrupting the periosteum demonstrated its necessity for
624 proper and timely healing.[129] Moreover, a tissue-tracing strategy in mice using transplantation
625 of bone grafts from Rosa26 donors (expressing the LacZ reporter gene) into a fracture site after
626 removing either the periosteum or the BM/endosteum, revealed that the periosteum and
627 BM/endosteum both contribute osteoblasts during bone repair, but the periosteum contributes
628 more, and specifically constitutes the prime source of chondrocytes in the callus.[130] Recent
629 studies revealed that periosteal cells not only repair large defects but also regenerate bone and
630 hematopoiesis-supporting BMSCs following injury.[131-133] The injury responses of periosteal
631 SSPCs are controlled by the factors secreted in the early phases of fracture healing, including
632 as mentioned BMP, PDGF, VEGF, Notch, Wnt, and FGF signals, many of which are believed
633 to be induced by hypoxia due to the ruptured blood vessels and hematoma formation, and
634 elicited by endothelial, bone, and immune cells in the fracture environment.[101,104,121] Cell-
635 intrinsically, Hox genes also play important roles in specific anatomical regions, for instance
636 Hoxa10 and Hox11 in the zeugopod, in maintaining periosteal stemness and determining cell
637 fate.[134-137] Besides its prominent role in fracture healing, the postnatal periosteum is generally
638 acknowledged to contribute to periosteal bone thickening and cortical bone maintenance under
639 physiological conditions, although much of the mechanisms and mediators remain to be
640 clarified.
641 Studies employing lineage mapping and tracing showed that the adult periosteum contains long-
642 term, slow-cycling, self-renewing SSPC pools with a high capacity for rapidly expanding in
643 response to fracture and producing osteoblasts and chondrocytes mediating the repair
644 process.[33,110] As such, periosteal SSPC subpopulations with the ability to respond to bone
645 fracture by proliferative expansion and multi-lineage differentiation *in vivo* (producing
646 osteoblasts and chondrocytes) have been genetically marked in lineage mapping/tracing studies
647 by their expression of Ctsk[33], PDGFRβ[57,108], Osx[57], Gli1[131,133], Axin2[138], Sox9[139,140],
648 PDGFRα[141], Prrx1[46,142], or α-smooth muscle actin (αSMA)[143,144], and as combined αSMA^{+}

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myxovirus resistance-1 $(Mx1)^+$ cells that have high colony formation potential and long-term repopulating capacity[110]. It is important to note, however, that most or all cell populations delineated by these single genetic markers are very heterogeneous, likely consisting of multiple independent lineages and/or differentiation states within those lineages, and that they represent at least partly overlapping rather than discrete cell subsets. In addition, none of these markers are exclusively specific for the periosteum or even for bone. For instance, a newly generated Prrx1-CreER^{T2} mouse model recently underscored that, besides its global marking of the limb bud mesenchyme, induced Prrx1-CreER^{T2} also targets a broad range of SSCs and SSPCs within the appendicular and cranial bones at postnatal ages, while also labeling stem cells in subcutaneous white adipose tissue and skin.[145]

Periosteal SSPC populations have also been described based on cell surface marker expression, such as the fracture-induced bone-cartilage-stromal progenitors (f-BCSPs) characterized in mice by integrin alpha-6 (CD49f) expression[146] and human periosteal SSPCs expressing markers such as PDPN, CD73, and CD164.[8]

Recently, PDGFRα^+ periosteal stem cells from both murine and human sources were reported to be important for bone regeneration, albeit not inclined to participate in chondrogenesis.[141] This touches upon the fact that it still remains enigmatic which signaling pathways trigger SSPCs into endochondral versus intramembranous ossification, and if all SSPCs possess the intrinsic capacity to participate in each of these ossification processes – as opposed to the existence of lineage-restricted (unipotent) SSPC populations. Periosteal cells marked via Ctsk-Cre are specialized in intramembranous bone formation at baseline, and upon *in vivo* transplantation of the Ctsk-labeled $CD200^+CD105^-$ subpopulation under the kidney capsule these cells form intramembranous bone; yet, while normally only generating osteoblasts, they are also able to initiate chondrogenesis and endochondral ossification in response to injury.[33] Similarly, $Mx1^+$ αSMA^+ periosteal cells undergo intramembranous ossification upon a drill-hole injury and are a main supply for osteoblasts, while they also differentiate into chondrocytes

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676 in larger defects.[110] In the calvarium, which forms and heals only via intramembranous
677 ossification, quiescent Axin2-expressing SSPCs in the sutures contribute to osteolineage cells
678 during skeletal development and upon calvarial injury, although transplantation into the kidney
679 capsule and treatment with BMP could trigger chondrogenesis.[29] It is well known that there is
680 a profound influence of stabilization at the fracture site on the induction of intramembranous
681 (when stably fixed), versus endochondral (when mechanically unstable or only partially fixed)
682 repair mechanisms, likely implicating some mechanical clues.[147-149] Reports suggested that
683 BMP and Ihh signaling could also be implicated in this differential response.[150,151] However,
684 overall, our knowledge on the mechanisms of action of mechano-transduction pathways is
685 limited.
686
687 The periosteum has unique mechanical properties, due to collagen and elastin fibers, which are
688 reinforced by collagenous Sharpey's fibers attaching to the bone surface at a 45-degree angle,
689 enabling cells in the environment to sense tensional stress.[152] Given that the periosteum
690 consists of different layers adjoining multiple cell types, blood vessels, nerves, adjacent
691 muscles, and tendons, SSPCs within the periosteum are exposed to many different
692 environmental stimuli, including mechanical stress modulated by the fibroelastic layer. This
693 environment likely provides a nurturing niche regulating quiescence, renewal, and
694 differentiation of periosteal SSPCs, with supportive roles exerted by diverse extrinsic cues and
695 by the ECM.[153] For instance, when periostin, a matricellular protein whose expression is
696 increased by axial compression[154], was deleted using *Prrx1*-Cre mice, this resulted in
697 periosteal SSPC loss and failed activation of the tissue repair program upon fracture.[142] Further
698 research will undoubtedly expose more details on the intricate intercellular communication and
699 regulatory pathways occurring across the various layers of the periosteum, including the
700 involvement of mechano-sensing cues, cell-cell crosstalk, and cell-matrix interactions in
701 periosteal SSPC maintenance and activation.

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Better insights into the cell biology of periosteal SSPCs and into the niche-derived signals that activate quiescent stem cells into regeneration hold great promise for the development of new approaches to enhance compromised bone healing and facilitate the repair of non-union fractures. Especially human periosteal SSPCs, due to their surgical accessibility and robust expansion and differentiation capacities, bear enormous therapeutic promise as a cell source for bone tissue engineering purposes.

Contributions to fracture repair by bone marrow-resident SSPCs and considerations on the use of BMSCs for skeletal cell therapies

Besides the critical importance of periosteal SSPCs in fracture repair, several studies have documented that various populations of endogenous, bone marrow-resident SSPCs are mobilized and contribute to fracture healing.[55,155,156] Specifically, mouse lineage tracing studies documented that quiescent CXCL12-CreER^{T2}-marked BMSCs (CAR cells) transform into activated, proliferative osteogenic progenitor cells upon injury and functionally contribute to bone regeneration.[155] Another stromal marker, Mx1, delineates a pool of mostly osteogenesis-restricted progenitor cells located at the endosteal surface and within the bone marrow, which respond to fracture by proliferation and migration to the site of injury, supplying new osteoblasts during the healing process.[156] BMSCs marked by adiponectin (Adipoq)-Cre/CreER, previously reported as marrow adipogenic lineage precursors[157], were recently found to be prime sources of reparative cells in drill hole injuries (small and stable defects), in contrast to bicortical fractures (large and unstable defects) that were primarily repaired by periosteal cells.[131] Other fate-mapping studies in transgenic mice also allocated roles in fracture healing for Osx-, PDGFRβ–, LepR-, and Grem1-lineage cells, contributing to both the chondrogenic and osteogenic lineages during endochondral bone repair.[57,95,158] Yet, besides cells located in the bone marrow, these Cre lines also target cell populations located in the periosteum and/or the growth plate, making it difficult to delineate the exact spatial origin of the reparative cells.

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Besides delivering osteogenic progenitors, bone marrow SSPCs probably also contribute to the repair process through their high expression of M-CSF and RANKL.[159-162] These signals induce osteoclastogenesis, which is required for remodeling the callus tissue after the stabilizing osteo-anabolic phase to re-establish the bone marrow space within the healed bone.[163]

Many studies support the capacity of bone marrow aspirates or BMSCs to enhance fracture healing in experimental bone regeneration studies. Human BMSCs are being used in clinical trials for craniofacial bone repair, distal tibial nonunion, and osteonecrosis of the femoral head, with relatively promising results, although there are many scientific, technical, practical, and regulatory limitations, that stand in the way of the integration of human BMSC/SSPC-based therapies into clinical practice.[19] These include the poor definition of the cells and the lack of molecular-mechanistic insights. Obtained by plastic adhesion or by cell sorting, most human BMSCs comprise highly heterogeneous populations of stromal cells, and it is at present unclear which fraction of BMSCs are true SSCs and how these cells can be purified.[3] Their isolation with cell sorting is often based on varying and relatively generic marker sets, and usually followed by *ex vivo* expansion and characterization. Currently, according to the International Society of Cellular Therapy, MSCs are defined as cells exhibiting plastic adherence, clonal expansion potential (CFU-F formation), the ability for trilineage differentiation *in vitro*, and expression of CD73, CD90, and CD105, while being negative for haematopoietic markers (CD34, CD45, CD14, CD19, HLA-DR).[164,165] Denoting also the tissue of origin, for instance, 'BM-MSC' for cells harvested from bone marrow, will be important for building toward well-founded cell therapies.[3] Besides more precise identification and in-depth mechanistic studies to better characterize human BMSCs and SSPCs from various sources as cell therapeutics, there is also a critical need for standardized guidelines and procedures for expansion/upscaling, shipment, and storage of cell-based products in good manufacturing practice (GMP) facilities in order to enable their safe and predictably effective use in future regenerative medicine.[19]

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CELLULAR DYNAMISM AND PLASTICITY IN THE SKELETAL SYSTEM

The strong endogenous capacity of bone to repair defects is remarkable. This capacity may perhaps be explained not only by the activity of a limited subset of specialized SSPCs, but, in fact, by the broader ability to elicit orchestrated tissue-regenerating responses whereby multiple endogenous skeletogenic cell sources become engaged into the process and cell-autonomously contribute to rapid and effective healing. This hypothesis is supported by experimental fracture healing experiments that focused on the early cellular dynamics in the callus environment, using Osx- and PDGFRβ-driven lineage mapping and tracing strategies in combination with high-resolution confocal and deep-tissue microscopy performed at short time intervals after bone fracture.[57] Analysis of the fractured area 12, 24 and 48 hours after injury showed that traced Osx-lineage cell subsets at various locations within the environment of the bone defect and tissue damage, including the periosteum, endosteum, bone marrow stroma, and local trabecular bone surfaces, were massively activated, indicated by their increased number, acquirement of vigorous PDGFRβ-expression and a spindle-shaped morphology, and altered spatial orientation with cells attaining a directionality towards the defect site. At subsequent stages, an extensive mobilization of reparative SSPCs was evident, with increasing numbers of the traced cells populating the bone defect site, first as fibroblast-like cells and subsequently as differentiated chondrocytes and osteoblasts.[57] Cells with a history of Osx-expression labeled just before the fracture in adult mice, or dating back from early postnatal life (P5) or fetal stages (E14.5), all had the capacity to participate vigorously in the repair process. Moreover, virtually all cells contributing to the callus derive from the PDGFRβ-lineage, and many reparative Osx-lineage and PDGFRβ-expressing SSPCs closely associate with blood vessels.[57].

These fate-mapping and lineage tracing observations suggested that bone trauma can trigger a fast, abundant, and broad (re-)activation and proliferation response in multiple endogenous cell populations residing in or close to the local injury environment, to orchestrate a common

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reparative response and ensure quick and successful mending of the defect, possibly also including contributions by differentiated skeletal cells such as mature osteoblasts.[57] Regarding this latter aspect, it has been shown that differentiated chondrocytes can generate osteoblasts during endochondral bone repair.[68] Also, mature bone marrow adipocytes, although their excessive presence in ageing and obesity is considered to negatively impact fracture healing[166], were recently found to be highly dynamic and plastic and to bear osteogenic potential during bone repair.[167] Moreover, some studies suggest that previously differentiated osteolineage cells that subsequently became retreated or quiescent, may still have the capacity to be reactivated and provide a (lineage-restricted) source of active osteoblasts in adult bone upon trauma, although these conclusions are not at present universally supported and are challenged by the difficulty in mature cell tracing studies to exclude any activity of the lineage maker in undifferentiated cells.[144,168,169] Altogether though, the observations indicate that various cell populations can functionally act as SSPCs upon bone injury or under stress. As elaborated on below, the idea is thereby emerging that cellular lineage specification and differentiation processes, traditionally seen as organized accordingly to a hierarchical tree with unidirectional downstream branches, might be more dynamically and flexibly occurring in the skeleton, especially in settings of repair/regeneration and pathology (**Figure 6**).

Postnatal tissue stem cells and their descendants are usually viewed as hierarchically organized, with the most primitive, self-renewing, and multipotent stem cells standing at the apex of the chart. From this top position unidirectional fate map lines are depicted, going towards the downstream more lineage-committed and fate-restricted progenitors, and ultimately the differentiated progeny (**Figure 6A**). For instance, in hematopoiesis, a small number of multipotent HSCs can give rise to all blood cell lineages upon repopulation of the bone marrow. Yet, although even a single HSC can rebuild the system after transplantation, more recent works have shown that, instead of the classically defined HSCs giving rise to all progeny, rather several long-lived progenitors and lineage-biased or lineage-restricted cells are the main drivers

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of steady-state hematopoiesis and mediators of stress responses.[170-172] Thus, fundamental differences are being uncovered between the physiological maintenance of the haematopoietic system, its regulation during pathology or stress, and its behaviour after transplantation.[170] Similarly, efforts have recently been directed towards building the lineage hierarchy map for the skeletal system and the identification of the primitive self-renewing and multipotent SSC at the apex of the chart, based on cell surface marker-based isolation and functional characterization through transplantation assays, which continue to be refined with additional markers.[7,8,173,174] Such cells are highly fitting to instruct tissue-engineering applications effectively. Yet, the functional contributions to *in vivo* life-long tissue maintenance and native injury repair in the skeleton appear to be spread over various individual endogenous stem/progenitor cell compartments rather than mediated by a single primitive SSC entity (**Figure 6B**). Especially during bone injury repair, and in settings of intensive bone growth and (patho-)physiological or induced osteogenesis – i.e., when the need to expand the active skeletogenic populations is large or even critical for reinstalling tissue integrity – multiple cell subsets appear to bear skeletal progenitor characteristics and provide sources of osteogenic potential, including cells in the periosteum, endosteum, muscle, growth plate, primary spongiosa, and bone marrow. Moreover, ample evidence indicates that the lineage relationships between the differentiated progeny of SSCs/SSPCs are more complex and dynamic than previously thought, particularly in pathological conditions. As such, questions on de-/re-/trans-differentiation mechanisms and cellular plasticity are increasingly arising in the field, as illustrated by the following examples.

The **first** example concerns the fate of terminally differentiated chondrocytes. As mentioned, lineage fate mapping and tracing experiments in mice have indisputably shown that apoptosis is not always the endpoint of the chondrocyte lineage, as at least fractions of the borderline chondrocytes and hypertrophic chondrocytes can evolve into bone marrow SSPCs, giving rise to osteoblasts and adipocytes for prolonged periods of time (see[88]).

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Secondly, mature bone marrow adipocytes, the fate of which was recently lineage-traced using a perilipin (Plin1)-CreER;tdTom mouse line, appear to be able to convert to BMSCs and to give rise to osteoblast lineage cells – most pronouncedly, but not exclusively, in contexts of injury and tissue regeneration in the bone and marrow environment.[167] Concretely, in this study, mature adipocytes were first massively formed in the bone marrow by irradiation-induced injury and then labeled via tamoxifen administration. Upon the subsequent bone marrow regeneration, the traced cells Plin1-lineage cells dedifferentiated to $LepR^+$ BMSCs; later on, following a cortical bone drill-hole injury, these adipocyte-derived cells (or adipocytes themselves, although direct transdifferentiation seems less likely) could become Col1-GFP^+ osteoblasts participating in bone repair.[167]

Thirdly, although bone lining cells have long been presumed to be descendants from mature osteoblasts that became inactive with respect to bone formation, or even quiescent, it has been shown in mice that such flat, bone surface-associated cells may not fit that description or that they can be re-activated to cuboidal, actively bone-forming osteoblasts. This phenomenon has been described, for instance, upon bone-anabolic intermittent PTH treatment[175,176], sclerostin antibody administration[177], mechanical loading[178], irradiation[179], and other settings[180]. Conversely, bone lining cell activation was reported to be inhibited by glucocorticoids, known inducers of osteoporosis.[180] Interestingly, activated bone lining cells expressed cell surface markers characteristic of SSPCs, including Sca-1 and LepR.[180] However, since no lineage tracing experiments specifically pulse-chasing bone lining cells have been performed so far, the above observations have to be interpreted with caution.

Fourthly, the concept of de-differentiation and reprogramming of mature cells toward multipotent progenitors has been described in skeletal systems of more primitive animal species, e.g., as part of fin regeneration in zebrafish and in axolotls during limb regeneration upon amputation.[181,182] But also in mice, following adult digit tip amputation, mature osteolineage cells (labeled using Dmp1-$CreER^{T2}$ transgene) have been documented to divide and acquire a flexible mesenchymal precursor-like blastema phenotype, and contribute to digit

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tip regeneration.[183] In the same mammalian context, nail mesenchyme cells were also found to make a direct and essential contribution to digit tip regeneration.[184] These studies provide support for the idea that a traumatic, regenerative environment can promote the acquisition of a precursor state that allows plasticity, i.e., for cells from one tissue type to contribute to the regeneration of another tissue type, at least between mesenchymal cell types.[183,184]

Other suggestions of *in vivo* cellular plasticity between cell types in the mammalian skeletal system have emerged in settings of disease and tissue repair studied in mouse models. For instance, tumor-induced conversion of endothelial cells into osteoblasts has been reported to contribute to osteoblastic bone metastases in prostate cancer.[185] Moreover, endothelial-to-mesenchymal transition towards stem-like cells, which could be triggered to differentiate into osteoblasts, chondrocytes, or adipocytes, was shown in fibrodysplasia ossificans progressiva (FOP), a disease in which heterotopic ossification occurs due to activating mutations in the BMP receptor ALK2.[186] Lastly, as mentioned, during fracture repair, muscle-derived cells have been shown to contribute to bone regeneration.[124,125,187]

Albeit incompletely understood at present, these examples of unconventional lineage relationships among skeletal cell types challenge the traditional view of a single, hierarchical SSC-driven model of progressive, linear fate specification and maturation into the differentiated progeny. Perhaps these *in vivo* examples are illustrating that skeletal cell fates could be more flexible and dynamic, and that multiple cell sources can be triggered to operate as functional SSPCs to mediate adult bone tissue anabolism and facilitate bone repair and regeneration in response to injury (**Figure 6**). While further research will be needed to unravel these fate capacities and lineage relationships, it is interesting to see that similar thoughts and paradigm movements have been rising in other stem cell fields.[188-191]

TAKE-HOME MESSAGES AND OUTLOOKS FOR FURTHER RESEARCH

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Osteoporosis and non-healing fractures pose an increasing clinical problem worldwide, echoing the need for safe and effective bone anabolic drugs and therapies for bone repair. Basic insights into the origin and maintenance of developmental and regenerative stem/progenitor sources for bone-forming osteoblasts will facilitate the development of new cell biology-based approaches, including pharmacological treatments and stem cell therapies, that can be translated into clinical bone regenerative medicine applications. Current research is expanding the investigation of SSPC populations from physiology toward clinically important conditions and aspects relevant thereto, such as the impact on SSPC maintenance and reparative functionality of ageing, osteoporosis, sex hormones, inflammation and cellular senescence, diabetes, and other metabolic diseases, malignancies, and genetic disorders.[174,192-200] While a lot of work still needs to be done in these directions, this area of research will advance our understanding of the pathological mechanisms underlying skeletal diseases. Concomitantly, increased molecular insights in the regulatory control of developmental skeletal progenitors and reparative SSPCs will undoubtedly help uncover strategies to repair bone and cartilage defects caused by injury or disease. A thorough understanding of the identity, characteristics, and molecular regulation of osteogenic and chondrogenic progenitors that play critical roles in establishing the skeleton during embryogenesis, mediate its intense growth, and organize tissue repair, may offer vital new directions for diagnostics and prediction of compromised fracture healing potential, for intervening therapeutically in situations of impaired repair, and for informed design of tissue-engineered regenerative strategies. The therapeutic prospects towards the enhancement of failing fracture healing are extensive. New solutions to treat delayed-union fractures could encompass pharmacological stimulation of endogenous repair mechanisms, by rejuvenating endogenous cells or mobilizing and expanding SSPC subsets. Non-unions are envisioned to be cured in the future in efficient and patient-friendly ways by off-the-shelf solutions based on engineered micro-tissues, generated via autologous or allogeneic cell populations or iPSCs, or through cell therapy using exogenous SSPC populations that are primed to home to the site of the defect through molecular or genetic modifications.

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The field is rapidly progressing, and over the last years exciting new insights have been obtained on the origin of bone-building osteoblasts in intramembranous as well as endochondral bones at various locations, including calvaria, vertebra and long bones, and on the reparative SSPC populations mediating bone repair. An important lesson taken from these recent developments and discoveries is that the skeleton harbors (at present still mostly heterogenous) sets of skeletal stem and progenitor cells that can differ between and within bones, stages in life, and settings. Further, we learned that disturbed interactions between co-existing SSPC subsets at a single site, or between SSPCs and the niche components surrounding them, can underly mechanisms driving skeletal disorders (such as craniosynostosis).[34] Moreover, as very recent work uncovered that the formation and maintenance of vertebral bones is established by SSPCs that transcriptionally and functionally differ from those found in long bones, an explanation could concomitantly be postulated for the longstanding clinical question of why disseminating breast cancer cells spread more often to vertebral bones than to the long bones of the limbs, which was reported to be linked to the selective secretion by vertebral SSCs of a metastatic trophic factor driving the migration and recruitment of the tumor cells.[173]

Much of the new findings and progress in dissecting the origin of bone-forming and -regenerating osteoblasts have been obtained with the use of sophisticated cell lineage tracing approaches in mice, which allows investigators to follow the fate of selected cell subsets over time, using high-resolution microscopy to enable visual readout of the *in situ* location, phenotype, cellular neighbors, and interactions. Further advances in spatial multi-omics and the combined use of FACS-assisted identification and isolation of SSPCs with transplantation and high-resolution molecular profiling methodologies are providing the tools to address currently existing areas of uncertainty in the field. As such, major further questions remain to be answered in future research regarding the heterogeneity of SSPC populations existing at the distinct anatomical locations and niches. At present, much also remains unclear about the relative contributions of distinct SSPC subsets to tissue formation and maintenance in various settings, their therapeutic applicability and responsiveness, and their disease-causing potential. It is also

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not yet known whether osteoblasts derived from different SSPC sources are functionally different or predisposed to distinct fates or sensitivities. But a main challenge remains the translation to human biology and pathology. To bridge this gap, it will be important to dissect whether and how the much-studied murine skeletal stem and progenitor populations correlate with human counterparts and to investigate the role of particular stem cells and skeletal progenitor subsets in the development of rare genetic diseases, congenital limb malformations, and bone-centered malignancies in humans. These efforts are actively being initiated.[20,21] For instance, recent work presented the first detailed spatiotemporal characterization of human embryonic limb development, established by combining single-cell and spatial transcriptomics.[21] Moreover, the study included efforts toward the assembly of an integrated cross-species developmental limb atlas, by comparing human data with scRNA-seq on mouse embryonic limbs, which indicated substantial homology between the two species at least in this developmental context.[21] Finally, priorities for the upcoming years should also include untangling the cellular-molecular crosstalk between SSPCs and their respective micro-environmental niches, addressing the influence of endocrine, mechanical, metabolic, and epigenetic control mechanisms, as well as the impact of oxygen and nutrient availability, ageing and inflammatory cues, on SSPC behavior. Such further insights from basic research, complemented with more intensive studies on human samples, will open the door towards clinical translation and undoubtedly facilitate important future clinical applications.

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Author contributions (CRediT format)

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Seppe Melis: Writing – original draft, Investigation, Writing – review & editing.

Andrei S Chagin: Writing – review & editing, Investigation, Funding Acquisition.

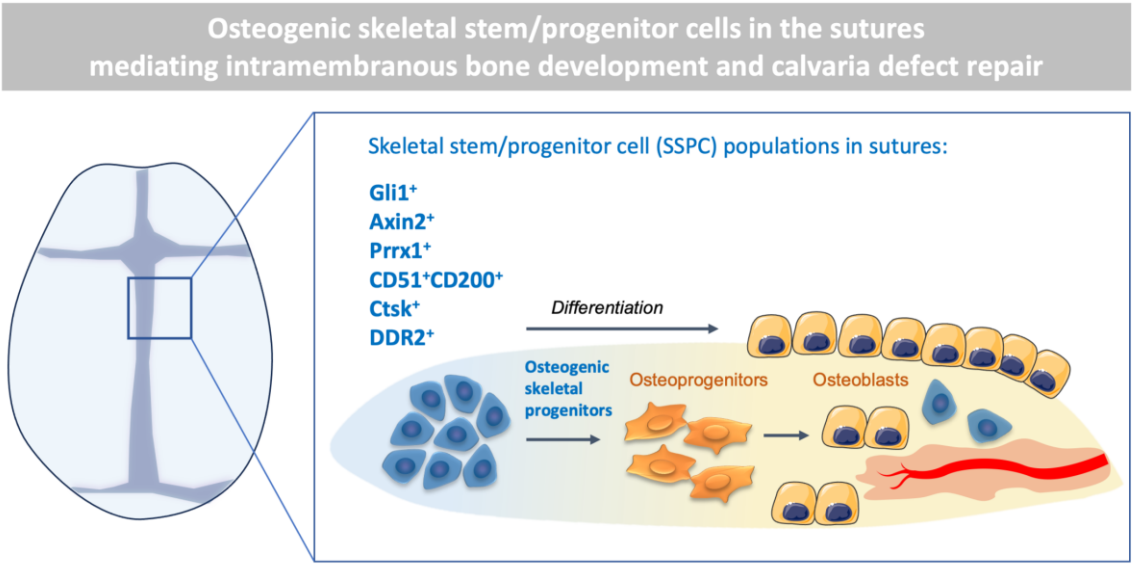
Christa Maes: Conceptualization, Supervision, Writing – original draft, Investigation, Writing – review & editing, Funding Acquisition, Resources, Project administration.

All authors discussed and revised the content and approved the final version. CM conceived and supervised the project.

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989 **FIGURE LEGENDS**



990

991 **Figure 1. Stem and progenitor cells for intramembranous bone formation in the sutures.**
992 In fetal skeletogenesis, flat bones are formed through intramembranous ossification. This
993 process starts with the condensation of mesenchymal cells at sites of future bone formation, and
994 their subsequent osteogenic differentiation without the formation of an intermediate cartilage
995 template. Elongation of the flat bones is driven by the proliferation and differentiation of
996 skeletal progenitors at the osteogenic front, and the bones are separated by unossified sutures,
997 which remain active sites of bone formation and constitute stem cell niches. Several skeletal
998 stem/progenitor cell (SSPC) populations for intramembranous bone formation and repair have
999 in recent years been identified in the sutures, based on expression of the indicated markers.

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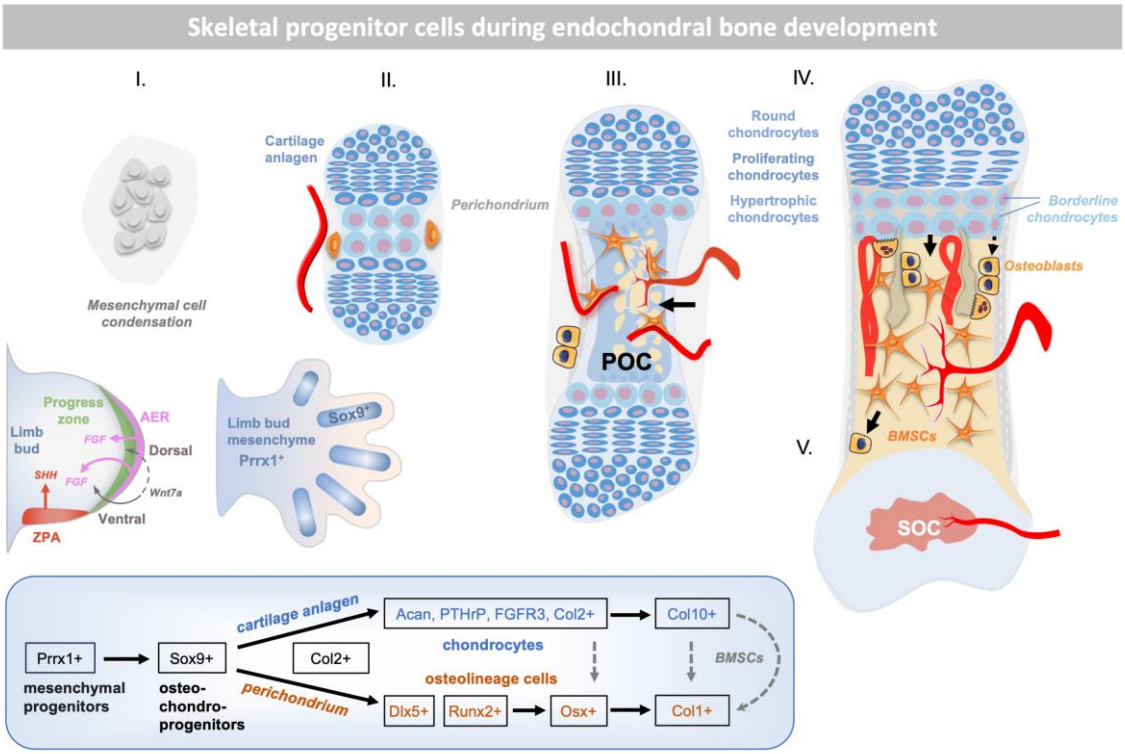


Figure 2. Fetal and early postnatal endochondral bone development is driven by transient sources of chondrogenic and osteogenic progenitor cells.

After early fetal limb bud arising and determination along the three main axes, long bone development starts with the condensation of Prrx1$^+$ mesenchymal progenitors within the limb bud, giving rise to Sox9-expressing osteo-chondroprogenitors (I). Cells in the center differentiate into Sox9$^+$ and Col2$^+$ chondrocytes, depositing the cartilaginous template for the future bone. Cells in the periphery (perichondrium) first remain undifferentiated and then become Runx2$^+$ and Osx$^+$ osteolineage cells (II). Primary ossification center (POC) formation next initiates by the co-invasion of the avascular cartilaginous template by blood vessels and perivascular Osx$^+$ osteoprogenitors, which give rise to reticular stromal cells and trabecular osteoblasts in the nascent bone marrow cavity (III). The POC separates the two remaining growth cartilage masses at both ends of the skeletal element, which are now called cartilaginous epiphyses or fetal growth plates. Growth plate chondrocytes can undergo apoptosis after hypertrophy or continue to contribute descendant cells to the pools of BMSCs and osteolineage

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cells in the metaphysis during perinatal and juvenile growth and further in adulthood (IV). After the formation of the secondary ossification center (SOC) (V) the growth cartilage obtains its typical 'plate'-like structure positioned between the SOC and the POC, and long-term postnatal cartilage SSPCs appear in the murine growth plate. LepR^{+} BMSCs become the main source of osteoblasts inside the bone shaft from post-adolescence (stage V is detailed in Figure 3).

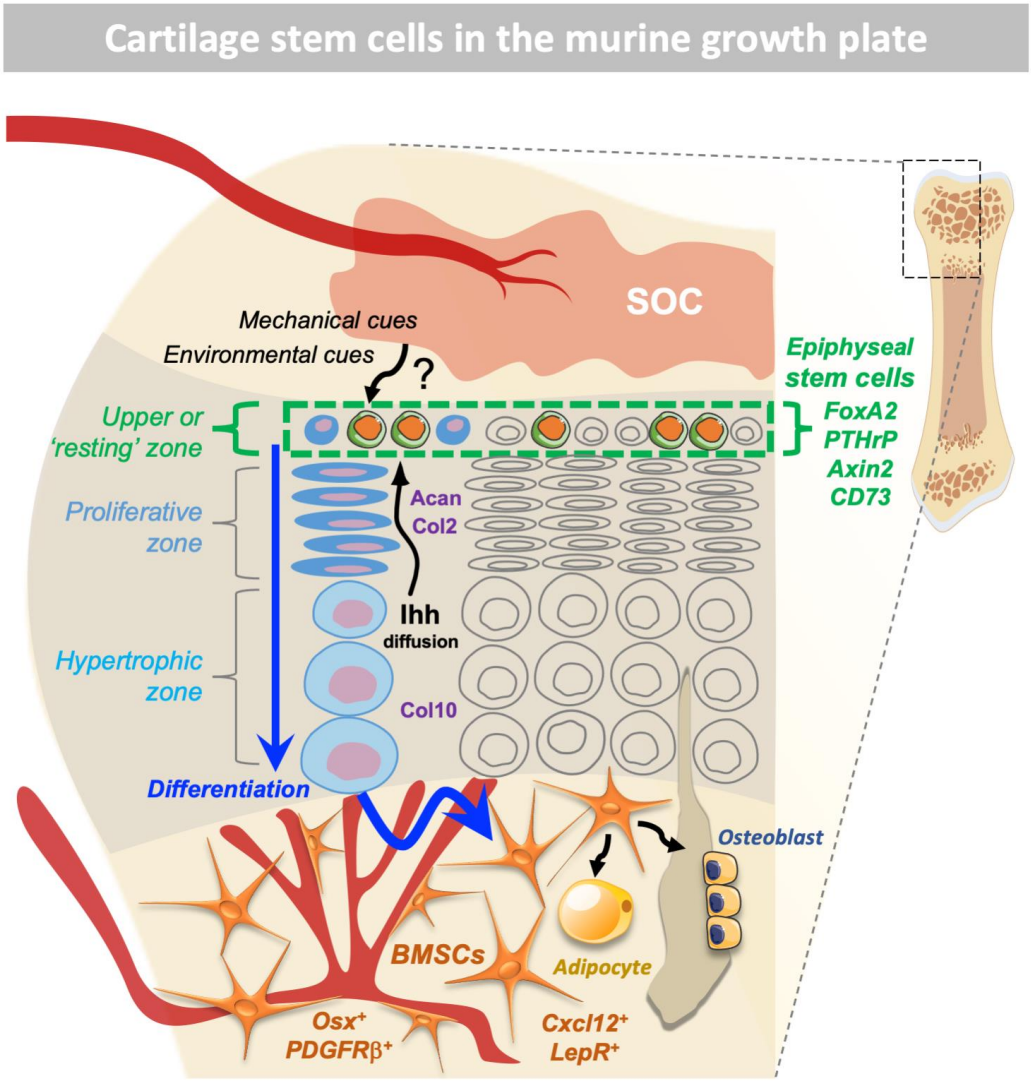


Figure 3. SSPCs in the post-adolescence murine growth plate.

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Epiphyseal stem cells reside in the upper resting zone of the growth plate, closest to the secondary ossification center (SOC). Populations of SSPCs marked by expression of FoxA2, PTHrP, Axin2, and/or CD73 have been identified in this cartilage region. They give rise to proliferative chondrocytes and hypertrophic chondrocytes, which either undergo apoptosis or further evolve into perivascular and reticular bone marrow stromal cells (BMSCs) (differentiation route marked with blue arrow). BMSCs can become osteoblasts and adipocytes in the metaphysis. Typical cartilage extracellular matrix components are indicated in purple.

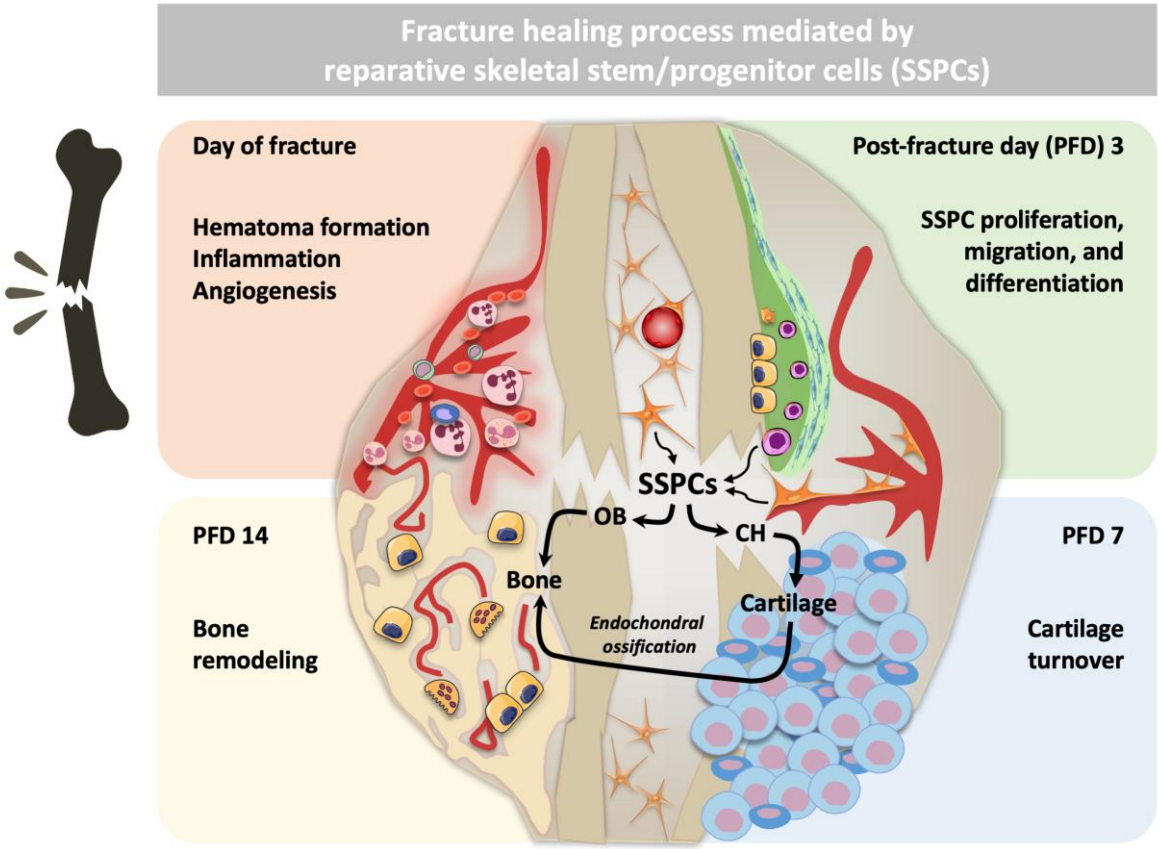


Figure 4. The endochondral bone fracture healing process mediated by reparative SSPCs.

Immediately following fracture of bone (upper left), hematoma formation and an inflammatory reaction is initiated. Tissue hypoxia due to vascular disruption recruits cells to induce neo-

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angiogenesis at the fracture site, and is accompanied by the release of growth factors and cytokines that initiate the activation, recruitment, and proliferation of reparative SSPCs in the periosteum and bone marrow (post-fracture day (PFD) 3, upper right). Prominent expansion of periosteal SSPCs results in locally thickened periosteal tissue layers (also see Figure 5). The resulting pool of reparative SSPCs bears the capacity to differentiate into chondrocytes (CH) or osteoblasts (OB), forming cartilage or bone, respectively. In endochondral fracture healing, a transient cartilaginous soft callus is initially formed (PFD7, lower right), which subsequently undergoes tissue turnover and replacement by woven bone through endochondral ossification (PFD14, lower left). Finally, osteoclasts and osteoblasts continue to remodel the callus, ultimately restoring the original shape of the bone cortex.

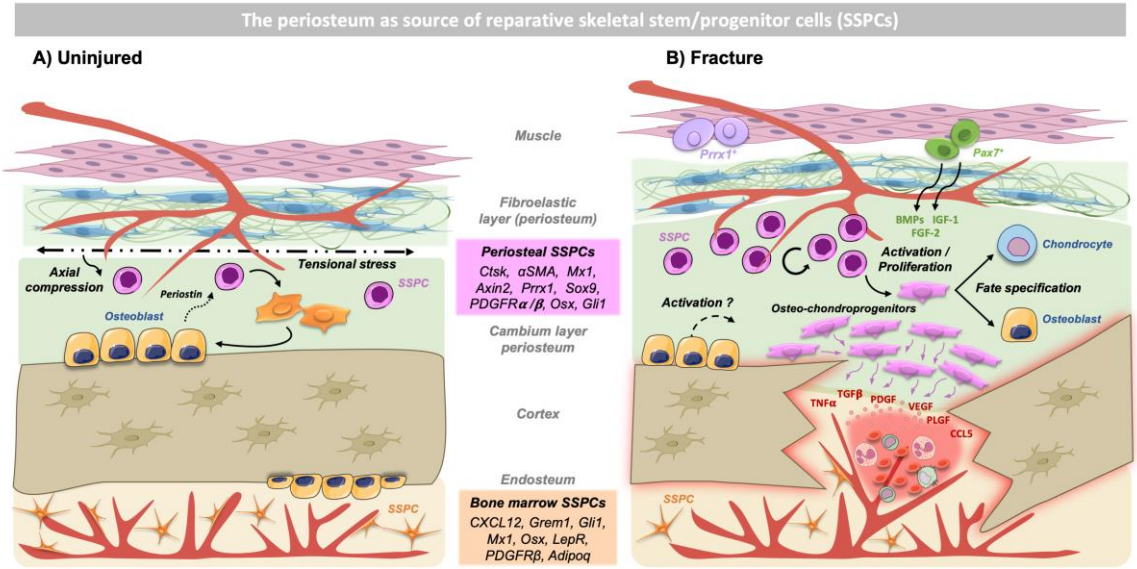


Figure 5. The periosteum as key source of reparative SSPCs.

The prime sources for SSPC mobilization to large bone fracture sites are the periosteum, a composite connective tissue lining the outer surface of the bone, and to a lesser degree subsets of BMSCs and local muscle-derived cells. In homeostatic settings (A), periosteal SSPCs are thought to maintain cortical bone by giving rise to osteoprogenitors and ultimately osteoblasts. Due to the fibroelastic layer and muscle, periosteal SSPCs receive mechanical cues that can

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influence their maintenance, activation, and differentiation. Upon fracture (B), the periosteum serves as crucial source of regenerative SSPCs, which become activated to vigorously proliferate, leading to pronounced periosteal thickening. Periosteal SSPCs establish pools of osteo-chondroprogenitors, that have the capacity to subsequently give rise to chondrocytes and osteoblasts in the healing fracture callus. The proliferation, migration, and differentiation of periosteal SSPCs and osteo-chondroprogenitors is driven by a myriad of signals, elicited at the fracture site from the early hematoma stage, including VEGF, PDGF, TGFβ, and many others. For further details, see text.

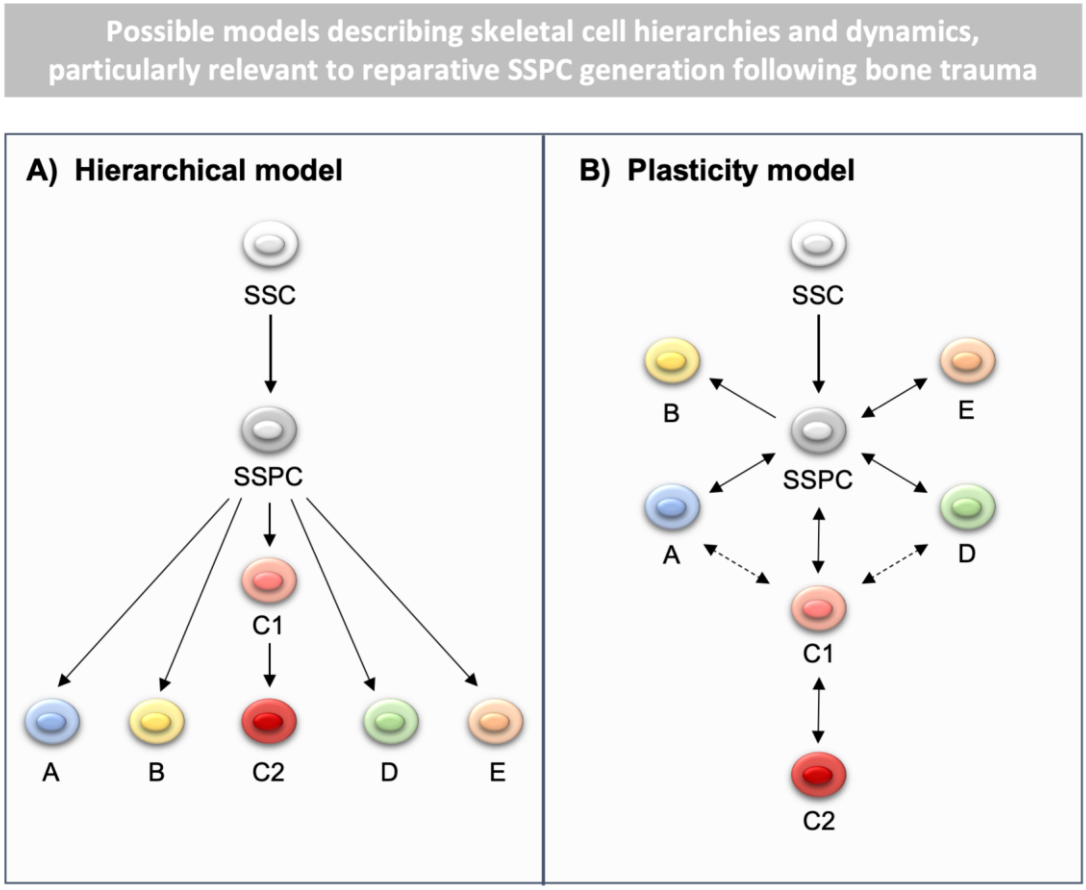


Figure 6. Hypothetical models describing possible relationships between skeletogenic cells in their cellular lineage specification and differentiation processes.

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(A) Traditionally, stem cell functioning is seen as organized according to a hierarchical tree with the most primitive, self-renewing and multipotent, stem cells at the apex, driving postnatal tissue maintenance and regeneration by leading the process according to unidirectional downstream branches towards the differentiated cell progeny. (B) Recent observations suggest that, at least upon bone or marrow injury or under stress, multiple cell populations can functionally act as osteogenesis-competent SSPC sources, and give rise to less differentiated and/or more differentiated skeletal cells. These findings raise the currently largely speculative idea that there might be considerable diversity, dynamism, flexibility, and possibly plasticity among the lineages in the skeleton, especially in settings of repair/regeneration and pathology.

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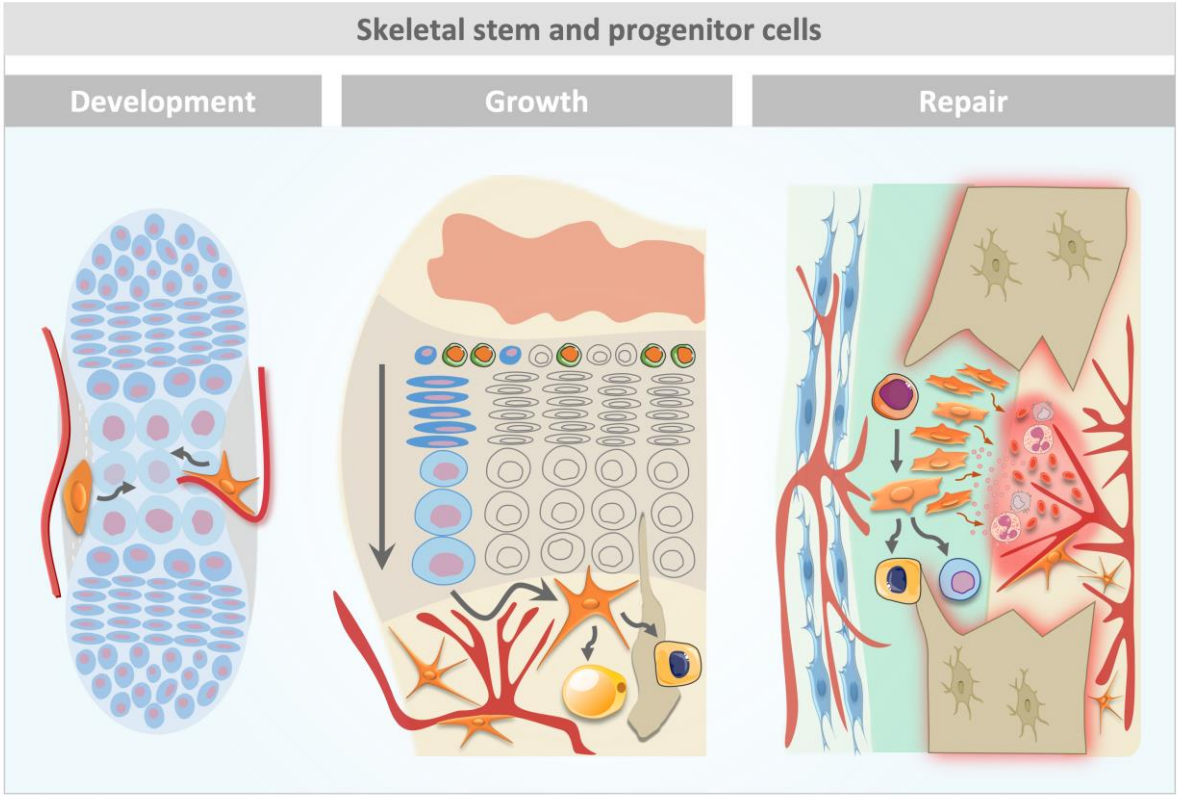
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