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REVIEW

Skeletal stem cells: insights into maintaining and regenerating the skeleton

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ABSTRACT

Skeletal stem cells (SSCs) generate the progenitors needed for growth, maintenance and repair of the skeleton. Historically, SSCs have been defined as bone marrow-derived cells with inconsistent characteristics. However, recent *in vivo* tracking experiments have revealed the presence of SSCs not only within the bone marrow but also within the periosteum and growth plate reserve zone. These studies show that SSCs are highly heterogeneous with regard to lineage potential. It has also been revealed that, during digit tip regeneration and in some non-mammalian vertebrates, the dedifferentiation of osteoblasts may contribute to skeletal regeneration. Here, we examine how these research findings have furthered our understanding of the diversity and plasticity of SSCs that mediate skeletal maintenance and repair.

KEY WORDS: Stem cells, Skeleton, Skeletal repair, Periosteum, Bone marrow, Growth plate

Introduction

Identifying the cells that maintain and repair the skeleton has been an area of intense recent investigation. A number of research groups have identified cell types that when engrafted give rise to new skeletal tissue, as well as specific tissue-resident cells with varying multilineage potential that participate in skeletal homeostasis and repair. It has also been shown that, in some non-mammalian vertebrates that are capable of large-scale skeletal regeneration, osteoblasts may dedifferentiate into a progenitor state to replace missing bone. Many of these cell populations come under the umbrella of skeletal stem cells (SSCs) – a population of cells that can self-renew and generate osteoblasts, chondrocytes, adipocytes and stroma – although only a few of these populations have been rigorously tested.

SSCs from the bone marrow were initially defined by their ability to adhere to tissue culture plastic, clonally expand, differentiate into multiple cell types *in vitro*, and generate skeletal tissue upon subcutaneous transplantation into mice (Friedenstein et al., 1966; Tavassoli and Crosby, 1968). Later, fluorescence-activated cell sorting was used to isolate subpopulations of marrow cells based on combinations of cell surface markers (Bianco and Robey, 2015). Recently, genetic Cre-mediated lineage-tracing experiments, primarily within the postnatal mouse, have revealed diverse populations of cells with SSC properties. These putative SSCs are located not only within the bone marrow but also within the periosteum (the connective tissue surrounding bone) and in the resting zone of the growth plate (a cartilaginous structure separating the primary and secondary ossification centers in growing bones) (Fig. 1). These populations vary in their lineage capabilities, their prevalence in embryonic through adult stages, and their participation in repair, highlighting that there are likely multiple types of SSCs.

Several attempts to define SSCs have taken inspiration from studies of the hematopoietic system, where a rare population of apex stem cells gives rise to lineage-committed intermediate progenitors and eventually all blood cell types (Becker et al., 1963). Recent investigations suggest that a similar hierarchical system may exist for SSCs (Chan et al., 2015, 2018), yet other reports suggest that the skeletal system is surprisingly plastic, more akin to the intestinal system where differentiated cells can adopt a stem cell state when normal stem cells are ablated (Choi et al., 2018). For example, osteoblasts have been observed to dedifferentiate into a progenitor state during regeneration of the fin and skull bones of zebrafish (Geurtzen et al., 2014; Knopf et al., 2011; Stewart and Stankunas, 2012) (Box 1), as well as during murine digit tip regeneration (Storer et al., 2020).

Although strategies to identify prospective SSCs are rapidly improving, current techniques suffer from limitations in their interpretation. Cells can change their properties after tissue isolation and prolonged culture, and thus multilineage differentiation and self-renewal after transplantation does not necessarily mean that the cells originally isolated had these stem properties. Methods to isolate cells can also vary from one laboratory to another, making it challenging to make direct comparisons. As an alternative, researchers have used tissue-specific expression of Cre recombinase to induce DNA recombination and drive permanent expression of fluorescent proteins in Cre-expressing cells and all their descendants. However, determining the precise temporal and spatial expression of Cre activity can be challenging. The regulatory sequences that drive Cre expression can be active at multiple stages of development and/or activated in response to injury. To obtain tighter control, inducible systems can be used to produce a pulse of Cre activity at a defined time, typically relying on fusions of Cre to the tamoxifen-dependent estrogen receptor (CreER). However, non-tamoxifen-treated controls are essential to ensure the absence of 'leaky' activation of CreER, which can create confusion when interpreting results (see Song and Palmiter, 2018 for guidelines on proper Cre/Lox experiments). Even when tight temporal control is achieved, CreER may still be expressed in more than one cell type. In addition, many CreER lines used in skeletal research target regulators of major signaling pathways, such as WNT (*Axin2*), BMP (*Grem1*) and HH (*Gli1*); given the widespread activity of these pathways in non-skeletal tissues, such CreER lines are likely not specific to a single cell population. Fortunately, emerging technologies, such as single cell RNA sequencing and cellular barcoding, should allow creation of a complete catalog and lineage tree of cell types in the skeletal system.

Here, we review and critique recent studies that have identified various populations of SSCs within vertebrates. We highlight how a

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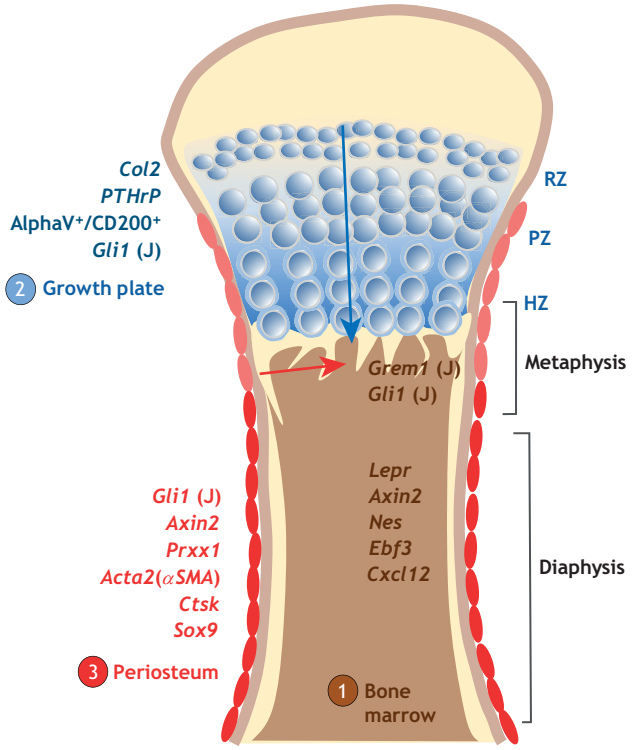

Fig. 1. Skeletal stem cell populations and niches. Several populations of skeletal stem cells (SSCs) have been identified to date. (1) SSCs can be identified in the marrow cavity (brown) with some populations being enriched in the metaphysis region, particularly at early postnatal or juvenile stages. These populations can be identified using various Cre lines for the genes indicated (with 'J' indicating labeling at juvenile stages). (2) SSCs can also be found in the resting zone (RZ) region of the growth plate (blue), expressing the genes indicated. These cells contribute to more lineages than just cartilage. In a growing bone, the chondrocytes of the growth plate proliferate (in the proliferation zone, PZ) and become larger and hypertrophic (within the hypertrophic zone, HZ) near the juncture with the marrow cavity. Some of these cells do not undergo apoptosis but are ejected from the growth plate into the marrow cavity (represented by blue arrow) where they contribute to osteoblasts, adipocytes, other marrow cells, and potentially marrow SSCs. (3) The periosteum (indicated in dark red) is also known to contain SSCs (marked by expression of the genes indicated) involved in homeostasis and repair. During development, progenitor cells within the perichondrium (light red) translocate into the marrow (represented by red arrow) during initial vascularization of the bone.

major challenge that remains is to understand the lineage relationships between diverse SSCs, as well as the specialized functions of SSCs in homeostasis and repair. We also discuss the role of niche factors in specifying and maintaining SSCs and examine how stem cell plasticity may underlie the degree of skeletal repair in different contexts and organisms. Such knowledge will allow the field to develop improved targeted cellular therapies that enhance skeletal repair in the clinic.

SSCs in bone development and homeostasis

Bone marrow-derived SSCs

The bone marrow is a common site for the extraction of 'mesenchymal stem cells' (MSCs), a highly heterogeneous mixture of cells, only a fraction of which may have stem properties. Unfortunately, these cells are being tested for treatment of a wide range of conditions, often in non-scientific, exploitative, and potentially dangerous ways (Baker, 2005; Fitzsimmons et al., 2018; Sipp et al., 2018). 'Mesenchyme' is a broad term referring to cells in the embryo with a connective tissue morphology and broad differentiation capacity, as opposed to the more specialized connective tissue in the postnatal animal. In the bone marrow, connective tissue has a prominent supportive or 'stromal' function for hematopoiesis and bone formation, and hence the connective tissue cells within bones are often referred to as bone marrow stromal cells (BMSCs), a more accurate term than 'MSCs'. Initial attempts to specifically isolate SSCs within the broader BMSC population in both mice and humans have involved sorting cells based on the presence of a few cell surface makers [e.g. CD146 (also known as MCAM) and PDGFRα] and the absence of markers of hematopoietic (e.g. CD45; also known as PTPRC) and endothelial (e.g. CD31; also known as PECAM1) lineages. The function of these populations is then often assessed by their ability to differentiate into osteoblasts and other skeletal cell types *in vitro*, as well as *in vivo* upon transplantation under the skin, intravenously, or into sites invested with substantial vasculature such as the kidney capsule (Chan et al., 2015; Morikawa et al., 2009; Park et al., 2012; Sacchetti et al., 2007).

However, cell properties can change when cells are isolated and placed *in vitro*, for example due to selection biases during cell sorting or in response to the non-physiological conditions of cell culture. In addition, the sorting procedure can often kill subpopulations of cells. Hence, complementary efforts have focused on defining endogenous SSC populations within skeletal tissues *in vivo*. To do so, fate mapping using Cre-mediated DNA recombination is often employed. In this way, a number of markers of putative bone marrow SSCs have been identified in mice (summarized in Tables 1 and 2). One such marker is the Bmp antagonist gremlin 1 (Grem1) (Worthley et al., 2015). *Grem1*$^{+}$ cells are located in the growth plate and marrow of long bones, primarily in the metaphysis (i.e. the region just below the growth plate), and do not express markers of perivascular stromal cells such as *Nes* and *Cxcl12*. Using a conditional *Grem1-CreER* transgenic line, it was

Box 1. Osteoblast dedifferentiation during zebrafish bone regeneration

Several lines of evidence indicate that, in response to injury, osteoblasts in the adult zebrafish fin and skull can revert to a progenitor state to generate new osteoblasts. For example, following amputation of the bony fin skeleton or drill lesions of the calvarial bone, osteoblasts downregulate expression of the mature osteoblast marker *bglap* (also known as *osteocalcin*), upregulate expression of the osteoprogenitor gene *runx2b* and the connective tissue marker Tenascin, re-enter the cell cycle, and produce new osteoblasts (Geurtzen et al., 2014; Knopf et al., 2011). Moreover, lineage tracing with an *sp7(osterix)*-CreER transgene has shown substantial contributions of pre-existing osteoblasts to new osteoblasts in the fin regenerate (Knopf et al., 2011; Singh et al., 2012). In order to rule out *sp7(osterix)*-CreER activity outside of osteoblasts (Ono et al., 2014a; Zhang and Link, 2016), Knopf and colleagues employed time-lapse imaging of CreER-converted cells, as well as osteoblasts in which the *entpdf5*:Kaede transgene is photoconverted from green to red. In both cases, osteoblast-derived cells are observed to migrate into the blastema, consistent with their later differentiation into new osteoblasts at a distance (Geurtzen et al., 2014; Knopf et al., 2011). However, even when pre-existing osteoblasts are ablated, the fin bone still regenerates, suggesting that an additional reserve pool of progenitors may also contribute to bone regeneration (Singh et al., 2012). Good candidates for such a reserve population are *mmp9*$^{+}$ cells, as these are found in uninjured fin ray joints and contribute to new osteoblasts after fin amputation (Ando et al., 2017). In the future, it will be important to understand the mechanisms by which osteoblasts dedifferentiate in response to injury in fish, and whether similar mechanisms operate in mammals, such as during digit tip regeneration (Storer et al., 2020).

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Table 1. Comparative summary of reported skeletal stem cells

Cre	Location	Cre conversion	Contributions	Requirement	References
Acta2(αSMA)-CreER	PO, BM	Tam at 3-5 m, day before and after fracture	Tibia callus (C, B)	ND	Matthews et al., 2014
Axin2-CreER	PC	Tam at P6, chase to P9	Tibia (C)	*Axin2-CreER*;β-cat$^{fl/fl}$: ectopic cartilage near PC (P13)	Usami et al., 2019
	PO, BM	Tam at 8 w, 1 w or 3 m before injury	Tibia callus (C, B)	DTR ablation; reduced cartilage callus	Ransom et al., 2016
Axin2-CreDox	Su	Dox at P25-P28, chase for 1 m, 3 m and 1 year	Calvarium (B)	ND	Maruyama et al., 2016
	Su	Dox at P25-P28, 2 days before injury	Calvarial injury (B)	ND	
Col2a1a-CreER	GP	Tam at perinatal and juvenile stages	Tibia (C), humerus (C)	ND	Newton et al., 2019
	GP	Tam at P3, chase for 1 m (B, S) or 2 m (A)	Femur (C, B, A, S)	ND	Ono et al., 2014b
Ctsk-Cre	PO	Constitutive	Kidney capsule transplant (B)	*Ctsk-Cre;Osx$^{fl/fl}$*; reduced mineralization of calvarium and femur	Debnath et al., 2018
	PO	Constitutive	Femur callus (C, B)	*Ctsk-Cre;Osx$^{fl/fl}$*; reduced repair and bone volume	
	PC	Constitutive	PC at P7	*Ctsk-Cre;Ptpn11$^{fl/fl}$*; dwarfism, scoliosis, metachondromatosis	Yang et al., 2013
Cxcl12-CreER	BM	Tam at P3 and P21, chase to 1 year	Femur (S)	ND	Matsushita et al., 2020
	BM	Tam at 8 w, chase to 1 year	Femur (B-trabecular, A, S)	ND	
	BM	Tam at 4-9 w, 7 days before injury	Femur callus (C, B-cortical, S)	*Cxcl12-CreER;Ctnnb$^{fl/fl}$* or DTA ablation; reduced repair and bone volume	
Ebf3-CreER	BM	Tam at 10 w, chase for 13 m	Femur (B, A, S)	ND	Seike et al., 2018
Gli1-CreER	BM	Tam at E13.5, chase for 2 m	Femur (C, B, A, S), calvarium (B)	DTA ablation; reduced cancellous bone	Shi et al., 2017
	BM	Tam at 1 m, fracture at 10 w	Femur callus (C, B)	ND	
	Su	Tam at 1 m, chase for 1-8 m	Calvarium (B)	DTA ablation; suture fusion after 2 m	Zhao et al., 2015
	Su	Tam at 1 m, 5 days before injury	Calvarial injury (B)	DTA ablation; reduced bone repair	
Grem1-CreER	BM	Tam at P1, chase for 6 w	Femur (C, B, S)	DTA ablation; reduced body size, reduced bone volume	Worthley et al., 2015
	BM	Tam at >8 w, 1 w before fracture	Femur callus (C, B)	ND	
Lepr-Cre	BM	Constitutive	Femur (B, A, S)	DTR ablation; increased bone and fat	Zhou et al., 2014a
	BM	Constitutive	Tibia callus (C, B)	ND	
Mx1-Cre	BM	pIpC at 6-8 w, chase for 20 days	Femur (B), calvarium (B)	ND	Park et al., 2012
	BM	pIpC, time unknown	Femur callus (B), calvarial injury (B)	ND	
	PO	pIpC at 4-6 w, chase for 2 m	Tibia (B, S)	ND	Ortinau et al., 2019
	PO	pIpC and DT before injury	Tibia callus (C, B), calvarial injury (B)	DTR ablation; reduced bone repair	
Nestin-CreER	BM	Tam at 3 m, chase for 8 m	Femur (C, B, S)	DTR ablation; HSCs affected, not bone	Mendez-Ferrer et al., 2010
Prrx1-CreER	PO, GP	Tam at E9, E15.5-E16.5, or P19-P23 with chase to E17, E18.5 or P26	Radius, ulna, tibia (C, B)	ND	Kawanami et al., 2009
	PO, GP	Tam at P52 and P53, fracture at P49	Ulna callus (C, B), femur callus (C, B)	ND	
	Su	Tam at P7 or P28	ND	DTA ablation; reduced femur/tibia length	
	Su	Tam at 8 w, 5 days before and after injury	Calvarial injury	DTA ablation; reduced repair	
Prrx1-Cre	Su	Constitutive	Calvarium (B)	DTA ablation; no calvarial or limb development	Wilk et al., 2017
	Su	Constitutive	Calvarial injury (B)	ND	
PTHrP-CreER	GP	Tam at P6, chase to P12-P36	Femur (C, B)	DTA ablation; increased GP hypertrophic zone	Mizuhashi et al., 2019
Sox9-CreER	PO	Tam at 12-16 w, chase for 14 days	Femur (C, B)	ND	He et al., 2017
	PO	Tam at 12-16 w, 2 w before injury	Femur callus (C, B), rib callus (C, B)	*Sox9-CreER;Smo$^{fl/fl}$*; reduced callus	He et al., 2017; Kuwahara et al., 2019
	PO, GP, BM	Tam at P3, chase to P30 and P60	Tibia (C, B, A, S)	*Sox9-CreER;PTH1R$^{fl/fl}$*; reduced Sox9-derived osteoblasts	Balani et al., 2017
Sox9-Cre	PO, GP	Constitutive	Entire limb (C, B), E10.5-E17	*Sox9-Cre;Osx $^{fl/fl}$*; no bone mineralization	Akiyama et al., 2005

Alternate shading indicates different lineages. A, adipocyte; B, bone; BM, bone marrow; C, cartilage; Dox, doxycycline; DTA, diphtheria toxin; DTR, diphtheria toxin receptor; GP, growth plate; m, months; ND, not determined; PC, perichondrium; pIpC, polyinosinic:polycytidylic acid; PO, periosteum; S, stromal cell; Su, suture; Tam, tamoxifen; w, weeks.

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shown that *Grem1*$^+$ cells marked at postnatal day (P) 1 give rise to growth plate chondrocytes, osteoblasts, marrow stromal cells and periosteal connective tissues by 1 month of age. Furthermore, diphtheria toxin-mediated ablation of *Grem1*$^+$ cells at P9 results in decreased bone formation by P23. *Grem1*-mediated recombination at 1 month also labels stromal and periosteal cells in the femur at 1 year, suggesting long-term self-renewal. Together, these data argue that *Grem1*$^+$ cells are a type of SSC, given their multilineage differentiation capacity *in vivo*, their requirements for postnatal bone formation, and their capacity for long-term self-renewal.

In contrast to *Grem1*$^+$ cells, cells expressing leptin receptor (LepR) and traced with *Lepr-Cre* exhibit SSC properties at later postnatal stages (Zhou et al., 2014a). *Lepr*$^+$ cells also differ from *Grem1*$^+$ cells in that they are concentrated near blood vessels (i.e. they are perivascular) and they are able to generate marrow adipocytes. Indeed, *Lepr*$^+$ cells contribute to ∼75% of marrow adipocytes at 2 months of age and ∼95% by 14 months, and can give rise to the majority of colony-forming unit-fibroblasts (CFU-Fs), an *in vitro* measure of stem cell activity. The contribution of *Lepr*$^+$ cells to osteoblasts is minimal until about 6 months, at which point they constitute ∼20% of osteoblasts in the tibia, and by 14 months ∼90% of osteocytes in the femur. One caveat to these studies is that endogenous *Lepr* is abundantly expressed in chondrocytes, in contrast to the *Lepr-Cre* transgene, which appears to be more specific for marrow cells, perhaps due to the insertion of Cre in a specific Ob-Rb splice form of the *Lepr* transcript (Giovannone et al., 2019; Zhao et al., 2009; Zhou et al., 2014a). *Lepr*$^+$ cells can also be found in the periosteum (Gao et al., 2019), and recent single cell analyses of bone marrow have revealed distinct subpopulations of *Lepr*$^+$ cells that exhibit differential lineage biases (Baryawno et al., 2019; Tikhonova et al., 2019). These findings indicate that *Lepr-Cre* marks a broad and heterogeneous population of BMSCs, of which only a subset likely represent true SSCs. In summary, *Grem1* and the *Lepr* Ob-Rb splice form appear to mark distinct early and late populations of SSCs, respectively, with their timing of emergence perhaps underlying their different lineage potentials.

It should be noted that BMSCs fulfill a dual role in the marrow, acting not only as a source of skeletal lineage cells but also supporting hematopoiesis (Greenbaum et al., 2013). In this context, many other surface marker combinations and transgenic mouse lines have been used to label BMSCs. For example, a *Nes-GFP* transgene marks BMSCs that have been proposed to serve as osteoblast precursors (Méndez-Ferrer et al., 2010), although a separate group found little contribution of *Nes*$^+$ cells to osteoblasts during early mouse postnatal life (Worthley et al., 2015). *Mx1-Cre* also labels a population of BMSCs with osteogenic, but not adipogenic or chondrogenic, potential *in vivo* (Park et al., 2012). Recently, *Ebf3-CreER* was shown to mark a subset of *Lepr*$^+$ BMSCs that express *Cxcl12* and are self-renewing. When *Ebf3* is deleted in *Lepr*$^+$ stromal cells (using *Lepr-Cre*), cells lose their HSC-supportive stromal function and prematurely differentiate into osteoblasts, suggesting that *Ebf3* functions to maintain an immature 'stromal' phenotype in a subset of *Lepr*$^+$ BMSCs (Seike et al., 2018). Further, a recent study specifically tracked the perisinusoidal Cxcl12-expressing cells using a *Cxcl12-CreER* mouse line, and saw that, although normally quiescent, these cells participated in repair after a drill hole injury or fracture (Matsushita et al., 2020). The timing, contributions and requirements of these and other BMSCs marked by various genes are summarized in Tables 1 and 2.

Growth plate SSCs

Longitudinal growth of long bones is accomplished by the growth plates, in which slow-cycling cells (which form a 'resting zone') give rise to columns of proliferating chondroblasts (within a 'proliferative zone'), which then mature into hypertrophic chondrocytes (in a 'hypertrophic zone'). At the limit of the hypertrophic zone, the growth plate cartilage is eroded and replaced by bone and marrow tissues via the process of ossification (Box 2). Marrow osteoblasts are derived in part from progenitors from the perichondrium (the fibrous layer surrounding the cartilage template) that migrate into the marrow space along with the newly forming vasculature (Maes et al., 2010). It is also now recognized that hypertrophic chondrocytes are another significant source of osteoblasts in the postnatal animal. In mice (Bianco et al., 1998; Jing et al., 2015; Mizuhashi et al., 2018; Park et al., 2015; Roach, 1992; Yang et al., 2014; Zhou et al., 2014b) and zebrafish (Giovannone et al., 2019), histological analyses and lineage tracing show that a proportion of hypertrophic chondrocytes escape cell death and differentiate into osteoblasts and potentially also marrow adipocytes. Hypertrophic chondrocytes also re-enter the cell cycle in both mouse (Park et al., 2015) and zebrafish (Giovannone et al., 2019), and in zebrafish they also express *lepr*. As *lepr* is broadly expressed outside of putative SSCs, including in chondrocytes of mouse and zebrafish (Giovannone et al., 2019), whether hypertrophic chondrocytes acquire progenitor characteristics needs to be tested more thoroughly. Recent studies on growth plate chondrocytes have therefore been aimed at defining the stem cells within the resting zone that fuel continued growth plate expansion, as well as the potential transformation of hypertrophic chondrocytes into SSCs that will later reside in the marrow cavity.

Labeling of resting growth plate chondrocytes with *Col2a1-CreER* and a multicolor fluorescent reporter has recently revealed that columns of chondrocytes shift from being multiclonal in embryos and neonates to being monoclonal at postnatal stages (Newton et al., 2019). At postnatal stages, mTORC1 signaling was found to fine-tune chondrocyte cell divisions to achieve the correct balance of asymmetric divisions that optimally maintain chondrocyte stem cells in the resting zone (Newton et al., 2019). A separate study used *PTHrP-CreER* to label a subset of resting zone chondrocytes and found that they are a major source of chondrocytes for several months after labeling (Mizuhashi et al., 2019). In agreement with this observation, diphtheria-toxin-mediated ablation of *PTHrP*$^+$ cells disrupts bone elongation. In addition,

Table 2. Skeletal stem cells isolated using FACS

Marker	Location	Analysis	Contributions	Species	References
Itgav$^+$, CD200$^+$	GP	Sorted, transplanted into mouse kidney capsule	Ectopic (C, B, S)	Mouse	Chan et al., 2015
CD164$^+$, PDPN$^+$, CD73$^+$	GP	Sorted, transplanted into mouse kidney capsule	Ectopic (C, B, S)	Human	Chan et al., 2018
Sca-1$^+$, PDGFR-α$^+$	BM	Sorted, transplanted into mouse, intravascular	Differentiated (B, A, S)	Mouse	Morikawa et al., 2009
CD146	BM	Sorted, transplanted into mouse, subcutaneous	Ectopic (B, S)	Human	Sacchetti et al., 2007

A, adipocyte; B, bone; BM, bone marrow; C, cartilage; GP, growth plate; S, stromal cell.

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Box 2. Endochondral versus direct ossification

Bone development occurs via two main pathways: endochondral ossification or direct (or intramembranous) ossification. Endochondral ossification involves the formation of a transient cartilage template in which skeletal progenitor cells condense, differentiate into chondrocytes, and then progress through hypertrophy. Apoptosis of hypertrophic chondrocytes is thought to create a marrow cavity, with osteoprogenitors from the perichondrium then migrating into this cavity along with the vasculature. However, recent studies have shown that hypertrophic chondrocytes are also a source of marrow osteoblasts and stromal cells (Jing et al., 2015; Mizuhashi et al., 2018; Park et al., 2015; Yang et al., 2014; Zhou et al., 2014b), although the percentage of marrow cells deriving from hypertrophic chondrocytes likely differs depending on bone type. During the process of direct ossification, skeletal progenitors proliferate, condense, and differentiate directly into osteoblasts without a cartilage template. In mammals, most of the appendicular, spine and thoracic skeleton forms via an endochondral pathway, whereas most of the skullcap and facial skeleton form through direct ossification.

During repair, bone also forms through endochondral or direct ossification. In some contexts (e.g. small injuries), repair appears to occur through direct ossification. Larger injuries with more soft tissue trauma correlate with the formation of a cartilage callus – a healing tissue that forms in response to injury. Although this cartilage callus may simply provide a supportive role before sufficient ossification has occurred, and may even help align the fracture (Rot et al., 2014), some of the callus cells appear to have hybrid cartilage/bone osteochondral properties. These hybrid cells then mature into bone-producing osteoblasts, therefore actively participating in building new bone tissue (Kuwahara et al., 2019; Paul et al., 2016).

PTHrP-CreER-labeled cells were shown to contribute to *Col1a1*$^{+}$ osteoblasts and *Cxcl12*$^{+}$ stromal cells in the marrow, consistent with the eventual transition of resting zone cells into the hypertrophic chondrocytes that transdifferentiate into osteoblasts and stromal cells. However, it cannot be ruled out that some or all *PTHrP-CreER*-labeled cells bypass the hypertrophic state, as non-hypertrophic chondrocytes closest to the bone collar (‘borderline chondrocytes’) may selectively undergo transdifferentiation (Bianco et al., 1998; Mizuhashi et al., 2019; Roach, 1992). A salient feature of monoclonal *Col2a1*$^{+}$ and *PTHrP*$^{+}$ cells is that they do not appear in the resting zone until after birth (approximately P6). In addition, induction of *Col2a1-CreER* at P3 reveals a contribution of the marked cells to marrow adipocytes in 1-year-old animals (Ono et al., 2014b), whereas induction of *PTHrP-CreER* at P6 and *Col2a1-CreER* at P28 does not label adipocytes at any time point assayed (Mizuhashi et al., 2018; Newton et al., 2019). The labeling of adipocytes by early *Col2a1-CreER* induction may be attributed to activity of *Col2a1-CreER* outside the growth plate, such as in osteochondroprogenitors. Overall, these findings suggest that embryonic growth plates are fueled by a distinct chondroprogenitor pool, with the onset of *PTHrP* expression coincident with the acquisition of self-renewal ability within the postnatal resting zone. Thus, resting zone SSCs do not appear to be simply remnants of a developmental growth plate population.

Whereas the above studies focused on the *in vivo* potential of resting zone cells, a separate study used a panel of surface markers [CD45^{-}/TER119 (LY76)$^{-}$/Tie2 (Tek)$^{-}$/Thy^{-}/6C3 (Enpep)$^{-}$/CD105 (Eng)$^{-}$/AlphaV (Itgav)$^{+}$/CD200^{+}] to purify cells from the growth plate of murine bones (Chan et al., 2015). Isolated cells could be serially passaged and induced to differentiate into bone, cartilage, and stromal cells upon transplantation into the kidney capsule of recipient mice. Moreover, self-renewing cells could be extracted from these ectopic ossicles and used for serial transplantation through several rounds of recipient mice, while maintaining their multilineage differentiation capacity. The same group subsequently reported that an analogous SSC could be isolated from the fetal and adult growth plates of human bones, although a different set of surface markers was utilized compared with the mouse study (Chan et al., 2018) (Table 2). Although these studies did not pinpoint where within the growth plate these proposed SSCs might be located, it is possible that they correspond to cells similar to the *Col2a1*$^{+}$/*PTHrP*$^{+}$ monoclonal resting zone cells found *in vivo* (Mizuhashi et al., 2018; Newton et al., 2019), although transcriptomic analysis did not reveal significant expression of *PTHrP*. A mystery is how these purified cartilage cells are able to acquire self-renewal, osteoblast and adipocyte differentiation capacity in culture. Might this reflect that chondrocytes can normally transdifferentiate into osteoblasts and stromal cells within the bone marrow? Or might this reflect selective sorting of rare, non-chondrocyte cells or the induction of cell plasticity upon culture? Of note, it has long been appreciated that chondrocytes can readily adopt a mesenchymal state upon *in vitro* culture (Holtzer et al., 1960), and hence the culture conditions could in theory apply selective pressure for self-renewal and multilineage differentiation capacity. It will thus be important to understand how these isolated cells, which show clear SSC characteristics upon serial transplantation, relate to endogenous SSCs within the growth plate.

Periosteal SSCs

The periosteum is a complex tissue that lines the outer surface of bones and is composed of fibroblasts, blood vessels, nerves and, particularly in the inner layer, osteoprogenitors. Whereas the growth plate plays a major role in longitudinal bone extension, cells in the periosteum contribute to bone thickening and cortical maintenance during development and homeostasis (Allen et al., 2004). The importance of the periosteum for bone growth and repair has been appreciated for over a century, yet the identity and regulation of periosteal progenitor cells are just beginning to be unraveled (Chang and Knothe Tate, 2012; Colnot, 2009; Murao et al., 2013; Roberts et al., 2015; Salazar et al., 2019; Wang et al., 2017).

During development, the periosteum arises from the perichondrium as mesenchymal cells become primed to generate osteoblasts. *Gli1* is a transcriptional target and effector of the Hedgehog (Hh) signaling pathway, which has well-known roles in bone and cartilage development. Several groups have therefore used *Gli1*-based transgenes to mark cells with skeletogenic potential. *Gli1-CreER* induction at embryonic day (E) 13.5 in mice results in labeling primarily in the perichondrium, with tracing of these cells until 2 months of age showing major contributions to cortical and trabecular osteoblasts, bone marrow adipocytes, and bone marrow stromal cells of the femur. Notably, ∼75% of Gli1-labeled cells in the marrow (traced from E13.5 until 2 months old) express *Lepr*, suggesting that *Gli1*-expressing cells in the embryo give rise to most *Lepr*$^{+}$ SSCs in the postnatal marrow (Shi et al., 2017). However, *Gli1-CreER*-labeled cells are also found within the growth plates, making it unclear whether lineage-traced *Lepr*$^{+}$ cells originate from periosteal progenitors, growth plate chondrocytes, or some other *Gli1*$^{+}$ source. Induction of *Gli1-CreER* at 4 months highlights a small contribution of marked cells to the periosteum but decreased contribution to osteoblasts and marrow cells, whereas induction at 12 months results in little to no labeling within the femur. Thus, *Gli1-CreER*-labeled cells are similar to *Grem1*$^{+}$ SSCs in being a transient SSC population supplying cells for juvenile growth but less so for long-term bone homeostasis. As *Lepr*$^{+}$ SSCs increase their contributions through adulthood, it may be that those *Lepr*$^{+}$

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SSCs not derived from embryonic *Gli1*-expressing cells have a preferential role in adult bone homeostasis. In addition, it has been shown that *Gli1* marks mesenchymal cells within postnatal cranial sutures, which separate intramembranous bones of the skull and face (see Box 2), with *Gli1-CreER*-labeled cells contributing to and being required for growth and repair of skull bones (Zhao et al., 2015). Similarly, *Gli1-CreER*-labeled cells act as long-lived stem cells for the continuous growth of the mouse incisor (Zhao et al., 2014). Thus, Hh activity, and in particular *Gli1* expression, may generally mark stem cells within a range of skeletal tissues, including the developing perichondrium, the growth plate, the metaphysis bone marrow compartment, and the periosteum. This broad contribution is similarly observed using *Axin2-CreER*, suggesting that SSCs may also be Wnt responsive, though it is unclear whether these two transgenes mark the same populations (Maruyama et al., 2016; Ransom et al., 2016; Usami et al., 2019).

A recent study using a constitutive *Prrx1-Cre* line, which broadly labels mesenchymal cells in the limbs and elsewhere, has provided more evidence that self-renewing, multipotent SSCs exist within the periosteum (Duchamp de Lageneste et al., 2018). In this study, *Prrx1-Cre*-marked cells were isolated from the periosteum and shown to express a panel of markers associated with BMSCs, including *Pdgfra*, *Grem1*, *Cxcl12* and *Nes*. In addition, following transplantation of *Prrx1-Cre*-labeled periosteum into a fracture site, *Prrx1*$^{+}$ periosteal cells re-establish a pool of progenitor cells after a second injury, highlighting their self-renewing quality. Similar data to support the existence of a periosteal SSC have been obtained using the broadly expressed transgene *Acta2(αSMA)-CreER*, expression of which is highly enriched in the periosteum (Matthews et al., 2014; Matthews et al., 2016). However, as these Cre lines label broad mesoderm-derived populations, it has not been possible to pinpoint which cells within the periosteum behave as SSCs.

Another marker of periosteal SSCs is *Ctsk* (Debnath et al., 2018; Yang et al., 2013), which encodes the cysteine protease cathepsin K. *Ctsk* as a marker of periosteal cells came as a surprise given its historical use as a marker of bone-resorbing osteoclasts (a hematopoietic lineage cell). Indeed, the majority of *Ctsk-Cre*-marked cells in the marrow cavity express tartrate-resistant acid phosphatase (TRAP; also known as ACP5), indicative of osteoclast identity. However, when isolated from the femur periosteum of juvenile mice, cultured *Ctsk*$^{+}$ cells exhibit self-renewing properties, with single cells able to form clones with multilineage differentiation potential (bone, cartilage and adipocyte, but not stromal cells). A similar *Ctsk*$^{+}$ periosteal stem cell was also identified in human periosteal tissue, suggesting conservation of Ctsk as a periosteal SSC marker across species (Debnath et al., 2018). In mice, *in vivo* deletion of the crucial osteogenic transcription factor *Sp7* (also known as osterix) in *Ctsk-Cre*-marked cells results in a profound loss of cortical bone, highlighting the requirement for *Ctsk-Cre*-marked cells in osteoblast production. In addition, long-term lineage tracing revealed that *Ctsk-Cre*-marked cells contribute to osteoblasts in the cortical bone but not to osteoblasts or stromal cells in the marrow. Thus, *Ctsk-Cre* appears to mark a population of SSCs distinct from those that migrate into the marrow with the invading vasculature from the perichondrium (Maes et al., 2010). However, it should be noted that, similar to *Prrx1* and *αSMA*, *Ctsk-Cre* marks a broad population of periosteal cells, only some of which are likely to have SSC activity.

Sox9-CreER induction labels a smaller population of cells with stem cell-like properties within the periosteum of adult femur and rib bones in mice (Akiyama et al., 2005; Balani et al., 2017; He et al., 2017; Kuwahara et al., 2019). In the femur, induction of *Sox9-CreER* at P42 with a 2-day chase results in labeling of the growth plate, the adjacent perichondrium, and cells in both the periosteal layer and the endosteal layer (an osteogenic connective tissue lining the inner bone surface) (Balani et al., 2017; He et al., 2017). However, in the rib, induction at 3-4 months results in labeling predominantly in the periosteum (~6% of cells), with many fewer cells labeled in the endosteal compartment of the diaphysis – the shaft portion of the bone (Kuwahara et al., 2019). RNA sequencing of *Sox9*$^{+}$ periosteal cells shows enrichment for a panel of bone genes such as *Bglap*, *Bglap2*, *Col1a1* and *Col1a2*, and also cartilage genes such as *Sox9* and *Col2a1* (He et al., 2017). Osteochondral progenitors have been previously shown to co-express *Sox9*, a major cartilage transcription factor, and *Runx2*, a major bone transcription factor, potentially reflecting dual lineage potential of these common bone/cartilage progenitors (Eames et al., 2004). It is therefore possible that, within the periosteum, *Sox9* similarly marks cells that have retained multilineage skeletogenic potential from development. Whereas these *Sox9*$^{+}$ periosteal cells normally contribute only to bone during homeostasis, they can be induced to form cartilage in response to injury, as discussed in more detail below.

An integrated view of SSC populations

The studies described above underscore the complexity of the SSC system throughout the lifetime of the organism (summarized in Fig. 1). The varying temporal emergence and extinction, differing lineage potentials, and distinct spatial distributions of proposed SSC populations point to heterogeneous pools of SSCs working together to construct and maintain the skeleton, rather than the existence of a single apex SSC, as proposed for the hematopoietic system. Defining the relationships between proposed SSC populations will be needed to clarify hierarchies and plasticity in the skeletal system. Fortunately, gene expression data for many of the above populations already exist, and will allow us to begin formulating testable hypotheses of 'SSC relatedness'. For example, RNA sequencing of periosteal *Ctsk-Cre*$^{+}$ cells shows high expression of *Sox9*, suggesting that *Sox9-CreER* marks a subpopulation of *Ctsk*-expressing cells within the periosteum. As marrow SSCs appear to be derived from both early periosteal cells that migrate into the marrow and hypertrophic chondrocytes that dedifferentiate (and/or borderline chondrocytes that escape hypertrophy), it will be interesting to examine heterogeneity in marrow SSCs in relation to these two different developmental origins (Fig. 2). For example, do marrow SSCs of a growth plate origin have different lineage capacities (e.g. chondrocyte-biased) than those from the invading periosteum? Reciprocally, osteoblasts of a growth plate chondrocyte lineage can also be found in the periosteum of adult zebrafish (Giovannone et al., 2019), suggesting that both the perichondrium and growth plate may contribute to the mature periosteum. Clearly, the distinctions between periosteal, marrow, and growth plate compartments are fluid. Elucidating how cells transition between these compartments and how developmental origins influence later behavior will be key to understanding the distinct roles of SSCs in maintaining and repairing adult skeletal tissues.

Regulators of the SSC niche

Although *PTHrP-CreER* and *Col2a1-CreER* can be used to mark growth plate cells in the embryo, it is not until postnatal stages that they clearly mark cells within the growth plate that have distinct SSC properties. This suggests that these postnatal populations only acquire SSC function under the appropriate

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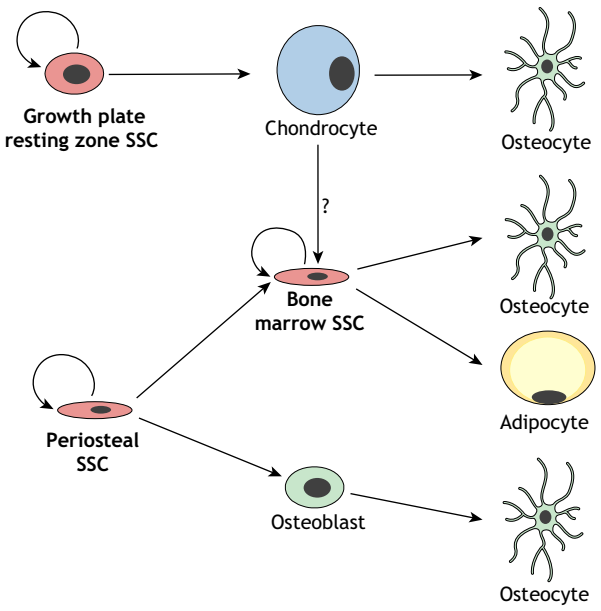

Fig. 2. Redundant pathways to make bone during development and homeostasis. An SSC (red) in the growth plate resting zone is proposed to self-renew and give rise to hypertrophic chondrocytes (blue) that can undergo transdifferentiation to give rise to osteocytes (green) (Park et al., 2015; Yang et al., 2014; Zhou et al., 2014b) and possibly (indicated by '?') to bone marrow SSCs (Giovannone et al., 2019; Mizuhashi et al., 2019). Bone marrow SSCs can then give rise to osteocytes and adipocytes (yellow) in the bone marrow compartment (Zhou et al., 2014b). Better evidence for the origin of bone marrow SSCs comes from a study (Maes et al., 2010) showing that periosteal/perichondrial SSCs contribute to the marrow compartment during development. Whether this also happens postnatally is not clear. Osteocytes can also arise (via an osteoblast intermediate) from periosteal SSCs at the periosteal surface (Debnath et al., 2018).

niche conditions. Further, several groups have recently shown that dysfunction in the niche can lead to skeletal dysfunction. For example, inflammation via enhanced NF-κB and TNFα signaling reduces SSC abundance and function but can be counteracted pharmacologically to rejuvenate SSCs to improve fracture healing in models of aging and diabetes (Josephson et al., 2019; Tevlin et al., 2017). Thus, gaining a better understanding of the niche factors required for healthy SSC biology is an important direction of research.

Using transcriptomic analyses to identify highly expressed morphogens and their cognate receptors, it is possible to infer a potential role for these pathways in regulating SSC activity. For instance, transcriptome analyses of *Col2a1*$^+$ growth plate chondrocytes has revealed the dynamic regulation of several key pathways (Newton et al., 2019). Notably, negative regulators of the Wnt signaling pathway are downregulated, suggesting that Wnt signaling promotes SSC establishment in the growth plate. This idea is supported by *in vitro* studies in which active Wnt signaling was shown to support the undifferentiated state of SSCs; however, once these cells become committed to an osteogenic fate, Wnt signaling enhances their differentiation (Quarto et al., 2010). Wnt signaling may also function to maintain SSCs in the periosteum, as many Wnt ligands are enriched in *Ctsk*$^+$ SSCs based on RNA sequencing (Debnath et al., 2018). A role for Wnt signaling is further reflected by the ability of *Axin2-CreER* to mark periosteal SSCs in the cranial and appendicular skeleton, as *Axin2* is a direct transcriptional target of Wnt signaling in many tissues (Maruyama et al., 2016; Ransom et al., 2016).

One possibility is that interactions between SSCs and their downstream progeny serve to maintain an appropriate pool of SSCs through feedback signaling. Bone morphogenetic protein (BMP) signaling is one attractive candidate pathway that may play a role in such feedback due to its well-known roles in skeletal differentiation. As stated earlier, the BMP antagonist Grem1 is a marker of SSCs in the marrow compartment (Worthley et al., 2015). Isolated Itgav$^+$/CD200$^+$ cells also express several BMPs and their receptors, whereas downstream progenitors express high levels of two other BMP antagonists, Grem2 and noggin (Chan et al., 2015; Chan et al., 2018). *In vitro*, BMP2 addition enhances and Grem2 inhibits SSC expansion, suggesting a feedback role for BMP signaling in maintaining SSCs. A recent study has also shown that BMP2 is required in immature *Prrx1*$^+$ periosteal progenitors, but not mature osteogenic cells, to drive appositional bone growth during early life, with its upregulation in the periosteum in response to injury accelerating fracture repair (Salazar et al., 2019). Given the widespread use of BMPs in clinical application and trials, more studies are needed to tease out the specific roles of BMP signaling in SSC biology (Krishnakumar et al., 2017).

The Hh signaling pathway also likely regulates the SSC niche. During the time when *Col2a1*$^+$ SSCs appear in the growth plate, high expression of sonic hedgehog (*Shh*) can be observed in the secondary ossification center along with Indian hedgehog (*Ihh*) expression in growth plate cartilage cells (Newton et al., 2019). Strong expression of the Hh mediator Gli2, as measured by RNA *in situ* hybridization, can be seen in these *Col2a1*$^+$ cells; this is consistent with strong recombination of *Gli1-CreER* in the region of the resting growth plate where *Col2a1*$^+$ SSCs reside. Pharmacological inhibition and/or activation of the Hh pathway, as well as genetic removal of the obligate Hh mediator smoothened, further support a role for Hh signaling in the regulation of growth plate SSC proliferation (Long et al., 2004; Newton et al., 2019). Hh signaling promotes proliferation of growth plate SSCs without premature induction of differentiation (Long et al., 2004; Newton et al., 2019), which is somewhat surprising given the wide body of literature describing a role for Hh signaling in driving the differentiation of skeletal lineage cells in development and regeneration (reviewed by Alman, 2015). Moving forward, it will be important to untangle the temporal and spatial role of Hh signaling in chondrogenesis from its potential role in SSC maintenance and/or expansion.

Although many signaling pathways have been identified as potential niche regulators, further research is needed to identify the precise ligands, receptors and target genes involved. In addition, it is likely that other non-skeletal cell types may modify the niche environment. For example, studies have shown the importance of a perivascular niche for skeletal progenitor cells during bone development and regeneration, including the action of endothelial-derived VEGF on hypertrophic chondrocytes at the edge of the growth plate (Colnot et al., 2004; Gerber et al., 1999). In a recent study, it was elegantly shown that periosteal endothelial cells secrete PDGF ligands to attract PDGFRβ$^+$, Sp7$^+$ osteoprogenitors, with PDGFβ signaling keeping these progenitors in an undifferentiated, proliferative state (Böhm et al., 2019). Nerve cells may also influence repair. For example, it has been shown that Shh emanating from nerves maintains tooth incisor stem cells, and that Schwann cells from nerves in the mandible potentially provide important paracrine factors for bone repair in the jaw (Jones et al., 2019; Zhao et al., 2018). The influence of cells from the immune system may also be crucial. Indeed, TRAP$^+$ macrophages (derived from the monocyte lineage) drive periosteal osteoblast differentiation via a PDGF-BB signal (Gao et al., 2019),

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with potentially other immune cell types and signaling pathways still to be discovered. It is likely that even moderate alterations to the niche environment could have major impacts on SSC potency. Thus, identifying and characterizing niche regulators may aid the development of new clinical strategies to combat skeletal degeneration in disease or aging.

SSCs in repair

In mammals, simple fractures of bone often exhibit scarless healing, and in certain non-mammalian vertebrates, large pieces of missing bone and cartilage, and even entire skeletal appendages, can undergo regeneration. Which cells fuel these regenerative processes? One possibility is that the same SSCs involved in bone homeostasis function to repair bone after injury. Alternatively, or in combination with this, skeletal injury may create an abnormal microenvironment (e.g. inflammation, altered mechanical properties, cell death and migration) that results in certain cell populations contributing to new bone that would not normally do so during homeostasis (Wang et al., 2017). Current evidence supports the local periosteum and bone marrow being the two primary sources of bone-forming cells during repair; indeed, parabiosis experiments do not support significant contributions from circulating cells (Chan et al., 2015; Colnot, 2009; Colnot et al., 2006; Yu et al., 2010; Zhang et al., 2005; Zhou et al., 2014a).

Bone repair can occur either through a cartilage callus intermediate or through direct ossification (Box 2). Recent data indicate that the cartilage callus during bone repair differs substantially from the growth plate cartilage that builds endochondral bones developmentally. During development, growth plate chondrocytes begin to express genes associated with osteoblast differentiation [e.g. *Runx2*, *Sp7*, *Spp1* and *Bglap* (also known as *Ocn*)] only after expression of *Col10a1* at the pre-hypertrophic stage. In contrast, during bone regeneration in the zebrafish jaw and the mouse rib, callus chondrocytes co-express high levels of chondrocyte and osteoblast genes at much earlier stages of differentiation (Kuwahara et al., 2019; Paul et al., 2016). For example, *Sox9* and *Runx2* are co-expressed at initial stages of chondrogenesis in the callus, and the expression of *Col1a1* precedes that of *Col10a1*, a sequence never seen in normal development of zebrafish or mouse rib growth plates. This acceleration of osteoblast gene expression relative to the chondrogenic program during repair may reflect altered regulation in the wound setting, and has led to callus cells being termed 'hybrid osteochondral cells'. The mechanics of the fracture environment have also been shown to influence the mode of bone repair. Whereas repair of unstabilized fractures typically involves a robust cartilage callus, rigidly stabilized injuries, such as focal lesions in the intramembranous bones of the skull or surgically fixated long-bone fractures, heal primarily through direct ossification (Le et al., 2001; Thompson et al., 2002). Recent insights into the SSCs contained within the fibrous sutures separating the intramembranous skull bones are providing interesting contrasts with the SSCs of endochondral bones (Box 3). It may be that the same SSC populations react differently to mechanical stimuli (i.e. preferentially forming osteoblasts over chondrocytes in a stiff environment), or that distinct SSC populations with unique properties are activated depending on the injury type. SSC heterogeneity may also be found across different bone types, as each bone has unique biophysical requirements (i.e. weight-bearing versus non-weight-bearing), different vascularization and innervation statuses, and distinct developmental histories. For example, different bones express different Hox genes and, at least in the context of the ulna (but not the humerus) and the tibia (but not the femur), paralogs of *Hoxa11/d11* are required for normal fracture healing (Rux et al., 2017). In addition, bone healing could involve a significant contribution of cells that do not fit the strict definition of a stem cell, as suggested by the observed dedifferentiation of osteoblasts in zebrafish bone regeneration (Geurtzen et al., 2014; Knopf et al., 2011; Singh et al., 2012) (Box 1) and murine digit tip regeneration (Storer et al., 2020). Probing the behavior of SSCs and other populations across a variety of injury types and bones should help clarify the diversity of SSCs and repair mechanisms throughout the skeleton.

To date, the same transgenic tools used to track the contribution of SSCs to homeostasis have been used to examine the contribution of these cells to repair. In 3-month-old mice, *Acta2 (αSMA)-CreER* induction marks a broad population of periosteal cells that proliferate rapidly and generate almost all chondrocytes and osteoblasts within the repair callus 6 days after tibial fracture (Matthews et al., 2014). However, in these studies, tamoxifen induction was performed both before and after tibia fracture, and thus the contributions of pre-existing αSMA^{+} cells and those that potentially switch on *Acta2(αSMA)* expression after injury cannot not be distinguished. Similarly, *Axin2-CreER* has been used to mark periosteal cells that participate in bone repair. For example, periosteal-specific induction of *Axin2-CreER* (via local injection of tamoxifen) marks a small population of periosteal cells in the tibia of 2-month-old mice (Ransom et al., 2016). Following a 1-mm drill injury, *Axin2-CreER*-labeled cells are observed in the repair site and contribute to ∼11% of callus chondrocytes but to virtually no osteoblasts 1 week after injury. When *Axin2-CreER* is induced systemically, a higher percentage of periosteal (∼37%) and endosteal (∼42%) cells are labeled, with contributions now seen (presumably from endosteal cells) to the bony portions of the repair callus. *Axin2-CreER* may therefore mark at least two populations of cells: a periosteal population biased toward endochondral bone formation and an endosteal population biased toward direct

Box 3. Repair in cranial bones

Suture mesenchyme is a unique connective tissue that can be found at the junctures between cranial bones. Complications of bone growth at the suture have been implicated in craniofacial defects such as craniosynostosis. SSCs are concentrated in the suture region and contribute extensively to new bone during skull growth and repair (Zhao et al., 2015), with efficiency of regeneration decreasing as injury distance from the suture increases (Park et al., 2016). In mice, suture SSCs can be marked using a *Gli1-CreER* line following induction at 1 month of age. Postnatal *Gli1-CreER*-traced cells give rise to parts of the periosteum, dura, and osteocytes of the calvaria (Zhao et al., 2015). Ablation of this population postnatally results in a complete loss of sutures, halted bone growth and a malformed skullcap (Zhao et al., 2015). Suture SSCs can also be labeled by *Prrx1-CreER* and *Axin2-CreER* lines (Maruyama et al., 2016; Wilk et al., 2017). Although both populations overlap with *Gli1*-expressing cells, it is unclear whether they overlap with each other as the *Prrx1* population does not express *Axin2* unless stimulated with a WNT agonist (Wilk et al., 2017). As with the $Gli1^{+}$ population, both the $Axin2^{+}$ and $Prrx1^{+}$ populations have been shown to participate in repair. Ablation of the $Prrx1^{+}$ population does not cause craniosynostosis, suggesting differences from the *Gli1* population. However, $Axin2^{-/-}$ mutants do display synostosis of a subset of sutures (Yu et al., 2005). Notably, markers for $Axin2^{+}$ suture SSCs overlap with those for periosteal SSCs in long bones in the appendicular skeleton, but not those of bone marrow SSCs (*Grem1*, *Nes*) with the exception of *Lepr*, which is highly enriched in suture SSCs (Maruyama et al., 2016). This suggests parallels between suture and long bone periosteal SSCs that will be interesting to pursue.

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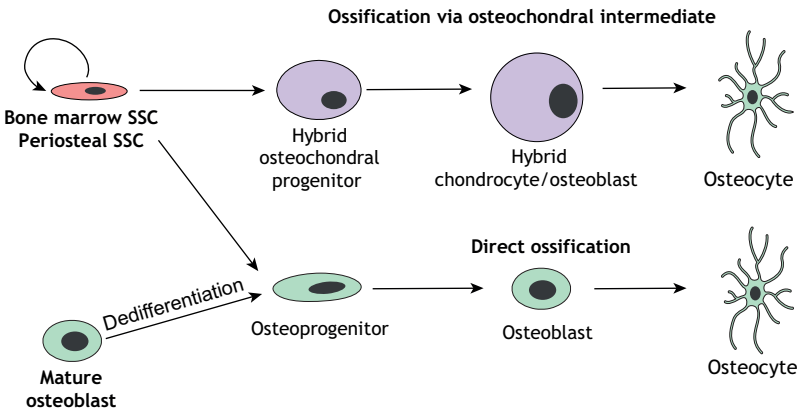

Fig. 3. Redundant pathways to make bone during repair. In response to injury, SSCs (red) from the periosteum and/or the bone marrow compartment generate bone through via an osteochondral intermediate (giving rise to cells with cartilage/bone properties, i.e. hybrid osteochondral progenitors, purple) or through direct ossification (giving rise to osteoprogenitors, green). In some contexts, such as the zebrafish fin and murine digit tip, osteoblasts can dedifferentiate and re-differentiate to produce new bone.

osteogenesis. *Axin2*$^+$ cells are required for repair, as diphtheria toxin-mediated ablation of these cells prior to injury inhibits bony but not cartilage callus formation, suggesting that *Axin2*$^+$ cells play only a minor role in cartilage callus formation. Another periosteal marker *Ctsk-Cre* marks half of all chondrocytes in the femur fracture callus 6 days after injury (Debnath et al., 2018). However, as *Ctsk-Cre* is constitutive, it is not possible to distinguish between repair cells derived from the periosteal lineage and those that may have switched on *Ctsk* expression in response to injury.

Recent studies of the mouse rib bone, which shows extraordinary regeneration capacity compared with other bones, have revealed a role for periosteal cells not only in forming repair tissue but also in organizing the regenerative response. Before injury, *Sox9-CreER* induction marks a population of periosteal cells (~6% of the periosteum) in the rib diaphysis, with many fewer cells labeled on the endosteal surface (Kuwahara et al., 2019). Following resection of 3 mm of rib bone, *Sox9-CreER*-labeled cells contribute to ~20% of callus cells in both the cartilage and bony portions 10 days after injury. In addition, upon deletion of the Hh co-receptor smoothened in *Sox9-CreER*-labeled cells prior to resection, progenitor cells fail to differentiate into the hybrid osteochondral cells that build the cartilage callus. Strikingly, loss of Hh signaling in pre-injury *Sox9*$^+$ cells blocks cartilage differentiation in the 80% of cells not derived from the *Sox9*$^+$ lineage. This suggests a special organizing function of periosteal *Sox9*$^+$ cells in not only contributing to the cartilage callus but also recruiting other cells into the callus. Hh signaling (mediated by *Ihh*) has been shown to play a similar role in promoting large-scale regeneration of the zebrafish jawbone, although in this context it was not investigated whether Hh was acting on *Sox9*$^+$ periosteal cells (Paul et al., 2016).

Markers for endosteal-specific populations in the marrow compartment can help determine whether periosteal versus endosteal cells have distinct roles during skeletal repair. In juvenile mice, marrow cells below the growth plate (i.e. in the metaphysis) can be marked using a *Gli1-CreER* line at P30 (Shi et al., 2017). Following femur fracture, these cells show substantial contributions to both the cartilage and bone portions of the callus 10 days after injury. However, because of broad labeling by *Gli1-CreER* beyond the marrow cavity, it remains unclear which of the P30 *Gli1*$^+$ populations (or their descendants) give rise to each of the skeletal lineages observed. In addition, the number of *Gli1*$^+$ cells markedly decreases at 4 months, and disappears completely at 12 months. Thus, in juvenile mice, marrow-resident *Gli1*$^+$ cells serve as a reservoir for femur fracture repair, whereas contribution in older animals is likely minimal. *Lepr-Cre* labeling can also be used to mark marrow-specific cells in the adult mouse tibia, although sparse labeling can be found in the periosteum of other bones (e.g. the sternabrae) (Zhao et al., 2015). *Lepr*$^+$ cells contribute substantially to both the cartilage and bone callus 2 weeks after tibial fracture, with abundant osteocytes retaining label even after 8 weeks. Owing to the constitutive Cre, however, new induction of *Lepr* expression after injury could also explain the presence of labeled cells. The *Grem1-CreER* line, by contrast, is conditionally inducible and can therefore be used to label metaphyseal cells prior to injury (Worthley et al., 2015). When Cre is induced in 8-week-old animals and followed 1 week later by femur fracture, *Grem1*$^+$ cells generate ~14% of *Sox9*-expressing chondrocytes and ~28% of *Col1a1*-expressing osteoblasts in the 1-week repair callus, providing the best evidence thus far for the contribution of marrow cells to the early repair callus.

Together, these studies highlight a wide range of Cre lines that can be used to observe the contributions of cells during homeostasis and repair in the mouse (summarized in Tables 1 and 2). Cells from various compartments contribute during repair, through a cartilage-like intermediate, via direct ossification, or both (Fig. 3). Specific subpopulations may already be biased toward one path versus another. Alternatively, or in addition, specific niche factors, the unique properties of specific bones (their developmental history), and the mechanical environment may influence the lineage outcome. Furthermore, osteoblasts may also dedifferentiate to repair bone in certain contexts. Although initially described in non-mammalian vertebrates (Box 1), this process of osteoblast dedifferentiation has recently been observed in a study of murine digit tip regeneration (Storer et al., 2020). This analysis revealed that, when osteoblasts are pre-labeled with *Dmp1-CreER* (which was confirmed to be osteoblast/osteocyte specific via single cell RNA sequencing) and then subjected to digit amputation, *Dmp1*-lineage cells generate proliferative blastema cells that lose the expression of differentiated osteoblast markers and acquire a progenitor-like signature, including expression of *Grem1*. These osteoblast-derived blastema cells can then contribute extensively to the regenerated digit bone. However, a separate study found that *Ocn-CreER*-traced osteoblasts do not contribute to regeneration in a murine calvarial microfracture model (Park et al., 2012), suggesting that, in mammals, osteoblast dedifferentiation may be specific to appendage regeneration. In the future, it will be interesting to determine whether dedifferentiation can be induced and used as a method to build new skeletal tissue clinically.

Conclusions and future directions

There has been an enormous effort in recent years to identify and characterize rare, discrete populations of bona fide, self-renewing, multipotent SSCs. The search for SSCs has undoubtedly been

influenced by studies that have uncovered a hierarchy of lineage-restricted stem cells in the hematopoietic system. Although recent work lends support for such a hierarchy in both the mouse and human skeletal systems (Chan et al., 2015, 2018), it is also clear that diverse types of SSCs can be isolated from distinct spatial locations. These heterogeneous populations likely coordinate their activities to build, maintain and repair the skeleton. Further, there appears to be considerable plasticity in SSCs, which may help to ensure that the required skeletal cells are efficiently replaced in response to different types of injuries.

In the future, a major challenge will be to relate diverse populations of SSCs to one another. In addition, do the distinct embryonic origins of SSC types prefigure their unique properties with regards to building, maintaining and repairing the skeleton? Emerging techniques should help to address both the lineage relationships and potential of SSCs. As a complement to Cre/Lox-based systems, it is now possible to generate new animal models rapidly using Flp/Frt or Dre/Rox recombinases to expand the toolbox and enable *in vivo* lineage tracing of multiple cell populations in parallel (Plummer et al., 2015). Genetic barcoding could potentially allow tracking of thousands of individual cells *in vivo* (McKenna and Gagnon, 2019). Resolving SSC lineage relationships should help resolve major questions regarding the relative contributions of cells from the bone marrow, growth plate and periosteum in the skeletal system. In parallel, identifying essential niche factors will help determine how SSC populations stay quiescent, become activated due to injury, and undergo the specific transitions needed to build new skeletal tissues. We will need to complement studies in model organisms with those in human tissues, as markers may differ between species. This will allow us to improve our understanding of how cell populations and niche factors are altered in dysmorphology, injury, disease, and aging of the human skeleton. Overall, these inquires will no doubt give us a greater understanding of the plasticity of the skeletal system and ultimately will help bring innovative therapeutic approaches to the clinic.

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