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In vivo dynamics of hard tissue-forming cell origins: Insights from Cre/loxP-based cell lineage tracing studies☆

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ABSTRACT

Bone tissue provides structural support for our bodies, with the inner bone marrow (BM) acting as a hematopoietic organ. Within the BM tissue, two types of stem cells play crucial roles: mesenchymal stem cells (MSCs) (or skeletal stem cells) and hematopoietic stem cells (HSCs). These stem cells are intricately connected, where BM-MSCs give rise to bone-forming osteoblasts and serve as essential components in the BM microenvironment for sustaining HSCs. Despite the mid-20th century proposal of BM-MSCs, their *in vivo* identification remained elusive owing to a lack of tools for analyzing stemness, specifically self-renewal and multipotency. To address this challenge, Cre/loxP-based cell lineage tracing analyses are being employed. This technology facilitated the *in vivo* labeling of specific cells, enabling the tracking of their lineage, determining their stemness, and providing a deeper understanding of the *in vivo* dynamics governing stem cell populations responsible for maintaining hard tissues. This review delves into cell lineage tracing studies conducted using commonly employed genetically modified mice expressing Cre under the influence of LepR, Gli1, and Axin2 genes. These studies focus on research fields spanning long bones and oral/maxillofacial hard tissues, offering insights into the *in vivo* dynamics of stem cell populations crucial for hard tissue homeostasis.

1. Introduction

Although bone tissues may seem dormant, they undergo constant remodeling and are stringently regulated by osteoclastic bone resorption and osteoblastic bone formation, ensuring the maintenance of both quantity and quality [1–3]. Because the lifetime of mature osteoblasts is limited, a constant supply of osteoblasts from undifferentiated progenitor cells is required to maintain osteoblasts and compensate for resorbed bone tissues [4,5]. The notion that the stem cell fraction responsible for osteoblast generation resides in the bone marrow (BM) has been a longstanding concept dating back to the mid-20th century. Since the 1960 s, intensive investigations by Friedenstein et al. [6–9] have progressively unveiled the presence of stem cells within the BM. Their seminal work demonstrated that subcutaneous transplantation of BM cells induces the formation of bone tissue with hematopoiesis, proposing the existence of an undifferentiated cell fraction within the BM capable of regenerating both the bone and marrow environment. Additionally, culturing BM cells at low density revealed the emergence of colonies from a single fibroblast-like cell, identified as colony-forming unit fibroblasts (CFU-F). This experimental outcome suggests that individual cells form colonies owing to their inherent ability to self-renew under culture conditions. Furthermore, cells derived from a single CFU-F exhibited multipotent differentiation into various lineages, including osteoblasts, adipocytes, and chondrocytes; consequently, these CFU-F-forming cells were classified as stem cells within the BM. Caplan [10] and Pittenger et al. [11] later coined the term “BM mesenchymal stem cells (MSCs)” for these BM cell fractions with CFU-F capacity. However, objections arose as not all cells derived from CFU-F displayed self-renewal and multidifferentiation abilities, raising concerns about the definition of CFU-F as a stem cell [12]. Addressing this issue, the International Society for Cellular Therapy (ISCT) recommends the term “Multipotent Mesenchymal Stromal Cells” for fibroblastic culture dish-adherent cells exhibiting CFU-F capacity, irrespective of tissue origin [13]. Additionally, in cases where the cell population is anticipated to encompass both progenitor and stem cells, the term “Mesenchymal Stem and Progenitor cells” is employed as an analogy to the hematopoietic system [14]. The designation “skeletal stem cells” has long been proposed based on their *in vivo* properties [15,16], and this

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term has been utilized in studies identifying the osteogenic stem cell population in humans and mice through the combination of cell surface protein markers [17,18]. Based on this background, this review adopts the term "Skeletal Stem and Progenitor cells (SSPCs)" to denote stem cells contributing to hard tissue formation, encompassing the diverse nomenclature and evolving understanding within this field.

The described *in vitro* approach for assessing cell stemness through the analysis of CFU-F and pluripotency remains a valuable method frequently employed by researchers. Nonetheless, its limitations become apparent when attempting to demonstrate stemness *in vivo*. The advent of cell lineage tracking techniques, employing Cre/loxP-based strategies in genetically modified mice, has led to a new era of stem cell research by enabling the direct demonstration of *in vivo* stemness [19]. In this method, cells can be labeled depending on the expression of Cre recombinase under the control of a promoter for specific cell markers, and the stemness (self-renewal and multipotency) of the labeled cells was examined using *in vivo* tracking. Over the past few years, researchers have attempted to understand the hierarchical relationships between BM mesenchymal cell populations *in vivo* using this technique and have identified specific markers to detect SSPC populations in bone tissues [20,21].

In addition to limb bones, hard tissue-forming cells also originate in oral and cranio-maxillofacial areas, such as dental pulp (DP) [22–24], periodontal ligament (PDL) [25–29], alveolar bone (AB) [30,31], and cranial sutures [32]. Recent advancements in cell lineage tracing approaches have provided insights into the *in vivo* dynamics of these tissues. Various Cre-expressing genetically modified mice have been employed in hard tissue stem cell research, with a particular emphasis on the utility of leptin receptor (LepR), glioblastoma (Gli)1, and Axin2-induced Cre-expressing mice [33–35]. These models prove especially beneficial for conducting a wide range of hard tissue experiments, including limb and oral/cranio-maxillofacial bone studies.

This review begins by outlining the methodology of cell lineage tracing analysis using genetically modified mice, followed by a historical overview of BM SSPC research, which has significantly progressed with the integration of cell lineage tracing technology. Additionally, the review presents recent findings on the *in vivo* dynamics of SSPCs within limb and oral/cranio-maxillofacial hard tissues, focusing on LepR-, Gli1-, and Axin2-induced Cre-expressing mice. Table 1 provides a comprehensive list of representative studies analyzing the origin of hard tissue-

Table 1
Representative studies of cell lineage-tracing analysis in LepR$^+$, Gli1$^+$, or Axin2$^+$ populations.

A. Long bones

Driver	Time point of induction	Cre-expressing cells	Contribution	References
LepR-Cre	• N/A	• Perivascular BM stromal cells	• Osteoblasts, osteocytes, adipocytes, and regenerative chondrocytes	[57,58]
Gli1-CreER^{T2}	• Three consecutive days at 4 weeks old [99] • Every other day for 7 days at 8 weeks old [104]	• MMPs beneath the growth plate [99] • Periosteum [99,104]	• Osteoblasts, LepR$^+$ stromal cells, and adipocytes in the BM of growing bone [99] • Fracture callus [99,104]	[99,104]
Axin2-CreER^{T2}	• One time at P1 [132] • Five consecutive days at 8 weeks old [133] • One time at P6 [134]	• Perivascular BM stromal cells [132] • Periosteum [133] • Outermost layer of the growth plate [134]	• Osteoblasts, osteocytes, and adipocytes in the BM of growing bone [132] • Fracture callus [132,133] • Growth plate chondrocytes [134]	[132–134]

B. Dental pulp

Driver	Time point of induction	Cre-expressing cells	Contribution	References
Gli1-CreER^{T2}	• Three consecutive days at 4–6 weeks old	• Dental mesenchyme around cervical loop of incisors	• Whole dental mesenchyme of incisors	[111]
Axin2-CreER^{T2}	• Three consecutive days at 6 weeks old	• Dental pulp cells at the site of damaged dentin in the molars	• Reparative odontoblast-like cells	[141]

C. Periodontal ligament

Driver	Time point of induction	Cre-expressing cells	Contribution	References
LepR-Cre	• N/A	• Perivascular mesenchymal cells in PDL	• AB osteocytes and cementocytes • Regenerated bone in the tooth extraction socket	[87]
Gli1-CreER^{T2}	• Two consecutive days at 5–8 weeks old [113] • Two consecutive days at 3 weeks old [114] • Two consecutive days at 4 or 8 weeks old [115–117]	• PDL in the apical region surrounding the NVB	• PDL fibroblasts, cementoblasts, and osteoblasts [113–115] • Regenerated bone in the tooth extraction socket [116] • Osteoblasts, osteocytes, and fibroblasts on the traction side of orthodontic treatment [117]	[113–117]
Axin2-CreER^{T2}	• Three consecutive days at 5 weeks old [158] • One time at 4 weeks old [162,163]	• Mesenchymal cell population randomly distribute throughout the PDL	• Regenerated bone in the tooth extraction socket [158] • Cementoblasts and cementocytes [162] • Osteoblasts and osteocytes on the traction side of orthodontic treatment [163]	[158,162,163]

D. Alveolar bone

Driver	Time point of induction	Cre-expressing cells	Contribution	References
LepR-Cre	• N/A	• Mesenchymal cells in alveolar BM	• Osteoblasts and osteocytes in AB, and regenerated bone in the extraction socket	[90]
Gli1-CreER^{T2}	• Two consecutive days starting at 6 weeks old	• Perivascular mesenchymal cells in alveolar BM	• Osteoblasts and osteocytes in regenerated bone in the extraction socket	[125]

E. Calvarial suture

Driver	Time point of induction	Cre-expressing cells	Contribution	References
Gli1-CreER^{T2}	• Four consecutive days at 4 weeks old	• Suture mesenchymal cells	• Calvarial osteoblasts, periosteum, and dura	[109]
Axin2-rtTA	• Three consecutive days starting at P25	• Mesenchymal cells localized in the center of the suture	• Suture mesenchyme, osteoblasts, and osteocytes • Suture mesenchyme and osteocytes in regenerated calvarial bone	[135]

BM, bone marrow; MMPs, metaphyseal mesenchymal progenitors; P, postnatal; AB, alveolar bone; PDL, periodontal ligament; NVB, neurovascular bundle.

forming cells using the mouse lines discussed in this review.

2. Cell lineage tracing analysis using Cre/loxP techniques

The advent of Cre/loxP-based genetic modification technology has facilitated the specific labeling of cells *in vivo*, enabling the monitoring of their dynamics throughout their lifespan. The DNA recombinase Cre can excise the DNA region flanked by loxP sequences. Consequently, when a stop codon, positioned between loxP sequences, is situated upstream of a reporter gene, such as tdTomato (tdTom), the excision of the stop codon occurs upon Cre expression, inducing the expression of the reporter gene. The gene inserted at the Rosa26 locus is expressed systemically [36,37]. Therefore, mice harboring the specified gene (loxP-stop-loxP-tdTom) at the Rosa26 locus possess a Cre/loxP-based reporter system in a systemic manner (ROSA26-loxP-stop-loxP (R26)-tdTom) [38]. Consequently, only the Cre-expressing target cells are specifically labeled in the mouse body (Fig. 1A). Additionally, the timing of cell labeling can be artificially controlled using CreER^{T2}, a mutant of the estrogen receptor, combined with Cre (Fig. 1B). CreER^{T2} remains outside the nucleus in the absence of tamoxifen (Tam); however, upon binding to Tam, it translocates to the nucleus and cleaves the loxP site. As a result, the target cells are labeled in response to Tam administration in mice [39]. Owing to the half-life of Tam being less than 48 h, cell labeling is transiently induced. This system allows analysis of the persistence (self-renewal) and differentiation of labeled cells into progeny (pluripotency) to demonstrate their stemness *in vivo*.

3. Development of SSPC research in parallel with hematopoietic stem cell niche research

BM is a unique tissue containing two types of stem cells, SSPCs and hematopoietic stem cells (HSCs). The field of SSPC research has progressed alongside HSCs, as illustrated in the subsequent historical account.

The stem cell niche is believed to create a microenvironment that sustains stem cell properties, such as self-renewal capacity and multilineage ability [40]. In 1978, Schofield [41] proposed the existence of a stem cell niche in the BM tissue that regulates HSCs. Since then, hematologists worldwide have actively sought the identity of cells comprising the HSC niche. In the early-2000 s, multiple research groups reported that osteoblasts were responsible for the HSC niche [42–44]. However, ongoing controversy surrounds whether osteoblasts truly function as niche cells for HSCs [45–48]. Méndez-Ferrer et al. [49] observed the BM tissue of transgenic mice expressing GFP under the control of the nestin (Nes) promoter and enhancer, detecting Nes-GFP^{+} cells as perivascular stromal cells. Depleting Nes^{+} stromal cells *in vivo* using the Cre/loxP-based strategy significantly reduced the number of HSCs in the BM, supporting the conclusion that Nes^{+} stromal cells serve as HSC niche cells. In contrast, in 2003, Ara et al. [50] reported that the chemokine C-X-C chemokine ligand 12 (CXCL12) (also known as SDF-1: stromal cell-derived factor-1) is a necessary factor for BM engraftment of HSCs in developing BM tissue. Perivascular cells, identified as a source of CXCL12 in GFP knock-in mice in the second exon of CXCL12, were associated with BM HSCs. Depletion of CXCL12^{+} perivascular cells (referred to as CXCL12-abundant reticular (CAR) cells) significantly

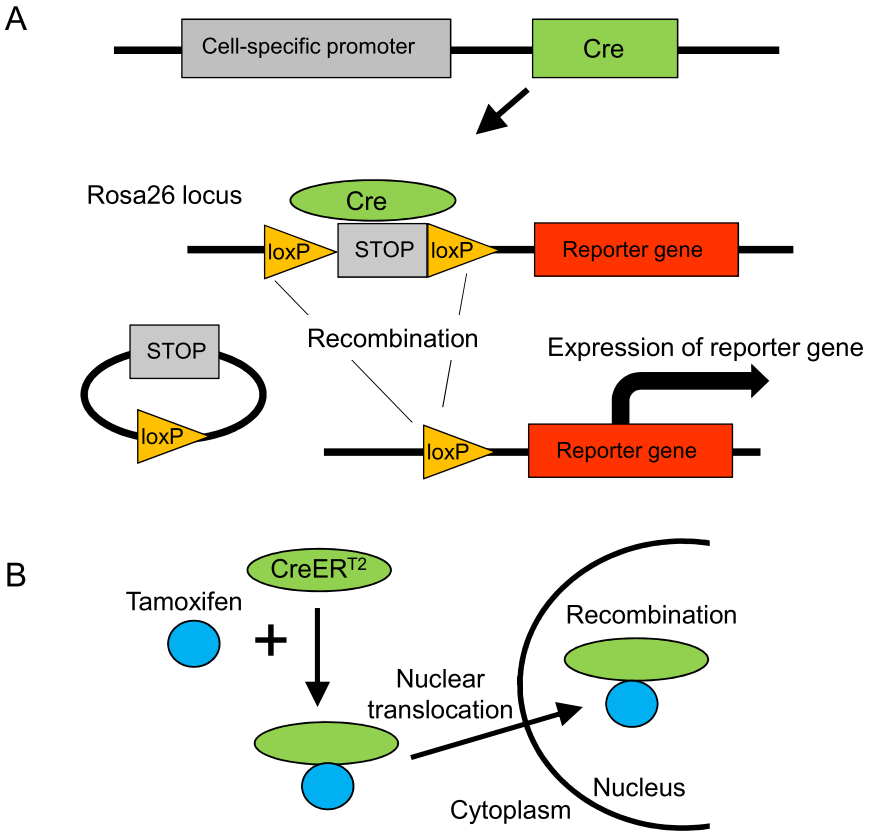

Fig. 1. System for cell lineage tracing analysis using Cre/loxP strategy. (A) When a stop codon sandwiched by a *loxP* sequence is cut out *via* Cre recombinase controlled by a cell-specific promoter, the expression of the reporter gene is induced. The gene inserted at the Rosa26 locus is systemically expressed. Therefore, the mice with the gene (*loxP-stop-loxP-reporter gene*) at the Rosa26 locus will have the Cre/loxP-based reporter system in a systemic manner. Thus, only Cre-expressing target cells can be specifically labeled by the induced reporter gene in the mouse body. (B) The timing of cell labeling can be artificially controlled using the CreER^{T2} system. CreER^{T2} resides outside the nucleus without tamoxifen (Tam); however, when bound to Tam, it migrates into the nucleus and cleaves the *loxP* site. Hence, the target cells are labeled in response to the Tam administration to the mice. The half-life of Tam is less than 48 h, so cell labeling is induced transiently.

decreased BM HSCs, suggesting that CAR cells also function as HSC niche cells [51]. Crucially, both Nes^{+} stromal and CAR cells were shown to possess SSPC capacities and exhibit roles as the components of the HSC niche [49,51]. Similar to mouse BM tissue, human-derived melanoma cell adhesion molecule (MCAM/CD146)$^{+}$ BM perivascular cells demonstrated the capacity for both SSPCs and the HSC niche, as evidenced by cell transplantation experiments in immunocompromised mice [52]. Therefore, the experimental histories of SSPCs and HPCs are closely intertwined. In the pursuit of HSC niche cells, researchers have successfully pinpointed populations of SSPCs.

4. Identification of SSPCs using LepR-Cre mice

Leptin is a peptide hormone expressed by adipocytes that has been identified as a causative gene of obesity in ob/ob mice [53]. Similarly, LepR has been identified as the cause of early obesity in db/db mice [54]. Leptin exerts antifeedant activity by binding to LepR expressed on neurons in the hypothalamus [55]. Lineage tracing analysis performed on LepR-Cre knock-in mice [34] revealed that LepR can be used as a marker of SSPCs in the adult stage.

4.1. Identification of long bone-derived LepR^{+} SSPCs

In addition to the Nes^{+} stromal and CAR cells described above, Ding et al. reported LepR^{+} stromal cells as HSC niche cells in the BM [56]. LepR^{+} stromal cells are perivascularly localized and function as an HSC niche through stem cell factor (SCF) expression, which is necessary for HSC maintenance in the BM. LepR^{+} stromal cells were detected as tdTom^{+} cells in the BM of LepR-Cre; R26-tdTom mice (Fig. 2A). *In vivo* lineage tracing analysis revealed that LepR^{+} stromal cells differentiate into osteoblasts, adipocytes, and fractured callus chondrocytes, indicating that these cells have the capacity for SSPCs, similar to Nes^{+} stromal cells and CAR cells [57,58]. Researchers have shown that most LepR^{+} stromal and CAR cells overlap [59,60]. In addition, BM Nes-GFP^{+} stromal cells have been classified into two subpopulations, Nes-GFP^{bright} and Nes-GFP^{dim}, based on their GFP expression levels. It has also been shown that Nes-GFP^{dim} stromal cells overlapped with LepR^{+}/CAR cells [61]. In contrast, Nes-GFP^{bright} stromal cells were found in peri-arterial and metaphyseal BM tissues [62] (Fig. 3). Periarterial Nes-GFP^{bright} stromal cells are positive for the pericyte marker neural/glial antigen 2

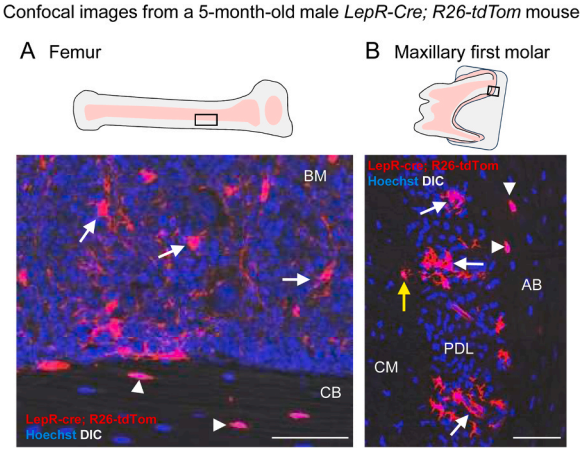

Fig. 2. LepR^{+} SSPCs labeled by LepR-Cre. Z-stack confocal images of thick femur (A) and maxillary first molar (B) sections from 5-month-old male LepR-Cre; R26-tdTom mice. The squares in the upper schema indicate the area of the Z-stack confocal images. White arrows: LepR^{+} SSPCs; White arrowheads: LepR^{+} SSPC-derived osteocytes; yellow arrows: LepR^{+} SSPC-derived cementocytes. BM, bone marrow; CB, cortical bone; CM, cementum; AB, alveolar bone; PDL, periodontal ligament; DIC, differential interference contrast. Scale bar = 50 μm (A and B).

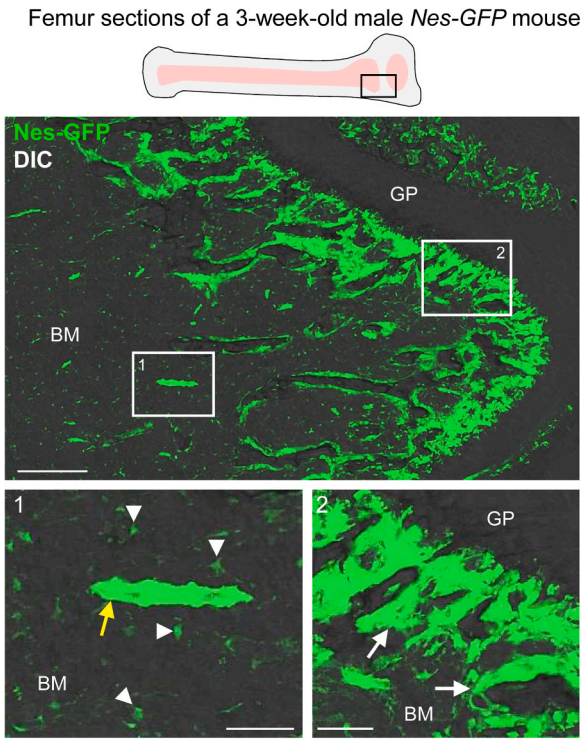

Fig. 3. Subpopulation of Nes-GFP^{+} cells in long bone detecting as Nes-GFP^{bright} and Nes-GFP^{dim}. Z-stack confocal images of thick femur sections from 3-week-old male Nes-GFP mice. The square in the upper schema indicates the area of the Z-stack confocal image. Numbered panels represent the magnified views of the boxed areas. Nes-GFP^{+} stromal cells were distinguished as Nes-GFP^{bright} or Nes-GFP^{dim} based on their GFP expression levels. Nes-GFP^{dim} stromal cells overlap with LepR^{+}/CAR cells. Nes-GFP^{bright} stromal cells were observed in the periarterial and metaphyseal regions. Periarterial Nes-GFP^{bright} cells are suggested to be HSC niche cells. The characteristics of Nes-GFP^{bright} stromal cells in the metaphyseal BM tissue remain unclear. White arrows: Metaphyseal Nes-GFP^{bright} stromal cells; white arrowheads: Nes-GFP^{dim} stromal cells; yellow arrows: Periarterial Nes-GFP^{bright} stromal cells. BM, bone marrow; GP, growth plate; DIC, differential interference contrast. Scale bar = 200 μm (upper panel), 50 μm (lower panels).

(NG2) and have been suggested to be HSC niche cells [61]. However, the characteristics of Nes-GFP^{bright} stromal cells in metaphyseal BM tissue remain unclear; therefore, further analysis is warranted to comprehensively understand BM stromal populations.

4.2. Differences in the characteristics of LepR^{+} SSPCs between growth and adult stages

Although LepR^{+} stromal cells demonstrate the capability of SSPCs, the presence of LepR^{+} SSPC-derived osteoblasts is rarely observed in neonatal bone tissue [57,58]. These observations imply that neonatal osteoblasts may originate from cell populations distinct from LepR^{+} SSPCs, indicating a developmental stage-dependent variation in the source of osteoblasts. Mizuhashi et al. [63] reported that growth plate resting zone chondrocytes, labeled with parathyroid hormone-related protein (PTHrP)-CreER^{T2}, serve as the source of osteoblasts in developing bone tissue. These resting zone chondrocytes undergo subsequent differentiation into proliferating and hypertrophic chondrocytes, ultimately migrating to the BM through osteoblastogenesis. Furthermore, Shu et al. [64] demonstrated that osteoblasts in the early postnatal period differentiate from growth plate chondrocytes labeled with Aggrecan-CreER^{T2}. However, their origin shifts to LepR^{+} SSPCs after adolescence, as determined using dual-recombinase fate-mapping systems. This technique involves labeling two cell fractions with different

fluorescent dyes, enabling the simultaneous tracing of their lineages. These results suggest that LepR$^+$ SSPCs predominantly contribute to bone remodeling with relatively modest bone formation rather than playing a significant role during the bone growth phase characterized by substantial bone formation. Consequently, LepR$^+$ SSPCs may collaboratively participate in bone remodeling alongside osteoblasts and osteoclasts in adulthood to old age. Additionally, the association between age-related loss of osteogenic potential and cellular senescence in LepR$^+$ SSPCs is intriguing; however, the details remain unknown and necessitate further investigation.

4.3. Regulatory mechanism of LepR$^+$ SSPC differentiation

Teriparatide, a biologically active amino acid 1–34 fragment of human PTH [hPTH (1−34)], demonstrates bone anabolic activity and is clinically utilized for osteoporosis treatment [65,66]. Teriparatide treatment significantly increases the number of mature osteoblasts in bone tissue, contributing to the mechanisms of bone anabolism [67]. Although one mechanism involves the induction of osteoblastic differentiation of SSPCs by teriparatide, the details remain unclear. Cell lineage tracing analysis revealed that teriparatide treatment expedites the differentiation of LepR$^+$ SSPCs into osteoblasts [68]. Conversely, teriparatide inhibits differentiation into adipocytes, another lineage of LepR$^+$ SSPCs [69]. Consistent with these findings, conditional deletion of the PTH/PTHrP receptor in mesenchymal cells throughout bone tissue reduced bone formation increased BM adipocytes [70]. This suggests that part of teriparatide's bone anabolic effect involves lineage switching from adipocytes to osteoblasts.

Does the LepR expressed in SSPCs influence the lineage differentiation? Deletion of LepR in mesenchymal cells throughout long bone tissue using Prx1-Cre; floxed-LepR mouse lines reportedly increased bone formation rate, elevated bone mass, and reduced adipocyte numbers when compared with those in the controls [71]. These findings indicate that peripheral leptin/LepR signaling in SSPCs negatively regulates osteoblastic differentiation and positively influences adipocytic differentiation. Similarly, leptin has been reported to inhibit osteoblast differentiation *via* the sympathetic nervous system [72,73]; however, the relationship between leptin signaling in peripheral and central pathways remains unclear. In contrast, bone mass in leptin-dysfunctional ob/ob mice is significantly reduced when compared with that in wild-type mice [74], and leptin reportedly promotes osteoblastogenesis in the periphery [75]; therefore, further investigations are required to determine the regulatory mechanisms of leptin signaling in lineage differentiation.

Additionally, certain transcription factors governing the lineage differentiation of LepR$^+$ SSPCs have been identified. Conditional deletion of forkhead box c1 (Foxc1) in LepR$^+$ SSPCs (LepR-Cre; floxed-Foxc1) resulted in increased numbers of BM adipocytes, indicating that Foxc1 negatively regulates adipocyte differentiation in LepR$^+$ SSPCs [60]. Conversely, the transcription factor early B-cell factor 3 (Ebf3) inhibits osteoblast differentiation from LepR$^+$ SSPCs [76]. In pathological conditions, LepR$^+$ SSPCs serve as the origin of myofibroblasts induced in primary myelofibrosis, a disorder of BM hematopoiesis [77], and runt-related transcription factors (Runx) 1 and 2 have been shown to suppress their differentiation [78].

The regulatory mechanism of the lineage differentiation of LepR$^+$ SSPCs into various cell types *in vivo* is gradually becoming clear at the molecular level. Further exploration of the signaling cascades and age-dependent epigenetic changes of these molecules is expected to enhance our understanding of the pathological mechanisms underlying bone regulation by LepR$^+$ SSPCs.

4.4. Heterogeneity of LepR$^+$ SSPCs

Single-cell RNA sequencing (scRNA-seq) revealed that long bone-derived LepR$^+$ SSPCs comprise heterogeneous cell populations with distinct gene profiles [79–83]. CAR cells/LepR$^+$ SSPCs were shown to be classified into "Adipo-CAR cells" and "Osteo-CAR cells" based on their genetic profiles [81,82]. These two populations have different origins in developmental cartilage primordia, with adipo-CAR cells derived from Distal-less homeobox 5 (Dlx5)$^+$ perichondrial cells and osteo-CAR cells derived from Fibroblast growth factor receptor 3 (Fgfr3)$^+$ chondrocytes [84]. The Fgfr3$^+$ cells are also detected in the endosteum and represent the origin of osteoblasts during young stages [85]. Interestingly, Adipo-CAR was localized only in the central long axis of the BM and contributed to osteoblasts in the trabecular bone but not in the cortical bone [82]. These findings provide an intriguing suggestion that the origin of osteoblasts differs between the cortical and trabecular tissues. Furthermore, a subpopulation of LepR$^+$ SSPCs was identified as an osteogenic growth factor, osteolectin$^+$, localized in the periarteriolar region [86]. Periarterial osteolectin$^+$ LepR$^+$ cells not only generate osteoblasts but also regulate bone volume and lymphocytic differentiation by sensing mechanical stress. Altogether, the BM cell population labeled with LepR-Cre is composed of heterogeneous subpopulations that can be distinguished by genetic profiling, each of which may cooperate to maintain bone and BM homeostasis.

4.5. LepR$^+$ SSPCs localized in PDL and AB

The PDL is a connective tissue required for the attachment of the tooth to the jawbone by penetrating the AB and cementum. PDL contains SSPCs that provide hard tissue-forming cells such as osteoblasts and cementoblasts [29]. Similarly, the marrow space of the AB contains specific SSPCs, which are known to have lower differentiation potential than chondrocytes and adipocytes and higher angiogenic potential than iliac BM-derived SSPCs [30,31]. It has been suggested that SSPCs contribute to jawbone maintenance; however, their *in vivo* dynamics remain unknown. Cell lineage tracing studies have identified LepR$^+$ cells in the PDL and AB marrow and demonstrated the contribution of their lineage to hard tissue maintenance.

LepR$^+$ cells were detected as tdTom$^+$ cells in the PDL of the LepR-Cre; R26-tdTom mice (Fig. 2B). The number of LepR-Cre-labeled PDL cells (LepR$^+$ PDL cells) increased with age and differentiated into cementocytes and AB-embedded osteocytes [87]. The contribution of LepR$^+$ cells to cementocytes has also been demonstrated using inducible LepR-CreER^{T2} mice [88]. Furthermore, a depletion of low-density lipoprotein receptor-related protein 1 (LRP1) in LepR$^+$ cells has been shown to reduce their osteoblastic differentiation and the AB mass [89]. However, the frequency of LepR$^+$ PDL cell-derived lineages in hard tissues was less than 20%, suggesting that cell populations other than LepR$^+$ PDL cells also provide hard tissue-forming cells in parallel [87]. By contrast, Zhang et al. detected LepR$^+$ cells in the AB marrow of LepR-Cre; R26-tdTom mice and showed that these cells differentiated into osteoblasts in response to tooth extraction and contributed to bone regeneration [90]. Furthermore, socket regenerative bone healing was delayed by the depletion of LepR$^+$ cells using the Cre/LoxP system. However, the frequency of LepR-Cre-labeled cell-derived osteocytes in the regenerative bone of the extraction sockets was extremely low (<9% of the total), suggesting that the LepR$^+$ cell lineage may play an essential role in bone regeneration in addition to providing bone-forming cells [87]. Alternatively, this finding suggests that in addition to LepR$^+$ cells in the PDL and AB, other SSPC populations may contribute to bone regeneration in the extraction socket.

In summary, LepR$^+$ cells localized in both the PDL and AB marrow may contribute to jawbone maintenance. However, differences in the characteristics of these cell populations are not well understood. Additionally, most cell lineage tracing analyses of the LepR$^+$ cell population described above have been performed using non-inducible LepR-Cre mice. In the future, it will be necessary to reanalyze the dynamics of LepR$^+$ SSPCs in both limbs and jawbones using LepR-CreER^{T2} mice [64,88,91].

5. Identification of SSPCs using Gli1-CreER^{T2}

Indian Hedgehog (Hh), one of the three Hh family proteins, is a key regulator of chondrocyte and osteoblast differentiation during endochondral bone development [92–95]. Hh binds to the 7-transmembrane receptor smoothened (smo) and regulates gene expression through the activation or repression of the transcription factor Gli1–3 [96]. Gli1 acts as its own transcriptional target downstream of Hh signaling and upregulates its expression [97,98]. Thus, cells that received Hh signals and contributed to bone development were identified as Gli1-expressing cells. Cell lineage tracing analyses using Gli1-CreER^{T2} knock-in mice [33] have contributed to our understanding of the *in vivo* dynamics of SSPCs, which are detected as Gli1^{+} cells in limb and oral/cranio-maxillofacial hard tissues.

5.1. Gli1^{+} SSPCs contributing to long bone growth

Shi et al. performed a lineage tracing analysis of Gli1^{+} cells using Gli1-CreER^{T2} mice and analyzed their roles in long bone growth [99]. Gli1^{+} cells were only observed just below the growth plate and differentiated into osteoblasts, adipocytes, and LepR^{+} SSPCs during bone growth, and therefore termed "metaphyseal mesenchymal progenitors (MMPs)." These MMPs may act as the origin of osteoblasts subsequent to PTHrP-CreER^{T2}-labeled resting zone chondrocytes during bone development [63]. Osteoblastic differentiation of MMPs was accelerated in response to treatment with teriparatide [100] as well as the aforementioned LepR^{+} SSPCs [68,69]. In addition, gene profiling analysis at the single-cell level revealed that MMPs are composed of four subpopulations characterized by chondrocyte-like osteoprogenitors (COP), preosteoblasts, osteoblasts, and BM adipogenic lineage progenitors. Among these four subpopulations, COP is hierarchically at the top of cell differentiation [100]. Importantly, MMPs are observed only during the growth stage and disappear during adulthood. This suggests that MMPs are SSPCs that specifically contribute to bone growth. These transient SSPC populations may enable the generation of more hard tissue-forming cells and respond to drastic bone formation during bone growth.

5.2. Gli1^{+} SSPCs in the periosteum of long bones

Both BM- and periosteal-derived SSPCs have been proposed to play a role in regenerating fractured limb bones [101,102]. However, the primary question is which populations are predominantly responsible for this healing process. In long bones, there are two pathways for fracture healing: intermembranous ossification, where SSPCs directly differentiate into osteoblasts, contributing to bone formation, and endochondral ossification, occurring through chondrogenic differentiation from SSPCs. The activation of these pathways depends on the degree of bone damage [57,82,101,103]. Drill hole-induced bone defects are mainly repaired through intermembranous ossification, while non-stabilized bicortical fractures induce endochondral ossification, resulting in the formation of a cartilaginous callus outside the bone, replaced by a bony callus and eventually regenerating cortical bone. Although SSPC dynamics during the healing process have been controversial, studies using a Cre/loxP-based strategy have revealed that BM- or periosteum-derived SSPCs are selectively activated depending on the fracture type [104]. Periosteal SSPCs can be specifically labeled using Gli1-CreER^{T2} mice during adulthood [99,104]. In contrast, adiponectin (Adipoq)-Cre-labeled cells overlap specifically with LepR^{+} SSPCs in the BM but not in the periosteum. Chondrocytes and osteoblasts in the fracture callus induced by bicortical fractures originate from periosteal SSPCs, not SSPCs in the BM. Conversely, BM-derived SSPCs, not periosteal SSPCs, contribute to new trabeculae formation within the BM at the fracture site. Similarly, only BM-derived SSPCs contribute to repairing BM trabeculae after drill injuries. Notably, the origins of osteocytes in repaired cortical bone are periosteal SSPCs in bicortical fractures and BM-derived SSPCs in drill injuries. Additionally, muscle-derived mesenchymal progenitors labeled by Prx1-Cre are suggested to contribute to chondrocytes and osteoblasts in the fracture callus [105].

In summary, various types of SSPCs near the injured site during the fracture healing process sense the extent of damage and flexibly respond to bone healing. It is suggested that SSPCs possess a sensing system for tissue injury and are responsible for appropriate regeneration; however, future studies are required to clarify the details of this mechanism.

5.3. Gli1^{+} SSPCs in the suture regulating the craniofacial bone development

Unlike long bones, which form through endochondral ossification, craniofacial bones are flat bone tissues formed primarily by intermembranous ossification [106]. The cranial bone is composed of flat, dish-shaped bones joined together; these joints are called sutures, which serve as growth centers for intermembranous ossification. Calvarial sutures in humans fuse as they grow; however, in craniosynostosis, the sutures fuse prematurely in infancy. This disease causes delayed brain development owing to abnormal skull growth, resulting in mental retardation, learning disabilities, and cognitive impairment, which significantly reduce the quality of life of the patients [107,108]. It has been suggested that sutures contain SSPC populations that regulate skull growth by osteoblast generation at the osteogenic front, and the disruption of this system leads to the development of craniosynostosis [32]. Although the characteristics of SSPCs in the sutures have long been unclear, Zhao et al. identified SSPCs as Gli1^{+} cells in Gli1-CreER^{T2} mice [109]. Importantly, the depletion of Gli1^{+} SSPCs by the Cre/loxP system induced suture fusion, indicating that Gli1^{+} SSPCs suppress synostosis. Consistent with this finding, the number of suture-derived Gli1^{+} SSPCs was significantly reduced in Twist1 heterozygous deficient mice, a mouse model of Saethre–Chotzen syndrome that presents with craniosynostosis. Furthermore, a therapeutic strategy for craniosynostosis was proposed, showing that cranial suture reconstruction in a craniosynostosis mouse model could be induced by the transplantation of suture-derived Gli1^{+} SSPCs [110]. This reconstructed suture improved the cranial deformities, resulting in mitigated delayed brain development and neurocognitive abnormalities. These findings suggest that Gli1^{+} SSPCs may play a role in maintaining suture homeostasis. Future studies should elucidate their regulatory mechanisms as niche cells and lead to fundamental therapeutic strategies for craniosynostosis.

5.4. Gli1^{+} SSPCs localized in DP, PDL, and AB

Although the presence of SSPC populations in the DP has long been suggested, their *in vivo* dynamics remain unknown [22–24]. As mouse incisors grow continuously throughout life, pulpal SSPCs in the incisors have been assumed to permanently give rise to lineage cells, including odontoblasts, which form dentin tissue. Using Gli1-CreER^{T2} mice, Zhao et al. showed that Gli1^{+} cells detected in the dental mesenchyme around the cervical loop of incisors, which acted as SSPCs that provided the entire pulp mesenchyme to sustain incisor growth [111]. The sensory nerve in the neurovascular bundle (NVB) acts as a microenvironmental niche supporting Gli1^{+} SSPCs by expressing sonic hedgehog (Shh).

However, some studies have reported that Gli1^{+} cells in the PDL around the molars also act as SSPCs [112–117]. PDL-derived Gli1^{+} SSPCs are mainly localized adjacent to the NVB of the root apex and differentiate into osteoblasts, cementoblasts, and PDL fibroblasts to maintain jawbone homeostasis [113–115]. In this process, the activation of Wnt/β-catenin signaling is required for the differentiation of Gli1^{+} SSPCs into progeny cells [113,114]. Sclerostin is secreted by osteocytes [118], and acts as a Wnt inhibitor by directly binding to the Wnt receptor and LRP5/6 [119–122]. Osteocyte-derived sclerostin expression decreases in response to mechanical loading but increases following release of mechanical stress [123]. Consistently, the release of occlusion force-derived mechanical stress increases the expression of sclerostin in

cementocytes and osteocytes, reducing the lineage differentiation of Gli1$^+$ SSPCs and decreasing cementum and AB mass [113]. Conversely, sclerostin levels have been reported to be reduced on the orthodontic traction side [124], suggesting that Wnt/β-catenin signaling is activated there. Consistent with this finding, osteoblastic differentiation of PDL-derived Gli1$^+$ SSPCs was induced on the traction side during orthodontic tooth movement [117]. Furthermore, Gli1$^+$ SSPCs in the PDL contribute to bone regeneration after tooth extraction [116]. Meanwhile, perivascular Gli1$^+$ SSPCs have also been detected in AB marrow tissue which contributes to extraction socket repair and implant osseointegration in a Wnt/β-catenin signal-dependent manner [125].

Altogether, Gli1$^+$ SSPCs in the jawbone are localized in the DP, PDL, and AB marrow, and they share a similar localization in the vicinity of blood vessels [111,113,125]. Differences in their characteristics remain unclear, and future studies are required to clarify this question.

6. Identification of SSPCs using Axin2-CreER^{T2}

Wnt signaling is an indispensable pathway in bone development [126]. The canonical Wnt signaling is triggered upon the binding of Wnt ligands to the co-receptor, LRP5/6, and frizzled (FZD), leading to β-catenin stabilization. In contrast, in the absence of Wnt ligands, β-catenin is phosphorylated by the multiprotein destruction complex and degraded by the ubiquitin-proteasome, resulting in the signal being turned off. Axin2, a multiprotein destruction complex, is upregulated downstream of the canonical Wnt signaling and creates a negative feedback loop [127,128]. Based on this mechanism, lineage tracing analysis of Wnt signaling-activated cells (Wnt-responsive cells) has been performed using Axin2-CreER^{T2} knock-in mice, and their stemness has been confirmed in various tissues [35,129–131].

6.1. *Axin2$^+$ SSPCs contributing to long bone growth*

To investigate the dynamics of Wnt-responsive cells (Axin2$^+$ cells) during long bone growth, cell lineage tracing was performed using Axin2-CreER^{T2} mice [132]. In the early neonatal stage, Axin2$^+$ cells were primarily found as BM stromal cells; however, Axin2$^+$ cells were not detected in osteoblasts or osteocytes. Subsequently, Axin2$^+$ stromal cells differentiate into bone-forming cells with age, and their lineage contributes to bone healing; neonatal BM-derived Axin2$^+$ cells are considered SSPCs. Mechanistically, Axin2$^+$ SSPCs activate Wnt signaling in an autocrine manner *via* the secretion of Wnt ligands, leading to proliferation and osteoblast differentiation. Similarly, Axin2$^+$ cells have been found in the periosteum of long bones as SSPCs, which contribute to bone repair [133]. Additionally, Axin2$^+$ cells are detected in the outermost layer of the growth plate in the early neonatal stage, and these cells have been shown to contribute to the lateral growth of cartilage by differentiating into chondrocytes in the growth plate [134]. These Axin2$^+$ cartilage progenitors express Wnt, which activates their own canonical Wnt signaling in an autocrine manner, similar to neonatal BM-derived Axin2$^+$ SSPCs [132]. As the number of Axin2$^+$ cells in the outermost layer of the growth plate decreases with age [134], it is conceivable that Axin2$^+$ cells supporting bone development in the perichondrium and BM may reduce their own Wnt expression and complete the bone growth when they transition to the adult stage.

6.2. *Axin2$^+$ SSPCs in the suture regulating the craniofacial bone development*

Maruyama et al. generated Axin2-rtTA (reverse tetracycline transactivator); TRE (tetracycline response element)-Cre; R26-LacZ mice that expressed LacZ in Axin2$^+$ cells in response to doxycycline administration and identified cranial suture-derived Axin2$^+$ SSPCs [135]. Axin2$^+$ cells at postnatal day 28 localize to the center of the suture in a quiescent state and differentiate into sutured mesenchymal cells, calvarial osteoblasts, and osteocytes with aging. In addition, Axin2$^+$ SSPCs migrated to the injury site and contributed to bone healing in response to calvarial bone injury. Furthermore, the deletion of Bmpr1a in Axin2$^+$ SSPCs causes craniosynostosis because of decreased Axin2$^+$ SSPCs and increased osteoblasts, suggesting that the dynamics of Axin2$^+$ SSPCs in sutures are related to the pathogenesis of craniosynostosis [136]. Importantly, among the sutural mesenchymal cells, the Axin2$^+$ population had significantly higher expression of Gli1 and LepR, which are known markers of SSPCs [135]. Further studies are needed to clarify the relationship between these marker-positive populations in the suture.

6.3. *Axin2$^+$ SSPCs in the pulp of molars*

Wnt signaling pathways significantly contribute to tissue regeneration processes regulated by tissue-resident stem cells [137–139]. Studies have suggested that Wnt signaling positively regulates damaged dentin regeneration mediated by SSPCs in the DP [140–144]. As described above, odontoblasts are continuously supplied by SSPCs in rodent incisors. However, SSPCs in the molar pulp differentiate into odontoblast-like cells only when dentin is damaged and contribute to dentin healing. Hunter et al. [140] demonstrated that reparative dentin formation is induced in response to molar damage with pulp exposure and that this healing process is enhanced in Axin2 knockout mice, wherein canonical Wnt signaling is activated. Furthermore, treatment with Wnt3a accelerates the formation of both reparative dentin [140] and reactionary dentin (repairing dentin induced without pulp exposure) [144] in rodents. Tideglusib is a small molecule that activates canonical Wnt signaling by inhibiting glycogen synthase kinase-3 beta (GSK-3β), a key component of Wnt/β-catenin signal transduction [145]. To date, the clinical application of tideglusib has been attempted for the treatment of progressive supranuclear palsy, congenital/juvenile-onset myotonic muscular dystrophy, and Alzheimer's disease [146–149]. Neves et al. reported that tideglusib treatment of damaged dentin promoted reparative [142] and reactionary dentin formation [143]. In addition, Wnt-responsive pulpal SSPCs in the molars were detected as Axin2$^+$ cells in Axin2-CreER^{T2} mice [141]. These Axin2$^+$ SSPCs differentiate into odontoblast-like cells in response to dental damage by activating their own Wnt signaling pathway in an autocrine manner and contribute to reparative dentin formation. In addition to Axin2$^+$ populations, alpha-smooth muscle actin (αSMA)-CreER^{T2} labeling is recommended for pulpal SSPCs [150–152]. Furthermore, the cell-rich zones adjacent to the odontoblast layer detected with Nes-GFP have been suggested to be fractions that contribute to dentin repair in molars [153–157]. It has been suggested that there is a hierarchical relationship between these pulpal cell populations; however, further analyses are needed to answer this question.

6.4. *Axin2$^+$ SSPCs localized in PDL*

Several research groups have detected Axin2$^+$ cells in the PDL using Axin2-CreER^{T2} mice and demonstrated their ability to contribute to hard tissue homeostasis. Yuan et al. showed that (1) the PDL-derived Axin2$^+$ cells are quiescent in the steady state but proliferate in response to tooth extraction and differentiate into osteoblasts that contribute to socket healing [158], and (2) during the process of immediate post-extraction implantation, the PDL-derived Axin2$^+$ cells are responsible for osseointegration [159]. Additionally, Axin2$^+$ cells in the PDL have been shown to sense occlusal hyperloading of teeth and contribute to PDL reorganization *via* canonical Wnt signaling [160,161]. It was reported that Axin2$^+$ cells in the PDL differentiate into cementoblasts, contribute to root cementum growth [162], and differentiate into osteoblasts and osteocytes on the traction side of the orthodontic treatment [163]. Zhao et al. [164] suggested that during postnatal root formation, both CD90$^+$ perivascular cells and Axin2$^+$ cells in the PDL give rise to cementoblasts. However, in adult tissues, their sources are limited to Axin2$^+$ cells. In contrast, in periodontal disease, the origin of cementoblasts shifted from Axin2$^+$ cells to CD90$^+$ cells. Taken together, these studies suggest that

the PDL contains diverse origins of hard tissue-forming cells, each of which contributing to the maintenance of dental tissue at the same or different appropriate time points.

7. Conclusions

This review provides an overview of the SSPC population involved in hard tissue formation in both long bones and the oral/craniomaxillofacial area. It emphasizes the insights gained from employing the Cre/loxP-based cell lineage tracing strategy with LepR-Cre, Gli1-CreER^{T2}, and Axin2-CreER^{T2} mice. In addition to these mouse models, there are useful genetically modified mice that mark the SSPC population. PDL-derived SSPCs have been identified using αSMA-CreER^{T2} [165] and periodontal ligament associated protein-1 (Plap-1)-CreER^{T2} [166]. Alternatively, long bone periosteum SSPCs can be labeled with Cathepsin K (CTSK)-Cre [167]. Notably, the CTSK-Cre and discoidin domain-containing receptor 2-CreER^{T2} labels an independent SSPC population in the calvarial suture [168]. Herein, we focused on DP, PDL, and calvarial suture-derived SSPCs in the oral/maxillofacial region; however, temporomandibular joint-localized SSPC is also an area of interest in this research field. Labeling of the SSPCs localized in the mandibular condylar bone with Sox9-CreER^{T2} has been suggested, and they potentially contribute to jawbone regeneration [169]. These developed cell labeling techniques facilitate an in-depth exploration of cellular dynamics *in vivo*, ultimately revealing the diverse origins of hard tissue-forming cells.

The discussion on the necessity of diverse origins for hard tissue maintenance delves into the intricacies of bone formation processes in different *in vivo* contexts. For instance, rapid bone growth necessitates continuous osteoblast supplementation and activation, while bone tissue in the adult stage relies on remodeling with relatively modest bone formation. Conversely, damaged bone tissues prompt the reactivation of osteoblast supplementation to achieve swift bone healing. To adapt to these varying bone dynamics *in vivo*, the origin and supply system of osteoblasts may be dynamically altered based on the specific hard tissue requirements. Future research endeavors are anticipated to provide further clarity on these adaptive mechanisms. The review speculates on age-related bone loss, proposing that a disruption in the supply system of hard tissue-forming cells, potentially caused by the senescence of SSPCs, could be a contributing factor. Further studies are expected to unveil the intricate regulatory mechanisms governing the supply systems of hard tissue-forming cells responding to diverse tissue environments. The overarching goal is to pave the way for artificially controlling SSPCs, enabling effective bone tissue therapy in clinical settings.

Conflict of interest

The author declares no competing interests.

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