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Review Article

Identification and function of periosteal skeletal stem cells in skeletal development, homeostasis, and disease

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ABSTRACT

Background: Periosteum-resident skeletal stem cells (SSCs) are essential for the growth, maintenance, and repair of the skeletal system. These cells exhibit self-renewal ability and clonal pluripotency. Compared to the diverse bone marrow mesenchymal stem cells (BMSCs), periosteal skeletal stem cells (P-SSCs) represent a purified stem cell population and are preferable for bone tissue engineering.
Methods: This review covers the histological structure of the periosteum, process of isolating and characterising P-SSCs, and spatiotemporal distribution and characteristics of P-SSCs from different lineages. Additionally, the roles of P-SSCs in bone injury, disease, and periosteal niche regulation are discussed.
Results: Intramembrane and intraconal ossification of P-SSCs exhibits favourable therapeutic potential. Osteogenesis using P-SSCs is an ideal process for bone repair.
Conclusions: P-SSCs are vital for bone formation, maintenance, and repair. P-SSCs are essential components of the periosteal microenvironment. Therefore, it is essential to investigate their critical clinical applications and translational functions. By targeting and inducing endogenous stem cells, the in situ repair of bone defects can be facilitated, leading to the development of more effective novel therapies.
The translational potential of this article: To enhance our understanding of the function of P-SSCs in bone repair and skeleton-related diseases, it is imperative to elucidate the current research status of P-SSCs and ascertain the prospective trajectory for their advancement and refinement in bone tissue engineering. P-SSCs are expected to play an expanded role in treating bone abnormalities, leading to the optimisation of bone tissue treatment.

1. Introduction

Recently, the number of skeletal tissue injuries has increased significantly owing to various factors. Consequently, in situ bone formation, or bone tissue engineering, has emerged as an effective option to compensate for tissue defects [1,2]. Traditional bone tissue engineering methods show many areas for improvement, mainly regarding bioactive materials, cells, and biological factors [3–5]. Therefore, targeted induction of endogenous skeletal stem cells (SSCs) is a potential alternative therapy for in situ bone regeneration [6,7]. This approach fully utilises the regenerative capacity of cells without relying on exogenous cells [8,9].

The periosteum, located in the outer layer of the cortical bone, contains a rich supply of endogenous cytokines and stem cells. Therefore, they are highly promising for bone regeneration [10,11]. Periosteal skeletal stem cells (P-SSCs) are a diverse group of mesenchymal cells with stemness characteristics that reside in the periosteal tissue [12].

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Abbreviations:

αSMA	α-smooth muscle actin
BMSC	bone marrow mesenchymal stem cell
BMP	bone morphogenetic protein
CCL5	C–C motif chemokine ligand 5
CCR5	C–C motif chemokine receptor 5
CGRP	calcitonin gene-related peptide;
CFU-F,	Colony-forming unit-fibroblast
CREB1	cAMP responsive element binding protein 1
CPT	Congenital pseudarthrosis of the tibia
Ctsk	cathepsin K
HNSCC	head and neck squamous cell carcinomas
Hox	Homology box
IHH	Indian hedgehog
Itm2a	integral membrane protein 2A
Lkb1	liver kinase b1
miRNA	microRNA
MSCs	mesenchymal stem cells
mTORC1	mammalian target of rapamycin complex 1
NGF	nerve growth factor
Osx	osterix
PDGFRβ	platelet-derived growth factor receptor β
P-SSC	periosteal skeletal stem cell
Postn	periostin
Ptpn11	protein tyrosine phosphatase, non-receptor type 11
Runx2	runt-related transcription factor
SFRP4	Secreted frizzled receptor protein 4
scRNA-seq	single-cell RNA sequencing
SSC	skeletal stem cell
SSC-SimulTracer	SSC simultaneous tracing system
TIMP1	tissue inhibitor of metalloproteinase 1
TrkA	tropomyosin receptor kinase
A	ZNF, zinc-finger protein

They have the capacity for self-renewal and potential to differentiate into several lineages. Previous studies on mesenchymal stem cells (MSCs) and SSCs have primarily focused on bone marrow. However, studies on stem cell subsets in the periosteum are limited. Recent advancements in lineage tracing, flow cytometry, and single-cell RNA sequencing (scRNA-seq) have enabled scientists to identify specific stem cell populations in periosteal regions. By isolating and expanding these populations *in vitro* using particular markers, researchers can visualise them *in vivo* and study their properties simultaneously.

However, our understanding of P-SSCs is still lacking. Additional research is required to ascertain the exact functions of these cells in bone formation and development, as well as their involvement in the local microenvironment, bone damage healing, bone-related illnesses, and governing regulatory processes. This review focuses on the novel information regarding the origin and histological structure of the periosteum, the definition and biological characterisation of P-SSCs, and the spatiotemporal distribution patterns of several subpopulations of P-SSCs. In addition, we discuss their essential roles in bone injury repair and bone-related diseases. Finally, we discuss the role of periosteal ecological niches in the maintenance of P-SSCs. This review can serve as a valuable reference for the development of targeted cell therapies for bone diseases.

2. Histologic features of the periosteum

The periosteum is a bilayered connective tissue that envelops the surface of the cortical bone, excluding the joints, tendon attachments, and seed bones (Fig. 1) [13]. It is highly vascularised and serves as a transition zone between the skeletal muscle and bone tissue. The structure of the periosteum varies depending on the species, age, and anatomical position [14,15]. Typically, researchers divide the periosteum into superficial outer and deeper inner fibrous layers [13,16]. These layers are indistinguishable under a light microscope.

The outer fibrous layer is thicker and mainly composed of collagen fibres, elastic fibres, and fibroblasts [17,18]. It penetrates deep into the bone matrix, using Sharpey's fibres to firmly fix the periosteum to the cortical bone. The primary function of these fibres is to support and protect the periosteum. The inner cambium layer adjacent to the bone surface contains fewer fibres and sparse tissue but harbours many cell types, including P-SSCs, osteoprogenitor cells, osteoblasts, pericytes, and fibroblasts [5,19]. This layer has the remarkable ability to induce osteogenesis. The skeletal system undergoes dynamic maintenance

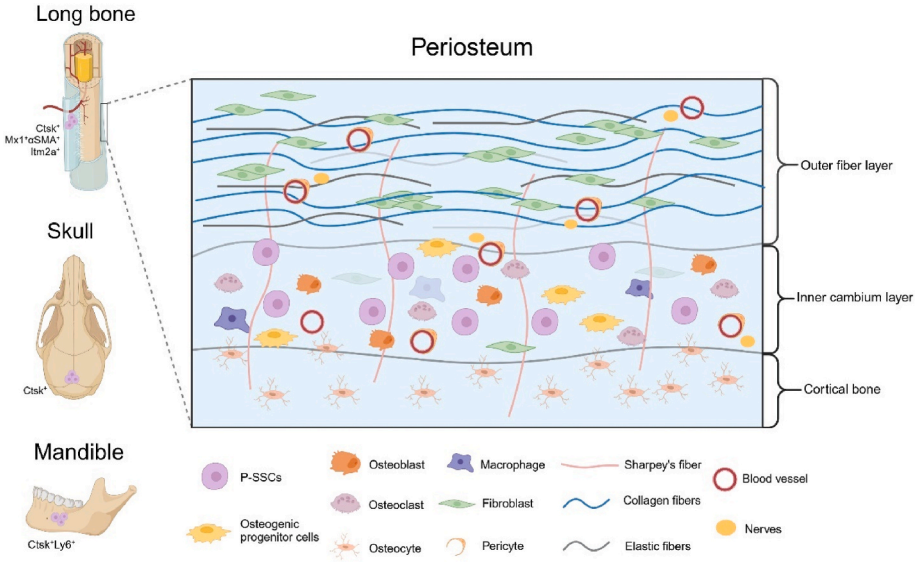

Fig. 1. Histological structure of the periosteum. The periosteum is divided into the outer fibrous layer and the inner cambium layer adjacent to the cortical bone. (Created with BioRender.com.)

during adulthood, with the endosteal layer gradually thinning and periosteal stem and progenitor cells changing from an aligned to a dispersed distribution. However, they still possess a robust capacity to maintain bone equilibrium and regeneration. Furthermore, the periosteum contains abundant microcirculatory systems and is densely innervated by sensory and sympathetic nerves [20]. The bilayer structure of the periosteum is strongly linked to multiple possible activities, including creating ecological niches for multipotent cells and molecular components that control cellular responses [21]. Therefore, maintaining the structural integrity of the periosteum and utilising it for bone healing is crucial for preserving bone homeostasis and facilitating bone repair.

Overall, the periosteum, specifically its inner layer, is a substantial reservoir of SSCs and indispensable for the development, growth, and maintenance of skeletal tissue.

3. Definition and biological characterisation of P-SSCs

3.1. Definition of P-SSCs

Bone homeostasis is maintained through the concerted efforts of osteoblasts, which are involved in bone formation, and osteoclasts, tasked with bone resorption [22]. Osteoclasts are derived from haematopoietic stem cells or yolk sac progenitor cells, whereas osteoblasts, traditionally thought to be solely responsible for bone formation, are derived from MSCs [23,24]. However, MSCs exhibit marked heterogeneity, originating from various tissues, and have yet to undergo fractionation to identify subpopulations with definitive stem cell attributes, such as self-renewal and *in vivo* pluripotency [25,26]. Consequently, Bianco and Robey introduced the term "skeletal stem cells" (SSCs) to denote cells found within skeletal tissues that possess the capacity for self-renewal and differentiation into multiple cell types, including cartilage, bone, haematopoietic-supporting stroma, and bone marrow adipocytes [27]. This terminology was adopted to delineate specific subpopulations with osteogenic potential, distinguishing them from earlier research on MSCs. Chan et al. identified a population of SSCs at the apex of differentiation in mice using a combination of cell surface markers ($CD45^-Ter119^-Tie2^-CD51^+Thy1^-6C3^-CD105^-$) [28]. Subsequently, in 2018, they identified similarly characterised SSC subpopulations in human bone tissue using the same combination of markers [29]. Their efforts have established pivotal templates for contemporary SSC research.

Furthermore, recent investigations have revealed that SSCs do not constitute a homogeneous population; rather, they encompass a spectrum of related yet distinct cell types that function as stem cells across various skeletal sites and stages of bone development [30–32]. This diversity among SSC types underpins the myriad characteristics observed during bone ontogeny. The advent of advanced technologies, including flow cytometry, lineage tracing, and scRNA-seq, has significantly facilitated the tracking and characterisation of SSCs across different skeletal regions and developmental stages [33,34]. Recent studies have identified physiological precursors of periosteal osteoblasts within the periosteum of murine long bones and skulls, referred to as P-SSCs [12]. These cells exhibit clonal multipotency and self-renewal capabilities, positioning them at the highest tier within the differentiation hierarchy. Consequently, they are indispensable for the generation of the bone's outer layer, cortical thickening, and fracture healing.

It is noteworthy that P-SSCs, as a subset within the realm of SSCs research, have a relatively nascent history. Early studies primarily concentrated on the overarching characteristics and functions of stem cells. However, as research has progressed and technological advancements have emerged, the examination of P-SSCs has gained increasing attention in recent years, resulting in a series of significant breakthroughs.

3.2. Research methods for isolation and characterisation of P-SSCs

The methods currently employed for the isolation and identification of P-SSCs are primarily based on the protocols defined by Chan and colleagues for mouse and human SSCs [28,29]. The core focus of this research is to characterise the stemness properties of P-SSCs and elucidate their differentiation trajectories. Subsequently, this information will be integrated to gain a deeper understanding of the changes and roles of P-SSCs during skeletal development and in the context of skeletal diseases. Additionally, the study aims to identify the key factors influencing the differentiation and self-renewal of these P-SSCs (see Table 1).

Complete removal of the periosteum is crucial for obtaining periosteum-derived cells. Using a periosteal stripper or similar surgical instrument is the best method to secure the periosteum [35]. Periosteal-derived cells are obtained via enzymatic digestion or tissue mass culture. Isolated P-SSCs can adhere to plastic culture dishes and proliferate in media containing foetal bovine serum and antibiotics (Fig. 2A) [36,37]. Continuous spheroplastisation of P-SSCs in culture provides solid evidence for their clonogenicity and self-renewal ability *in vitro* (Fig. 2B). Moreover, by adding differentiation cocktails to the culture medium, P-SSCs predominantly differentiate into mature osteoblasts, adipocytes, and chondrocytes *in vitro*. Serial transplantation experiments conducted *in vivo* have also further validated the ability of P-SSCs to continuously renew and re-differentiate into different cell populations (Fig. 2C) [12,38,39]. This is considered the gold standard for defining stem cells and demonstrates that P-SSCs represent a genuine stem cell-like population. Examination of cell surface markers via scRNA-seq and flow cytometry revealed that P-SSCs are devoid of haematopoietic and endothelial cell markers but express conventional mesenchymal markers, including CD29, CD51, CD105, CD90, and Scal-1 in mice, and CD73, CD90, CD146, CD105, and CD166 in humans (Fig. 2D) [38,40–42]. P-SSCs have been shown to exhibit pluripotency *in vitro*, having the ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages. Debnath et al. revealed that, unlike BMSCs (which are involved in endochondral ossification), P-SSCs directly differentiate into osteoblasts through an intramembranous pathway under normal physiological conditions *in vivo*, playing a pivotal role in bone formation and maintenance [12]. However, under pathological conditions, P-SSCs acquire an endochondral osteogenic capacity after periosteal damage through endochondral ossification and participate in fracture healing and repair [12,38,43,44].

4. Distinct distribution and identity of P-SSC subpopulation

P-SSCs have been primarily investigated in transgenic mouse models using spectral tracer technology. From this data, different P-SSC subpopulations have been identified that exhibit comparable traits, although they are not identical and do not fully coincide with each other in space or time (Table 2). This highlights the diversity of P-SSCs. Thus, to accurately define and characterise P-SSCs, it is imperative to use a combination of several markers for labelling.

4.1. Distribution and characteristics of Ctsk $^+$ P-SSCs

For several decades, it was believed that osteoclasts express Ctsk, a cysteine protease [47–49]. Recently, Ctsk was found to mark MSCs in the perichondrial groove of Ranvier cartilage [50]. This indicates that Ctsk serves as a marker for osteoclasts and plays a role in osteoblast lineage cells. Debnath et al. previously conducted a study using *Ctsk-Cre; mtmg* fluorescent reporter mice to identify a group of mesenchymal cells in the periosteum of mouse long bones and skulls [12]. These mesenchymal cells, labelled with Ctsk-Cre, can be divided into three subsets: P-SSCs ($CD200^+$ $CD105^-$), periosteal progenitor 1 (PP1) ($CD200^-$ $CD105^-$), and periosteal progenitor 2 (PP2) ($CD105^+$ CD200variable). Among them, only the $Ctsk^+$ P-SSCs exhibit true 'stemness' and possess

Table 1
Methods for isolation and identification of P-SSCs.

Method	Application	Advantages	limitations	References
Flow cytometry	Isolation and collection cells	Constructing a hierarchy of differentiation to determine which cell type is stem cell	Requires specific cell surface markers; may not capture all subpopulations	[34]
Lineage tracing	Cre-LoxP systems	Tracking the transformation and fate of P-SSCs	Limited by the availability of Cre-recombinase driver lines; may not capture all cell divisions or fate decisions	[33]
In vitro	Colony-forming unit-fibroblast (CFU-F)	Assess proliferative capacity	May not reflect *in vivo* behavior; limited to a two-dimensional environment	[27]
	Tri-lineage differentiation assays	Assess the multilineage differentiation potential of P-SSCs	Single *in vitro* culture environment, not a substitute for *in vivo* environment	[34]
In vivo	Serial transplantation	Flow cytometry combined with *in vivo* transplantation serves as the current gold standard for evaluating the differentiation potential of P-SSCs	Requires surgical procedures; time-consuming and labor-intensive; may be influenced by the host environment	[12]
ScRNA-seq	—	To investigate cellular heterogeneity, differentiation trajectories and cellular transcriptome analyses	Technically demanding, dependent on cell state, high cost, requires experimental support	[12,45]

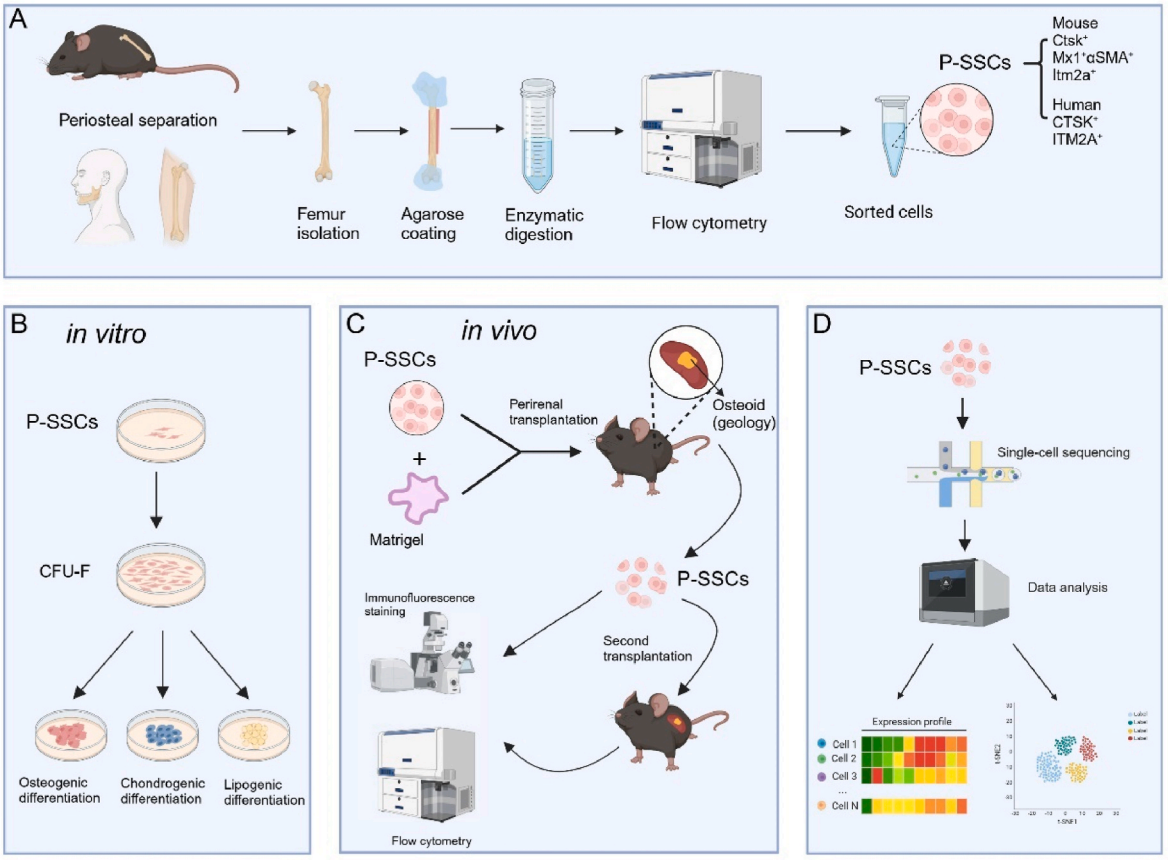

Fig. 2. Methods for isolation and characterisation of P-SSCs. (A) P-SSCs were isolated from periosteal tissue through mortar and pestle grinding and digestive enzyme dissociation, followed by sorting using flow cytometry. (B) Isolated P-SSCs exhibit proliferation and a trilineage differentiation capacity *in vitro*. (C) Successive *in vivo* transplantation of isolated P-SSCs demonstrates stemness. (D) Flowchart of single-cell sequencing and data analysis of isolated P-SSCs. (Created with BioRender.com.)

the capacity to differentiate into PP1 and PP2, positioning them at the apex of the differentiation hierarchy. Ctsk$^+$ P-SSCs were initially observed in the perichondrium of the long bones at E14.5 and continued to be present at the postnatal stages P7, P15, and P32. Excluding the long bones, Ctsk$^+$ P-SSCs were mainly observed in the sutures of the skull at P15 and P32 in mice. As animals mature, these cells migrate from the sutures to the cranial periosteum. Another study conducted by Debnath et al. revealed that Ctsk$^+$ P-SSCs exhibit the unique ability to differentiate into osteoblasts directly through an intramembranous pathway rather than the typical endochondral ossification process observed in other SSCs. These findings establish the cellular foundation for bone formation and maintenance under physiological conditions.

By tagging them with *Ctsk-Cre,* P-SSCs were shown to play a crucial role in maintaining the structure of the postnatal growth plate and promoting the extension of longitudinal bones through the secretion of Indian hedgehog (IHH) [51]. While IHH generated by postnatal P-SSCs promotes SSC multiplication in the growth plate resting zone, IHH produced by the developing growth plate gradually diminishes with age, indicating a potential communication between the periosteal and growth plate stem cells.

4.2. *Distribution and characteristics of Mx1$^+$αSMA$^+$ P-SSCs*

α-SMA, encoded by the *ACTA2* gene, is a key cytoskeletal

Table 2
Distinct distribution and identity of P-SSC subpopulation.

Gene	Transgenes	Location	Features	References
Ctsk$^+$	*Ctsk-Cre; mtmg*	Long bone and calvarial periosteum	Clonal pluripotency and self-renewal and located at the apex of the hierarchy of differentiation; Direct intramembranous route to osteogenesis.	[12]
Mx1$^+$αSMA$^+$	*Mx1-Cre; Rosa26-Tomato;α SMA-GFP*	Long bone and calvarial periosteum	Mx1 and αSMA combination selectively labels P-SSCs; Specific expression of CCL5 receptor CCR5; CCL5 induces Mx1$^+$αSMA$^+$ P-SSC migration *in vivo*, and its loss delays bone healing.	[46]
Itm2a$^+$	*Itm2a-CreER*; *Itm2a-DreER*	Long bone	Membrane protein ITM2A as a marker that labels P-SSCs; Itm2a$^+$ cells were mainly distributed in the outer fibrous layer of the periosteum; Contributed about 37 % of chondrocytes in the fracture healing tissue.	[45]

CCL5, C–C motif chemokine ligand 5; CCR5, C–C motif chemokine receptor 5; Ctsk, cathepsin K; Itm2a, integral membrane protein 2A; αSMA, α-smooth muscle actin; P-SSC, periosteal skeletal stem cells.

component. It is commonly used as an indicator of pericytes and myofibroblasts [52]. Matthews et al. discovered that *αSMA-CreERT2* could be used to identify periosteal progenitor cells in mouse long bones; these cells play a role in repairing bone injuries because of their ability to generate bone and cartilage [53]. Nevertheless, a significant drawback of such monogenic lineage-tracing methods involves considerable variability within the identified cell population and the inability to differentiate tagged SSCs from their offspring. Thus, Ortinau et al. conducted a study where they bred Mx1-Cre Rosa26 tomato mice with an αSMA-GFP reporter mouse line [46]. They then used polycytidic acid to activate *Mx1-Cre* expression consistently. This allowed them to identify a specific group of mesenchymal cells labelled with *Mx1* and *αSMA* in the periosteum and suture line of the mouse tibia. Subsequent investigations revealed that the majority of Mx1$^+$αSMA $^+$ periosteal cells exhibited key characteristics resembling those of SSCs, including long-term regeneration and differentiation within a living organism.

The current understanding of the extent of overlap between Ctsk$^+$ P-SSCs and Mx1$^+$αSMA$^+$ P-SSCs is incomplete. While a subset of αSMA$^+$ cells may be present in developing Ctsk$^+$ periosteal cells produced by mice, Ctsk$^+$ periosteal cells lack expression of *LepR* and *CD140α*, traits found in Mx1$^+$αSMA$^+$ cells [12,46]. Hence, the specific process that accounts for the disparity in expression between the two requires further elucidation.

4.3. Distribution and characteristics of Itm2a$^+$ P-SSCs

Integral membrane protein 2A (ITM2A), a 263-amino acid protein with a single transmembrane domain, acts as a thymocyte development activation marker [54,55]. Previously, ITM2A could be obtained through subtractive hybridisation from a complementary DNA (cDNA) library derived from in vitro-cultured mouse mandibular condyles, and its expression has been detected in osteogenic tissues [54].

Recently, Zou et al. utilized single-cell transcriptomics to identify membrane protein ITM2A as a marker for P-SSCs for the first time [45]. They validated the stemness of Itm2a$^+$ P-SSCs through a series of rigorous *in vivo* and *in vitro* experiments, demonstrating that this subpopulation resides at the apex of its differentiation hierarchy. Intriguingly, the researchers discovered that Itm2a$^+$ periosteal cells are predominantly located in the outer fibrous layer of the periosteum and contribute approximately 37 % of chondrocytes in the fracture callus during the process of fracture healing [45]. This suggests a high proportion of endochondral ossification among other stem cell populations in other parts of the periosteum during fracture healing. Furthermore, Itm2a$^+$ P-SSCs exhibit enriched expression of genes associated with the bone morphogenetic protein (BMP) pathway. Mice lacking BMP2 in the Itm2a-expressing lineage exhibited impaired fracture repair. Mice lacking the ability to produce BMP2 within the Prrx1 lineage displayed spontaneous fractures that did not resolve over time [56]. These data indicate that Itm2a$^+$ cells may serve as a source of BMP2 during fracture healing. Additionally, Itm2a$^+$ cells are enriched in WNT signaling, which may be crucial for the involvement of Itm2a$^+$ P-SSCs in fracture repair, necessitating further experimental validation.

5. Role of P-SSCs in bone repair and disease

5.1. Repair of P-SSCs in long bone fractures

The procedure for fracture healing is intricate and can be broadly categorised into three distinct stages. During the early phase, a hematoma develops and initiates an inflammatory reaction that catalyses bone repair [57]. Subsequently, SSCs become active and travel towards the damaged area in the bone. These cells then multiply and transform into chondrocytes and osteoblasts, which combine to form the main calli. With revascularisation and calcification, the callus gradually remodels and returns to its typical bone structure without scar tissue (Table 3).

During the initial phases of fracture healing, Mx1$^+$αSMA$^+$ P-SSCs rapidly mobilise to the location of injury [46]. These cells invaluably contribute to new osteoblasts and the limited number of chondrocytes in the callus through two robust ossification processes. Matthews et al. showed that ablation of αSMA$^+$ cells during fracture healing reduces the fracture size and cell number, decreases the callus area, delays mineralisation, and, ultimately, significantly decreases bone mass [58]. Importantly, although originating from the same embryonic mesenchymal lineage, P-SSCs and BMSCs exhibit unique functions in bone regeneration. P-SSCs mediate endochondral ossification to generate chondrocytes, whereas BMSCs support this osteogenic process. Therefore, P-SSCs have dual functions in chondrogenesis and osteogenesis and thus exhibit more vital regenerative abilities than BMSCs [38]. In addition, Ctsk$^+$ P-SSCs exhibit plasticity under pathological conditions and promote fracture callus formation via endochondral bone formation [12] (Fig. 3A). A conditional knockout of osterix (*Osx*), a marker of mature osteoblasts, blocked the osteogenic capacity of Ctsk$^+$ P-SSCs and severely impaired cortical bone formation and fracture repair in mice.

The endosteal formation layer has long been considered the only osteogenic layer of the periosteum, whereas the function of the outer fibre layer in bone formation remains unclear. Liu et al. recently constructed a novel dual homologous recombinase (Pdgfra-CreER and Osx-Dre) reporter gene, *R26^{ZT1}*, and screened a variety of lineage-tracer mice capable of labelling SSCs [59]. Thus, an SSC simultaneous tracing system (SSC-SimulTracer) was established that could simultaneously trace two types of heterogeneous substances. This system could discern the lineage fate of bone stem cells in the bone marrow (BMSCs), periosteal inner cambial layer (CL-PC), and outer fibrous layer (FL-PC) by utilising the tdTomato and ZsGreen fluorescent proteins. The experimental results revealed that the outer fibrous layer cells in the mouse periosteum,

Table 3
Role of P-SSCs in bone repair and disease.

Disease Model	Cell type	Function	Reference
Long bone fractures	$Mx1^+\alpha SMA^+$ P-SSCs	Early and rapid migration to the site of injury; support bone repair through both intramembranous osteogenesis and endochondral ossification	[46]
	$Ctsk^+$ P-SSCs	Promote fracture callus formation via endochondral bone formation	[12]
	Outer fibrous layer cells	Plays an important role in fracture repair, although not involved in homeostatic osteogenesis; Challenges the traditional understanding that the periosteal fibrous layer lacks osteogenic capacity	[59]
Craniofacial bone injuries	$Mx1^+\alpha SMA^+$ P-SSCs	CCL5 promotes cell migration to the damaged site and differentiation into osteoblasts	[46]
	$Ctsk^+Ly6a^+$ stromal cells	$Ctsk^+Ly6a^+$ stromal cell deficiency will lead to impaired healing of jaw deformities	[60]
	$CTSK^+$ P-SSCs	$CTSK^+$ P-SSCs mediating bone repair in orbital reconstruction	[65]
Congenital pseudarthrosis of the tibia (CPT)	P-SSCs	Dramatic decrease in circZNF559 expression in CPT-derived P-SSCs may contribute to tibial development	[70]
Bone tumour	$Ctsk^+$ periosteal cells	Knockdown of *Lkb1* by *Ctsk-cre* results in osteosarcoma	[75]
		Knockdown of *Ptpn11* by *Ctsk-cre* results in chondromatosis	[50,76]
	Periosteal cells	HIF1α from HNSCC upregulates TIMP1 in periosteal osteoblasts, inhibiting matrix-degrading enzymes and thickening the periosteum to resist cancer cell invasion	[77]

αSMA, α-smooth muscle actin; CCL5, C–C motif chemokine ligand 5; CPT, congenital pseudarthrosis of the tibia; Ctsk, cathepsin K; Lkb1, liver kinase b1; HIF1α, hypoxia-inducible factor 1α; HNSCC, head and neck squamous cell carcinomas; P-SSCs, periosteal skeletal stem cells; Ptpn11, protein tyrosine phosphatase, non-receptor type 11; TIMP1, tissue inhibitor of metalloproteinase 1.

assumed to lack osteogenic capacity, are the most important contributors to injury repair in osteoblasts after fracture, although they do not participate in steady-state osteogenesis [59]. This finding challenges the previous understanding that the periosteal outer fibrous layer cells lack osteogenic capacity and identifies their actual role in fracture injury repair.

In conclusion, P-SSCs are the primary cell source for bone repair, and their osteogenic and chondrogenic differentiation capacities are essential for fracture healing.

5.2. Repair of P-SSCs in craniofacial bone injuries

In a mouse model of skull injury, it was observed that chemokine (C–C motif) ligand 5 (CCL5) exhibited a similar effect to that observed in long bone fracture repair. CCL5 facilitated the migration of $Mx1^+\alpha SMA^+$ P-SSCs to the injury site, where they centralised and subsequently differentiated into a subset of osteoblastic lineage cells crucial for defect repair [46] (Fig. 3B and Table 3).

Ding et al. identified a unique population of Ctsk $^+Ly6a^+$ stromal cells explicitly enriched in the jawbone periosteum of mice [60]. This subpopulation was enriched during the growth phase of mice but gradually decreased with age. $Ctsk^+Ly6a^+$ stromal cells effectively promote the healing of jaw defects. However, after depleting Ctsk cells with diphtheria toxin, a substantial decline in the proportion of $Ctsk^+Ly6a^+$ periosteal cells was observed, resulting in impaired healing of jaw abnormalities [60]. Although this study did not characterise the stemness of $Ctsk^+Ly6a^+$ periosteal stromal cells, other studies have reported that mice with systemic Ly6a knockouts exhibit significantly reduced bone mass [61]. In addition, $PDGFR\alpha^+Ly6a^+$ MSCs exhibit enhanced proliferative and multidirectional differentiation compared to standard culture-selected MSCs [62]. Therefore, the combined labelling of *Ctsk* and *Ly6a* is practical for the identification of jawbone P-SSCs.

The orbital bone primarily consists of thin cortical bone containing minimal marrow, and the periosteum widely envelops it [63,64]. This unique anatomical feature allows the orbital bone to generate new bone tissue inside the membrane. In contrast to orthodox views, Liu et al. offered a novel viewpoint by challenging the notion that the healing process following orbital bone fractures relies primarily on BMSCs as a source of endogenous stem cells [65]. Their research unveiled that the human orbital periosteum harbours a significant population of $Ctsk^+$ P-SSCs, a versatile cell type capable of multidirectional differentiation *in vitro* and promoting bone formation within living organisms. These cells rapidly multiply and relocate to the location of the orbital fracture to aid in the recovery process. Subsequent investigations revealed notable disparities between Ctsk + P-SSCs and BMSCs through a comparative examination of their transcriptional traits. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses revealed that $Ctsk^+$ P-SSCs exhibited greater proliferative activity and possessed more specialised functions than BMSCs. These characteristics align closely with the cellular features necessary for intramembranous osteogenesis. The genes identified in the analyses were mostly involved in signal transduction pathways and pathways associated with tumours. These variations may be attributed to the distinct ecological niches in which these two organisms reside. Differences in their microenvironments and intercellular interactions result in discrepancies between their respective functions. However, this method requires further investigation.

5.3. Role of P-SSCs in bone disease

Congenital pseudarthrosis of the tibia (CPT) is a rare disease in children, and its pathological changes are mainly characterised by periosteal thickening [66–68] (Fig. 3C and Table 3). Numerous studies have demonstrated that a diminished ability for P-SSCs to differentiate into osteoblasts plays a significant role in the development of CPT. However, the precise molecular pathways underlying this phenomenon remain unknown [69]. According to recent studies, circRNAs and microRNAs (miRNAs), which are non-coding RNAs extensively distributed in eukaryotic cells, are crucial for the pathophysiology of P-SSC-mediated CPT [70]. One of the critical roles for circRNAs is for them to act as molecular sponges to inhibit miRNA activity, which can adversely affect target gene replication and thereby prevent protein synthesis [71]. Notably, miR-338-3p overexpression promotes osteoclast formation and significantly inhibits osteogenic differentiation of BMSCs, thereby disrupting the balance of bone metabolism [72–74]. Li et al. discovered that circZNF559, which originates from the zinc-finger protein (*ZNF*) gene, hinders the function of miR-338-3p and enhances the production of its target gene [70]. Thus, the viability of P-SSCs and their capacity to differentiate into bone cells are enhanced. Hence, a substantial decrease in circZNF559 expression in CPT-derived P-SSCs may contribute to the development of the tibia, offering a possible approach for treating CPT.

Osteosarcoma is caused by the deletion of liver kinase b1 (*Lkb1*) via *Ctsk-Cre,* which activates mammalian target of rapamycin complex 1 (mTORC1) [75] (Fig. 3D and Table 3). Furthermore, in cells expressing *Ctsk*, knockdown of *Ptpn11*, the gene that encodes the tyrosine

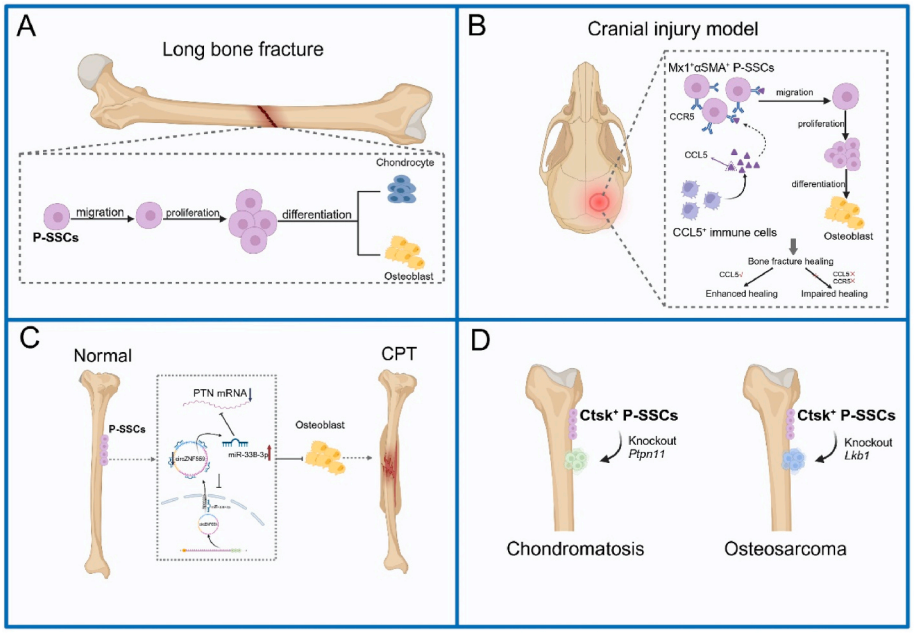

Fig. 3. Role of P-SSCs in bone repair and disease. (A) In long bone fractures, P-SSCs migrate early, proliferate and differentiate to participate in the repair of the injury site. (B) In a cranial injury model, CCL5 promoted the migration of $Mx1^{+}\alpha SMA^{+}$ P-SSCs to the injury site and differentiation of osteoblasts involved in repair. (C) Reduced circZNF559 expression in P-SSCs impedes miR-338-3p function, which may cause CPT osteochondral thickening. (D) Knockdown of *Ptpn11* in Ctsk $^{+}$ P-SSCs resulted in chondromatosis, whereas lack of *Lkb1* resulted in osteosarcoma. (Created with BioRender.com.) αSMA, α-smooth muscle actin; CCL5, C–C motif chemokine ligand 5; CCR5, C–C motif chemokine receptor 5; CPT, congenital pseudarthrosis of the tibia; Ctsk, cathepsin K; *Lkb1*, liver kinase b1; *Ptpn11*, protein tyrosine phosphatase, non-receptor type 11; P-SSC, periosteal skeletal stem cells.

phosphatase SHP2, causes chondromatosis resulting from the hyper-activation of Hedgehog signalling in the perichondrial groove of Ranvier [50,76]. Therefore, these data indicate that cancers arise when there is a dysregulation of postnatal periosteal cells expressing *Ctsk*. Furthermore, a recent study has revealed that hypoxia-inducible factor 1α (HIF1α), originating from the tumour microenvironment of head and neck squamous cell carcinomas (HNSCC), induces an upregulation of TIMP1 (tissue inhibitor of metalloproteinase 1) expression in periosteal cells

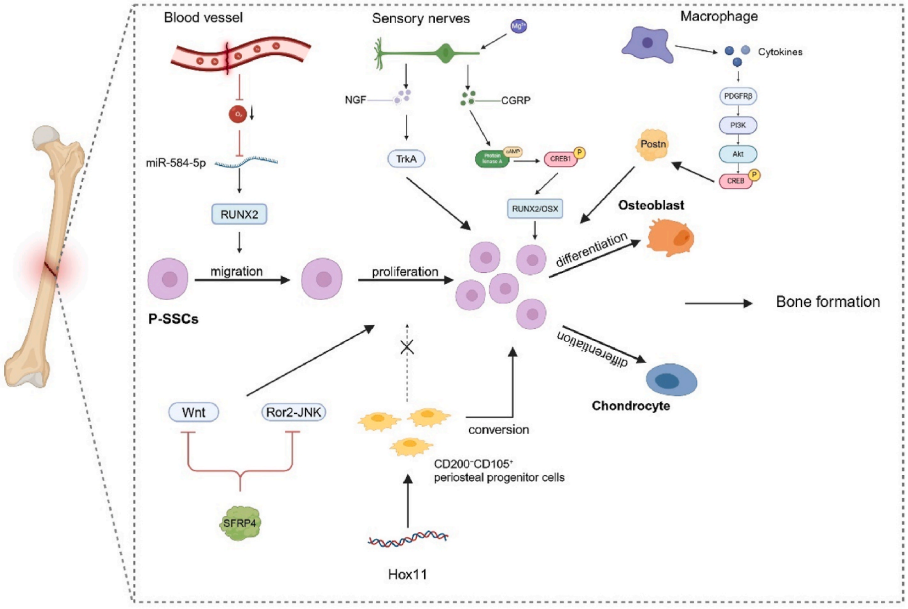

Fig. 4. Regulation of periosteal microenvironment on P-SSCs. (A) Damage to the periosteal vasculature results in oxygen deprivation, which promotes the expression of *Runx2* in P-SSCs. (B) Sensory nerves secrete NGF, activating TrkA and facilitating ossification by PSSCs. (C) Mg^{2+} stimulates sensory nerves to secrete CGRP, which activates CREB1, promoting the expression of *Runx2/Osx* and ossification by PSSCs. (D) $Trap^{+}$ periosteal macrophages secrete factors that interact with PDGFRβ in P-SSCs, activating the Pi3K–Akt–CREB pathway and enhancing ossification. (E) Overexpression of Hox11 does not enhance the proliferative capacity of P-SSCs; however, it converts $CD200^{-}CD105^{+}$ periosteal progenitor cells into P-SSCs, thereby increasing their population. (F) SFRP4 promotes ossification by PSSCs through antagonism of the Wnt signaling pathway or local inhibition of the Ror2-JNK signal. (Created with BioRender.com.) CGRP, calcitonin gene-related peptide; CREB1, cAMP responsive element binding protein 1; Hox11, homology box 11; NGF, nerve growth factor; Osx, osterix; PDGFRβ, platelet-derived growth factor receptor β; P-SSCs, periosteal skeletal stem cells; Runx2, runt-related transcription factor 2; TrkA, tropomyosin-related kinase A.; SFRP4, secreted frizzled receptor protein 4.

adjacent to bone surfaces, particularly within the subpopulation of periosteal osteoblasts [77]. This upregulation subsequently inhibits matrix-degrading metalloproteinases, initiating a thickening of the otherwise thin periosteum as a defensive mechanism against cancer cell invasion.

6. Regulation of periosteal microenvironment on P-SSCs

The P-SSC niche, also known as the P-SSC microenvironment, promotes stem cell self-renewal and maintains stemness. The components of the stem cell microenvironment vary widely from site to site in terms of the interactions between stem cells and neighbouring cells, vascular neural networks, signalling pathways, the extracellular matrix, and various secretory factors and mechanical signals [78]. The niche supporting SSCs can be categorised into the bone marrow and periosteum. Here, we summarise and discuss studies on the regulation of P-SSCs in periosteal microenvironments (Fig. 4).

6.1. Effects of vascularisation on P-SSCs

The periosteum has an abundant blood supply that nourishes the cortical bone and provides nutrients and oxygen to P-SSCs. Fractures can disrupt the periosteal blood vessels, leading to hypoxia. Hypoxia is critical for P-SSC development into osteoblasts. Lu et al. found that hypoxia mediates the downregulation of intracellular miRNAs (miR-584-5p) in P-SSCs [79]. This facilitates the activation of Runx2, which is associated with bone differentiation. Consequently, it induces the osteogenic mineralisation of P-SSCs and enhances ALP expression. miRNAs are crucial biomolecules that control the development of MSCs into osteoblasts and chondrocytes [80,81]. During the osteogenic differentiation of human periodontal ligament stem cells, the expression of miR-584-5p is altered and suppressed [82]. Furthermore, miR-584-5p is pivotal in regulating osteosarcoma cell growth and preserving bone tissue equilibrium [83]. These findings indicate that controlling the activity of miR-584-5p and miR-584-5p/Runx2 may serve as a novel approach for promoting bone healing and regeneration by facilitating the osteogenic differentiation of P-SSCs. However, this study only replicated the effect of hypoxia on bone formation *in vitro* [79]. The complexity of the fracture microenvironment in living organisms necessitates further exploration of the crucial elements involved in the formation of P-SSCs generated by fracture-induced hypoxia.

6.2. Effects of sensory nerves on P-SSCs

Sensory nerves originating from the dorsal root ganglia are widely distributed throughout the periosteum [84]. They control the generation of osteochondral lineage cells in long bones during the developmental stage of the body and sense pain signals when the bone is injured [85]. In addition, they secrete neuropeptides that promote repair. These functions depend on the action of nerve growth factor (NGF). Fracture injuries can stimulate nerve sprouting, upregulate NGF expression, and activate tropomyosin receptor kinase A (TrkA) signal transduction in sensory nerves to transmit bone pain signals [85–87]. These reactions occur prior to vascular reconstruction. According to previous studies, blocking NGF/TrkA signalling with paclitaxel or deleting the *TrkA* gene significantly reduces the bone-healing ability of mice. Furthermore, Zhang et al. demonstrated that the Mg ions generated by the degradation of Mg *in vivo* could stimulate the discharge of calcitonin gene-related peptides (CGRPs) via sensory nerve terminals within the periosteum [88]. Elevated levels of CGRP in the periosteum activate the cyclic adenosine monophosphate signalling pathway, resulting in the phosphorylation of the transcription factor CREB1. This then triggers the activation of *Osx* and *Runx2*, encouraging the osteogenic differentiation of P-SSCs and resulting in a substantial quantity of fresh bone in the periosteum.

During the inflammatory stage of bone regeneration, activated bone macrophages assemble at the injury site [39]. Furthermore, these cells not only eliminate bone debris and dead cells at the fracture location but also release cytokines to stimulate the endochondral ossification in P-SSCs, thus facilitating the regrowth of bone tissue. The work conducted by Gao et al. revealed a significant role for $Trap^{+}$ periosteal macrophages in the secretion of PDGF-BB [39]. This factor interacts with the PDGFRβ present in P-SSCs, thereby triggering activation of the Pi3K–Akt–CREB pathway and subsequently promoting the production of periostin (Postn).

6.3. Specific genes and proteins

Several proteins and genes are essential for modulating P-SSC function. Homology box (*Hox*) genes are transcription factors that have been highly conserved throughout evolution [89]. They act as regulators of embryonic development and determine the fate of adult P-SSCs after birth [89–91]. *Hox* genes are expressed in P-SSCs to maintain their initial state; silencing these genes reduces their osteogenic capacity [90]. *Hox11* overexpression does not enhance the proliferative capacity of P-SSCs; however, it converts $CD200^{-}CD105^{+}$ periosteal progenitor cells into P-SSCs, increasing their abundance [91]. Additionally, *Hox11* improves the bone-healing capacity of various senescent mouse models, possibly due to the enhanced self-renewal capacity and altered osteogenic fate of P-SSCs [91]. However, the mechanism by which *Hox* genes mediate this process requires further investigation.

Secreted frizzled receptor protein 4 (SFRP4) is a decoy receptor and antagonist of the Wnt signalling pathway [92]. SFRP4 dysfunction leads to Peyer's disease, which manifests as limb and cranial deformities, particularly thinning of the cortical bone [93–95]. SFRP4 regulates intraperitoneal remodelling through local inhibition of Ror2-JNK signalling and is expressed in $Ctsk^{+}$ P-SSCs [96]. Its absence impedes the differentiation and repair of P-SSCs. Notably, Sfrp4 is necessary for PTH to promote the involvement of $Ctsk^{+}$ P-SSCs in cortical bone thickening and osteogenesis [96].

Extracellular-matrix-produced Postn has previously been shown to communicate with other proteins to support cell migration, expansion, and anti-apoptosis [97–99]. These proteins are crucial for tissue regeneration and cancer progression. Duchamp et al. demonstrated that Postn has a more pronounced effect on stimulating the regeneration of P-SSCs than the regeneration of BMSCs [38]. In the bone transplant model used in this study, Postn enhanced the proliferation and migration of P-SSCs towards the bone marrow. This finding presents an intriguing avenue for further investigation.

7. Conclusion

This review provides an overview of recent research on P-SSCs, encompassing their definitions, their biological characteristics, the spatial and temporal distribution of distinct subpopulations, their pivotal roles in bone repair and bone-related diseases, and their potential regulatory mechanisms within the periosteal microenvironment. P-SSCs conform to stringent modern definitions and are acknowledged as true SSCs. However, the limited availability of P-SSCs impedes their clinical translation and hinders the development of bone regenerative medicine. Further studies on P-SSCs and stem cell niches are required to elucidate the cellular and molecular mechanisms regulated by P-SSCs and the osteogenic pathways involved in P-SSC-mediated intramembranous and endochondral ossification. Additionally, it is critical to identify the causative link between skeletal diseases and the dysregulation of P-SSCs for the therapeutic use of this promising population of cells. Therapies based on P-SSCs are expected to become reliable biological solutions for addressing bone abnormalities and diseases.

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Conflicts of interest

A conflict of interest occurs when an individual's objectivity is potentially compromised by a desire for financial gain, prominence, professional advancement or a successful outcome. The Editors of the *Journal of Orthopaedic Translation* strive to ensure that what is published in the Journal is as balanced, objective and evidence-based as possible. Since it can be difficult to distinguish between an actual conflict of interest and a perceived conflict of interest, the Journal requires authors to disclose all and any potential conflicts of interest.

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