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Concise Review: Conceptualizing Paralogous Stem-Cell Niches and Unfolding Bone Marrow Progenitor Cell Identities

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ABSTRACT

Lineage commitment and differentiation of skeletal stem cells/bone marrow stromal cells (SSCs/BMSCs, often called bone marrow-derived "mesenchymal stem/stromal" cells) offer an important opportunity to study skeletal and hematopoietic diseases, and for tissue engineering and regenerative medicine. Currently, many studies in this field have relied on cell lineage tracing methods in mouse models, which have provided a significant advancement in our knowledge of skeletal and hematopoietic stem-cell niches in bone marrow (BM). However, there is a lack of agreement in numerous fundamental areas, including origins of various BM stem-cell niches, cell identities, and their physiological roles in the BM. In order to resolve these issues, we propose a new hypothesis of "paralogous" stem-cell niches (PSNs); that is, progressively altered parallel niches within an individual species throughout the life span of the organism. A putative PSN code seems to be plausible based on analysis of transcriptional signatures in two representative genes that encode *Nes*-GFP and leptin receptors, which are frequently used to monitor SSC lineage development in BM. Furthermore, we suggest a dynamic paralogous BM niche (PBMN) model that elucidates the coupling and uncoupling mechanisms between BM stem-cell niches and their zones of active regeneration during different developmental stages. Elucidation of these PBMNs would enable us to resolve the existing controversies, thus paving the way to achieving precision regenerative medicine and pharmaceutical applications based on these BM cell resources. STEM CELLS *2017; 00:000–000*

SIGNIFICANCE STATEMENT

We propose a new concept relating to "paralogous" stem cell niches (PSNs), that is, progressively and functionally transformed niches within an individual species throughout the life span of the organism. We aim to systematically untangle the complicated biology of skeletal stem cells/bone marrow stromal cells under this new concept. We would like to shed light on: (a) the putative PSN codes that underlie lineage commitment and differentiation and (b) the conceptual significance of PSNs in bone marrow (i.e., paralogous BM niche [PBMN]). [Correction made here after initial online publication.] Unraveling of PBMNs would enable us to provide decisive marker panels for PBMN cell identity, solve significant stem cell controversies, and provide new insights into precision regenerative medicine and pharmaceutical application.

INTRODUCTION

Pluripotent and adult stem-cell biology provide endless possibilities for regenerative medicine, disease modeling, and pharmaceutical applications [1–4]. The precision clinical use of these valuable cell resources relies on a thorough understanding of some fundamental issues in stem-cell biology, including origins and composition of various stem-cell niches, stem-cell identities, and their physiological roles in a clinical sitting. Still, there are considerable controversies, experimental discrepancies, and data reproducibility issues to be resolved to ensure their successful therapeutic applications.

Misunderstandings and disagreements in one area of the stem-cell field encompass an elusive and misleading concept regarding "mesenchymal stem cells," which was initially based on "bone marrow stromal cells" (BMSCs) [5, 6] and its subset of multipotent skeletal stem cells (SSCs) [7]. Mesenchymal stem cells are thought by many to be ubiquitously distributed in adult tissues, having substantial plasticity and multilineage differentiation potentials [8–11]. During the past two decades, the term mesenchymal

stem cell (and more recently, mesenchymal stromal cell) has gained wide popularity, but its use has also raised a number of issues based on the fact that "MSCs" from different tissues are not the same [7, 12–14]. Other challenging questions related specifically to the bone marrow (BM) stem-cell field are: (a) the contribution of regional neural crest cells (besides the cranial neural crest) to colony-forming unit-fibroblasts (CFU-Fs) or SSCs [15] and (b) the exact locations of hematopoietic stem cell (HSC) niches within BM [16, 17]. All of these issues are, in fact, related to origin, cell identifies, and differentiation potentials of mesenchyme, which is an embryonic connective tissue of varied embryological origins, and the subsequent postnatal cell fates of its progeny. It is also unclear what fundamental mechanisms control cell lineage commitment and differentiation. Thus, there is an urgent need to address these important questions.

To precisely define diverse mesenchymal cell lineage derivation and differentiation is a challenging task due to the diffuse-and-complex nature of this particular stem-cell field. Virtually, all three-germ layers contribute directly or indirectly to the development of miscellaneous embryonic mesenchymal lineages. During gastrulation, the first mesenchyme or mesenchymal layer in the primitive streak is formed by an epithelial-to-mesenchymal transition (i.e., EMT). Mesenchyme that will form the skeletal lineage can be derived either from the cranial neural crest of neuroectoderm or from paraxial and somatic lateral-plate mesoderm, or both neuroectoderm and mesoderm [18]. Interestingly, the reverse process of EMT enables the conversion of mesenchyme to epithelium or epithelium-like cells, a process known as the mesenchymal-epithelial transition (MET) [19–24]. Thus, there are multiple waves of interchangeable EMT-MET events, which drive delineation of distinct cell phenotypes and thus make it difficult to discern cell identities. Additionally, the field suffers from an over-reliance on artifactual and less than rigorous assays, a lack of definitive stem-cell markers, the absence of a conceptual consensus for postnatal mesenchymal biology, and the consistent use of misleading terminologies such as mesenchymal stem cells in a postnatal setting.

Here, we propose a new concept relating to "paralogous" stem-cell niches (PSNs); that is, progressively and functionally transformed niches within an individual species throughout the life span of the organism. We aim to systematically untangle the complicated biology of SSCs/BMSCs under this new concept. We would like to shed light on: (a) the role of multi-temporal and -dimensional EMT-MET dynamics in the development of PSNs in vertebrates, (b) the conceptual significance of the putative PSNs in BM (i.e., paralogous BM niches, PBMNs) and prospective technological challenges, (c) dysregulation of PSNs and diseases. Unraveling of PBMNs would enable us to provide decisive marker panels for PBMN cell identity, solve significant stem-cell controversies, and provide new insights into precision regenerative medicine and pharmaceutical application.

Conceptualizing Paralogous Stem-Cell Niches

It is conceivable that homologous stem-cell niches denote the existence of similar or identical cellular compartments that nurture stem-cell growth, self-renewal, and homeostasis among homologue species. Hence, the term "homologous" niche was previously used to depict the similarity of BM HSC niches among different species (e.g., in humans and mice) [4]. However, the concept of PSNs has neither been previously conceptualized nor described in the literature. Paralogue is a genetic term that is frequently used to depict gene developmental products with a common ancestral origin. However, its derivative paralogous could be extended to designate a group of similar things (e.g., stem cells) with certain lineage associations. Here, we suggest that progressive diversifications of cell identity after the first EMT in the primitive streak of each individual species lead to the formation of a cluster of regional PSNs for nurturing specific types of tissue-specific stem/progenitor cells (Fig. 1). However, the mechanisms underlying these default cell fates are only partially elucidated in developmental biology. Perceptibly, epigenomic changes during different developmental stages might play a pivotal role in the regulation of these PSNs. However, in the following sections, we will briefly provide some developmental insights, with a focus on taxonomical analyses for two distinct PSNs, representative of neural crest and mesodermal derivatives.

Developmental Insights

Prior to the establishment of a fully functional blood circulation (e.g., after E10, the embryonic day 10 in mice) [26], morphogen signaling gradients along the cranial-to-caudal axis are essential for the establishment of the anterior-to-posterior (AP) axis and for commitment of subsequent cellular states (Fig. 1A). Dominant morphogen signaling molecules (e.g., BMP4, Wnt1 and 3a, Shh, and Pax1) create such acceptor sites for the anchorage of PSN cells. At a multi-somite stage in vertebrate embryos, neural crest cells migrate along the cranial-to-caudal axis to form diverse tissues or organs (e.g., facial bones and cartilages, portions of the tooth, adrenal medulla, and epidermal pigment cells) (Fig. 1A). Such a wide range of neural crest cell diversifications represents complicated, but fascinating stem-cell biology, regardless of the existence of largely unknown taxonomical properties of these PSN cells.

Taxonomy of Diverse PSNs

Indeed, taxonomical analysis of various PSNs would certainly illuminate lineage expansion, specification, and diversification in each individual organism, particularly in vertebrates. PSN development in vertebrates includes embryonic mesenchymal niches (i.e., mesenchyme), and prenatal and postnatal counterparts. Physically, PSNs are associated with neural crest lineage development (e.g., facial bone and tooth development), and that of mesoderm (BM SSC and HSC niches). Despite the differences of PSN locations and components, PSNs appear to share some basic structural properties, including extracellular matrices (subdivisions), blast-stage cellular boundaries, immature and/or mature cellular compartments, and condensed or organized functional tissues (Fig. 1B). Here, we focus on two representative PSNs as described above.

Craniofacial PSNs

At the cranial level, embryonic mesenchymal cells, derived from the neural crest, generate various tissues that include cranial ganglia, glia, pigment cells, smooth muscle, facial cartilage and bone, and teeth (Fig. 1B, upper panel). Interestingly,

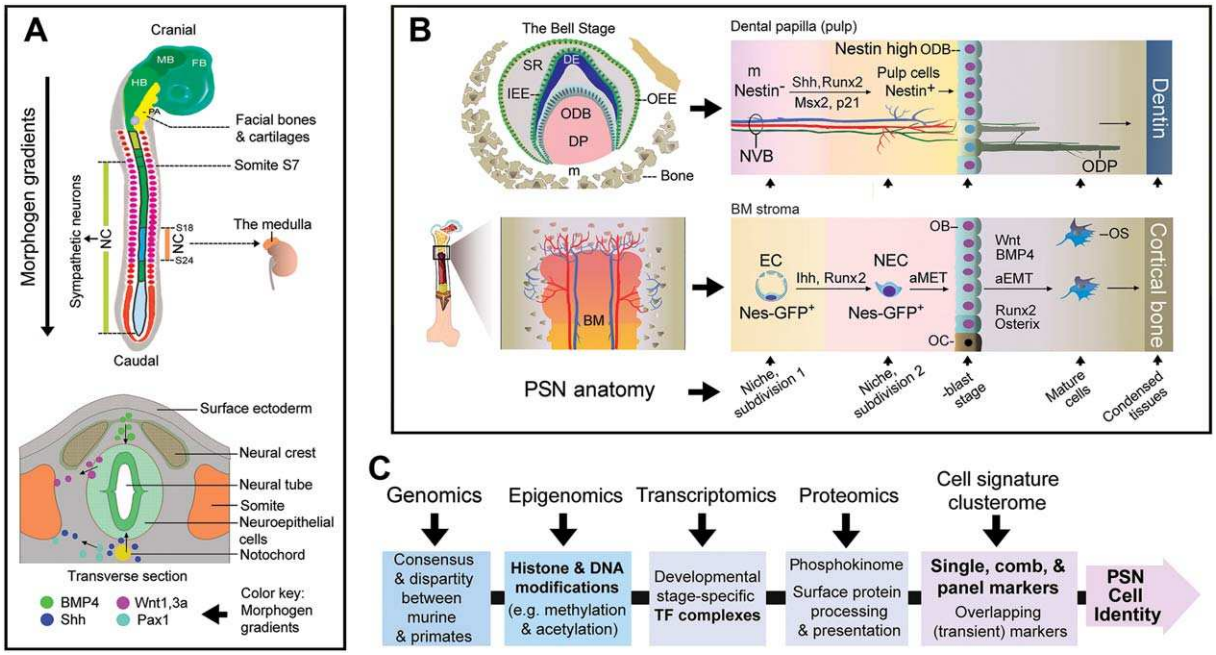

Figure 1. Conceptualizing PSN development in vertebrates. **(A)**: Developmental insights of morphogen signaling gradients along the cranial-caudal axis into the commitment of cellular states at various developmental stages (adapted from reference 18): Upper panel presents a 28-somite embryo and migratory NC cells along the cranial-caudal axis to form various tissues or organs (e.g., the maxillofacial bones and cartilages, the sympathetic neurons, and the medulla of the suprarenal gland). Lower panel depicts a transverse sectional view of dominant morphogen signaling molecules (e.g., BMP4, Wnt1/3a, Shh, and Pax1) at a somite region after folding the neural plate. **(B)**: Representatives of various PSNs in the dental papilla and the BM: Top panel illustrates a dental stem-cell niche, in which nestin is regulated by indicated factors. Lower panel presents a BM SSC niche that is regulated by Ihh and Runx2. *Nes*-GFP$^+$ ECs become *Nes*-GFP$^+$ NECs after induction by both Ihh and Runx2 [25]. These NECs may be early progenitor cells of osteoblasts. **(C)**: A scheme is shown for unraveling paralogous niche cell identities through hierarchy cell marker discovery. Abbreviations: aEMT, atypical epithelial-to-mesenchymal transition, a term designated here to describe the transition from fibroblast- or neural crest-like cells to pseudo-epithelium-like cell layers (e.g., the arrays of odontoblasts and osteoblasts in their confined locations); aMET, atypical mesenchymal-to-epithelial transition analogous to aEMT; BM, bone marrow; BMP4, bone morphogenetic protein 4; comb, combined sets; DP, dental papilla (pulp); ECs, endothelial cells; FB, forebrain; HB, hindbrain; Ihh, Indian Hedgehog; IEE, inner enamel epithelium; m, mesenchyme; MB, midbrain; the medulla, the medulla of suprarenal gland; NC, neural crest; Msx2, Msh homeobox 2; NECs, nonendothelial cells; OB, osteoblast; OC, osteoclast; ODB, odontoblast; ODP, odontoblast process; OEE, outer enamel epithelium; OS, osteocyte; PA, pharyngeal arches; p21, cyclin-dependent kinase inhibitor 1A (CDKN1A); Pax1, paired box 1; PSN, paralogous stem cell niche; Runx2, Runt-related transcription factor 2; Shh, Sonic Hedgehog; SR, stellate reticulum; TF, transcriptional factor; Wnt, wingless-type MMTV integration site family.

the teeth are a complex organ that is created by a composite niche that involves both oral ectoderm and neuroectoderm (i.e., neural crest). The oral ectoderm gives rise to the dental epithelium that generates enamel-producing ameloblasts. The neural crest cells make dentin-producing odontoblasts (Fig. 1B, upper panel). Developmentally, the nestin protein, encoded by the *NES* gene, is absent from the bud and cap stages, but is expressed in many pulp cells at the cusp region of the dental papilla and highly enriched in the odontoblasts of the bell stage [27]. Structurally, the odontoblast layer may be considered a pseudo-neuroepithelial layer, whose MET-EMT dynamics transforms tooth morphologies. At the molecular level, a common morphogen module (that incorporates BMP4, FGFs, WNTs, and SHH) at the dental papilla mesenchyme appears to play a decisive role in tooth development [28, 29] (Fig. 1B, upper panel).

Paralogous BM Niches

Bone with its marrow is an intriguing organ, which nurtures multiple stem cell niches. BM stromal and perivascular spaces contain HSCs and their differentiating progeny, endothelial cells, smooth muscle cells, pericytes, fibroblasts, and adipogenic cells. The BM stroma also contains a subset composed of multipotent SSCs [30–32]. Importantly, these self-renewing SSCs, adjacent to BM sinusoids, consolidate to form a portion of the HSC niche [33]. Moreover, SSCs express a subset of specific osteogenic factors such as Ihh and Runx2. Abluminal SSCs are believed to be identifiable by expression of indicator genes from promoters for *Nes, Lepr, Prx-1,* and *Mx-1* in mice, and additionally, by CD146 in humans. For example, a *Nes*-GFP reporter (*Nes*-GFP) mouse strain has been widely used to trace neuroectodermal and endothelial lineages [25, 31, 34]. To study the regulation of the endochondral osteogenic niche by Ihh and its combined regulators, Kronenberg and colleagues observed that *Nes*-GFP$^+$ endothelial cells became *Nes*-GFP$^+$ nonendothelial cells after induction by both Ihh and Runx2 [25]. These *Nes*-GFP$^+$ nonendothelial cells may be considered as early progenitor cells of osteoblasts or chondrocytes (Fig. 1B, lower panel). Not surprisingly, an increase in *Nes*-GFP$^+$ cells was also found in the ossification center [25]. However, it is difficult to establish a lineage relationship based on the *Nes*-GFP reporter, which is not a permanent genetic marker. To accurately define a lineage relationship, some permanent genetic markers, such as the *loxP*-flanked tdTomato, for the

Nes gene are needed for further analysis. Recently, combing leptin receptor-Cre (*Lepr*-Cre) with tdTomato (a photostable red fluorescent protein), Morrison and colleagues found that *Lepr*-expressing stromal cells are the major osteoprogenitor source in the adult mouse BM [31]. These studies suggest that both *Nes*-GFP and leptin receptor are informative markers for studying endothelial and mesenchymal cell lineages in mice.

Collectively, comparative analyses of representative PSNs (i.e., craniofacial PSNs and PBMNs) yield insights into their similarities and disparities with respect to their physical and cellular niche components. Although established by cells of different germ-layer origins, the BM niche shows similarities to the dental papilla niche in terms of their organization of niche compartments (Fig. 1B). We further hypothesize that the PBMN cell identities are highly correlated with their developmental stages. However, this correlation could be altered or highly masked in in vivo animal models due to the presence of numerous variables (e.g., DNA epigenomic, mRNA transcriptomic, and protein translational alterations). To accurately understand the putative PBMN cell identity code, we must appreciate systems biology that integrates large genomic, epigenomic, and proteomic datasets into a manageable scheme (Fig. 1C). Importantly, we should gain the capacity to transform this data-informatics into simple-and-robust assays to precisely define a particular cellular state (Fig. 1C).

Likewise, the relationship between the PBMN cell identity and developmental stages could be polynomially or nonlinearly associated. The PBMN code and its complexity might lie in the interplay between multiple core signaling pathways (combined with tissue-specific regulators) and effectors in distinct PBMNs. Notably, embryonic mesenchymal signals can be derived from diverse and functionally distinct fibroblasts of the three-germ layers. [Correction made here after initial online publication.] Clearly, such spatiotemporal interplay instructed by tissue-specific signal molecules enables the regulation and formation of diverse PBMNs for subsequent cell-type determinations.

The Significance of Regulation of PBMN Cell Fates in BM

The BM accommodates numerous PBMN cell types based on lineage tracing. Notably, there are CXCL12-abundant reticular (CAR) cells [35], *Nes*-GFP^{high}/*Lepr*-Cre^{-} and *Lepr*-Cre^{+}/*Nes*-GFP^{low} cells [17, 31], PαS (PDGFRα^{+}/Sca-1^{+}/CD45^{-}/Ter119^{-}) cells [36, 37], pericytes [38], and SSCs [7]. However, among these cell types, some are likely to be completely overlapping (i.e., the same cell types) or partially overlapping (i.e., at different cell transition phases). Currently, various mouse genetic models using transgene-based promoter/enhancer reporter strains have become the predominant method to map cell lineage in vivo. Consequently, there are emerging controversial concepts, inconsistent data, and inappropriate data interpretation due to a lack of understanding of many mouse genetic systems. Many technical reasons may underlie the inconsistency of different transgenic mouse lines, which comprise the designs of transgenes or targeting vectors used for generating transgenic mice, different methods of gene expression (e.g., constitutive vs. inducible gene expression systems), and cellular cytotoxicity and side effects associated the experimental systems. Noticeably, many transgenes have different expression patterns in BM in terms of their genetic manipulations and chromosomal integrations. For example, *Nes*-GFP expression often differs from that of *Nes*-CreER^{T2} (Fig. 2) [25, 34, 39, 47]. It is worth noting that gene reporter (e.g., GFP) expression is not always consistent with its mRNA and protein expression patterns. It is known that *Nes*-GFP does not regularly mimic the expression of the endogenous *Nes* gene (Fig. 2) [31]. Even *Nes*-GFP^{high} cells may lack expression of the endogenous nestin protein at a specific developmental stage [16]. Therefore, it is particularly important to consider each individual mouse reporter strain (e.g., *Nes*-GFP or *Nes*-CreER^{T2}) as an independent assay resource. Careful tracing of BM microcirculation routes and accurately marking miscellaneous cell types would certainly yield positive answers for resolving the controversies such as the origin, identity, and spatial localization of both SSC and HSC niches in the BM.

Diverse PBMNs and SSC Origins

In principle, prior to the naming of any postnatal adherent fibroblastic cell as a mesenchymal stem/stromal cell, certain stringent in vitro assays and in vivo differentiation assays must be used. In vivo assays were initially developed in immunodeficient mice that receive ectopic transplants of single-colony derived BMSCs [48–50]. Due to their mesodermal origin (except for facial bones), BMSCs were subsequently thought to differentiate into muscle, tendon, ligament, and so forth, and were given the name mesenchymal stem cell [8]. Based on subsequent studies using BMSC surface markers for nonskeletal adherent fibroblastic cells, many studies were conducted under the inappropriate terminology of mesenchymal stem cells or "MSCs." It is widely believed that MSCs are ubiquitously presented in adult tissues characterized as fibroblast-like cells under in vitro culture conditions. However, MSCs from different human tissues (i.e., the BM, muscle, and cord blood) display quite different transcriptomes, concomitantly with different differentiation capacities under stringent assays as mentioned above [51].

Since the transcriptome data were generated from total RNA isolated from multi-clonal cultures of CD146^{+} cell populations [51], we should be beware that some marker gene expression patterns could be altered under certain cell culture conditions. This is especially important when we compare transcriptome analysis (based on cell culture assays) with in vivo cell-fate mapping data. In the case of CD146^{+} MSCs, we found these cells still had inherently distinct transcriptomic signatures and differentiation capacities in in vivo assays after a short-term (2-week) culture in basal medium, consistent with their diverse developmental origins [51]. [Correction made here after initial online publication.] Interestingly, these specific MSCs from different tissues do share the structural organization abilities to form functional vasculature and endothelial cells-associated pericytes [51].

Pericytes are ubiquitous cells found in micro-blood vessels and peri-endothelially located along the vascular basement membrane under electron microscopy (reviewed in reference 52). Importantly, pericytes should be distinguished from many other perivascular cells and smooth muscle cells. Thus far, no definitive single marker has been found to confidently identify pericytes. CD146, also known as MCAM, primarily found in neural crest-derived melanoma cells, sheds some light on a general recruitment mechanism in humans. Knock-down of

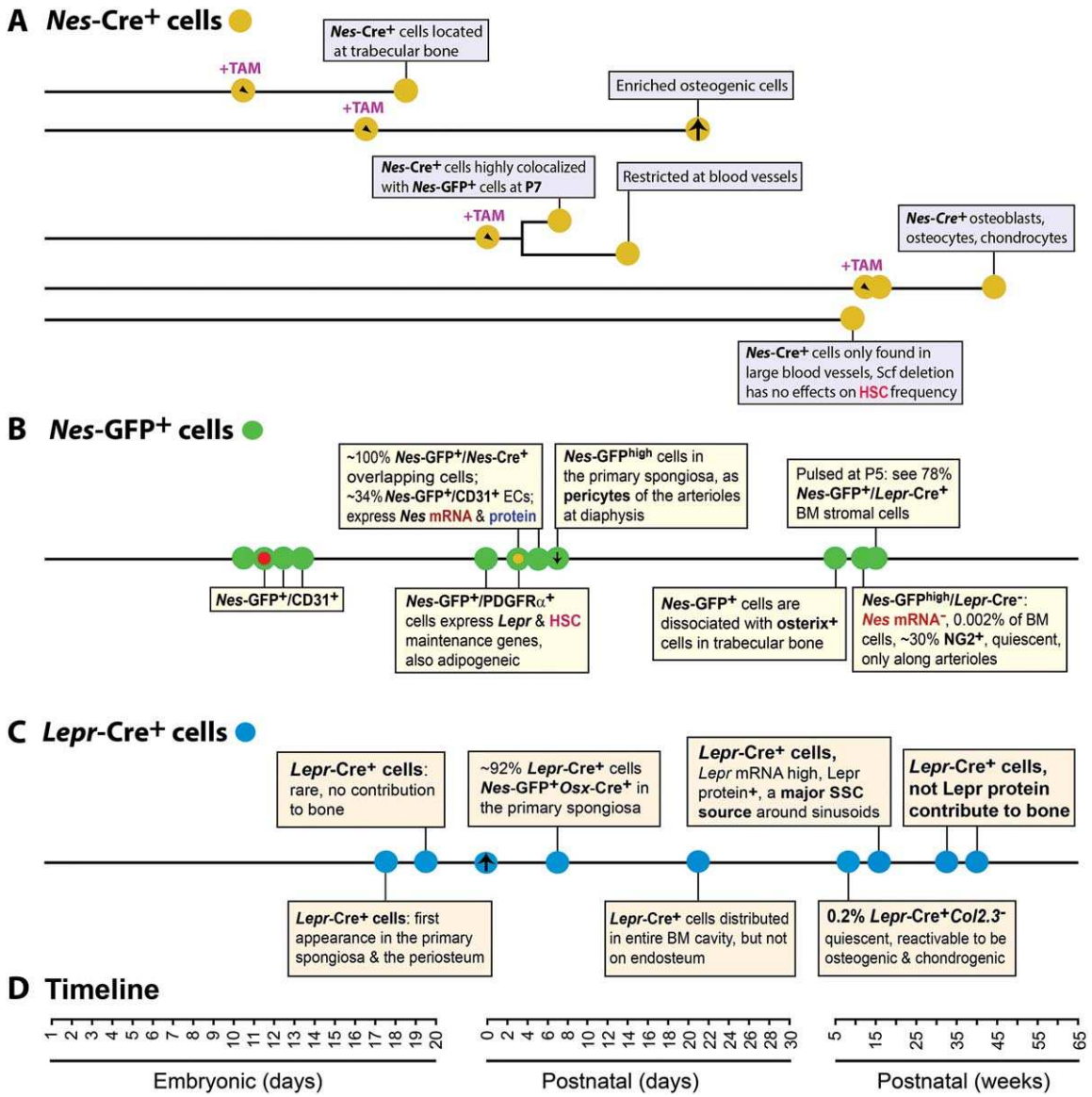

Figure 2. Transcriptional activity of the *Nes* and *Lepr* genes in mouse models at different developmental stages. **(A)**: Cell fate analysis of *Nes*-CreER^{T2} (*Nes*-Cre) transgenic mice that express the T2 mutant form of a Cre-estrogen receptor fusion (Cre-ER^{T2}) under the control of the 1.8-kb rat *Nes* intron-2 enhancer (i2E) element and a 160-bp HSV TK promoter followed by an SV40 poly A site [39]. Cre-ER^{T2} fusion protein activity is inducible to high levels at the nucleus following binding of TAM, thus deleting the floxed sequences in cells of bred mice [40]. Cre recombinase activity in transgenic mice was induced by TAM at indicated developmental stages and *Nes*-Cre marked cells were analyzed subsequently. **(B)**: Cell fate analysis of *Nes*-GFP transgenic mice (*Nes*-GFP) that express a GFP (green fluorescence protein) reporter driven by the 5.8-kb promoter and 1.8-kb i2E of the rat *Nes* gene [34]. **(C)**: Cell fate analysis of *Lepr*-Cre knock-in mice that bear the targeting vector that contains an IRES-NLS-Cre and a neo (flanked by *frt* sites) inserted immediately 3′ of the stop codon in the last exon of the *Lepr* gene [41]. **(D)**: Timeline that depicts developmental stages of mice, aligned with all above experimental schema. The information summarized in this figure is predominantly based on the following references 15–17, 25, 30, 31, 42–46. Abbreviations: BM, bone marrow; CD31, known as PECAM-1 (platelet/endothelial cell adhesion molecule 1), a platelet surface and endothelial cell intercellular junction protein; Col, collagen; Cre, Cre recombinase; HSC, hematopoietic stem cell; *Lepr,* leptin receptor; *Nes,* nestin; NG2, neural/glial antigen 2 or NG2 proteoglycan; *Osx*-Cre, osterix-Cre expression in mouse models; PDGFRα, platelet-derived growth factor receptor, alpha; P7, postnatal day 7; SSC, skeletal stem cell; TAM, tamoxifen.

CD146 resulted in abnormal blood vessel tube formation in vitro [51]. Teleologically, a differentiation potential of adult SSCs should be consistent with their primary regenerative needs after tissue damages. Not surprisingly, CD146^{+}/CD45^{-}/CD34^{-} defined MSCs in different tissues (i.e., perinatal core blood, BM, skeletal muscle, and periosteum) showed various tissue-specific transcriptomes and differentiation capacities [51]. Besides osteogenic and chondrogenic potentials from cord blood MSCs, which have more embryonic traits, the MSCs from BM and muscles exhibit osteogenic and myogenic restricted potential, respectively [51].

Furthermore, unbiased transcriptome analysis revealed that a 21-pericyte-gene "clusterome" was significantly different in the above four human MSC cells (Supporting Information Fig. 1) [51]. Interestingly, *NES* and *LEPR* lie in the two separate clusters with a reciprocal gene expression pattern.

Notably, *NES,* clustered with *CNN3, ABCC9,* and *NG2* (*CSPG4*), is highly enriched in perinatal cord blood, consistent with the preferential expression patterns of *NES* in embryonic tissues (Supporting Information Fig. 1). Similarly, a reciprocal regulation of *Nes*-GFP and *Lepr*-Cre was also evident in the BM of the developing mouse embryos and of postnatal stages (Fig. 2). For example, *Nes*-GFP$^+$ cells represent 8% of endothelial cells in limb buds at the endochondral condensation stage (E10.5), giving rise to distinct HSC niche-forming stromal cells in vivo at the perinatal stage (P0), and began to decline at P7 (Fig. 2) [15, 25]. However, the first appearance of *Lepr*-Cre$^+$ cells was in the BM primary spongiosa and the periosteum at E17.5, suggesting a possible origin of *Lepr*-Cre$^+$ cells from *Nes*-GFP$^+$CD31$^{+/-}$ cells [25, 42]. Furthermore, the emergence of *Lepr*-Cre$^+$ dominance (in the developing bone and BM) coincides with the decrease of *Nes*-GFP transcriptional activity [42]. Thus, coordination between *NES (Nes)* and *LEPR* (*Lepr*) gene regulation suggests a potentially important mechanism that regulates differential BM SSC origins.

The Contribution of Neural Crest Cells to SSCs

Of interest, several neuroectodermal derivatives, including peripheral sympathetic neurons and Schwann cells, are involved in the regulation of BM SSCs. Using a neural-crest lineage tracing method, Morikawa and colleagues observed a higher frequency of *P0*-Cre-labeled PDGFRα^+/Sca-1$^+$/CD45$^-$/Ter119$^-$ (PαS) cells [36], indicting a partial neural-crest origin of BM stromal cells. But, using a *Wnt1*-Cre mouse strain, in which Cre recombinase activity is under the control of the mouse *Wnt1* promoter and enhancer, Morrison and colleagues failed to label CFU-Fs [31]. They suggest that *Wnt1*-Cre labeled neural crest cells may represent a separate population from *Lepr*-Cre$^+$ cells in the BM [31].

The myelin protein zero (P0), a type I transmembrane glycoprotein expressed specifically in Schwann cells of the peripheral nervous system, is a structural protein of the peripheral myelin sheath. Defects of *P0* in Schwann cells led to nerve cell demyelination [53]. P0-Cre transgenic mice express Cre recombinase driven by the *P0* promoter. It has been used as a genetic tool for labeling neural crest cell lineages such as Schwann cells [54]. Moreover, *Wnt1*-Cre transgenic mice were generated by introducing the Cre recombinase cDNA into a modified 10-kb *Wnt1* genomic sequence between the promoter and enhancer [55]. It has been widely used in the study of Wnt signaling in middle brain development and in the early migratory neural crest cell populations and its derivatives [55, 56].

One possible explanation for the above discrepancy is that *P0*-Cre labels a different neural crest cell population that might not be labeled by *Wnt1*-Cre and *vice versa*. Although *P0*-Cre is expressed mainly in Schwann cells, it is also found in a subset of periventricular cells that yield cells in the cortex and in a small subset of neural-crest derived progenitors in the heart [54]. It is known that *P0*-Cre and *Wnt1*-Cre are differentially expressed, in which *Wnt1*-Cre is distributed extensively in the midbrain and *P0*-Cre in the hindbrain of embryos [57]. Hence, the variability of *P0*-Cre expression and the distribution differences between *P0*-Cre and *Wnt1*-Cre might also explain their discrepancies in labeling CFU-Fs in BM.

Another possible explanation for the inconsistency of the above studies is that *P0*-Cre-labeled neural crest cells may be amongst endothelial cells of the capillaries descended from the metarterioles in the medullary cavity. Thus, these endothelial cells, which should be *Nes*-GFP$^+$/CD31$^+$ cells, are able to migrate to perivascular sites to become CFU-Fs. Colocalization of *P0*-Cre-labled cells with *Nes*-GFP$^+$/CD31$^+$ cells around the capillary bed would clarify this issue. Since these metarterioles are sparsely distributed within the BM, their contribution to CFU-Fs may also be limited. Moreover, some perivascular pericytes, likely derived from the neural crest, may contribute to the progenitors of SSCs.

Finally, we cannot rule out that the above experimental discrepancies might be due to the use of different cell labeling techniques (i.e., *P0*-Cre vs. *Wnt1*-Cre). More robust analytic tools and markers should be used to resolve these issues. Thus, it is imperative that the cells with both CFU-F and neural crest features should be examined using freshly isolated single CFU-F-derived colonies from adult mouse BM under identical genetic experimental conditions. Taken together, neural crest cells in the BM might directly or indirectly contribute to osteogenesis via the formation and maintenance of SSCs, which are believed to be inseparable with HSC niches in BM.

Origin and Spatial Localization of HSC Niches in the BM

Currently, there are several useful makers, which include CD150 (SLAMF1), c-kit, and α-catulin-GFP, to define HSCs in the BM [16, 58]. However, the exact locations of the HSC niche are still under debate. Early studies provide some genetic evidence, implicating osteoblast lineage influences on primitive hematopoietic cells [59, 60]. It is conceivable that both HSC and SSC niches are distinct but inseparable, and nurture each other in the BM. HSCs are widely distributed in the BM, but concentrated in the trabecular region. Approximately 20% of mouse HSCs are within 10 μm of the endosteum [4], which may be directly regulated by factors secreted from the cells on endosteal surfaces. Moreover, HSCs could be greatly purified by sorting CD150$^+$/CD48$^-$/CD244$^-$ cells, which are found virtually within a 10-μm cell diameter area from sinusoids in both BM and spleen [61–64]. Using *Nes*-GFP, *Nes*-CreER^{T2}, and *Lepr*-Cre reporter strains, researchers have provided new results to elucidate the origins of BM HSC niches (Fig. 2). The HSC niches are suggested to reside at two distinct perivascular regions within the BM. One is believed to be a periarteriolar niche and another one a perisinusoidal niche [16, 17, 58, 65].

Frenette and colleagues initially found that rare NG2$^+$ (chondroitin sulfate proteoglycan or CSPG4$^+$) pericytes (based on the *NG2*-CreER^{TM} mouse stain) have a high *Nes*-GFP signal. These *NG2*-CreER^{TM+}/*Nes*-GFP^{high} pericytes ensheath small arterioles exclusively (preferentially in the endosteal region), thus creating a periarteriolar niche to maintain quiescent HSCs [17]. This periarterial niche hypothesis was supported by genetically activating the HSC cycle (through the depletion of NG2$^+$ cells), which altered the distribution of quiescent HSCs from periarteriolar to perisinusoidal regions [17]. Furthermore, ablation of periarteriolar *NG2*-CreER^{TM+} cells by diphtheria toxin also drove HSCs into cell cycling, thus depleting long-term repopulating HSCs in the BM [17]. This result was also supported by a recent study from the same group, in that *NG2*-CreER^{TM+}/*Nes*-GFP^{high} cells are shown to maintain the periarteriolar HSC niche, presumably by secreting the HSC

factor, Cxcl12 [65]. Noticeably, *NG2*-CreER^{TM} (with a tamoxifen-inducible Cre-fusion protein) and *NG2*-Cre (with a constitutive nuclear Cre) have different expression patterns in BM, in which *NG2*-Cre marks cells at both periarteriolar and perisinusoidal niches, thus exerting differential regulation of HSC niches [65]. [Correction made here after initial online publication.] For example, it was revealed that *NG2*-Cre, but not *NG2*-creER^{TM}, marked cells that were the source of Scf in BM [65]. Furthermore, knock-out of Scf or Cxcl12 in *NG2*-CreER targeted mice did not result in multilineage reconstitution defects (only T cell reconstitution defects observed), which contradicts with the multilineage reconstitution results obtained from *NG2*-Cre targeted cells [65]. Thus, different experimental approaches may produce dissimilar and meaningful results even under a slightly different genetic make-up.

However, the periarteriolar HSC niche location was not supported by the experimental data from Morrison and colleagues [16, 58]. They conditionally deleted *Scf* simultaneously in endothelial cells (using *Tie2*-Cre mice) and in *Lepr*$^{+}$ cells (using *Lepr*-Cre mice), resulting in depletion of the majority of CD229$^{-/low}$ HSCs. However, *Scf* depletion in hematopoietic cells, osteoblasts, *Nes*-CreER^{T2-} and *Nes*-CreER^{T2+} cells showed no major effects on BM HSCs [43]. The *Lepr*$^{+}$ cells largely surrounded sinusoids throughout the BM [58]. Thus, these data support a perisinusoidal HSC niche that maintains the dormant HSCs. Furthermore, using a deep imaging analysis of α-catulin-GFP knockin mice, the same group found both dividing and quiescent HSCs are concentrated around sinusoids of the central BM, physically interacting with *Lepr*-Cre^{+}/CxCL12^{+} stromal cells [16]. Under their experimental conditions, HSCs were distant from arterioles, transition zone vessels, the endosteal surface, and metaphyses of the long bone [16]. Therefore, this study further strengthens their perisinusoidal niche conclusion.

Evidently, one of the major factors that lead to the different conclusions is the type of manipulation of experimental systems, including the use of different mouse genetic strains, cytotoxicity from cell lineage ablation experiments using a Cre-inducible diphtheria toxin receptor [17, 66], tamoxifen induction-related side effects [67, 68], Cre-ER^{T2} activation-mediated hematological toxicity [69], and *NG2*-CreER^{TM} mediated recombination in brain [65, 70]. All the above conditions could directly or indirectly render nonspecific phenotypes on BM HSC biology. Nonetheless, the above studies provide substantial evidence, illustrating distinct microenvironmental niches that sustain quiescent HSCs. Still, the discrepancies between conclusions from different groups remain to be carefully analyzed based on available datasets from a tailored experimental system.

Visibly, current data indicate that there are two distinct cell populations (i.e., *NG2*-CreER^{TM+}/*Nes*-GFP^{high} and *Lepr*-Cre^{+}/*Nes*-GFP^{low} cells) that are essential for the determination of HSC niche. *NG2*-CreER^{TM+}/*Nes*-GFP^{high} cells represent a rare cell population that are mainly found near the arterioles (Fig. 3, A2), whereas *Lepr*-Cre^{+}/*Nes*-GFP^{low} cells are close to sinusoids (Fig. 3, A4). We speculate that *Nes*-GFP^{high} cells may represent either endothelial cells or smooth muscle cells or both within the arterioles in adult BM, thus physically separating themselves from perivascular niches. *Nes*-GFP^{low} cells are very close to *Lepr*-Cre^{+} cells in the perinatal BM [15, 25, 42]. But, they represent two separate populations in the BM perivascular niche. However, at the postnatal stage (1 week to 16 weeks), *Nes*-GFP^{low} cells are largely overlapping with *Lepr*-Cre^{+} cells near endosteal surface and endothelial cells [25] and around sinusoids [31, 43] (Fig. 2). Apparently, *Lepr*-Cre^{+}/*Nes*-GFP^{low} cells, which are also *Scf*-GFP^{+}, Cxcl12^{+}, *Nes*-CreER^{-}, and *NG2*-CreER^{TM-}, represent approximately 0.3% of adult BM cells [31]. Nevertheless, we need to use an experimental system with controllable variables, minimal cellular cytotoxicity, and permanent genetic markers (such as tdTomato) to implement cell lineage tracing. Moreover, *NG2*-Cre^{+}/*NG2*-CreER^{TM+}/*Nes*-GFP^{high} and *NG2*-Cre^{+}/*Lepr*-Cre^{+}/*Nes*-GFP^{low} cell populations, together with tdTomato-labeled cell fates, would provide important clues to map the cell lineages associated with HSC-niche supporting functions, thus likely facilitating the resolution of different opinions on distinct HSC niches. Likely, above two cell populations within their microenvironment might constitute an interactive interface niche, which secretes cytokines and signaling molecules, for maintenance of HSC niches and for cell-fate determinations under different stress conditions.

New Hypotheses: Coupling and Uncoupling of BM Stem Cell Niches Within Their Regenerative Zones

To facilitate the resolution of the above debates, we propose a stem-cell-dynamic niche model to illuminate potential mechanisms for niche development at various stages and to resolve the existing controversies under a unified consensus (Fig. 3). In this model, skeletal progenitor cells at the embryonic (prenatal) stage converge at sites undergoing endochondral bone formation (e.g., the primary spongiosa). At the perinatal stage, a rare and quiescent SSC population emerges from a skeletal progenitor pool, likely via an asymmetrical cell division mechanism. Moreover, a niche interface environment between the periarteriolar and perisinusoidal niches might function as a Yin-Yang niche for both SSCs and HSCs (Fig. 3, A3). Several lines of evidence support the Yin-Yang niche model [15, 17, 31, 42, 65, 71]. For example, periarteriolar NG2^{+} pericytes, possibly derived from the neural crest, strongly expresses *Nes*-GFP [15, 17]. These *Nes*-GFP^{high}/*Lepr*-Cre$^{low/-}$ cells may be converted to *Lepr*-Cre^{high}/*Nes*-GFP$^{low/-}$ at the Yin-Yang niche. This speculative cell-state conversion is deduced from the existence of intermediate and overlapping states of the above two cell types that span from periarteriolar to perisinusoidal niches (Fig. 1B, Fig. 2B, 2C) [17, 25, 31, 42, 65]. In the future, nestin^{+} cell tracing with a permanent genetic marker is essential to provide a clear lineage relationship. Nonetheless, the Yin-Yang niche likely provides an environmental cue that could regulate this cell-state conversion, possibly through reciprocally regulating *Nes*-GFP and *Lepr*-Cre epigenomes and transcriptomes. Thus, the epigenetic signatures on transcriptional complexes on both *Nes* and *Lepr* loci might provide additional molecular clues to the proposed model in the future [72, 73].

Furthermore, postnatally and in adulthood, both SSC and HSC niches segregate from their regeneration sites, which are distal from the central cavity along the developmental pipeline (Fig. 3). Yellow marrow increases with aging and secretes leptin, which might stimulate *Lepr* expression. Neurological cells express high levels of Lepr proteins, which might in turn suppress HSC and SSC niches. In addition, BM endothelial cells with strong endomucin expression provide reasonable niche signals for

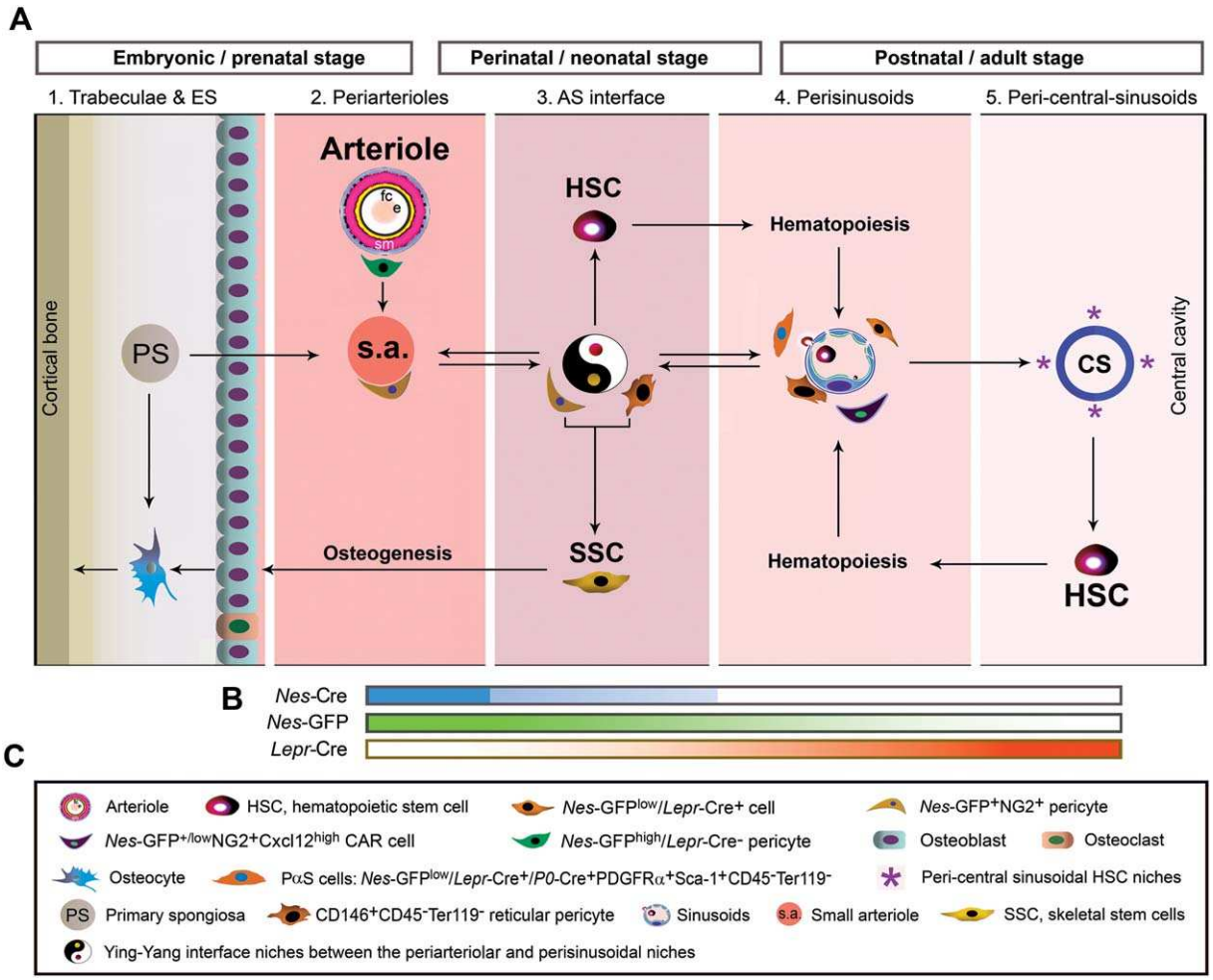

Figure 3. A dynamic niche model that underlies coupling and uncoupling of paralogous BM niches (PBMNs) with their regenerative zones at various developmental stages. **(A)**: At the embryonic (prenatal) stage, BM hematopoietic stem cells (HSCs), skeletal stem cells (SSCs), and embryonic progenitor cells are actively proliferating and converging at endochondral osteogenic sites (e.g., primary spongiosa and trabecular areas) near the ES. At the perinatal stage, both HSCs and SSCs are emerging into two distinct populations through asymmetrical cell division, with one rare and quiescent adult stem cell population and another one a progenitor pool. A Yin-Yang niche between the periarteriolar and perisinusoidal interface (i.e., the AS interface, Fig. 3A3) may be responsible for the above changes. For example, periarteriolar *Nes*-GFP^{high}/*Lepr*-Cre$^{low/-}$ cells may be altered to become perisinusoidal *Lepr*-Cre^{high}/*Nes*-GFP$^{low/-}$ cells at this Yin-Yang niche via an inverse regulation of the transcriptomes of both *Nes*-GFP and *Lepr*-Cre. At the postnatal and adulthood, non-overlapping SSC and HSC niches move toward the CS. **(B)**: *Nes*-Cre, *Nes*-GFP, and *Lepr*-Cre transcriptional activity in niche cells that are aligned to correspond to dynamic niche activities in mouse models depicted in Figure 3A. **(C)**: Notable cell types in PBMNs presented in Figure 3A might represent distinct or overlapping cells. The detailed descriptions of *Nes*-Cre, *Nes*-GFP, and *Lepr*-Cre reporter assays are available in the legend to Figure 2. Abbreviations: CS, central sinusoids; ES, endosteum.

perivascular osteoprogenitors in aged animals. Their cell numbers are associated with the formation of metaphysis and trabecular bone. In aged adult bone, the effector areas of SSCs are in trabecular and metaphyseal regions. However, the SSC niche moves closer to endothelial cells.

Alterations of PSNs and Diseases

Clearly, a thorough understanding of the influence of PSNs or PBMNs on disease conditions would facilitate the implementation of precision regenerative medicine. Such influences could be well exemplified by PBMN maintenance and homeostasis from fibrous dysplasia and from the development of refractory cancers such as acute myelogenous leukemia (AML) in BM.

Fibrous dysplasia is a severe disease that is caused by activating mutations of the *GNAS* (GNAS Complex Locus) gene [74] and now considered as an SSC disease. PBMNs have abnormal hematopoietic microenvironments with excessive misfunctioning SSCs/BMSCs in BM, thereby exhibiting deficiency of hematopoietic cells and marrow adipocytes [75, 76]. Moreover, SSC-like cells that express *Nes*-GFP and β2 adrenergic receptors were shown to expand significantly in BM during the development of acute AML. These expanded SSC-like cells were able to accelerate leukemogenesis by regulating leukemia stem cells, concurrently impairing NG2^{+} periarteriolar function [77].

With respect to the impact of alterations of stem-cell niches on aging, there is one convincing example from the intestinal PSN in *Drosophila*. In these intestinal stem cells, a decrease in insulin/IGF signaling activities concomitantly with an increased expression of stress-protective genes such as *Foxo* promotes cellular homeostasis and delays aging [78].

However, it is unclear whether there are similar niche-mediated protective mechanisms in BM.

Nevertheless, the above examples provide a basis for modeling of SSC diseases and raise hope for a cure for such diseases through mutation correction and possible pharmacological interventions. Enforcing stem cell quiescence by reducing cell proliferation, limiting stress-activated signaling responses, and enhancing cellular homeostasis (e.g., by autophagy) would likely prevent or delay stem-cell-based diseases, pathological aging, and carcinogenesis [79–81].

Concluding Remarks

Paralogous expansion seems to be a powerful approach by evolution to increase cell diversity and functionality in the vertebrate system. The concept of PBMNs may simplify our understanding of the complexity of this particular biological system. Moreover, this concept provides a consensus to integrate cell information from non-skeletal tissues. Deciphering of the PSN or PBMN code could be made possible through the analysis of transcriptional signatures and cell lineage analysis through transcription reporter-based animal models. Technically, each individual mouse reporter strain should be considered as an independent assay with a complete characterization at different developmental stages. We emphasize the role of a nontoxic, specific, and regulatable genetic system in minimizing experimental discrepancies. Thus, unfolding of the paralogous niche codes would enable us to handle prospective technological pitfalls and challenges with ease and to develop definitive marker panels for paralogous niche cell identity discrimination.

Accordingly, we shed light on the unique SSC identity with diverse PBMN origins from both mesoderm and neuroectoderm. We further propose a PBMN dynamic niche model that elucidates the coupling and uncoupling mechanisms between SSC and HSC niches and their cell regeneration zones. This Concise Review and perspective may aid to solve numerous stem cell controversies, thereby facilitating the way to disease modeling, precision regenerative medicine, and pharmaceutical applications.

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Author Contributions

K.G.C.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; K.R.J.: collection and/or assembly of data, data analysis and interpretation; R.D.M.: conception and design, data analysis and interpretation; P.G.R.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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