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REVIEW ARTICLE

Bone marrow niches for hematopoietic stem cells

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Abstract

Hematopoietic stem cells (HSCs) are the cornerstone of the hematopoietic system. HSCs sustain the continuous generation of mature blood derivatives while self-renewing to preserve a relatively constant pool of progenitors throughout life. Yet, long-term maintenance of functional HSCs exclusively takes place in association with their native tissue microenvironment of the bone marrow (BM). HSCs have been long proposed to reside in fixed and identifiable anatomical units found in the complex BM tissue landscape, which control their identity and fate in a deterministic manner. In the last decades, tremendous progress has been made in the dissection of the cellular and molecular fabric of the BM, the structural organization governing tissue function, and the plethora of interactions established by HSCs. Nonetheless, a holistic model of the mechanisms controlling HSC regulation in their niche is lacking to date. Here, we provide an overview of our current understanding of BM anatomy, HSC localization, and crosstalk within local cellular neighborhoods in murine and human tissues, and highlight fundamental open questions on how HSCs functionally integrate in the BM microenvironment.

HISTOLOGICAL ORIGINS OF BLOOD: A HISTORICAL PERSPECTIVE

Throughout human history, blood has been the object of continuous fascination, and the question of its histological and cellular origin remained enigmatic for centuries. Galen's medical theory established blood as one of the four essential humors, whose balance was key to a healthy life. He postulated that blood was continuously formed in the liver out of the processing of food, and this ancient theory prevailed for many centuries.[1] The development of the first rudimentary microscopes in the 17th century permitted the observation of circulating red "corpuscules," leading to the appreciation of the cellular nature of blood. William Hewson, considered one of the fathers of Hematology, further described a different type of white corpuscles and proposed the controversial hypothesis that both particles derived from the lymphatic system and entered the blood via the thoracic duct.[2] The refinement of microscopes and the use of natural stains by Ehrlich sharpened the notion that a plethora of cell types conformed blood, leading to their classification and initiating the morphological era in blood research. However, the origin of the production of blood cells remained elusive until 1868, when Ernst Neumann and Giulio Bizzozero almost simultaneously reported the existence of nucleated cells within the spongy marrow inside bones of humans and rabbits and postulated that blood cells originated in bone marrow (BM) tissues. Until then, marrow had been alternatively regarded as the nutrient source or waste product of the surrounding bony structures. Both Neumann and Bizzozero had trained as pathologists with Rudolf Virchow in Berlin at a time when the first notions of cellular theory emerged.[3] Virchow has been historically credited with contributing to this major conceptual breakthrough by coining one of its principal tenets, that every cell derives from a pre-existing cell (*omnis cellula e cellula*).[3] Most likely, building on these foundations and his own discoveries, Neumann not only placed the histological origin of hematopoiesis in BM but also presciently proposed the existence of a cellular precursor from which the repertoire of blood cells would be derived.[4] He termed this progenitor cell the "great lymphocyte," and this point has been considered by some authors as the birth of stem cell research.[5]

Formidable progress has been made since then in our understanding of the identity of hematopoietic stem cells (HSCs) and their inextricable functional relationship with their surrounding anatomical microenvironment. The effects observed in humans after the use of nuclear weapons in World War II propelled studies on radiation biology and hematological research and led to the crucial finding that transplantation of BM cells efficiently prevented the effects of whole-body irradiation by regenerating the entire hematopoietic system.[6] The seminal work by Till and McCulloch provided the first formal evidence that this potential was contained in progenitor cells, which clonally proliferated generating colonies in splenic tissues when transplanted into myeloablated mice.[7,8] Years later, the advent of flow cytometry and cell sorting would enable the isolation of bona-fide self-renewing multipotent HSCs, distinct from the colony-forming units observed in

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those initial experiments. Developments in the identification and quantification of HSCs were paralleled by numerous studies in the 1960s, which indicated that their functional properties crucially relied on extrinsic signals derived from a so-called *hematopoietic inductive microenvironment*, in which nonhematopoietic, stromal cells played a major role.[9,10] Hence, the concept of an *ecological niche* for HSCs gradually emerged.[11] Nonetheless, Robert Schofield has been most widely credited for laying the foundations of the niche hypothesis. Based on the different properties exhibited by progenitor cells in different tissue sites, he envisioned the niche as a fixed anatomical entity in which HSCs reside, with the ability to confer identity and determine fate.[12,13] His theoretical formulation has had a massive conceptual influence up to our days. Here, we provide an updated overview of progress made in describing preferential niches for HSC residence in the BM.

CELLULAR COMPOSITION AND STRUCTURAL ORGANIZATION IN THE BM

BM tissues distribute throughout the organism filling bone enclosures, which are highly heterogeneous in terms of developmental origin, morphology, and relative content in cortical and trabecular bone. Within cortical regions, such as those found in femoral shafts, marrow masses fill the elongated cavities, while inside trabecular bone tissues are found interspersed among a labyrinth of bony spikes or trabecules.[14] The bone surface to BM content ratio is significantly higher and bone remodeling processes are more active in trabecular areas, which potentially results in differences in the surrounding physiological milieu between both sites. Nevertheless, despite these strong structural variations, the hematopoietic and immune contents found in different bones and regions are remarkably homogeneous,[15,16] thereby pointing to the existence of finely tuned systemic regulatory mechanisms, that control the composition of hematopoietic tissue. Bona-fide multipotent, self-renewing HSCs are found at very low frequencies, and a large proportion of marrow content is made of multipotent, myeloid, and lymphoid-restricted progenitor cells, which progressively differentiate along the developmental hierarchy, continuously generating billions of mature cells to be released into circulation on a daily basis.[17] The BM is rich in mature cells of the innate and adaptive immune systems. Myeloid cells include various populations of DCs,[18] tissue-resident macrophages,[19] as well as recently generated and senescent neutrophils, which re-enter the BM for clearance.[20] In turn, BM tissues constitute a major hub for several long-lived cells of the lymphoid lineage, such as naïve, regulatory, and memory T cells and antibody-producing plasma cells.[21–23] Albeit their overall frequencies are relatively low, given that the BM is one of the largest organs in mice and humans, collectively marrow-residing fractions of these subsets are large, which makes it the dominant reservoir for immune regulation and memory.[21]

How is such a dynamic hematopoietic landscape spatially organized into a functional, blood-producing organ? As for most tissues, the hematopoietic fraction of the BM assembles around a complex stromal compartment made up of mesenchymal, endothelial, and neural cells, which are configured as cellular scaffolds that not only provide a stable infrastructure for hematopoietic development but also act as key functional regulators of hematopoiesis and bone metabolism[24,25,26] In the last decades, studies on the composition and function of this stromal fraction have flourished, fostered by the deep interest on the multiple roles of these cell types in the direct regulation of HSCs.[27,28] Detailed insight into the structural organization and interactions of stromal networks has been obtained using fluorescence-based immunohistological techniques and more recently three-dimensional (3D) microscopy.[29,30] With the development of genetic models to label and target different cell types and the advent of single-cell technologies, the emerging picture of BM stromal cell biology is one of overwhelming complexity.[31–33]

BM microvascular networks

The BM is densely vascularized by a unique microvascular tree, which accounts for 10%–20% of the tissue volume.[34,35] In long bones such as femurs, oxygenated blood penetrates BM cavities through bone foramina via large nutrient arteries.[36,37] Large infiltrating arterial vessels run longitudinally along the central axis and branch into arterioles of progressively smaller calibers, which migrate radially toward endosteal regions (Figure 1A–F). In the proximity of bone surfaces, arterioles merge with a network of transcortical capillaries, which traverse through bone inside narrow channels and fuse into so-called transitional (or type H) vessels.[38–40] These short vascular segments immediately give rise to the sinusoidal network, which is formed by wide and fenestrated structures that do not assemble hierarchically, but merge in a maze of interconnected vessels. Oxygen and nutrient exchange with surrounding tissues most likely actively starts in the transitional zones and the more permeable initial sections of sinusoids, which then drain venous blood from the endosteum toward a large central collecting sinus.[41,42] While this canonical structure has been mostly described and is best visualized in areas of cortical bones, such as the diaphysis of the femur, vascular networks in other trabecular areas, or sternal and cranial bones, appear more disorganized but follow very similar general structural principles.[15,35,43]

The endothelial walls of the different vascular districts are lined by endothelial cell (EC) subtypes, including arterial and sinusoidal ECs (AECs and SECs), which have distinct morphological, phenotypic, and molecular features (see Table 1).[44,45] The nature of lining ECs confers specific properties to the vessel in terms of permeability and nutrient exchange. Most importantly, through the production of a different set of angiocrine factors and the establishment of direct cellular interactions, ECs play pivotal roles in the orchestration of hematopoiesis and osteogenesis.[46,47] Recent data suggest that even within the well-defined sinusoidal tree, smaller subdistricts may differ in function and molecular profile depending on their relative positioning within the network. For instance, expression of the key myelopoietic factor CSF1 is restricted to defined sinusoidal sections, creating specialized domains for monocyte development.[48] Thus, the physiological milieu to which neighboring cells are exposed likely differs even between seemingly equivalent perivascular sites (Figure 2).

Mesenchymal infrastructure

In the BM, the term mesenchymal is used to refer to cells that form part of the skeletal system, which comprises mature cell types such as osteoblasts and adipocytes, as well as progenitor cells defined by their trilineage (osteoblastic, adipocytic, and chondrocytic) differentiation potential in vitro.[26] Of the latter, the largest fraction are so-called CXCL12-abundant reticular cells (CARc), collectively termed after their abundant expression of the chemokine CXCL12, and defined by expression of Leptin receptor (LepR^{+}).[49,50] CARc spread throughout the entire BM parenchyma as a compact network of spindle-shaped cellular bodies, which are highly interconnected through projections that stretch along matrix fibers (Figure 1D). Due to their high density and almost ubiquitous presence, CARc networks are interwoven with virtually all cells and constitute the basic fabric of marrow.[34] Yet, beyond their structural role, CARc are directly

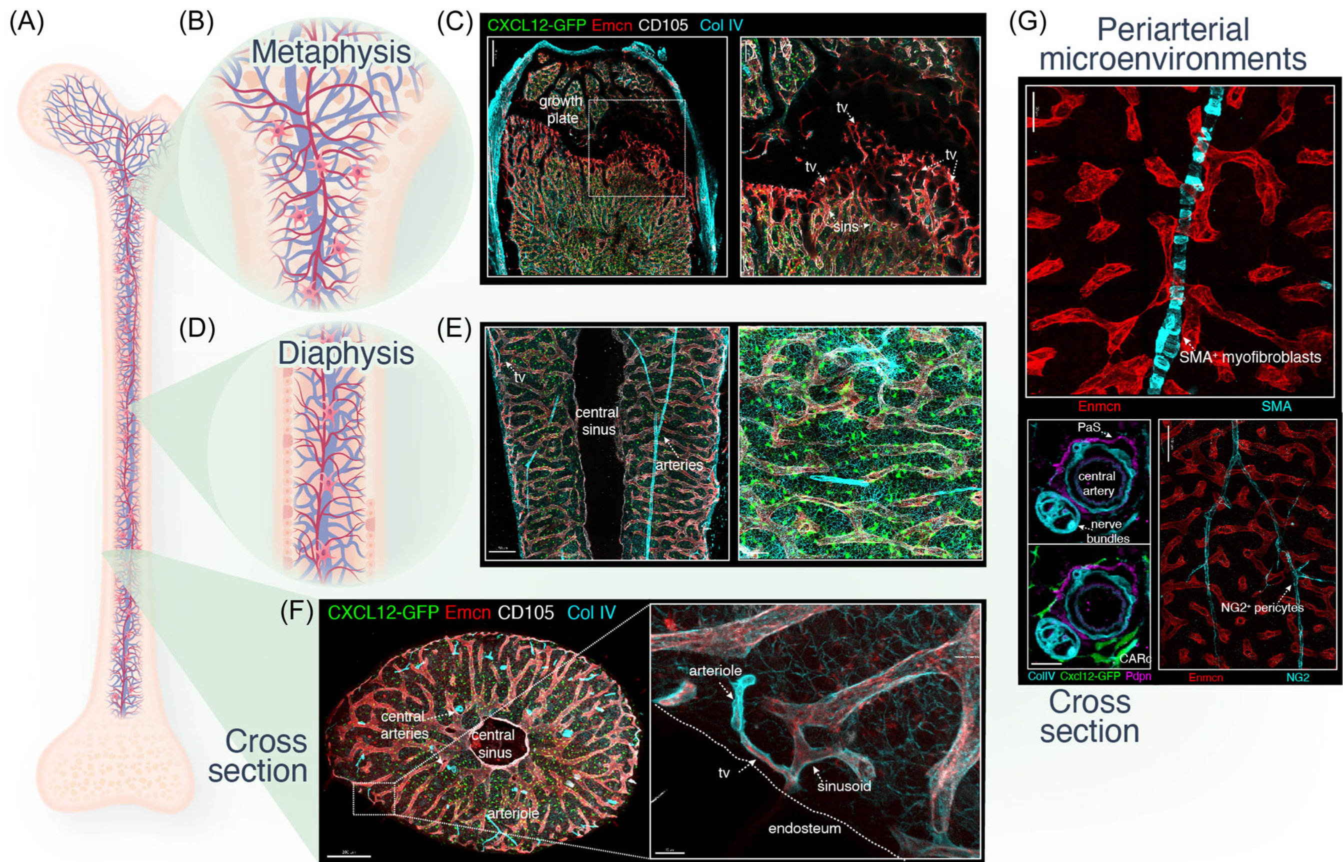

FIGURE 1 Structural organization of bone marrow tissues. Schematic representation and microscopic imaging of bone marrow (BM) tissues. (A) Femoral cavities contain regions representative of cortical (diaphysis) and trabecular (metaphysis) bone. (B) Schematic representation of metaphyseal regions including growth plate. (C) 3D microscopy of a femoral metaphysis; arteries marked by collagen IV (ColIV) expression (cyan), transitional vessels (tv) marked by a strong expression of endomucin (Emcn, in red), absence of CD105 and a straight and thin morphology. Sinusoids are wide irregular vessels labeled by the combined expression of Emcn and CD105. CARc present in metaphyseal areas are shown in green (*Cxcl12*-GFP). The outer layers of bone are shown in cyan due to labeling by ColIV. Scale bar 300 µm. High-resolution zoomed-in image of the growth plate showing details of transitional vessels ($Emcn^{bright}$) connecting to sinusoids (co-labeled by Emcn and CD105). Scale bar 100 µm. (D) Diagram of the vascular and mesenchymal structures of the diaphysis. (E) Microscopy depicts a large branching central artery, transitional vessels in endosteal regions, and the dense sinusoidal network which drains into a large central collecting sinus. Scale bar 150 µm. The image on the right illustrates the dense meshwork formed by interconnecting, spindle-shaped CARc and their interaction with the extracellular matrix (ECM) fibers (same color code as described in (B). Scale bar 50 µm. (F) Transversal section of the femoral diaphysis including six central arterial branches (cyan) arranged around a central sinus. In the proximity of endosteal regions, fragments of thin branching arterioles can be observed, in some cases directly connecting with sinusoids which drain blood back to central regions and the main collecting sinus. Scale bar 200 µm. Bottom image depicts the detail of the periosteal arteriole ($ColIV^+$) connecting to a transitional vessel ($ColIV^+Emcn^+$) and giving rise to a sinusoidal vessel. Scale bar 15 µm. (G) Images of periarterial microenvironments. Left: high-resolution cross-section of arterial vessel including periarterial layers of $ColIV^+$ pericytes, and an outer layer of $Pdpn^+$ PαSc (adapted from Helbling et al.[44]). Scale bar 10 µm. Periarterial CARc are shown in green (image below). Scale bar 50 µm. Center: arterial vessel surrounded by ring-like SMA^+ myofibroblasts. Right: $NG2^+$ pericytes along arterial trajectories in the BM. Scale bar 100 µm.

involved in orchestrating hematopoiesis through their prolific expression of factors involved in lympho-myeloid differentiation and stem cell regulation, as will be discussed later.[51–56] Besides their mesenchymal differentiation potential in vitro, CARc are the primary source of adipocytes and osteoblasts in vivo during homeostasis, as well as during fracture healing in adult mice.[57]

Given their abundance and multiple functions, it is not surprising that several studies have reported a high degree of heterogeneity within the CARc pool, including the presence of at least two subsets of cells displaying an obvious bias toward adipogenic or osteogenic differentiation (termed adipo- and osteo-CAR, respectively).[51,58–60] Whether these disparities in transcriptomic profiles are reflective of distinct, location-specific functional roles of CARc subtypes remains to be fully elucidated. However, recent studies seem to validate this notion. For instance, while adipo-CARc displayed preferential association to sinusoids, osteo-CARc expressing higher levels of Alkaline phosphatase (*Alpl*) tend to localize closer to arteriolar environments or not directly adjacent to any vascular structure.[51] More recently, expression of Osteolectin (Oln) has been reported to mark a subset of CARc, which is poised towards osteogenic differentiation, contributes to the generation of osteoblasts during bone healing, and resides in the vicinity of arterioles where they regulate early lymphocyte progenitor maintenance through expression of stem cell factor (SCF).[61] Altogether, effective ways to discriminate CARc phenotypically, genetically, and functionally are currently needed to understand their heterogeneity, functional specialization, spatial regionalization, and ontogenic relationships.

Other mesenchymal stromal cell (MSC) subsets have been found primarily localizing around the arterial vascular tree. Two of these cell

TABLE 1 List of the main endothelial, mesenchymal, and neural stromal components described so far.

Compartment	Stromal component		Markers		Marker genes (sc-RNA seq)		Mouse reporters/genetic tools		References
	AEC		Caveolin, Sca-1^{hi}, Col4a1^{hi}			*Cav1, Cd34, Ly6a, Ace, Ltbp4, Sox17, Nrp1, Efnb2*		*Flk1*^{GFP}	
Endothelial	**Transitional vessels (type H)**		Endomucin^{hi}	CD31, Cdh5	*Cdh5, Pecam1, Tek*		*Cdh5*-Cre, Cdh5-Cre^{ER}, *Tie2*-Cre^{ER}	-	43,44,51,58,59,99,152
	SEC		CD105, Endomucin			*Ephb4, Stab2, Flt4, Epor, Angpt2, Vcam1, Flt4, Sele, Selp, Emcn*		*Efnb2*-GFP, *Bmx*-Cre^{ER}	
	CARc	**Adipo-CAR**	-	Nestin^{lo}, CD140a/b, CD51, LepR	*Cxcl12, Kitl, Lepr, Ebf3*	*Hp, Lpl, Adipoq, Cd302, Gas6, Apoe, Tnem176b,*	*Adipoq*-Cre^{ER}	*Cxcl12*^{GFP}, *Scf*^{GFP}, *LepR*-Cre, *Prrx1*-Cre, *Ebf3*-Cre^{ERT2}, Cxcl12	49,51,52,58–62,153–155
		Osteo-CAR	Alpl			*Ibsp, Spp1, Alpl, Wif1, Bglap, Sp7, Runx2, Igfbp4,*	*Osteolectin*^{tdTom}		
	PαSc		(PDGFR-b^{+}Sca-1^{+}), Podoplanin		*Pdgfrb, Pdgfra, Thy1, Ly6a*		*Prrx1*-Cre		44,52,53,64
	Pericytes		NG2, CD140b/a		*Cspg4*		*Prrx1*-Cre, *Nestin*^{GFP}; *Ng2*-Cre^{ERTm}		35,63
Mesenchymal	**Myofibroblasts**		SMA, SM22		*Acta2, Myh11, Col1a1, Tagln, Rgs5*		*Myh11*-Cre^{ERT2}		51,63,156
	Adipocytes		Perilipin, BODIPY		-		*Plin1*-Cre^{ER}, *Prrx1*-Cre		75,157,158
	Osteoblasts		Osteopontin, Osteocalcin		*Sp7, Runx2, Col1a1, Ibsp, Bglap2, Dmp1*		*Col2.3*-Cre, *Col1a1*-Cre^{ER}, *Osx*-Cre		
	Osteocytes						*Dmp1*-Cre, *Dmp1*^{GFP}	*Prrx1*-Cre,	51,58,60,70,157,159–161
	Chondrocytes		Acan, sox9		*Sox9, Col2a1, Col10a1, Pth1r, Acan,*		*Col2.3*-Cre, *Col1a1*-Cre^{ERt}, *Acan*-Cre^{ERt}		
	Nociceptive nerves		Tubulin, Calcitonin gene related peptide		-		-		77,81
Neural	**Sympathetic nerves**		Tubulin, Tyrosine hydroxylase, Peripherin		-		-		35,77,161–163
	Cholinergic nerves		βIII Tubulin, GDNF family receptor alpha 2		-		*(ChAT)-IRES*-Cre		84
	Non-myelinating Schwann cells		GFAP, Nestin		*Mog, Mag, Mpz, Plp1, Mbp*		*Gfap*-Cre, *Nestin*^{GFP}		35,51,82,156

Note: The table includes: i) a selection of the most common the marker combinations employed for their identification with flow cytometry (pre-gated as CD45^{-}Ter119^{-}) or microscopy, ii) specific marker gene signatures validated in various single cell mRNA sequencing studies, and iii) transgenic murine models developed for specific cell population targeting and employed to characterize their roles in BM tissues.

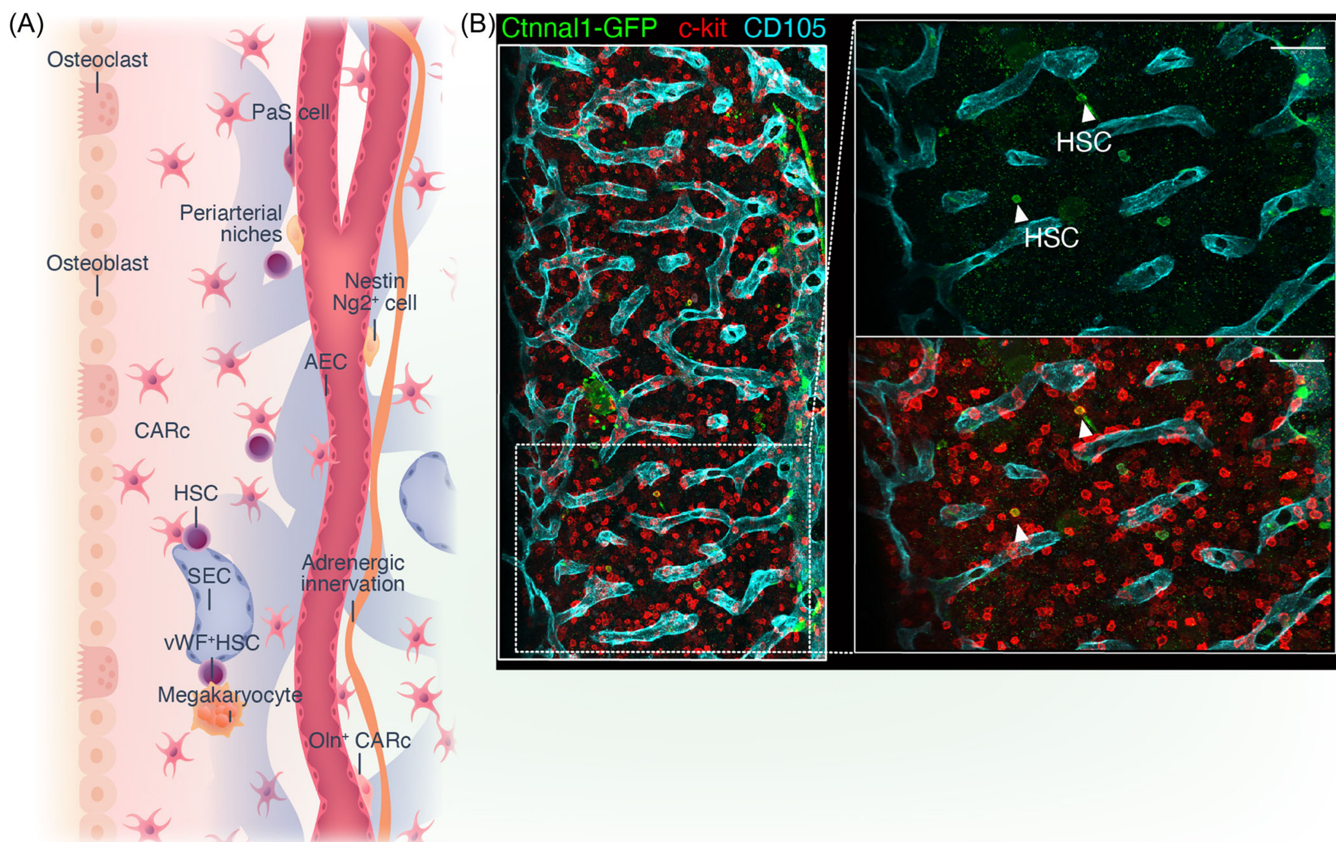

FIGURE 2 Bone marrow (BM) hematopoietic stem cells (HSC) niches. (A) Scheme depicting spatial localization and the main cellular interactions described for HSCs. Most HSCs have been observed scattered throughout BM parenchyma in the vicinity of sinusoidal vessels and directly adjacent to CARc. A minor fraction of HSCs lies near arterial or arteriolar vessels. PαSc, NG2$^+$ pericytes, Oln$^+$ CARc and SMA$^+$ myofibroblasts localize directly adjacent to the extraluminal layer of arterial and/or arteriolar endothelium. The principal neural structures of the BM run along arterial trajectories. Megakaryocytes (Mk) reportedly interact with a subset of vWF$^+$ HSCs. (B) 3D microscopy of HSCs using the *Ctnnal*-GFP reporter mouse line.[97] Image on the left depicts a large region of the BM containing several GFP$^+$c-kit$^+$ HSCs, which are found in the proximity of sinusoidal vessels (labeled with CD105). Details can be appreciated on the zoomed images of two HSCs (arrowheads) shown on the right. Scale bar 50 µm.

types exhibit the typical morphology of pericytes, extending physically adjacent and longitudinally along arterial trajectories. Periarterial MSCs were first observed by the Frenette group in the *Nestin*-GFP reporter mouse strain. Here, cells expressing low levels of GFP largely overlapped with CARc (GFP^{dim}), while those with the highest expression (GFP^{hi}) formed a distinct population, which was found attached to large arteries and smaller arteriolar branches.[35,62] Nes-GFP^{hi} cells express the pericyte marker NG2 and can be targeted using the *Ng2-Cre*ERT[2] mouse model (Table 1). Of note, this subset has been shown to also express factors such as CXCL12 or SCF, that are important in the regulation of HSCs and other progenitor cells, albeit at lower levels than CARc.[63] A second pericytic population is the so-called PαS cells, which are termed after their cell surface phenotype (PDGFR-a$^+$Sca-1$^+$).[64] Unlike NG2$^+$NesGFP^{hi} cells, PαSc also express Podoplanin and are confined to the thick and complex adventitial layers of large arterial branches (Figure 1), but have not been observed in smaller arterioles (Figure 1G).[44,65] PαSc have a distinct transcriptomic profile, including the conspicuous expression of markers typically associated to both osteoblastic and chondrocytic lineages.[44] Like Nes-GFP^{hi}, PαSc exhibit trilineage mesenchymal differentiation in vitro, and when transplanted in vivo, they can differentiate into osteoblastic and adipocytic lineages.[64] Thus, both subsets contain progenitors, although their precise differentiation potential and contribution in vivo to adult mesenchymal derivatives during homeostasis remains to be determined. Finally, Smooth Muscle Actin-expressing (SMA$^+$) cells directly ensheath larger arteries[66] (Figure 1G). Although their function in the BM has not been clearly delineated, as in other organs, these adventitial cells most likely regulate arterial flow. Based on single-cell transcriptomic studies, SMA$^+$ cells are categorized as myofibroblasts and can be targeted using the *Myh11*-Cre ER mouse strain (Table 1).[63]

Finally, mature mesenchymal derivatives include osteoblasts and osteocytes, adipocytes, and chondrocytes. While chondrocytes localize in growth plates and are generated mainly during postnatal development,[67] osteoblasts, and adipocytes are continuously formed throughout life from CARc as described above.[57,68] Terminally differentiated osteoblasts are confined to the inner surfaces of bone enclosure and are therefore directly exposed to hematopoietic cells residing in endosteal regions. Their influence on hematopoietic regulation, and more specifically in the control of early lymphoid progenitor populations and B-cell development is well-documented.[52,53,69] As part of the bone remodeling process, osteoblasts get gradually embedded in the bony matrix they produce, transforming into osteocytes, which being isolated by the bone enclosure, are not in direct

contact with hematopoietic cells. Nevertheless, evidence suggests that osteocytes could indirectly participate in the control of hematopoiesis via mechanisms poorly understood to date.[70,71] In turn, adipocytes are rare at early stages of postnatal life, but their frequency gradually increases with age and disease in a location-specific manner.[72] During adulthood and aging, the highest content of fat is found in marrow tissues of tail vertebrae and metaphyseal regions of long bones. At these sites, adipocytes are interspersed with marrow hematopoietic and nonhematopoietic components and have been shown to influence hematopoietic function through the secretion of different cytokines.[73–75] Nonetheless, much is still to be learned on the dynamics, reversibility, and functional consequences of BM adipocytic transformation at local and systemic levels. Of note, the age-dependent transition into yellow fatty marrow is significantly accelerated and increased in magnitude in humans compared to mice.

BM innervation

The largest and most conspicuous neural structures of murine femurs enter the BM through the nutrient foramen, a large transcortical channel, which is in the upper proximal metaphysis of femoral cavities, as well as inside transcortical channels in close association with the major nutrient artery and smaller transcortical vessels, respectively.[76,77] Thick nerve bundles surrounded by a dense layer of collagen-rich extracellular matrix (ECM) run parallel to the trajectory of the main central arteries, branching along the arterial tree as it transitions into smaller arterioles and projecting toward endosteal regions.[78,79] Terminals stemming from these bundles have been occasionally observed to project and directly associate with periarterial adventitial cells, creating a neuro-reticular interface.[80] However, the composition of these complex neural projections has not been fully elucidated yet. Electron and fluorescent microscopy studies suggest that large nerve bundles contain multiple myelinated and non-myelinated axons, which are strongly labeled by pan-neural markers such as Tubulin (Table 1).[79] At least some of the fibers enclosed in these structures express the noradrenergic marker tyrosine hydroxylase (TH), while others express the calcitonin gene-related peptide (CGRP), thus corresponding to nociceptive terminals.[81] In fact, recent quantitative analyses reveal that the trajectories of both nerve fiber types largely overlap in different BM regions.[77] Also included in these structures is a subset of non-myelinating Schwann cells characterized by the expression of Glial Fibrillary Acidic Protein (GFAP).[82] Independent from these thick neural projections, thin adrenergic fibers expressing TH can be visualized spiraling directly also around arterial and arteriolar vessels.[83] Finally, in homeostatic conditions, the presence of cholinergic nerves has been mostly observed in the outer layers of bone termed periosteum, but it is unclear whether these terminals reach central hematopoietic regions of the marrow.[84] Experiments employing pharmacological, surgical, or genetic ablation of neural structures and/or neurotransmitters reveal highly specific roles of the distinct types of innervation described in the control of BM function, and as explained below, on the regulation of HSC physiology.

HSCs IN THEIR NICHE

The conceptual framework directly formulated in Schofield's original theory defined niches as (i) discrete anatomical domains with a defined localization within the BM tissue ecosystem, (ii) units formed by a highly specialized set of interacting cell types, which nurture HSCs, (iii) deterministic fate regulators; cellular anchorage to the niche would confer stem cell identity, while physical detachment would result in differentiation and/or loss of stemness.[12,13] From these premises it can be also inferred that the number of stem cells in the BM is determined by the number of available niches. Altogether, these notions have critically shaped decades of research on niche identity, especially since niches in the ovaries and testes of *Drosophila melanogaster* were found to largely validate the core tenets of the model.[85] Consequently, most studies on BM have aimed at dissecting the anatomical configuration of niches through the visualization of HSCs, the analysis of their spatial distribution, and the identification of the cellular components with which they interact.[29] In turn, the potential functional relevance of observed spatial interactions has been validated by examining the impact on the HSC compartment of genetic or pharmacological depletion, interference, or manipulation of target putative niche players.

Challenges analyzing HSC niches

Substantial uncertainties still exist regarding HSC niches, which may be explained by the challenges intrinsic to the experimental strategies employed.[86] First, imaging pure populations of HSCs in a precise manner has been historically hindered by their scarcity and the lack of simple combinations of markers to tag them with high fidelity and confidence among the complex continuum of progenitors.[29,30] These issues become especially problematic when attempting to immunostain individual HSCs deep into tissues for 3D imaging studies. Also, there has been a lack of unified standards for the statistical methods employed to determine spatial dependencies between cell types in image data sets, which in some cases has led to erroneous interpretations of patterns of cellular proximity. Such technical hurdles are being overcome with the development of specific reporter mouse lines, the continuous improvement of volumetric imaging techniques, and the gradual implementation of sophisticated spatial statistical tests, which allow to identify HSCs and analyze their distribution with increasing precision.

Second, perturbations of a given step in the hematopoietic continuum rapidly propagate up- or downstream the developmental hierarchy, making it hard to isolate causality after niche cell-specific targeting. Similarly, neural, mesenchymal, and endothelial networks are highly interconnected, both anatomically and functionally. Thereby, manipulations of one subset will inevitably lead to immediate adaptations of connected components, which will influence the homeostasis of the system and blur specific effects.[87] It is therefore not entirely surprising that HSC proliferation is a general unspecific response observed after cellular depletion of most stromal or hematopoietic fractions.[27] While the limitations described above should be considered when critically interpreting the results obtained thus far, niche studies have yielded tremendous progress throughout recent years. Below, we will focus on work analyzing HSC niches in homeostatic, unperturbed settings. Yet, a large body of recent evidence suggests that, far from being static, the composition, structure, and function of the BM infrastructure and HSC niches are transiently or permanently remodeled in the context of aging, infections, regeneration, or hematological malignancies. We refer the interested reader to recently published, comprehensive reviews on these topics.[88–91]

Early studies: The osteoblastic niche

Pioneering attempts to map HSCs and uncover spatial patterns relied on the mechanical separation of regions along BM parenchyma, and functional readouts instead of imaging techniques to determine the local densities of colony-forming units (CFU-S), which were considered HSCs or earliest progenitors. These initial studies revealed that primitive hematopoietic stages accumulate in the proximity of

inner bone surfaces, thereby providing the first evidence of the nonstochastic compartmentalization in the marrow.[92] This spatial relationship to the endosteum, together with evidence from prior studies showing that osteoblasts could support human HSC maintenance in long-term ex vivo cultures,[93] gave rise to the hypothesis that bone-proximal osteoblasts could hold the potential to directly regulate HSC activity. In vivo imaging studies further showed that transplanted HSCs preferentially localized in the vicinity of endosteal surfaces. The analysis of the first mouse models allowing for genetic manipulation of osteoblast populations, revealed that alterations in the number and function of osteolineage cells were often mirrored by changes in hematopoiesis and HSCs, lending further support to the potential role of osteoblasts in HSC regulation.[94,95]

The advent of technical advances in the early 2000s transformed the way in which niches could be visualized. First, the identification of the specific expression profile of SLAM proteins (CD150 and CD48) in HSCs permitted the development of simplified immunostaining protocols to visualize nontransplanted, endogenous populations highly enriched in HSC content within histological sections. Together with improvements in methods for bone processing and the increasing resolution afforded by laser scanning confocal microscopy, these advances enabled the first analyses of the spatial distributions of HSCs. Using such approaches, Kiel and colleagues revealed that HSCs reside scattered throughout the deepest areas of BM, not necessarily in proximity to bone, but adjacent to sinusoidal vessels.[96] Thus, although quantitative imaging studies suggest that, as initially postulated, early progenitor cells spread along gradients with the highest concentrations found near bone,[38] the preferential physical association of HSCs with endosteum and adjacent osteoblasts was disproven.[28]

Perisinusoidal-CARc niches

Since the first report described that HSCs localize in direct contact or proximity with sinusoids, this relationship has been validated by multiple groups.[35,38,97,98] Within perisinusoidal spaces, HSCs also colocalize and directly interact with CARc, which as mentioned earlier, are highly abundant but specifically concentrate in the abluminal side of the sinusoidal endothelial wall.[34] The functional relevance of the interaction of HSCs at the SEC-CARc interface has been experimentally established. CARc are an essential source of SCF, which together with AECs, provide the necessary supply of this key cytokine to regulate maintenance and expansion of HSCs.[50,99] HSCs also rely on the growth factor pleiotrophin (PTN), which is produced by both CARc and SECs. While in steady state PTN depletion from CARc results in a profound decrease of HSCs, SECs transiently take over postmyeloablation and promote the regeneration of the HSC pool in this context.[100,101] Although targeting of *Cxcl12* expression in CARc does not result in detectable alterations of the size, cycling status, or function of the BM HSC pool, it does lead to increases in the numbers of extramedullary HSCs in blood and splenic tissues.[52,53] Yet, beyond their direct effects on HSCs, CARc produce other soluble factors with major relevance in myelo-erythorid differentiation, such as IL7 or CSF1.[44,56,58] Thus, CARc influence hematopoiesis at multiple progenitor cell stages through which they also contribute to indirectly maintain homeostatic balance of HSCs.

SECs also crucially contribute to perisinusoidal niches through their angiocrine function. For example, ECs are the major source of ligands stimulating Notch-receptor signaling, such as *Jagged 1* (JAG1), JAG2, and Delta-like ligands DLL1 and DLL4, which are required for HSC maintenance. EC-specific deletion of *Jag1* leads to reductions in HSC numbers,[102] while *Jag2* is upregulated during regenerative hematopoiesis and contributes to HSC expansion in these settings.[103] Targeting *Dll4* expression results in the abnormal upregulation of myeloid-specific genes in HSCs, leading to a biased specification and accumulation of myeloid cells in the BM.[58] However, given that not only SECs, but also AECs equally express both Notch ligands, it remains to be determined whether the observed effects are predominantly derived from the alteration of one EC subset or, alternatively, all vascular niches provide relevant cues for Notch-dependent regulation of HSCs. Beyond their direct involvement through the provision of cellular cues, SECs line the endothelial wall of sinusoids, which are the port of entry and exit of hematopoietic cells from and into circulation, as well as the physical barrier between perisinusoidal cells and blood-derived systemic factors. SECs have been shown to express *EphnB4*, which through its interaction with *EphrinB2* on the surface of HSCs, promotes trafficking via sinusoids. Blockage of this signaling axis reduces mobilization of HSCs and other myeloid subsets.[104] Thus, by regulating trafficking and tissue residence, SECs ultimately indirectly control HSC function and biology.

Periarterial niches

A minor though significant fraction of HSCs is consistently observed in the vicinity of arterial and/or arteriolar microvessels. However, while some studies have suggested that periarterial localization is a preeminent feature of quiescent HSCs,[35] the specific functional link between dormancy and periarterial location has not been confirmed in subsequent studies, in which noncycling HSCs were found scattered throughout BM parenchyma and equally associated with perisinusoidal locations.[97,98] Therefore, whether and to what extent periarteriolar neighborhoods imprint HSCs with distinct functional or proliferative properties remains contentious. Nevertheless, ample evidence demonstrates that the cellular and physiological features found in periarterial environments substantially differ from those encountered in perisinusoidal zones, which may point to the functional specialization of these microenvironments. First, the biophysical properties of arterial vascular walls and their associated pericytic layers render these vessels and their arteriolar branches less permeable than sinusoidal fenestrated endothelium. Hence, arterial endothelium restricts the passage of nutrients and decreases oxygen availability in periarterial areas, which are the most hypoxic regions within the BM parenchyma[41] and may protect HSCs from exposure to plasma-derived factors that increase oxidative metabolism.[42] Second, AECs and their associated periarterial mesenchymal components display cytokine and growth factor expression profiles, which largely differ from those of CARc and SECs.[44] Thus, it is conceivable that the signaling cues and physiological milieu in arterial neighborhoods distinctively modulate HSCs residing in their proximity. This notion is strongly backed by several genetic studies targeting AECs or periarterial fibroblastic subsets in murine BM. For instance, AEC-specific ablation of *Scf* expression leads to partial depletion of HSCs, thereby suggesting that a fraction of HSCs relies on AEC-derived SCF.[99] Supporting the existence of arterial HSC niches, studies by different groups demonstrate that expression of *Cxcl12* by periarterial NG2$^+$, and/or PαSc, is required to maintain quiescence and overall integrity of the HSC pool.[50,52,63] Furthermore, recent work identified Neogenin as a novel cell surface receptor predominantly expressed in dormant HSCs and involved in the regulation of quiescence.[105–107] Netrin-1, the ligand for Neogenin, is expressed in various stromal subsets and reportedly at the highest levels in AECs and SMA$^+$ cells.[105] Finally, comprehensive transcriptomic analyses have shown that both NG2$^+$ and PαS cells express relatively high levels of genes encoding for key cytokines associated with HSC maintenance or regeneration upon stress. In the case of PαSc, these include Angiopoietin like proteins (*Angptl*) 1, 2, and 7, which promote the proliferation and expansion of HSCs in vitro.[44] Thus, further work is

needed to uncover the precise mechanisms by which these cells impact hematopoiesis.

Neural-dependent regulation of HSCs

The effects of BM neural-derived signals in the control of HSC biology have been amply documented. Neural regulation is mostly exerted via the modulation of HSC trafficking throughout marrow cavities, which depends on the fine-tuning of expression of pro-retention and/or homing cues by key cell types.[79] For instance, the number of HSCs present in circulating blood exhibit circadian oscillations, which are controlled by light-dependent regulation of *Cxcl12* expression in the BM. In mice, sympathetic nerve activity and release of norepinephrine in the BM peak during the early phases after light exposure, thereby promoting the downregulation of *Cxcl12* via activation of the β_3-adrenergic receptor in MSCs. Nadirs in CXCL12 levels at this point correlate with the maximal release of HSCs to systemic circulation, while the inverse correlation is observed during exposure to the dark phases of the cycle.[108] Beyond their direct influence on MSCs, adrenergic nerves additionally control homing to the BM by modulating the cyclic expression of adhesion molecules in BM sinusoidal ECs via β_2-adrenergic receptor signaling.[109] In turn, nociceptor innervation collaborates with adrenergic signals in the process of maintaining the HSC pool in the BM, as well as during GCSF-enforced mobilization, through secretion of the calcitonin gene-related peptide (CRGP).[81] Finally, the basal sympathetic tone of the BM is influenced by cholinergic signals, which are not delivered locally but at the level of the central nervous system, and thereby modulate HSC mobilization and recirculation indirectly, through long-range cues.[110,111]

Neural-derived signals may also impact HSC functionality in the long term, either through the continuous modulation of their transit between BM and circulation, or alternatively via the direct control of niche cells and/or HSCs. For instance, surgical denervation of femoral and sciatic nerves results in the loss of TH^+ adrenergic fibers leading to the long-term loss of quiescence, expansion, and increased myeloid potential; traits that are reminiscent of aged HSCs.[112] Such premature aging was shown to also depend on impaired β_3-adrenergic receptor signaling on MSCs. Of interest, in another study surgical denervation was reported to induce a rapid proliferative response of HSCs, an effect that was attributed to the depletion of non-myelinating Schwann cells, which are at least partially responsible for producing TGF-β in the marrow.[82] However, although crucial aspects of neural regulation have been elucidated, how these distinct signals delivered to the different cellular players are collectively integrated to sustain balanced responses remains unresolved. Moreover, the microanatomical basis of cues derived via neural circuits is not entirely clear. Based on their direct spatial proximity, HSCs in periarterial neighborhoods would be most susceptible to direct regulation by the principal neural structures of the BM. However, how HSCs located far from nerve fibers and deep into tissues are rapidly reached by neural inputs is unclear. In this direction, further studies are needed to determine whether neural-derived signals are delivered directly to stromal cell networks through which they can be rapidly propagated across large BM regions, or alternatively, via diffusion of soluble neurotransmitters within intercellular spaces reaching relatively distant locations where HSCs reside.

Regulation of HSCs by their progeny

While most attention has focused on stromal cells, it seems logical that cues delivered by their progenitors will directly modulate HSC function and output, thereby establishing feedback regulatory loops, which transmit information on the downstream status of the hierarchy and elicit adaptation responses on HSCs. For instance, several groups have reported a direct spatial interaction of Mks to a substantial fraction of HSCs (~30%).[113] Depletion of Mks or conditional ablation of *Tgfb1* in this subset results in increased proliferation of HSCs, leading to the aberrant expansion of the pool.[113,114] Mks predominantly exert their influence on a subset of HSCs marked by the expression of Von Willebrand factor (Vwf), which exhibits myeloid and platelet-biased production.[115,116] Vwf-GFP^+ HSCs were found in close proximity to Mks and Vwf-GFP^- cells displayed a spatial bias toward arterioles. While Mk-depletion stimulated the proliferation and preferential expansion of Vwf-GFP^+ HSCs, depletion of NG2-Cre^+ periarteriolar MSCs caused a significant reduction of Vwf-GFP^- HSCs.[115] These results lend support to the notion that functionally heterogeneous and spatially distinct niches operate to control the cell fate of HSC subsets.

Macrophages also regulate HSC biology, yet the underlying molecular mechanisms remain unclear. At least two populations of macrophages, that express SMA^+ or DARC, have been suggested to spatially associate with HSCs and regulate their function.[117,118] However, confirmatory studies are required to validate the identity of these subtypes and their potential roles. Perhaps most importantly, macrophages indirectly mediate HSC retention in the BM microenvironment by modulating the expression of adhesion molecules and CXCL12 in MSCs.[119] Of note, complex multicellular feedback loops operate in the regulation of HSC niches by their mature progeny. For instance, senescent neutrophils cyclically entering the BM for clearance are phagocytosed by macrophages, which in turn control the rhythmic oscillations of expression of *Cxcl12* by CARc and the release of HSCs to circulation.[120] In addition, studies on mice displaying prolonged neutropenic states indicate that neutrophils contribute to the functional decline of niches via their regulation of proinflammatory signals derived from NK cells.[121]

Finally, studies have pointed to a potential involvement of regulatory T cells (T_{regs}) in the regulation of HSCs, especially in transplantation settings. T_{regs} suppress the rejection of transplanted allogeneic HSCs through the production of IL-10[122]. This protection may be articulated through the formation of immune-privileged niches where T_{regs} and HSCs colocalize. Furthermore, one study found that the paucity of T_{regs} in the BM causes the expansion of HSCs and that a subset of $CD150^{hi}$ T_{regs} directly influences HSC quiescence through the production of adenosine.[123] Yet, further research is needed to determine the specific nature and mechanisms of the crosstalk between T_{regs} and HSCs.

HSC NICHES IN HUMAN BM

Insight into the functional organization of marrow tissues and HSC niches has been almost entirely derived from studies in murine models. How much of this knowledge applies to human marrow? Obvious physiological differences exist, such as the age-dependent rate of transformation of red into white marrow through adipocytic differentiation of MSCs, which is accelerated and much more pronounced in humans.[124,125] Nonetheless, human and mouse HSCs rely on similar growth factor supplementation in vitro,[126,127] and murine BM has been long known to host and support engraftment human HSCs. Altogether, this suggests that understanding the physiological and anatomical principles operating in murine HSC niches may potentially translate into relevant knowledge in the human setting. Hence, research is needed to clarify whether and to what extent the cellular identities and functions of specific mesenchymal and endothelial cell types and the molecular mechanisms deployed in their regulation of HSCs are preserved across species.

Multiple studies have demonstrated the existence of distinct subsets of MSCs, which express CD271 and/or CD146 and exhibit

periendosteal or perivascular (arterial or sinusoidal) locations in human BM.[128–130] As in mice, when cultured in vitro, these cells exhibit trilineage differentiation.[129] Recent work identified a subset of CD271$^+$ cells, which shares a high degree of homology with murine CARc, based principally on the prominent expression of *Cxcl12* and *Scf*, as well as key CARc-specific transcription factors such as *Ebf3* and *Foxc1*.[131] These cells exhibit reticular morphology and extend throughout human tissues in dense web-like patterns, indicating that these structures are as pervasive in human marrow as they are in murine tissues and could strongly influence early stages of hematopoietic development.[131–133] This equivalence is further supported by the first single-cell sequencing studies of human BM stromal networks, which have revealed that CARc include heterogenous subsets and collectively express numerous factors involved in the control of HSC and progenitor cell (HSPC) maintenance and differentiation.[134–136]

Additionally, several attempts have been made to visualize HSC-enriched populations and describe their topological distribution and interactions in histological preparations of human BM. These studies revealed preferential accumulations of CD34$^+$ hematopoietic stem and progenitor cells (HSPC) in the proximity of bone surfaces.[133,137,138] However, such investigations performed in thin sections of human trephine biopsies are strongly limited by the lack of combinations of specific markers to label *bona fide* HSCs and discriminate them from progenitors with high confidence, the potential scarcity of HSCs present in restricted areas accessible for analyses, and the challenges associated to the simultaneous detection of HSCs and niche components. Alternative approaches have relied on the visualization of human HSCs residing in murine BM of xenografts. As observed in native human tissues, transplanted human CD34$^+$ HSPCs display a propensity to accumulate in the proximity of endosteal surfaces, and trabecular areas of murine long bones.[139] However, such a trend has also been observed upon transplantation of other cell types and may be explained by the fact that preferential homing occurs at these sites due to vascular anatomy.[140] Nonetheless, when tested in secondary transplantations, HSPCs residing in endosteal sites preserved improved reconstitution capacities compared to those present in cortical regions, which could reflect the existence of yet unidentified key cell types or growth factors that promote self-renewal in these areas.[139] In summary, much is still to be learned on the spatial logic underlying hematopoiesis and the topological arrangement of HSC niches in humans.

RETHINKING HSC NICHES

Moving away from the closed niche?

The large body of work described above has contributed to unequivocally establishing the cardinal role of the tissue microenvironment in supporting individual cellular fates and collective maintenance of the HSC pool. Nonetheless, a definitive and unified definition of the niche, as once envisioned by Schofield, has not been reached. In fact, recent evidence indicates that the interaction of HSCs with their surrounding environment may not exactly follow the principles of what has been termed a "closed niche," as a discrete and quantifiable anatomical entity to which stem cells remain anchored.[141]

First, the use of refined, highly accurate, and large-scale imaging approaches, together with the application of rigorous statistical methods, has revealed that the spatial relationships previously observed between HSCs and principal niche components are not specific, but purely stochastic. For instance, albeit most HSCs are in direct contact with CARc and sinusoids, this spatial proximity can be explained by the fact that both components are highly abundant and occupy a relatively large fraction of the BM space, inevitably interacting with very large proportions of cells, including HSCs.[34,98] Similarly, the fractions of HSCs in proximity or direct contact with arteries, Mks, or GFAP+ cells, do not differ from those expected from randomly distributed cells in the same tissue context, but are reflective of the sheer abundance of each component within marrow topography.[98] Therefore, although direct crosstalk with these cell types is necessary and most likely controls distinct aspects of HSC maintenance, the cellular interactions described so far are not driven by specific attractive forces bringing HSCs and cellular partners together in a confined anatomical unit. Second, the majority of putative niche cells studied so far populate the BM in much larger numbers than HSCs, which suggests that numbers of niches are not prefixed or limited in the BM by a given cell type or a combination thereof. Further supporting this notion, repeated transplantation of large numbers of HSCs into nonmyeloablated recipient mice leads to a sustained doubling of the numbers of functional HSCs,[142] which means that the hosting capacity of the BM for HSCs is malleable and not fully saturated in homeostatic conditions.

Finally, in vivo imaging of endogenous, nontransplanted HSCs within their unperturbed microenvironment has revealed that HSCs exhibit a previously unanticipated dynamic behavior.[143,144] Although to a much lower extent than HPCs and other motile cells such as lymphocytes, one study found that most HSCs continuously roam BM tissues, alternating periods of random walk with stretches of processive motion, which allow them to scan relatively large volumes of the tissue parenchyma.[144] Along their trajectories, HSCs appeared to engage in frequent but brief physical interactions with CARc. Together with the continuous and systemic recirculation of HSCs throughout distant BM cavities, this dynamic behavior suggests that reception of key regulatory signals by HSCs may not require durable anchoring within a fixed microanatomical spot, but rather the iterative and continuous collection of molecular input through dynamic screening of broad tissue areas. In summary, the evidence described above collectively calls for the existence of more complex mechanistic models of HSC maintenance.

Open niches and stem cell competition

Recent studies in other tissues have suggested the existence of alternative modes of somatic stem cell integration and control of numbers by local microenvironments. In what has been defined as "open niches," stem cells lack a strictly fixed position in tissue microanatomy and lie dispersed among their progeny throughout relatively large regions.[141] For instance, mammalian spermatogenic stem cells are relatively motile and distribute dynamically along seminiferous tubules, exhibiting a subtle spatial bias toward lymphatic endothelium. This topological arrangement is at least partially dictated by the secretion of the mitogenic factor Fibroblast growth factor 5 (Fgf5) by lymphatic endothelial cells.[145] The limited availability and continuous consumption by stem cells of this diffusible factor leads to fluctuations in its local concentration, which are used by stem cells to "sense" the proximal densities of their counterparts. In this setting, competition for Fgf5 among genetically equivalent cells, so-called *neutral competition*, coupled with its mitogenic activity, drives the spatial patterning of the stem cell pool, while also sustaining feedback loops that preserve stable stem cell numbers.[145]

Notably, obvious tissue-scale spatial biases of HSCs within the BM have not been observed. Yet, ample evidence in the hematopoietic system supports the notion that HSCs locally compete for survival signals. For instance, HSCs sense the presence of competitor cells sharing their same spatial territory and adapt their transcriptomic programs in response to the perceived fitness of their counterparts, which at least partially depends on the status of p53 activation.[146,147] Moreover,

during aging, HSCs with leukemia-associated somatic mutations expand and increasingly contribute to hematopoiesis in the absence of overt disease.[148] This age-related clonal hematopoiesis is a manifestation of nonneutral competition in which mutations confer increased competitive fitness that manifests in a BM microenvironment shaped by aging, inflammation, or prior cytotoxic therapies.[149] While the latter phenomena reflect the consequences of competition between genetically nonequivalent stem cells, neutral competition most likely also continuously operates to shape the clonal repertoire of HSCs, as reflected by the gradual but steady decrease in clonal diversity throughout the entire lifespan.[150] Of note, the slow kinetics of clonal scaling in the hematopoietic system seem consistent with an open niche model, in which cells contend for survival or mitogenic signals that are found distributed throughout considerably large tissue volumes, rather than confined within a defined physical location. Intriguingly, a recent study strongly suggests that HSCs may not strictly compete for stimuli among themselves, but also with immediate progenitors. This adds yet an extra layer of complexity to the ecological integration of primitive populations in the hematopoietic microenvironment.[151]

CONCLUDING REMARKS

Almost four decades after the HSC niche theory was put forward, a formal demonstration of the existence of HSC niches is still missing. Along the way, numerous studies have brought clarity to the functional and compositional complexity of the BM microenvironment and the indispensable roles that extrinsic cues play in shaping the hematopoietic process. Nonetheless, the dynamic nature of HSCs, their promiscuity in establishing interactions, and the cellular complexity of the stromal scaffold, call into question whether HSC identity and fate are deterministically linked to prolonged residency in fixed anatomical units. Answering this fundamental question will require the full exploitation of novel spatial, proteomic, and transcriptomic technologies, state-of-the-art high-dimensional 3D microscopy, and advanced mathematical modeling to integrate multidimensional data, both in murine models as well as in human samples. Such interdisciplinary approaches should provide higher granularity and reveal the potential existence of spatial units made of rare and heterogeneous combinations of specialized cellular subsets, which may not have been described to date. At this point, it seems plausible that such entities exist and are required to distinctively imprint functional properties on the diverse subsets that make up the HSC pool. Alternatively, novel theoretical frameworks may be needed to revise current paradigms and explain how the preservation of a stable HSC pool is achieved through the coordination of individual fate decisions of cells scattered throughout large tissue areas. This represents a fundamental step toward a broader understanding of whether the hierarchical progression from HSCs follows a defined spatial order that can be mapped into BM anatomy.

AUTHOR CONTRIBUTIONS

Serena Galli wrote the manuscript. Ana Luísa Pereira wrote the manuscript and contributed to the figures. César Nombela-Arrieta designed the figures, developed the concept, and wrote the manuscript. All authors approved the final version.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no data sets were generated or analyzed during the current study.

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