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Extrinsic regulation of hematopoietic stem cells in development, homeostasis and diseases

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Abstract

Lifelong generation of blood and immune cells depends on hematopoietic stem cells (HSCs). Their function is precisely regulated by complex molecular networks that integrate and respond to ever changing physiological demands of the body. Over the past several years, significant advances have been made in understanding the extrinsic regulation of HSCs during development and in homeostasis. Propelled by technical advances in the field, the cellular and molecular components of the microenvironment that support HSCs *in vivo* are emerging. In addition, the interaction of HSCs with their niches is appreciated as a critical contributor to the pathogenesis of a number of hematological disorders. Here, we review these advances in detail and highlight the extrinsic regulation of HSCs in the context of development, homeostasis and diseases.

Visual abstract

The interaction between hematopoietic stem cells (HSCs) and their environment is critical for the hematopoietic system in development, homeostasis and diseases.

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Introduction

Multicellular organisms evolved tissue-specific stem cells to generate, sustain and repair diverse tissue and organ types. Stem cells are maintained in tissues through life-long self-renewal divisions, where one or two stem cells are generated in each round of cell division¹. Stem cells also have multilineage differentiation potential. Thus stem cells are constantly balancing two seemingly opposed functions: maintaining the undifferentiated stem cell state and differentiating into cells of multiple lineages. Work from *Drosophila* has demonstrated that by providing adhesive interactions and biased signaling to stem cells, but not their immediate downstream progenies, stem cell microenvironmental niches provide a perfect solution to this issue². Understanding how stem cells are regulated by their local niche and by other extrinsic mechanisms is fundamental to the field of stem cell biology.

Hematopoiesis has been a fruitful model for the study of stem cell biology. Multiple cell types constitute the hematopoietic system, including myeloid cells, lymphoid cells, erythroid cells and megakaryocytes. All of these lineages are ultimately generated from multipotent HSCs through a differentiation hierarchy that includes multiple levels of progenitors throughout life³. HSCs are also capable of regenerating the hematopoietic system after transplantation. In fact, HSC transplantation is the only cure available for a number of hematologic diseases. Their enormous medical potential aside, HSCs have served as the model tissue stem cell, having defined the rigorous standards of self-renewal and multilineage potential that characterize all tissue stem cells. This definition has provided the framework for understanding stem cell biology in general. Not surprisingly, the proposal of a stem cell niche was first suggested in the hematopoietic system for HSC maintenance⁴.

The high medical value and scarcity of HSCs prompted searches for conditions to culture or expand HSCs *in vitro*. Despite decades of effort, no culture system is able to robustly maintain or expand HSCs. However, these stem cells can clearly thrive within their native environment *in vivo*. Thus, defining the *in vivo* extrinsic regulatory mechanisms is a key step that will allow us to expand and augment the therapeutic utility of HSCs. Hematopoiesis and HSCs change organ sites several times throughout life to meet distinct physiological demands. The dynamic nature of the interaction between HSCs and their environments presents a fascinating yet challenging opportunity to understand HSC regulation. The fluid nature of the hematopoietic tissue and a lack of morphological or positional differences between HSCs and other hematopoietic cells have made the identification of these cells and their *in vivo* environment difficult. Despite these roadblocks, significant advancements have been made regarding the extrinsic regulation of HSCs in recent years. Here, we will summarize our understanding of the extrinsic regulation of HSCs in the context of development, homeostasis and disease. We will also highlight some of the outstanding questions in the field.

Overview of technical history

Our knowledge of HSCs is built on experimental evidence made possible by a number of technical advances, including two key innovations: transplantation and flow cytometry. During World War II, it was discovered that people exposed to lethal irradiation could be

rescued by transplantation of cells from healthy donor bone marrow. This sparked the quest for cells that can replenish the hematopoietic system⁵. Work from Till and McCulloch showed that there are cells in the bone marrow that when transplanted can regenerate the blood system and form colonies on the spleens (colony forming unit-spleen or CFU-S) of mice exposed to lethal doses of irradiation⁶. It was later discovered that CFU-Ss are not HSCs but hematopoietic progenitors^{7,8}. Nonetheless, using cytological methods, Till and McCulloch provided convincing evidence that these colonies contained multiple hematopoietic lineages and were derived from a single hematopoietic progenitor⁹. These observations have conceptually shaped the field of stem cell biology. The capability to stably reconstitute lethally irradiated recipient mice upon transplantation has become the gold standard in defining HSCs. Throughout the review, HSCs are defined by this criterion. Based on limiting dilution transplantation assays, it was estimated that about 5 cells in every 10⁵ C57BL/6 bone marrow cells are HSCs¹⁰. But these rare stem cells are so potent that a single transplanted HSC can reconstitute the entire blood system of a lethally irradiated recipient mouse^{11,12}.

Although HSCs were in the mixture of bone marrow cells used in early *in vivo* experiments, their exact identity remained elusive. No morphological features can distinguish rare HSCs from other hematopoietic cells, which was a major hurdle in the field. The invention of monoclonal antibodies and fluorescence activation cell sorting (FACS) made possible the isolation of HSCs based on the expression of specific cell surface antigens. Cell sorting combined with functional transplantation assays allowed for the development of a series of surface marker profiles that can be used to sort HSCs to high purity, particularly in the bone marrow³. The purification of HSCs has facilitated extensive research on intrinsic molecular mechanisms that regulate their self-renewal and differentiation. But how these mechanisms are integrated with the *in vivo* environment had not been clear.

Localizing HSCs *in vivo* is a prerequisite to elucidating their environmental regulation. Much of the focus in the field has been on the adult bone marrow. The complex markers used in FACS to purify HSCs were not suitable to identifying them on bone marrow tissue sections using microscopy, requiring the use of alternative markers in many early HSC niche studies. Simpler markers with low purity for HSCs¹³ and tracing assays for transplanted FACS-sorted fluorescent marker-labeled HSCs were initially used to localize HSCs *in situ*^{14,15}. But it was not clear whether the localization of these cells reflected the niche of endogenous HSCs under steady state conditions. The development of Signaling Lymphocyte Activation Molecular (SLAM) markers allowed for the identification of HSCs on bone marrow sections *in situ* by simple two-color staining for cells with markers that also strictly defined HSCs by FACS and transplantation¹¹. More recently developed genetically encoded fluorescent markers have also allowed detailed studies of HSC localization^{16,17}. The advancement of other imaging techniques such as intra-vital live imaging and tissue clearing technology has provided even more comprehensive views of the natural environment of HSCs in the bone marrow^{18,19}. The imaging of HSC emergence during development has also provided critical information on the niches that maintain HSCs. For example, live imaging of the aorta-gonad-mesonephros (AGM) has provided definitive evidence of hemogenic endothelium^{20–22}. Similarly, imaging data regarding the niches in hematopoietic malignancies and other stress conditions are emerging.

Imaging has provided a solid framework for understanding the niche that regulates HSCs. However, functional studies *in vivo* are key to uncovering the mechanisms of HSC extrinsic regulation. Ideally, a defined perturbation of the candidate niche cell type should result in a perturbation of HSCs. Following this logic, several functional approaches have been taken to uncover the nature of bone marrow niche that maintains HSCs. We use the bone marrow as an example to highlight some functional methods to study HSC extrinsic regulation. Early genetic analysis of *Steel* (with mutated stem cell factor, SCF) and *White* (with mutated cKit receptor) mutants led to the identification of the key role of the SCF-cKit pathway in maintaining HSCs *in vivo*²³. Additional genetic gain-of-function and loss-of-function studies have been used to elucidate the roles of other cell types and pathways in HSC maintenance^{24,25}. Many of these studies used whole-body mutant mice where all cells are genetically perturbed. Thus, it is not clear what niche cells are relevant to observed HSC phenotypes. Cell ablation experiments have also been employed to functionally assess the requirement of distinct cell populations in supporting HSCs²⁶. However, eliminating specific cells by inducing cell death may provoke non-specific HSC phenotypes related to other functions of the candidate niche cells. Ectopic bone marrow formation is another method employed to show the sufficiency of a given cell population in initiating an HSC-supporting environment^{27–29}. Purified candidate niche organizing cells are transplanted subcutaneously or under the kidney capsule, sometimes with scaffolds. Over time, bone marrow will be developed and hematopoiesis will be sustained in these ectopic loci. But it is not clear whether these niche organizing cells or other cells recruited by them are directly supporting HSCs within the ectopic bone marrow. Ultimately, HSC maintenance factors need to be conditionally deleted from candidate niche cells to definitively test the hypothesis that these cells create the niche by elaborating key HSC-supporting factors^{30,31}. Some of these functional studies have also been used to define cells and pathways that regulate HSCs extrinsically during development and in hematological disorders. In the following sections, we will discuss extrinsic HSC regulation with the emphasis on evidence from functional studies.

Developmental extrinsic regulation and migration

HSCs are formed during a narrow time window early during development in multiple anatomical sites (Figure 1). Thereafter, HSCs are maintained by self-renewal throughout adult life. As a consequence, the extrinsic regulation of HSCs during development is highly dynamic. Primitive hematopoiesis occurs in the yolk sac blood islands where lineage-restricted hematopoietic progenitors, but not transplantable HSCs, are generated. Definitive hematopoiesis follows with the emergence of HSCs that have self-renewal and multi-lineage potential in the AGM region. HSCs then colonize the fetal liver, where they mature and expand. The fetal spleen also serves as a transient reservoir of HSCs until birth, when HSCs migrate to the bone marrow, the predominant site of hematopoiesis in adults. The migration of HSCs through several organs suggests the sequential establishment of niches for HSCs. These niches may facilitate the maturation and expansion of definitive HSCs while eliminating primitive HSCs and other unwanted hematopoietic progenitors. Regardless of the exact mechanisms, the main purpose of extrinsic regulation of HSCs during development is to generate sufficient definitive HSCs for lifelong hematopoiesis. Conversely, HSCs can

egress from the bone marrow to seed the liver and spleen and initiate extramedullary hematopoiesis (EMH) in adults, indicating that the niches can be reactivated in these extramedullary organs. The transient nature of these niches poses challenges to study the underpinning mechanisms, but it also provides an opportunity to study how a niche is switched on and off. This information will be important to devise means to amplify HSCs by activating niches *in vivo*.

Yolk sac

The first blood cells in vertebrate embryos arise in the yolk sac. At embryonic day (E) 8.0 in mice, primitive erythrocytes and precursors of erythroid and myeloid cells with *in vitro* colony-forming potential are found in the blood islands of the yolk sac^{32–34}. However, whether HSCs that contribute to adult hematopoiesis are generated in the yolk sac is controversial^{35–41}. Different time points of analysis and different experimental techniques might explain conflicting results from varying studies. Nonetheless, the yolk sac does not seem to hold a large number of HSCs. Little is known regarding the extrinsic regulation in the yolk sac, although hypoxia is proposed to be critical for hematopoietic progenitors⁴².

Aorta-gonad-mesonephros (AGM)

Numerous studies have reported that HSCs arise *de novo* from the hemogenic endothelium of the dorsal aorta in the aorta-gonad-mesonephros (AGM) region through morphological cell transition⁴³. Moreover, AGM-derived stromal cells can support HSCs and hematopoietic progenitors *in vitro*^{44,45}. Although HSCs originate from the AGM, their poor bone marrow engraftment potential suggests that they require further maturation before seeding the bone marrow niche⁴⁶.

Several pathways are important extrinsic regulators of HSC emergence in the AGM. Studies in zebrafish and mouse embryos have shown that BMP4^{47,48}, Hedgehog^{47,49}, Wnt⁵⁰, VEGF⁵¹ and Notch^{52,53} signaling is critical for the establishment of HSCs in the AGM. These factors are generally from the endothelial or mesenchymal cells that surround the hemogenic endothelium. Recently, sterile tonic proinflammatory signals have also been shown to promote the generation of HSCs in the AGM^{54–56}. Consistent with this, proinflammatory cytokines IL-1 and IL-3 have been shown to constrain HSC differentiation⁵⁷ and to promote HSC emergence⁵⁸ in the AGM, respectively. The absence of the thrombopoietin (TPO)/MPL signaling results in a delayed production of HSCs in the AGM⁵⁹, implying that this pathway regulates HSC emergence. Besides these cytokine pathways, several unconventional cues are also important for HSC emergence in the AGM. Biomechanical force generated by blood flow is also important to the production of HSCs in the AGM, likely through the nitric oxide pathway^{60,61} and cAMP-PKA-CREB pathway^{56,57}. Several cell types make up the AGM, including endothelial cells, gonadal cells, mesonephric cells and mesenchymal cells. Identification of the cellular sources of the key extrinsic signals is required to gain a comprehensive understanding of the AGM niche.

Placenta

The placenta may also be an essential site for the generation and expansion of HSCs^{62–64}. IL-3 seems to play a role in placental HSC emergence or maintenance as placental cells

from *IL-3* knockout mice failed to reconstitute adult recipients⁵⁸. PDGF β signaling in trophoblasts has been suggested to modulate erythropoiesis in the placenta by negatively regulating the expression of EPO⁶⁵. Examination of the localization of *Runx1*⁺ (a master regulator of hematopoiesis) cells suggested that the large vessels in the chorioallantoic mesenchyme may be the origin of HSCs while small labyrinth vessels may provide a niche for HSC expansion⁶⁶. Further investigation is needed to uncover how other pathways and cell types control the hematopoietic activity of the placenta.

Fetal liver

The fetal liver is the major site for HSC maturation and expansion. Circulating HSCs from different anatomical sites seed the fetal liver around E11.5⁶⁷. Fetal liver HSCs are actively cycling and have better repopulating capacity compared to adult bone marrow HSCs^{68–70}. The SCF-cKit pathway is important for fetal liver HSCs. Administering functional cKit blocking antibody to mouse embryos at fetal liver hematopoiesis stages disrupts fetal liver hematopoietic progenitors and hematopoiesis⁷¹. Consistent with this, fetal livers from *Scf* knockout mice harbor significantly less HSCs^{30,72}. However, it is not clear what cells are the cellular source of SCF in the fetal liver *in vivo*.

Many cell types have been suggested to be critical components of the fetal liver niche. Murine fetal liver stromal cell lines⁷³ and human fetal liver fibroblastoid cells⁷⁴ have hematopoietic-supportive activity. Hepatic progenitors have also been implicated as niche cells based on expression of key HSC niche factors and their ability to support HSCs *in vitro*⁷⁵. Cells with characteristics of undergoing epithelial-to-mesenchymal transition (EMT) have also been demonstrated to help maintain hematopoietic cells including HSCs *in vitro*^{76,77}. Furthermore, endothelial protein C receptor (EPCR)-expressing cells enriched for HSC activity have been shown to localize near the sinusoidal network in fetal livers⁷⁸, suggesting a perivascular niche. However, these studies were largely based on *in vitro* co-culture systems. Direct *in vivo* functional evidence supporting important roles of these putative fetal liver niche components still needs to be obtained.

Recently, Khan *et al.* have proposed Nestin⁺NG2⁺ periportal stromal cells as an important component of the fetal liver HSC niche through *in vivo* experiments. Fetal liver HSCs were found adjacent to portal vessels, and Nestin-GFP⁺ stromal cells had HSC maintaining activity *in vitro*⁷⁹. Genetic ablation of NG2⁺ stromal cells led to a two-fold reduction in the total number of phenotypic fetal liver HSCs with more quiescence. However, fetal liver cells from these mice had better lymphoid reconstitution activity when transplanted (with normal myeloid cell potential – an accurate readout for HSC activity)⁷⁹. It is not clear why ablation of a niche cell type reduces HSC numbers but increases lymphoid reconstitution activity of fetal liver cells. One possibility is that NG2⁺ cell ablation may selectively deplete some myeloid-biased or balanced HSCs, but does not affect lymphoid-biased HSCs. Nevertheless, as ablation of NG2⁺ pericytes did not lead to a profound change of HSCs and reconstitution activity in the fetal liver, other cellular components may also be critical contributors to the fetal liver HSC niche.

Fetal spleen

The fetal spleen is a transient niche for HSCs during embryogenesis. HSCs are detected in the spleen at E14.5 prior to their colonization of the bone marrow⁸⁰. Fetal spleens lack HSC expansion capacity and fetal spleen stromal cells have been reported to fail to maintain multi-lineage potential of HSCs *in vitro*⁸¹. However, it seems unlikely that HSCs detected in the fetal spleen are merely passing through circulation as HSCs are found in the blood stream at a significant lower level than in the fetal spleen during embryogenesis⁸⁰. It is not clear what cells or extrinsic factors regulate HSCs in the fetal spleen.

Fetal and neonatal bone marrow

HSCs start to seed the bone marrow at E17.5⁸⁰. This process depends on the expression of bone marrow C-X-C Motif Chemokine Ligand 12 (CXCL12), a known chemokine for HSCs⁸². Other than through attractive signals elaborated from the fetal bone marrow, the exit of HSCs from the fetal liver may be driven by the loss of HSC-supporting activity in the liver, although evidence for this has not yet been shown. *Osterix*⁺ cells⁸³, neural crest-derived *Nestin*⁺ cells⁸⁴, *Col2-cre*⁺ cells⁸⁵, and *Lepr*⁺ cells⁸⁶ have been suggested to be the cellular source of stromal populations that comprise the fetal bone marrow HSC niche. In addition, fetal mouse bone marrow CD105⁺Thy1⁻ stromal cells have been shown to form HSC-supporting bone marrow²⁸. However, the lineage and spatial relationship of these cells needs further investigation.

Bone marrow HSCs from 3-week old mice are more similar to fetal liver HSCs than adult bone marrow HSCs in their self-renewal capacity and gene expression⁸⁷. At 4 weeks old, HSCs acquire characteristics of adult bone marrow HSCs, including transition to quiescence⁸⁷. Therefore, it is speculated that the neonatal bone marrow niche undergoes a major transformation during this period. However, little is known about how this transition occurs, including the role of niche independent, HSC-intrinsic pathways.

Extramedullary hematopoiesis (EMH)

The developmental migration of HSCs from fetal liver to fetal spleen and bone marrow is reversible. In adults, HSCs can egress from the bone marrow to seed the spleen and liver to initiate EMH. This is proposed to be a response to hematopoietic stress, particularly when the bone marrow is not inhabitable for HSCs⁸⁸.

A perisinusoidal niche comprised of endothelial cells and *Tcf21*⁺ perivascular stromal cells is activated in the spleen during EMH⁸⁹. Key signals involved in HSC migration to the bone marrow during development, such as CXCL12, also play important roles in establishing spleen EMH^{89,90}. It would be interesting to investigate whether the adult EMH niche is comprised of the same cellular components as the fetal HSC niche. Furthermore, whether EMH niches regulate HSCs differently than the bone marrow warrants future studies.

Homeostatic extrinsic regulation

After development, mammalian HSCs take up residence in the bone marrow niche to sustain homeostatic hematopoiesis. The bone marrow niche is a specialized microenvironment that

exquisitely regulates the quiescence, self-renewal, and differentiation of HSCs⁹¹. Studies have shown that bone marrow HSCs preferentially localize near the vasculature, and subsequent loss-of-function experiments have confirmed that both the vascular endothelium and perivascular stroma are crucial sources of HSC regulatory factors. In addition, mature hematopoietic cells and adipo-lineage cells within the bone marrow are able to regulate HSC behavior. Local oxygen levels, the sympathetic nervous system, and both local and circulating cytokines have also been implicated as regulators of HSCs. In total, these extrinsic signals are crucial to maintaining a functional HSC pool and lifelong hematopoiesis (Figure 2).

The bone marrow HSC niche: location, location, location

Although the concept of an HSC niche was first proposed almost four decades ago⁴, robust characterization of the bone marrow niche was hindered by the lack of markers that could precisely label HSCs *in situ*. One of first studies performed by Lord *et al.* demonstrated that CFU-Ss (later shown to be hematopoietic progenitors, not HSCs) are enriched in endosteum, hinting this region may contain HSC niches⁹². Initial *in situ* visualization studies indeed showed that transplanted, flow cytometrically purified HSCs homed to the endosteum^{14,15}, where osteoblasts were proposed to be a critical component of the niche. Recent work using SLAM or other genetic markers demonstrates that a vast majority of HSCs are directly in contact with the vascular endothelium^{16,17,19,26,93,94}, with only a minority of them close to endosteum, pointing to the perivascular microenvironment as the bone marrow HSC niche.

While there is a growing agreement on the central role of the perivascular microenvironment, there is conflicting evidence as to whether HSCs prefer the endosteum. One should be careful to distinguish observations of endogenous HSCs from those of transplanted HSCs, since the niches may differ in these conditions in several ways. First, it is possible that the niche is perturbed by the myeloablation used in many transplantation experiments, which is known to damage the sinusoidal vasculature^{86,95}. Second, it has been shown that endosteal cells proliferate rapidly and up-regulate HSC maintenance pathways in response to myeloablation^{96,97}. Finally, it is not clear whether the transplanted HSCs are engaged with their niche or in the process of finding the niche. Thus, it is possible that the endosteal localization frequently seen of transplanted HSCs reflects experimental conditioning rather than homeostatic physiology, although understanding how the niche differs in these states has important clinical implications for transplantation therapies. Discrepancies in the literature thus may in part reflect subtle differences between the ‘homeostatic niche’ and ‘post-transplant niche’. However, the cell types and signaling pathways implicated in HSC maintenance seem to play significant roles in both the homeostatic and post-transplant bone marrow – for example, angiogenin from mesenchymal stromal cells (MSCs) regulates HSC proliferation in homeostasis and promotes hematopoietic regeneration following myeloablative conditioning^{98,99}.

Key cellular components of the bone marrow niche

Functional evidence suggests that a number of cell types contribute critically to the bone marrow niche. As the cellular components of the bone marrow niche have been extensively

reviewed elsewhere^{91,100,101}, we will only summarize some of the key *in vivo* functional evidence here. Early studies showed that osteoblast number was positively correlated with the number of HSCs in two mouse genetic models^{24,25}, which in part led to the initial proposal of an endosteal bone marrow niche. Osteoblasts have been proposed to regulate HSCs by a variety of mechanisms including granulocyte colony stimulating factor (G-CSF)¹⁰², Notch²⁴, Wnt¹⁰³, Angiopoietin-1 (ANGPT1)¹³, and thrombopoietin (TPO)¹⁰⁴ signaling and N-cadherin-mediated cell adhesion²⁵. However, careful functional studies reveal conflicting evidence over the roles of Notch¹⁰⁵, ANGPT1¹⁰⁶, and N-cadherin^{107–109}, and additional data complicates the picture of how osteoblasts regulate HSCs. In several other mouse models where osteoblast number was increased or decreased, HSC number was unchanged^{107,110–113}. Furthermore, conditional deletion of critical HSC maintenance factors SCF and CXCL12 from osteoblasts has no effect on HSC function^{30,31}. However, it has been shown that osteoblasts serve as an important niche component for early lymphoid progenitors by synthesizing CXCL12^{31,114}. It is possible that osteoblasts act through other mechanisms to modify HSC behavior, but their role as a niche cell is not as prominent as initially proposed.

While the evidence about the role of osteoblasts in HSC maintenance is conflicting, there is strong evidence that the vascular endothelium is a crucial cellular component of the niche. Endothelial cells (ECs) drive HSC self-renewal *in vivo* through Notch signaling^{115–118}, FGFR signaling¹¹⁹, gp130¹²⁰ and production of SCF³⁰ and CXCL12³¹, and HSC homing through release of CXCL12³¹, ROBO4^{121,122} and pleiotrophin^{123,124}. The vasculature also plays a critical role in mediating a physiologic response to hematopoietic stress; following myeloablation, regeneration of VEGFR2⁺ sinusoidal ECs is required for effective hematopoietic reconstitution^{95,125}. Moreover, endothelial cell-derived EGF is critical for HSC and hematopoietic regeneration after irradiation¹²⁶.

Perivascular MSCs that lie in close contact with the bone marrow vasculature have also been identified as major regulators of HSCs. Several markers with overlapping expression patterns have been used to define these perivascular stromal cells, including platelet-derived growth factor receptor ζ (PDGFRA)^{127,128}, leptin receptor (LEPR)³⁰, Prx1-cre¹¹⁴, Osx-cre¹¹⁴, Osx-creER, FAP¹²⁹, CD146 in humans²⁷, SCF³⁰, CXCL12^{31,114,130}, and the *Nestin*-GFP transgene²⁶. Using some of these markers as tools to manipulate these cells, a number of functional studies have provided strong evidence that the perivascular stromal cells are a critical component of the bone marrow niche. Ablation of *Cxcl12*-expressing or FAP-expressing bone marrow cells led to depletion of HSCs¹³¹ and compromised hematopoiesis^{131,132}. Conditional deletion of *Scf* from perivascular stromal using *Lepr-cre* resulted in HSC depletion³⁰, as did conditional deletion of *Cxcl12* from perivascular stromal cells using *Lepr-cre* or *Prx1-cre*^{31,114}. Interestingly, these stromal cells have mesenchymal progenitor activity with the capacity to differentiate into bone and adipocyte lineages *in vitro* and *in vivo*^{26,86}. Conditional deletion of *Foxc1*, a transcriptional factor involved in mesenchymal cell fate determination, from perivascular stromal cells led to excessive adipogenesis and depletion of HSCs¹³³. Recently, angiogenin has been identified as a novel factor from Osx⁺ mesenchymal cells for HSC maintenance⁹⁹. Besides these genetic studies, subcutaneously transplanted human CD146⁺ bone marrow stromal cells have been shown to

form ectopic HSC-supporting bone marrow²⁷. Thus, the bone marrow perivascular mesenchymal stromal cells are a critical component of the bone marrow niche.

Other non-hematopoietic cells have also been implicated in HSC maintenance. HSCs from adipocyte-rich bone have decreased function and treatment with adipogenic inhibitors increases the reconstitution potential of HSCs, suggesting that adipocytes may negatively regulate HSCs¹³⁴. Additionally, non-myelinating Schwann cells have been shown to activate latent TGF- β signaling required for HSC maintenance¹³⁵.

Mature hematopoietic cells have also been connected to HSC regulation. Megakaryocytes physically associate with HSCs, maintain HSC quiescence through secretion of factors such as CXCL4 and TGF- β 1, and promote hematopoietic recovery after myeloablation by driving HSC expansion^{136,137}. Like megakaryocytes, bone marrow macrophages also modify HSC behavior. Depletion of macrophages leads to increased egress of HSCs from the bone marrow, suggesting that macrophages help retain HSCs in the bone marrow niche through cell homing and adhesion pathways, such as CXCL12-CXCR4 signaling^{138,139}.

Macrophages are believed to drive this retention of HSCs indirectly by acting on other niche cells including MSCs^{138,139}. However, a recent study suggested that a subpopulation of macrophages which express Duffy antigen/receptor for chemokines (DARC) directly maintain HSC quiescence via binding of DARC to the HSC cell surface antigen KANGAI1 (KAI1)¹⁴⁰. Thus, macrophages can directly and indirectly impact HSCs. In addition, Treg cells have been shown to provide immune privilege in the bone marrow niche¹⁴¹. In summary, these findings highlight the complex and interconnected regulatory interactions of the bone marrow niche.

Hypoxia and bone marrow niche

The cells of the perivascular bone marrow niche are critical extrinsic regulators of HSCs, but they are not the only factors that influence HSC biology. Numerous studies suggest that oxygen levels in the bone marrow have a major impact on HSC homing and function. In poorly perfused regions of the bone marrow that presumably have lower oxygen tension, the HSC population is enriched, more quiescent, and better able to serially reconstitute irradiated recipient mice¹⁴². Consistent with these findings, HSCs with high levels of reactive oxygen species (ROS), a feature associated with aerobic metabolism, have diminished self-renewal potential¹⁴³. Indeed, HSC maintenance seems to require continuous activation of hypoxia-associated transcriptional and metabolic programs^{144–147}.

Interestingly, harvesting HSCs in a hypoxic environment better preserves transplantable HSCs¹⁴⁸. The importance of these pathways to HSC function has driven many labs to investigate the location of the putative ‘hypoxic niche’ through indirect⁹⁴ and direct¹⁴⁹ measurements of oxygen levels throughout the bone marrow. The most recent and direct evidence shows that the peri-sinusoidal region in the bone marrow is the most hypoxic region of the bone at steady state¹⁴⁹. However, it is possible that the association between HSCs and hypoxia is indirect. The ‘hypoxic’ profile of HSCs measured by pimonidazole may reflect their metabolic status independent of localization⁹⁴. In addition, the hypoxia master regulator HIF-1 α may be dispensable in HSCs¹⁵⁰, although HIF-1 α activity drives

growth and expansion of vascular endothelium¹¹⁷. Thus, hypoxia may promote the development of the HSC niche, rather than provide direct extrinsic regulation of HSCs.

Heterogeneity of the bone marrow niche

The above data strongly suggest that HSCs reside in a perivascular niche with endothelial cells and mesenchymal stromal cells as critical components. Recent evidence points to potential heterogeneity in the perivascular niche. Perivascular NG2⁺LEPR⁻Nes-GFP^{bright} mesenchymal cells that predominantly associate with bone marrow arterioles, not sinusoids, have been shown to play a role in HSC maintenance: partial ablation of NG2⁺ cells leads to decreased HSC quiescence and diminished capacity for functional reconstitution¹⁵¹. However, deletion of *Scf* and *Cxcl12* using *NG2-creER* did not significantly affect HSCs¹⁹. Further investigation is needed to clarify this discrepancy. Mechanisms other than SCF and CXCL12 production may account for the HSC phenotype observed in the mice with NG2⁺ cells ablated. Recently, heterogeneity among endothelial cells in the bone marrow has been suggested to play important roles in regulating distinct HSC behaviors - arterial blood vessels maintain HSCs while sinusoids promote HSC activation and trafficking¹¹⁹. The functional evidence supporting this conclusion is partly based on evidence that conditional deletion of *Fgfr* from endothelial cells using *Vecadherin-CreER* leads to vasculature leakage and HSC reduction. However, *Vecadherin-creER* recombines in all bone marrow endothelial cells including arteriolar and sinusoidal endothelial cells¹⁵². Cre drivers specific to distinct vascular domains are needed to further elucidate the roles of different subsets of the vasculature.

Beyond the bone marrow: systemic regulation of HSCs

The bone marrow microenvironment is the most well-studied component of HSC extrinsic regulation, but extramedullary signals from outside the bone marrow also have a significant impact on HSCs. One prominent example of an extramedullary regulator is the sympathetic nervous system, which has a major influence on the release of HSCs into the blood stream^{153,154}. Catecholaminergic signaling to both HSCs¹⁵⁵ and niche cells¹⁵⁶ drives HSC egress from the bone marrow by downregulating the chemokine CXCL12 and upregulating metalloproteinases that degrade the adhesive extracellular matrix. This signaling is linked to the body's circadian rhythm, which also drives clearance of aged neutrophils from the bone marrow niche¹⁴⁷.

There is growing evidence that systemically circulating factors also influence the biology of HSCs. New studies have shown that physiological circulating estrogen¹⁵⁷ and induced upregulation of endogenous erythropoietin (EPO)¹⁵⁸ can directly promote HSC proliferation or instruct HSC differentiation to specific cell fates. These molecular signals originate outside the bone marrow but are still able to regulate the fate and behavior of HSCs, raising the possibility that other endogenous hematopoietic cytokines synthesized outside the bone marrow may directly maintain the HSC pool.

Disruption of the extrinsic regulation

The bone marrow homeostatic niche must balance activating HSCs to replace lost progenitors while maintaining quiescent HSCs for future needs. This finite equilibrium can be disturbed in aging or other hematopoietic disorders. Aging of the hematopoietic system is associated with a decline of HSC function and hematopoiesis. This may be related to the demands of the an aging individual - to minimize oncogenic transformation at the cost of self-renewal activity¹⁵⁹. It is evident that the extrinsic regulatory mechanisms play a role in HSC aging. Just as the HSC extrinsic regulation is important in homeostasis and aging, its dysregulation also plays a key role in coping with or even initiating pathological hematopoiesis. Below, we will discuss illustrative examples of the extrinsic regulation of HSCs during aging, hematopoietic malignancies and other stress conditions (Figure 3).

Aging

The effects of aging on HSC function have been well described. Old HSCs have a decreased regenerative potential, are biased towards the myeloid lineage, and are more easily mobilized in response to cytokine cues¹⁶⁰. Some of these phenotypes are linked to intrinsic changes within the HSC, but others have been proposed to be the result of an aging microenvironment. The effects of aging specifically on the bone marrow HSC niche has only begun to be explored. Transplantation of HSCs from young mice to old recipient mice (or vice versa) shows that at least part of the aging HSC phenotypes can be attributed to an aging environment. Young bone marrow HSCs home inefficiently to old bone marrow compared with young bone marrow, showing that there is a relevant difference in interactions between HSCs and the old environment that affects HSC engraftment¹⁶¹. Details on which signaling interactions differ and how engraftment is altered have yet to emerge. Similarly, old HSCs are less myeloid-biased when transplanted into young mice, suggesting that the age of the environment plays a key role in HSC differentiation lineage bias, which could be explained by an increase in the inflammatory cytokine Rantes¹⁶². Systemic age-related changes might also influence HSCs. Parabiosis experiments between old and young mice have demonstrated blood-borne factors that vary with age can influence muscle satellite stem cell and neural stem/progenitor cells function^{163,164}. These studies suggest the possibility of systemic mechanisms for stem cell aging in general, but direct evidence for age-related systemic changes in HSC regulation is still needed. The question still remains of exactly how other age-related changes in HSCs are regulated by an old environment, both locally and systemically.

Age-related niche dysregulation may in part be explained by the increase in adipocyte content of old bone marrow, but the exact mechanisms are not yet clear. From adolescence to old age, the bone marrow progressively becomes more adipogenic with a higher fat content and decreased osteogenesis^{165,166}. These changes are thought to be derived from altered differentiation of aged bone marrow MSCs. As described above, MSCs are a key component of the HSC niche and can differentiate into adipocytes, osteoblasts and chondrocytes within the bone marrow^{86,167}. Old bone marrow MSCs are less proliferative, less likely to differentiate into osteoblasts, more likely to differentiate into adipocytes, and more apoptotic than young MSCs^{168,169}. Interestingly, age-related changes in HSC frequency have been

shown to be strain dependent, but in the strain most studies use, C57BL/6, HSC number increases with age¹⁷⁰. Many studies have shown that there is an age-related decrease in HSC function, an increase in HSC number and in the adiposity of older bone marrow. However, studies of adipogenesis (in which age was not controlled for) suggest that medullary adipose tissue reduces HSC self-renewal in non-elderly C57BL/6 mice, resulting in decreased HSC number and function^{133,134}. Furthermore, excessive adipogenesis from troglitazone, a drug that enhances adipogenesis, does not impact HSC⁸. Thus, it is unknown whether the correlations between adipogenesis and HSC dysfunction result directly from adipocyte-mediate effects or if they are due to indirect effects, such as a reduction in other stromal cell types. The functional differences between aged HSCs and HSCs from adipogenic models suggest that excess adipogenesis is not the only change in the old microenvironment affecting HSCs.

Other cellular components have also been examined to test whether aging leads to the loss of the normal homeostatic niche architecture. Old bone marrow has reduced vascularity and a decrease in PDGFR β ⁺NG2⁺ perivascular stromal cells, causing a decrease in key niche signals like CXCL12, and SCF¹¹⁷. Notch activation in endothelial cells rescued some of these phenotypes, causing an increase in capillaries and arterioles, more perivascular stromal cells, higher SCF expression, and a higher HSC number in mutant old mice than in wild-type old mice¹¹⁷. These studies only begin to delve into how HSC regulation is altered in an aged environment.

Role of the Niche in Hematologic Malignancies

Abnormalities in the niche are associated with hematologic malignancies. Leukemia-initiating cells (LICs) hijack the HSC mechanisms to persist and fuel the growth of leukemia. For an LIC to emerge and thrive, it must lose the restraints of HSC regulatory mechanisms keeping its growth and function in check. Some of those mechanisms are intrinsic to the LICs, but many are dependent on the niche. Whatever the inciting cause, as leukemia develops, there is ongoing crosstalk between the niche and LICs that eventually leads to further dysregulation and loss of normal hematopoiesis. Here, we will discuss some of the key features of the niche and malignant cells interactions, which have been described in more detail in other reviews^{101,171–173}.

Live imaging of the bone marrow niche post-transplantation with a B-ALL leukemic cell line or T-ALL cells has demonstrated the stable interaction of leukemic cells with the endothelial domain, where the CXCL12-CXCR4 pathway is critical for leukemia cell engraftment^{18,174}. However, a recent study has shown that the interaction of T-ALL cells and the niche is highly dynamic and not associated with bone marrow sub-compartments¹⁷⁵. More investigation is needed to resolve the discrepancy. Comparing with the homeostatic bone marrow, imaging data of the malignant niche is only beginning to surface; all of these studies have been focused on transplanted leukemia models rather than *de novo* leukemia models. Furthermore, niche information concerning LICs is lacking. Technical improvement on malignant niche imaging is expected to lead to more exciting new insights.

Regardless of the localization of leukemic cells or LICs, an abnormal environment is important for malignancy progression¹⁷². A growing number of papers have demonstrated that the niche can be the inciting factor in developing malignancy. Myeloproliferative neoplasms (MPN) or myeloproliferation can be induced by deletion of *Ikb α* (an inhibitor of NF κ B)¹⁷⁶, *Mindbomb-1* (a notch ligand regulator) or retinoic acid receptor- γ in the bone marrow microenvironment^{177,178}. Furthermore, conditional deletion of *Rbpj* (a notch pathway component) from endothelial cells also promotes myeloproliferation¹¹⁵. Moreover, conditional deletion of *retinoblastoma* protein (*Rb*) from both hematopoietic cells and stromal cells, causes a myeloproliferative-like disorder, but not when only deleted from myeloid progenitors or stromal cells¹⁷⁹. In a similar fashion, conditional deletion of *Dicer1* from osteoprogenitors but not more mature osteoblasts causes transplantable myelodysplasia and secondary leukemia¹⁸⁰. Finally, Kode *et al.* induced acute myelogenous leukemia (AML)-like disease by constitutively activating β -catenin in osteoblasts¹⁸¹. These studies directly demonstrate the causal role of the niche in hematopoietic malignancies.

The interaction of leukemia cells and their niche is reciprocal. Several studies have shown that the introduction of leukemia can alter the niche, and often in ways that support the progression of leukemia and suppress normal hematopoiesis. Live imaging of mouse bone marrow transplanted with human leukemia cells showed that leukemic cells alter the stromal niche¹⁸². These abnormal niches sequester normal HSPCs and impair normal hematopoiesis¹⁸². Chronic myelogenous leukemia (CML) cells have been shown to alter key niche factors – such as decreasing CXCL12 and SCF and increasing G-CSF – that prevent HSCs from occupying their normal niche and instead promote leukemic cell development^{183,184}. Similarly, the MPN bone marrow niche undergoes an alteration of niche signals and loss of bone marrow Schwann cells and nestin⁺ MSCs¹⁸⁵. Rescuing some of the lost sympathetic tone with a selective β 3 adrenergic agonist reduced the severity of disease¹⁸⁵. Besides leukemia, the niche can also be an active participant in disease progression in other hematopoietic disorders. It has been shown that myelodysplastic syndrome (MDS)-initiating hematopoietic cells have the ability to induce neighboring MSCs to propagate a diseased microenvironment and facilitate the development of MDS¹⁸⁶. These results demonstrate that abnormal hematopoietic cells may benefit from the changes they induce in the niche.

However, studies have also shown that the niche can act as a leukemia suppressor. Ablation of certain components of the microenvironment, such as catecholaminergic neurons, can lead to more severe disease in an aggressive MLL-AF9 AML model¹⁸⁷. Krause *et al.* also illustrate this well in their work showing that activation of parathyroid hormone in osteoblasts inhibits CML progression but worsens survival in a model of AML¹⁸⁸. Different types of hematologic malignancy may rely on and interact with the HSC niche very differently, reflecting not only the variety of ways that dysregulation of the niche may occur but also the complexity of signaling that fights to maintain hematopoiesis.

Recent understanding of how the bone marrow niche contributes to malignancy has led to the development of niche-targeted therapies with promising initial results. For example, AML cells exploit the CXCL12/CXCR4 axis for proliferation and survival, and high expression of CXCR4 on LICs correlates with worse prognosis. Inhibitors of CXCR4, most

notably plerixafor, in combination with traditional chemotherapy agents have improved outcomes in AML murine models and clinical trials¹⁸⁹. While niche-targeted agents have yet to become a part of standard treatments, research in this area is likely to grow.

Extrinsic regulation of HSCs in damage responses

Extrinsic regulatory mechanisms can also protect against infection and injury by modulating HSCs. During infection, the hematopoietic system must generate a sufficient immune response that often requires more mature hematopoietic cells from HSCs. Inflammatory signaling from Toll-like receptors (TLRs) and cytokines such as interferon- γ (IFN γ), interferon- α (IFN α), and tumor necrosis factor- α (TNF α) can activate HSCs¹⁹⁰. These signals can be sensed directly by HSCs, but the bone marrow hematopoietic environment also plays a key role in mediating some of these immune responses¹⁹¹. For example, it has been shown that bacterial cell wall products from *E.coli* infection activate TLR and nucleotide-binding oligomerization domain-containing receptors (NOD) signaling, which induces G-CSF expression and decreases CXCL12 expression from niche endothelial cells, which in turn mobilizes HSCs to the spleen¹⁹². Similarly, IFN γ has been linked to increased HSC proliferation and mobilization to the spleen¹⁹³. This effect can be mediated by MSCs expressing IL6 in response to IFN γ , which results in increased myeloid differentiation in response to infection¹⁹⁴.

The bone marrow also responds to injury induced by irradiation. After irradiation, sinusoids regress, osteoblasts expand and megakaryocytes cluster around the endosteum and sinusoidal endothelium to promote HSC niche recovery^{20,22,96,97,106}. Bone marrow vasculature regeneration plays a critical role in HSC recovery from irradiation. Targeting sinusoidal endothelial cells caused delay in hematopoietic recovery⁹⁵. Angiopoietin-1-mediated interactions between HSCs and the bone marrow vasculature are important for optimal hematopoietic recovery after irradiation¹⁰⁶ and cotransplantation of HSCs with endothelial cells results in faster hematopoietic recovery¹⁹⁵. Moreover, endothelial jagged-1, pleiotrophin and EGF have been shown to be important for HSC regeneration after irradiation^{118,124,126}.

Myelosuppression associated with chemotherapy is another stressor with clinical relevance. Similar to irradiation, recovery of the vasculature is critical for efficient hematopoietic recovery after myelosuppression. The endothelial VEGFR2 pathway is required for the restoration of hematopoiesis⁹⁵. In this setting, TGF- β pathway plays an important role in dampening HSC activity¹⁹⁶, suggesting that inhibiting this pathway could lead to better recovery after myelosuppression.

Perspectives

Significant advances have been made in the field over the last several years and it is expected that our understanding of the cellular and molecular components of extrinsic regulation of HSCs will continue to grow. This will allow us to compare and contrast the extrinsic regulatory mechanisms in different physiological and pathological settings. Interestingly, the sites of hematopoiesis are not conserved across vertebrates. For example, adult

hematopoiesis occurs in the kidneys of fish and the livers of amphibians. However, in adult birds and mammals, the bone marrow is the primary hematopoietic organ. These observations suggest that extrinsic HSC supporting mechanisms are not confined to a specific organ. This is also reflected in the migration of mammalian HSCs during development and EMH. When the adult bone marrow niche is compromised in leukemia, myelofibrosis, osteopetrosis, and other medullary diseases, extramedullary sites are often reactivated to support hematopoiesis. Understanding more about the developmental and EMH niche could help us improve the responses of hematopoietic niches to disease and stress. Emerging evidence suggests that the vasculature plays important roles in creating the niche across different organs. Investigating the common and unique vascular components in these niches will shed light on potential unifying HSC niche mechanisms. For example, while the majority of HSCs are kept quiescent in the adult bone marrow niche, fetal liver HSCs actively cycle. Understanding signals from the fetal liver microenvironment that allows HSCs to expand rapidly while maintaining their self-renewal potential could help devise novel strategies to amplify HSCs.

As the cellular components are better defined, studying the regulatory mechanisms of the niche cells will likely help generate more functional HSC niches. The markers currently used for identifying HSC niche cells require further improvement. Even in the best-studied bone marrow niche, the frequencies of perivascular stromal cells and endothelial cells are at least an order of magnitude greater than that of functional HSCs. It could be that only a subset of these niche cells maintains HSCs. Like the evolution of HSC markers, development of better markers that can be used to purify and target true niche cells will significantly improve the precision with which we can study the extrinsic regulation of HSCs, not only during homeostasis but also in hematopoietic disorders.

With the discovery of markers that can subdivide the niches, more exciting biology awaits. For example, HSCs are heterogeneous in their cell cycle status, marker expression, lineage output and response to extrinsic signals^{197,198}. This raises the question as to whether there is heterogeneity within the niches. Some recent studies have started shedding some light on the heterogeneity of the niche in regulating function of heterogeneous HSCs. But more investigation is needed regarding the niche heterogeneity in the bone marrow and other hematopoietic organs under homeostasis and pathological conditions.

Most studies of HSC extrinsic regulation have been focused on the local microenvironment. However, systemic regulation is also critical. Hematopoiesis and HSC function are tightly regulated by the overall physiological demands of the body. This provides a feedback mechanism essential to maintaining just the right amount of hematopoiesis. It appears that all of the known systemic regulators are geared to push HSCs along specific differentiation pathways. The systemic factors that determine the physiological homeostatic level of HSCs have yet to be discovered.

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Figure 1. Extrinsic regulation of HSCs during ontogeny

A. An illustration depicting hematopoietic organs in mouse embryos. **B.** Timing and location of hematopoiesis during development. Hematopoiesis takes place in several anatomical sites. Primitive hematopoietic progenitors are found in the blood island of the yolk sac. AGM is the predominant site of HSC emergence although the placenta may also play a key role. HSCs migrate to, mature and expand in the fetal liver. Subsequently, HSCs transiently seed the fetal spleen and around birth, migrate to the bone marrow where HSCs reside throughout adult life. Under stress conditions, HSCs can egress from the bone marrow and take residence in the liver and spleen to initiate EMH. **C.** Candidate cellular components and extrinsic signals that regulate hematopoiesis during development. AGM, aorta-gonad-mesonephros; EMH, extramedullary hematopoiesis; EMT, epithelial-mesenchymal transition.

Figure 2. Homeostatic HSC extrinsic regulation
A. Anatomy of a mammalian long bone, the major hematopoietic organ in adults. **B.** In the bone marrow, HSCs are regulated by a variety of cell types including endothelial cells, heterogeneous populations of mesenchymal stromal cells, non-myelinating Schwann cells, megakaryocytes, macrophages and osteoblasts. Signals from sympathetic nerves, circulating factors and possibly hypoxia (not pictured) are also important. Key locally synthesized factors for HSC maintenance include SCF, CXCL12, TGF-β1 and CXCL4. **C** and **D.** Major and other HSC regulatory cells in the bone marrow, where major regulatory cells are required to maintain the HSC pool, and other regulatory cells affect the cell cycle, localization and downstream progeny of HSCs through direct and indirect mechanisms.

Figure 3. Disruption of extrinsic regulation of hematopoiesis

A. The aged hematopoietic niche (left) features increased adipogenesis, decreased osteogenesis, decreased vascularity, decreased numbers of mesenchymal stromal cells (MSCs), and decreased niche signaling factors. HSCs are less functional. In states of malignancy (right), extrinsic regulation can be disrupted in many different ways. I) Aberrant niche cells can incite malignancy and leukemia-initiating cells (LIC). II) Malignant cells can inhibit normal functioning of niche cells and reduce normal niche signaling as well as induce leukemia-reinforcing phenotypes in niche cells. III) Normal niche cells can inhibit disease progression. **B.** Dysregulation of the niche varies across different of abnormal conditions – old age, malignancy, inflammation and irradiation. Common themes emerge, including the loss of cell types promoting critical niche signaling factors, pointing to the importance of these pathways in maintaining hematopoiesis.