Review

Haematopoietic stem cell niches: new insights inspire new questions

Fernando Ugarte and E Camilla Forsberg*
Department of Biomolecular Engineering, Institute for the Biology of Stem Cells, University of California Santa Cruz, Santa Cruz, CA, USA

Haematopoietic stem cell (HSC) niches provide an environment essential for life-long HSC function. Intense investigation of HSC niches both feed off and drive technology development to increase our capability to assay functionally defined cells with high resolution. A major driving force behind the desire to understand the basic biology of HSC niches is the clear implications for clinical therapies. Here, with particular emphasis on cell type-specific deletion of SCL and CXCL12, we focus on unresolved issues on HSC niches, framed around some very recent advances and novel discoveries on the extrinsic regulation of HSC maintenance. We also provide ideas for possible paths forward, some of which are clearly within reach while others will require both novel tools and vision.

What drives the desire to understand HSC niches?

Haematopoietic stem cell (HSC) niches are an intensely studied topic that aims at understanding the elusive cues from the microenvironment that supports HSC function in vivo. Combined with the intrinsic properties of HSCs, these signals are capable of maintaining a perfect balance of HSC quiescence and proliferation, self-renewal and differentiation to maintain haematopoietic homeostasis under drastically dynamic conditions throughout life. In a field of many ‘firsts’—prospective isolation of the stem cell, single cell transplantation, stem cell therapy—HSC biology also suffers from many unknowns. It is frustrating to admit that despite decade-old knowledge that adult HSCs reside primarily in the bone marrow (BM), we do not have a clear mechanistic understanding of the cues that attract them to and retain them in the BM, and we do not understand the complex inputs that enable HSC self-renewal within these niches (Figure 1). These gaps in understanding the basic biology of HSC regulation hamper our clinical treatment strategies. The difficulty in achieving robust ex vivo expansion of functional HSCs—considered the ‘holy grail’ of haematopoiesis (Sauvageau and Humphries, 2010)—and inability to derive robustly engrafting HSCs from pluripotent cells means that we cannot provide a reliable supply of HSCs for all patients, although many lives could be saved and painful diseases cured if we did. We also lack protocols for specific manipulation of HSC dislodgment out of and engraftment into BM niches. Thus, more than 50 years after the first successful haematopoietic transplantation in humans, we still have to use non-specific, broadly damaging and, too often, lethal approaches to enable transplanted HSCs to engraft and thrive long term in the recipient. By comprehending the function of HSC niches, we hope to solve these issues and many more, including how extrinsic cues affect lineage output and contribute to leukaemogenesis and other haematopoietic disorders.

Several excellent and comprehensive reviews on HSC niches have been published recently (Isern and Méndez-Ferrer, 2011; Frenette et al, 2013; Krause et al, 2013; Smith and Calvi, 2013). Therefore, although many factors are involved in HSC maintenance, we restrict the scope of this review to selected unresolved issues, using recent discoveries on the structural, cellular, and molecular regulation of HSC maintenance by extrinsic factors as the framework for discussion and future directions.

Technical and conceptual advances by recent publications

Defining HSC location relative to other components of the BM is a challenging task, with technical difficulties ranging from preparation of BM sections, cell-specific labelling, and high-resolution detection methods. Nevertheless, major advances have been made in the past years to narrow down the location of HSC niches (Figure 1). While HSCs are known to circulate and reside in multiple tissues, they primarily localize to the BM (Figure 1A). Within the BM, both perivascular and endosteal niches have been described (Kiel and Morrison, 2008), providing potentially different cellular contexts for HSCs (Figure 1B and C). Increasingly, specific molecules in different cellular environments are being probed for their contribution to HSC function (Figure 1D). A breakthrough in the ability to investigate HSC location was made when the Morrison laboratory established the SLAM markers to identify stringently defined HSCs by two-colour analysis, and locate them close to sinusoidal endothelial cells (SECs) in BM and spleen (Kiel et al, 2005). One technical hurdle in
moving forward from those studies has been limitations in microscopy capability. High-resolution analysis usually limits examination to only a few areas of a BM section, making the search for the rare HSCs prohibitively labour intensive and time consuming. The analysis of only a fraction of HSCs that reside in each bone may miss features of the comprehensive environment. A recent study by Nombela-Arrieta et al (2013) used novel technology to overcome these limitations. Laser scanning cytometry (LSC) is a slide-based microscopy technique that allows for the quantification of light emitted by all cells in a relatively large section of tissue, therefore not limiting analysis to a small field of view (see LSC review; Harnett, 2007). In their study, Nombela-Arrieta et al used a combination of LSC and confocal imaging to describe the three-dimensional vascular architecture of the BM at a more global level, revealing that the BM environment is highly vascularized, especially at the bone-proximal regions. They also comprehensively quantify the location and distribution of haematopoietic cells, demonstrating an enrichment of haematopoietic stem and progenitor cells (HSPCs) in perivascular areas of bone-proximal regions. This observation suggests that vascular and endosteal niches should not be viewed as two different compartments, but rather that highly vascularized endosteal regions may provide the complex cellular and molecular environment necessary for HSC maintenance. This finding also fits the idea of different types of niches in close proximity, supporting either quiescence or proliferation of HSPCs depending on demand (Kiel and Morrison, 2008). Interestingly, Nombela-Arrieta et al also observed that hypoxic HSPCs are evenly distributed throughout the BM, in contrast to previous observations that highlighted the endostium as a hypoxic compartment favourable for HSC maintenance (Eliasson and Jönsson, 2010; Mohyeldin et al, 2010; Takubo et al, 2010; Suda et al, 2011). The ability of this new technology to visualize single cells and structures in large, three-dimensional space will facilitate the creation of more comprehensive, architectural maps of the BM vasculature, with distribution of niche cells and HSCs. How such maps change under different conditions will be important for understanding the dynamics of HSC regulation by these environments.

New discoveries on the spatial organization of HSC niches were also made recently by Wang et al (2012). Using a dual-colour reporter mouse (mT/mG mice; Muzumdar et al, 2007) in combination with an endothelial-specific inducible allele (cdh5(PAC)-creERT2), they were able to distinguish SECs from perivascular stromal cells (PVCs). This led to the identification of a zone between SECs and PVCs containing clusters of HSCs, which they named hemospheres. These distinct hemosphere compartments were of variable size, but highly enriched for HSCs on the abluminal surface of a sinusoidal vessel (Figure 2). Deletion of VEGFR2 from endothelial cells disrupted the formation of hemosphere structures and led to reduced numbers of HSCs in the BM. This exciting new finding adds a spatial dimension to the cellular organization of HSC niches. Importantly, it provides a conceptual framework for testing how the BM is compartmentalized to provide the specialized context necessary for HSC function.

In addition to advances in structural organization, our understanding of both the cellular and molecular specificity of HSC maintenance has taken great strides forward. Several recent studies have capitalized on the well-established roles of the ckit and CXCR4 receptors and the corresponding ligands, SCF and CXCL12, in HSC function (Chabot et al, 1988; Williams et al, 1990; Nagasawa et al, 1996; Broudy, 1997; Williams et al, 1998; Peled et al, 1999; Nie et al, 2008; Kimura et al, 2011); to define the cellular and molecular environment necessary for HSC maintenance. Building on the pioneering studies of Sugiyama et al who used a CXCL12-GFP reporter mouse to identify a small fraction of perivascular cells in close proximity to putative HSCs, named CXCL12-abundant reticular (CAR) cells (Sugiyama et al, 2006), several groups have used similar strategies to label and functionally test the importance of potential niche cells and molecules. Mendez-
Figure 2 The hemosphere niche model. A novel view of the perivascular niche was proposed by Wang et al., where HSCs and hematopoietic progenitors are contained in defined areas termed hemospheres (Wang et al., 2012), delimited by SCFs and perivascular stromal cells. This area may be highly enriched for molecular cues that promote HSC maintenance and self-renewal, such as SCF and CXCL12. Broad acceptance of this model will likely require validation by alternative genetic model and imaging techniques.

Ferré et al. used a Nestin-GFP reporter mouse to identify a rare population of mesenchymal stem/progenitor cells (MSPCs) highly enriched for factors implicated in HSC maintenance, including CXCL12 and SCF (Méndez-Ferré et al., 2010). The Nestin- cells were located in perivascular regions close to putative HSCs. Deletion of the Nestin population significantly reduced the number of HSCs in the BM, demonstrating that these perivascular MSPCs play important roles in HSC maintenance. Then, the Morrison and Link labs turned the question around by focusing on specific molecules—SCF and CXCL12—and asking which cells are responsible for their production (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013). Intriguingly, the two groups independently demonstrated that deletion of either SCF or CXCL12 from endothelial and perivascular cells, but not from other stromal or hematopoietic cell types, led to impaired HSC maintenance (see Table I for Cre recombinase mouse lines used in these studies). In an impressive demonstration that cell type-specific production of secreted factors matters, they also observed that deletion of CXCL12 from osteoblast lineage cells (OBCs) resulted in decreased numbers of lymphoid progenitors, but not of HSCs. Although a few differences between the findings from the two groups need to be reconciled, their studies converge on a model where different niche cells support different stem and progenitor populations: HSCs are supported by SCF- and CXCL12-expressing vascular and perivascular cells, whereas lymphoid progenitors are maintained by CXCL12-expressing OBCs.

We anticipate that the powerful tools and concepts established in these studies will be applied to the many additional secreted molecules implicated in HSC function (for a more comprehensive list of factors, see Wilson and Trumpp, 2006). Integration of the findings on microvessel organization (Nombela-Arrieta et al., 2013), hemostatic structures (Wang et al., 2012), and cell type-specific production of SCF and CXCL12 (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013) promises to advance our understanding of HSC functions far beyond our current knowledge. In this context, we discuss some of the conceptual and technical challenges of addressing the complexities of HSC-supporting niches, and provide suggestions for further progress.

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Niche cell nomenclature, isolation, and classification strategies

The genetic reporter strategies mentioned above delineated several new cell populations (e.g., CAR cells and Nestin+ cells), while other BM cells have been categorized by functional characteristics (e.g., MSPCs), by location (e.g., PVCs), or based on the markers typically expressed by a cell lineage (e.g., OBCs). This has led to a rapidly growing list of BM cells suggested to play a role in supporting HSC maintenance (Table II). The different strategies used to identify new cell populations, combined with inconsistent nomenclature, have precluded clear delineation of the relationship, and in many cases significant overlap, between these populations. Improvements in stromal cell naming conventions and isolation protocols would avoid unnecessary confusion on the identity of BM cell types.

Inconsistencies in nomenclature range from simple naming conventions to more complex issues. For example, the term BM ‘stromal cell’ sometimes excludes osteoblasts (Shestopalov and Zon, 2012) and sometimes is used to refer specifically to the progeny of mesenchymal stem cells (MSCs) (Frenette et al., 2013). In turn, the term ‘MSC’ has widely been used to describe the heterogeneous populations of cells obtained from various tissues, often without rigorous assessment of the self-renewal or lineage potential expected of true MSCs (Cao et al., 2013). Similarly, ‘perivascular stromal cell’ has been used to describe several different
types of cells located in proximity to SECs, including CAR, Nestin-GFP, SCF-GFP, and Lepr-expressing cells. The extent of overlap between these cell types remains to be determined, but is likely significant. Clarifying the lineage relationship between the different cell populations will also be important, and is facilitated by the recent establishment of new Cre/lox reporter lines. Unfortunately, such studies are not without caveats. For example, it was proposed recently that haematopoietic progenitors can give rise to osteoblasts upon transplantation (Hofmann et al., 2013), contradicting the dogma that osteoblasts originate from mesenchymal progenitors. Additionally, the specificity of Nestin-mediated reporter activity varies between different transgenic lines (Ding et al., 2012), and it is clear that the populations of cells targeted by the different Cre lines (Tables I and II) are not yet completely mapped and may in some cases target several cell types or lineages (Hanoun and Frenette, 2013). To date, some of the newly identified PVCs are believed to be mesenchymal progenitors, based on their potential to differentiate into osteo- and adipocyte lineages, as shown for CAR cells (Omatsu et al., 2010), or based on the CFU-F potential and in vivo ossicle formation, as shown for Nestin-GFP cells (Méndez-Ferrer et al., 2010). In contrast, lineage tracing experiments showed that Lepr-expressing cells did not give rise to osteoblasts (Ding et al., 2012). The functional properties of other types of PVCs remain unknown. A productive approach to this issue was used by Sacchetti et al. (2007), who showed that a clonal population of human CD146+ stromal cells were capable of self-renewal and formation of a haematopoietic microenvironment upon transplantation into heterotopic sites in mice. Whether PVCs have this ability and whether HSCs can colonize these ectopic areas remain to be tested. An additional source of variation is caused by differences in cell extraction protocols. Recovery and relative frequency of cell types greatly depend on the isolation method, and the methods established for harvesting haematopoietic cells from BM preclude recovery of some of the more adherent cell types. Different techniques such as flushing, crushing, and enzymatic digestion will yield different numbers and types of cell types greatly depend on the isolation method, and the methods established for harvesting haematopoietic cells from BM preclude recovery of some of the more adherent cell types. Different techniques such as flushing, crushing, and enzymatic digestion will yield different numbers and types of cell types.
et al., 2012). Similarly, GFAP+ cells, though detected under the microscope in BM sections, were not recovered in BM cell suspensions (Yamazaki et al., 2011). Suboptimal and inconsistent cell isolation protocols make it difficult to accurately quantify the numbers of distinct cell type and therefore, as discussed below, their relative contribution to secreted ligands. In addition, reliable isolation of viable cells is important in order to couple phenotype with function, as has been so valuable for understanding the haematopoietic system. Differences in isolation protocols may account for some of the inconsistencies in expression levels of HSC-supporting factors by different cell types. For example, Ding et al. found the highest levels of CXCL12 expression in PVCs, followed by endothelial cells and then by OBCs (Ding and Morrison, 2013), whereas others have reported that OBCs express higher or equivalent levels of CXCL12 compared to endothelial cells (Semerad et al., 2005; Smith-Berdan et al., 2012; Greenbaum et al., 2013). In addition, failure to recover viable cells corresponding to the phenotype described in BM sections hampers genome-wide molecular characterization and precludes assessment of their lineage potential.

Although HSC niches are a rapidly evolving research area, a consistent nomenclature based on the lineage and functional characteristics would be hugely beneficial in building a comprehensive picture of BM stromal cells. Here, in an attempt to adhere to a consistent, yet admittedly incomplete, nomenclature, we use the term ‘stromal cell’ to refer to all non-haematopoietic cells (generally defined as CD45−Ter119− or CD45−Lin− cells) in the BM. Stromal cells will therefore include MSCs, perivascular and endothelial cells, osteoblasts, adipocytes, chondrocytes, non-myelinating Schwann cells, and other cell populations of these lineages yet to be defined (Figure 3). We refer to cells that have committed to the osteoblast lineage as OBCs, inclusively referring to osteoprogenitors as well as mature osteoblasts (Marie, 2003; Stein et al., 2004). We also use the term MSPC, as in Frenette et al. 2013, for cell populations believed to contain MSPCs, but not always well characterized or consistently referred to (for a recent and comprehensive review on mesenchymal cells and their role in HSC regulation, see Frenette et al., 2013).

Considering the high complexity of the BM microenvironment, we believe that it will be productive to establish the developmental hierarchy of different types of PVCs and other BM stromal cells. A recent study using genetic lineage tracing for splenic stromal cells identified a common, multipotent precursor of different types of spleen stromal subsets capable of initiating the formation of an artificial lymphoid structure upon transplantation (Castagnaro et al., 2013). It will also be important to establish protocols capable of dissociating viable cells without destruction of epitopes that define these cells in situ. GFP-driven transgenes can be a productive approach, but additional strategies that are less time consuming and labour intensive, and more amenable to the multi-marker cell labelling that will likely be necessary to understand the complexity of HSC niches, would be extremely valuable.

**Secreted factors may affect HSC function by both direct and indirect mechanisms**

The recent reports by Ding et al. and Greenbaum et al. confirmed the previously established roles for cKit and CXCR4 as essential regulators of HSC function. However, HSCs are not

![Figure 3](image-url)
the only cell type responding to SCF and CXCL12, and it is therefore possible that indirect effects, by these ligands acting on niche cells, also play important roles in HSC maintenance (Figure 4).

Germline deletion of either CXCL12 or CXCR4 results in severely impaired BM haematopoiesis and late embryonic lethality (Nagasawa et al., 1996; Zou et al., 1998; Tachibana et al., 1998). The similar phenotypes of CXCL12 and CXCR4 deletions have contributed to the prevailing notion that CXCL12 serves as the main ligand for CXCR4, and it is clear that HSCs directly respond to CXCL12 by expression of CXCR4 (Peled et al., 1999; Smith-Berdan et al., 2011; Wright et al., 2002). Similarly, the rapid mobilization of HSCs from BM to blood upon in vivo administration of either anti-ckit antibodies (Czechowicz et al., 2007) or CXCR4 inhibitors such as AMD3100 (Brommeyer et al., 2005; De Clercq, 2010; Smith-Berdan et al., 2011) provides convincing evidence that ckit and CXCR4 function directly in HSC retention to BM niches. However, several lines of evidence suggest that SCF/ckit and CXCL12/CXCR4 interactions on other cells affect HSC function indirectly. For example, two models of conditional CXCR4 deletion in adulthood, one in all cell types (Rosa26-CreER; Nie et al., 2008) and the other selectively (but not exclusively; Park et al., 2012) in haematopoietic cells (Mx1-Cre; Sugiyama et al., 2006; Nie et al., 2008) resulted in different effects on HSC numbers. In addition, Sugiyama et al. (2006) reported that, surprisingly, HSC location next to CXCL12-expressing cells did not depend on CXCL12 expression by HSCs. Lastly, global CreER-mediated deletion of CXCL12 in adulthood appeared to be more severe than the analogous deletion of CXCR4 (Sugiyama et al., 2006; Nie et al., 2008). The extent to which other surface receptors offset the loss of CXCR4 to ensure proper HSC function is not clear (Forsberg and Smith-Berdan, 2009), but such intrinsic compensatory mechanisms may be more efficient than the ability to compensate for the loss of a ligand secreted by neighbouring cells. It is also possible that CXCL12 acts on non-haematopoietic cells expressing CXCR4, and that these stromal cells are important for HSC function (Figure 4B). Indeed, there is evidence for expression and function of the CXCR4 receptor by BM stromal cells. Importantly, CXCR4 deletion from osteoprogenitors, using an Osx-Cre transgene, resulted in impaired osteoblast differentiation and proliferation, accompanied by altered bone formation and extracellular matrix composition (Zhu et al., 2011). CXCR4 was also shown to be essential for vascular endothelial cell development, as mice lacking CXCR4 have defective vessel formation, in addition to impaired haematopoiesis (Tachibana et al., 1998). Thus, deletion of CXCL12 may strongly influence the function of osteoblasts or endothelial cells, including their ability to support HSCs.

To resolve these issues, it will be important to determine which stromal cells express the CXCR4 and ckit receptors and whether these cells are affected, directly or indirectly, by deletion of SCF or CXCL12 (Figure 4). While Ding et al. (2012) demonstrated that the frequency of SCF-GFP BM cells was normal in Lepr-Cre:SCF<sup>fl/gfp</sup> mice, it is possible that the organization or expression profile of SCF-expressing cells is altered upon SCF deletion. By capitalizing on the technical and conceptual approaches used by Nombela-Arrieta et al. and Wang et al., microvessel organization and hemispheric integrity can be investigated upon deletion of SCF and CXCL12. Determining the consequences of receptor deletion in specific stromal cell types, as already done for CXCR4 in osteoblasts in the report mentioned above (Zhu et al., 2011), and the potential impact on HSC maintenance will likely yield important insights into the crosstalk between different types of BM cells. Additionally, time courses in conditional deletion models may reveal whether one or more types of putative niche cells are affected prior to reduction in HSC numbers or function. While the issues at hand are complex, the comprehensive analyses of cell type-specific deletion of SCF and CXCL12 by Ding and Greenbaum demonstrated that our capability to systematically investigate HSC regulation is rapidly expanding.

**Figure 4** CXCL12 may affect HSC function by both direct and indirect mechanisms. In (A), the primary function of CXCL12 (yellow triangles) is to support HSC function by acting directly on HSCs. In (B), CXCL12 also affects HSC function by indirect mechanisms, by acting on CXCR4-expressing niche cells (effect number 1). This action of CXCL12 may function to reinforce an HSC-supportive environment by either cellular mechanisms, such as niche cell function or organization, and/or by molecular mechanisms, by triggering release of additional HSC-supporting factors, such as SCF or angiopeptin (blue circles), from niche cells (effect number 2).
Genetic deletions in stem and progenitor cells are inherited by their progeny

Another type of indirect effect comes from the fact that genetic-deletion strategies initiated in stem and progenitor cells eventually affect the progeny of the targeted cells (Figure 5). Even if Cre expression is restricted to the stem cell, their descendants will inherit the deleted alleles and, over time, cease to express the targeted factor. Factor deletion in a stem cell is therefore expected to result in stronger effects than its deletion in a specific differentiated cell type. This poses important considerations for concluding which cell types support HSCs by direct interaction.

Considering the potential MSPC identity of PVCs, deletion of a particular factor in PVCs may not only lead to direct effects on PVC-interacting HSCs, but also to indirect effects from factor deletion in downstream osteoblasts, adipocytes, or chondrocytes. For example, Nestin-Cre-mediated deletion of CXCL12 specifically from MSPCs capable of differentiating into OBCs will first result in reduced CXCL12 secretion from MSPCs themselves, but later also in reduced CXCL12 production by their OBC descendants. These considerations are particularly important when the targeted factor is important in more than one cell population within a lineage. As demonstrated by Ding and Morrison (2013) and Greenbaum et al. (2013), CXCL12 expression by PVCs, but not by OBCs, was important for HSC maintenance, whereas CXCL12 expression by OBCs supported lymphoid progenitors. Interestingly, lineage tracing experiments suggested that Lepr-expressing cells did not give rise to OBCs despite their expression of MSPC markers (Ding et al., 2012) and significant overlap with other MSPC populations (Hanoun and Frenette, 2013). Deletion of CXCL12 using Lepr-Cre should therefore leave CXCL12 expression by OBCs intact. In contrast, Prx1-expressing cells are enriched for MSPCs with in vitro osteolineage potential (Greenbaum et al., 2013; Figure 5). Thus, Prx1-Cre-mediated deletion of CXCL12 should also affect CXCL12 production by OBCs. These results also indicate that although both Prx1- and Lepr-expressing cells are MSC-like PVCs, they have distinct differentiation potential.

These examples stress the importance of understanding the lineage relationships between BM stromal cells. Mapping the overlap and differentiation potential of different MSPC populations promise to be particularly productive, given their purported importance in HSC maintenance (Frenette et al., 2013). Such studies will hopefully include measures of cellular half-life to better understand how rapidly MSPC-derived progeny is replaced by progenitor differentiation. In germline deletion models, the targeted factor will be absent from all cells at all times. However, in inducible deletion models, the effect of factor deletion is closely tied to the in vivo turnover and replacement rate of cells expressing the factor. This highlights the importance of the time frame of gene deletion relative to analysis of effects on HSCs. Combined with accurate estimates of the MSPC turnover rate in vivo, such time courses will provide essential information for distinguishing cells that provide direct versus indirect support of HSC function.

Localized concentration of secreted factors in HSC maintenance

A variable not yet addressed directly is whether gradients of factor concentration are established to create HSC niches. While selective deletion of SCF and CXCL12 from different cell types provided strong evidence that cell type-specific expression matters, the extent of factor gradients has important implications for what conclusions can be drawn with regard to HSC localization relative to these cells.

Highly diffusible factors may be relatively evenly distributed in the BM, even if selectively expressed by only a subset of cell types. Diffusible molecules are also more likely to be provided by other cell types expressing the factor, and

Figure 5  Sequential effect of gene deletion in stem and progenitor cells. This cartoon depicts a hypothetical scenario where a secreted factor is normally expressed by both a stem/progenitor cell and at least one of its more differentiated progeny, and the effects of stem/progenitor-specific genetic deletion of the gene for this factor. (A) Prior to gene deletion, the factor is secreted by both stem/progenitor and progeny cells. (B) Soon after gene deletion, depicted here as activation of Cre recombinase specifically in stem/progenitor cells, progeny, but not stem/progenitors, will produce the factor. (C) Over time, as factor-deficient stem/progenitor cells differentiate and replenish the pool of progeny, the factor will also be missing from an increasing proportion of progeny. For example, Cre-mediated deletion of CXCL12 specifically from mesenchymal stem cells capable of differentiating into osteoblasts will first result in reduced CXCL12 secretion from MSC themselves (B), but later also in reduced CXCL12 production by their osteoblast descendants (C). This highlights the importance of the time frame of gene deletion relative to analysis of effects on HSCs. Inducible Cre models are capable of temporally more cell-specific deletion than constitutive deletion models. However, the effects of factor deletion depend on the expression pattern and levels of the factor in question, the relative cell number and expression levels in stem/progenitors versus more mature cells, and the in vivo turnover and replacement rate of progeny.
the expression level of the factor in different cell types will influence the effect of cell type-specific deletion. For example, if endothelial cells are responsible for the vast amount of CXCL12, whereas OBCs express relatively little, then CXCL12 deletion from endothelial cells is expected to have a greater effect than deletion in OBCs (Figure 6A–C). Thus, effects of cell type-specific deletion on HSC function do not prove interaction or proximity with HSCs, and redundant factor expression may mask the effect of deletion on another cell type.

In contrast, if a factor remains close to the cell producing it, maybe by association with the surrounding matrix or with membrane molecules of the host cell, then localized factor gradients are likely established (Figure 6D). In the gradient model, cell type-specific deletion may have dramatic effects on the local environment without affecting the overall concentration of the factor in the BM. Redundancy in factor expression among different cell types is less likely to play a role in this scenario, unless the different cell types expressing the factor are localized near each other.

Because of the difficulties in measuring the localization of secreted proteins in vivo, in particular in tissues such as the BM, direct evidence for how CXCL12, SCF, and other factors are distributed relative to the cells producing these factors is scarce. Importantly, SCF has been reported to exist in both soluble and membrane bound forms (Anderson et al, 1991; Broudy, 1997) while all described splice variants of CXCL12 are secreted as very small (<10 kDa), soluble molecules. Previous results indicated that membrane-bound SCF is important for haematopoiesis (Wolf, 1978), while the role and distribution of soluble SCF is less clear. Whether the cell types investigated by Ding et al (2012) express soluble and/or transmembrane variants of SCF was not reported. The lack of demonstration of factor gradients and the possibility of the indirect effects discussed above preclude direct evidence for HSCs residing in areas enriched for certain factors. Instead, the location of HSCs near factor-secreting cells has mainly been inferred from a combination of indirect measures. Several variables need to be considered in making valid conclusions on HSC localization in factor-rich zones, including the number and frequency of the cell type(s) expressing the factor; the level of expression of the factor in these different cell types; their proximity to other cells that may respond directly to the factor and therefore indirectly affect HSC function; the relative location of different niche cells expressing the same factor; the specificity and efficiency of the Cre-mediated deletion of the factor; and whether differential effects on different cell types can be detected.

Figure 6 Gradients and sources of soluble factors for HSC maintenance. (A–C) Effect of factor deletion on different cell types. In (A), HSC (red cell) maintenance is supported by CXCL12 expression by both osteoblasts (green cells) and (peri)vascular cells (PVCs; pink and purple cells). (B) CXCL12 deletion in OBCs fails to affect HSC numbers, as threshold levels of CXCL12 are maintained by the high CXCL12 expression by PVCs. (C) Deletion of CXCL12 in PVCs leads to overall lower levels of CXCL12. The relatively low CXCL12 expression by OBCs is insufficient to maintain threshold levels of CXCL12. The net result is a decrease in HSC numbers, either by reduced self-renewing and increased differentiating divisions (indicated by changes in arrow thickness) or by mobilization to the periphery. Thus, differential effects of cell type-specific factor deletion may reflect differences in expression levels or abundance of the expressing cell type, and does not, in itself, indicate the location of HSCs relative to a specific niche cell type. (D) Factor gradient established locally around high-expressing stromal cells. In this model, the high Cxcl12 expression in PVCs results in a high local concentration of Cxcl12 (yellow triangles), and therefore HSCs, close to PVCs. (E) Competition for CXCL12-abundant niches between HSCs and other CXCR4-expressing cells. In this model, HSCs are actively migrating towards a CXCL12 gradient, established by CXCL12-expressing BM niche cells. It is unclear how HSCs outcompete more abundant cells that express higher levels of CXCR4 and migrate with greater efficiency towards CXCL12, such as B cells (Smith-Berdan et al, 2011), for access to limited numbers of niches.
Impressively, and a major reason for their substantial impact on understanding both the cellular and the molecular regulation of HSC maintenance, the Ding and Greenbaum reports accounted for many of these variables. Expression levels of CXCL12 and SCF in different cell types were compared; factors were deleted in many different cell types and the resulting effects on the overall levels of CXCL12 protein were measured; floxing specificity and efficiency was assessed by analysis of reporter activity; and BM sectioning localized most HSCs immediately adjacent to SCF-expressing cells (Ding et al., 2012; Ding and Morrison, 2013; Greenbaum et al., 2013). Maybe most convincingly, CXCL12 deletion in different stromal cell types had distinct effects on HSC and CLP numbers and location. Collectively, these stringent and comprehensive analyses provided a convincing case for vascular and perivascular regulation, via SCF and CXCL12 production, of HSC function. While these studies raise the bar for analogous investigation of additional factors, they also lower the threshold by providing the conceptual framework and established tools. Additional characterization of stromal cell types, their expression profiles, their location relative to each other and to HSCs, and whether their numbers or organization are affected by deletion of specific factors will provide an increasingly comprehensive understanding of HSC niches. The hemosphere model provides a plausible opportunity to test the concept of factor gradients (Wang et al., 2012). As both endothelial cells and PVCs express CXCL12, hemospheres may establish and maintain high local concentrations of CXCL12. HSCs sandwiched between these layers would therefore be exposed to high levels of factors important for HSC maintenance (Figure 2).

**How do HSCs gain competitive access to limited niche space?**

Increases or decreases in BM stromal cell numbers can lead to the corresponding changes in HSC frequencies in the BM, suggesting that the number of sites capable of supporting HSCs depends on the number of niche cells (Kiel and Krause et al., 2013). Likewise, some (Czechowicz et al., 2007; Bhattacharya et al., 2009) though not all (Westerhuis et al., 2011) transplantation experiments suggest that niche space limits the number of HSCs in the BM, but the mechanisms behind potentially limited niche space are not clear.

One possibility is that many types of niches exist, each designed to support a specific cell type (Figure 7A). In this ‘specialized niche’ model, the number of niches capable of supporting HSCs is limited. Different types of progenitor cells have their own types of niches that provide cues necessary for progenitor function, but not HSC self-renewal. The differential effects on HSCs and lymphoid progenitors when CXCL12 is deleted in different stromal cell types support this model and suggest that HSCs and CLPs occupy niches that have a different cellular composition, although both types share CXCL12 expression as a molecular feature.

An alternative possibility is that there are many more niches than HSCs, but that HSC access to these niches is limited by competition with other cells capable of thriving in the same environment (Figure 7B). These ‘equivalent niches’ can support either HSCs or progenitor cells. An attractive aspect of the ‘equivalent niches’ model is that it helps envision how HSCs respond to cues that act on progenitor cells, but not on HSC directly, such as G-CSF, irradiation, or chemotherapy. These clinically relevant treatments primarily affect highly proliferating progenitor cells, as opposed to the relatively quiescent HSCs. While it is clear that HSCs eventually do respond to maintain homeostasis, the mechanisms inducing this response are unclear. In the ‘equivalent niche’ model, a decrease in progenitor cell numbers (by death or mobilization to the blood) would lead to vacant niches, allowing HSC expansion and subsequent replacement of the vanishing progenitor cells.

Regardless of whether the equivalent or specialized niche model is correct, CXCL12 and SCF are clearly important for HSC maintenance. However, while it has been demonstrated that HSCs express the corresponding receptors and directly respond to manipulation of both ligand/receptor interactions, neither axis alone nor the combination of the two is specific for HSCs. Both the ckit and CXCR4 receptor are widely expressed by haematopoietic BM cells that vastly outnumber HSCs. For example, compared to HSCs, BM B cells are ~300-fold more frequent, express higher levels of CXCR4, and migrate with higher efficiency towards CXCL12 (Smith-Berdan et al., 2011). Why are the CXCL12-enriched niches not filled with B cells (Figure 6E)? One possibility is that HSC specificity is accomplished by combinatorial cues, according to our previously proposed ‘niche code hypothesis’ (Forsberg and Smith-Berdan, 2009). In this model, HSCs gain competitive access to niche by expressing a combination of additional homing and/or adhesion molecules that direct them to and promote retention in specific sites. Alternately, B cells (and other cells expressing CXCR4) may be lured to alternative niches by chemokines that do not attract HSCs. Such factors may be provided by OBCs, thereby creating the distinct lymphoid niches described by Ding and Morrison (2013) and Greenbaum et al. (2013).

As the picture of combinatorial cues that regulate differential cell localization becomes clearer, by methods now...
established by Ding and Greenbaum, we will better understand the complex regulation of HSC maintenance. Cellular and molecular characterization of cells essential for HSC function should allow us to classify and then quantitate the number of different types of niches. Comparison to the known number of functional HSCs will establish the HSC:niche cell ratio and how this balance changes under different conditions (further discussed below). At steady state, cells expressing nestin (Méndez-Ferrer et al, 2010) or SCF (Ding et al, 2012) are very rare, and likely more limiting in providing HSC support than the more numerous CXCL12-expressing cells. Determining whether the expression levels in and frequency of Nestin + and SCF + cells increase in response to haematopoietic stress, as observed for CXCL12 (Smith-Berdan et al, 2012) should be straightforward with the established reporter mice and important for understanding how niche cell numbers change in dynamic situations. As an example, one recent study demonstrated how the daily clearance of aged neutrophils in the BM generates signals that affect the frequency of CAR cells and thereby the size and function of BM niches (Casanova-Acebes et al, 2013).

**Multitasking by molecular and cellular regulators of HSC retention and trafficking**

A complicating issue in understanding HSC niches is that HSCs are capable of trafficking into and out of these sites. HSC migration is essential for transplantation therapies, transition between haematopoietic organs during development, and likely also important for maintaining homeostasis in adult life (Wright et al, 2002; Christensen et al, 2004; Massberg et al, 2007). Some molecules and cell types seem to play several different roles during HSC trafficking. CXCL12, for example, has been reported to act as an HSC attractant (Wright et al, 2002), mediate HSC adhesion (Broxmeyer et al, 2005; Smith-Berdan et al, 2011), maintain HSC quiescence (Nie et al, 2008), and promote HSC self-renewal (Sugiyama et al, 2006).

While the Ding and Greenbaum reports define the cell types required for CXCL12-mediated regulation of HSC location, their findings also open up new questions. Maybe the most puzzling finding is that LeprCre-mediated deletion of CXCL12 led to increased numbers of HSCs in the blood and spleen, without changes in HSC numbers or frequencies in the BM (Ding and Morrison, 2013). Consistent with previous reports (Broxmeyer et al, 2005; Smith-Berdan et al, 2011), this indicates that CXCL12 is important for HSC retention in the BM, but the net increase in total HSC numbers also reflects HSC expansion. Whether increased HSC self-renewal in this model occurs within their normal niches in the BM or elsewhere has not yet been investigated. One possibility is that CXCL12 mediates HSC quiescence, and that its deletion leads to increased self-renewing division, saturation of limited numbers of BM niches, and subsequent ‘spill over’ into blood and spleen. The HSC cell-cycle status was not investigated in the LeprCre model, but the increased proliferation of HSCs in a model with overlapping Cre specificity (Pnx1Cre; Greenbaum et al, 2013) provides support for this idea. Additionally, retention in niches may itself maintain relative quiescence and, when cycling does occur, promote self-renewing over differentiating divisions. Thus, the many functions ascribed to CXCL12 may be thought of more simply as attraction to specific locations. Stable retention and self-renewal clearly require additional factors, including SCF, whose more selective expression therefore defines niches capable of supporting life-long HSC persistence from those that cannot.

Like secreted proteins, specific cell types may also regulate HSCs by more than one mechanism. Rapidly accumulating evidence has convincingly demonstrated that vascular and perivascular cells play important roles within the BM environment to maintain HSCs. In addition to molecular retention, one possible mechanism is that vascular structures form physical barriers that restrict HSC movement. During trafficking to and from the blood stream, HSCs cross vessel walls and must therefore interact with vascular cells (Kollet et al, 2007). While the majority of the vasculature can be viewed as restrictive barriers, the specialized cellular organization of microvascular sinusoids in the BM is likely responsible for the ability of HSCs and progenitor cells to traffic between blood and marrow compartments. The regulation of HSC transendothelial migration has not been investigated, but it is conceivable that some of the molecules that promote HSC retention do so by regulating vascular permeability. Borrowing the imaging technologies pioneered by Nombela-Arrieta et al, the concept of hemospheres from Wang et al, and strategies from the leukocyte trafficking field will likely be productive approaches to further understand how HSCs interact with vascular cells.

**Niche-independent HSC maintenance**

Although BM niches are essential for long-term HSC self-renewal capability, there are several instances of niche-independent HSC maintenance, at least over limited time frames. Examples include the fetal liver during development, disease-induced extramedullary haematopoiesis, and G-CSF-stimulated expansion and mobilization (Kim, 2010). Similarly, Ding and Morrison (2013) found that deletion of CXCL12 in Lepr-expressing cells led to increased numbers of HSC in the periphery without changes in numbers of HSC in the BM. It is unclear whether elevated levels of HSCs persist over time in this model, and what the fate of these HSCs is in the long term. In other models, HSCs found in ectopic sites or circulating in the blood stream are capable of reseeding BM niches and engraft recipients upon transplantation (Abkwitz et al, 2003; Wright et al, 2002). However, as extramedullary HSCs may be continually replenished by trafficking of HSCs generated in the BM, it is unclear whether HSCs are capable of self-renewal, or just maintenance, outside of BM niches. This is a topic of intense interest due to the difficulties in deriving efficiently engrafting HSCs from pluripotent cells or by in vitro expansion (Wang et al, 2005; Clarke et al, 2013; Pereira et al, 2013; Isern et al, 2013).

Because fetal haematopoiesis appears to be normal in both CXCL12- and CXCR4-deficient mice (Zou et al, 1998; Nagasawa et al, 1996), it seems that this axis is dispensable for HSC maintenance prior to adulthood. However, it is not known whether the fetal liver environment is capable of supporting self-renewal in the long term or just the rapid expansion that occurs during development. It is interesting to note that several secreted factors, including CXCL12, are upregulated upon irradiation, G-CSF treatment, and other haematopoietic stresses (Semerad et al, 2005; Smith-Berdan et al, 2012). While there is evidence that irradiation leads to increased CXCL12
expression level per cell (Smith-Berdan et al., 2012), it is also possible that the number of cells expressing HSC-supporting factors increase in other cases. Thus, to understand whether increased niche space is essential for HSC expansion, it would be important to quantify the number of HSC-supporting niches in response to G-CSF-induced HSC self-renewal and in the LeprCre-mediated CXCL12 model. Conversely, is the number of HSC niches decreased in models where HSC maintenance is impaired? The timing of niche space expansion or contraction relative to changes in HSC numbers will provide important clues on cause and effect. Similarly, if hemospheres or similar entities regulate HSC maintenance, then it will be imperative to determine how the number, integrity, composition, and function of these sites change upon haematopoietic stress and under disease conditions.

While it seems daunting to take on the molecular regulation of highly dynamic situations when we have not yet comprehended the complexities of steady-state HSC maintenance, comparing different conditions may be both productive and essential for continued progress. Shared features, both cellular and molecular, between different situations of HSC maintenance should reveal what factors are required and which ones are dispensable. Given that the levels and roles of factors may change in different contexts, and that compensatory mechanisms may offset the impact of perturbations, this is indeed a challenging endeavour. However, as haematopoietic disease, by definition, does not occur under steady-state conditions, understanding HSC regulation under highly dynamic situations will be necessary for efficient clinical intervention.

Inspiration for future endeavours

The recent papers that serve as the bases for this review demonstrate that exciting and novel discoveries can be made, both conceptually and technically, despite the many challenges facing niche-mediated regulation of HSC function. Throughout this review, we have provided ideas for how the field can continue to move forward at a rapid pace. Important experiments range from simple characterization of BM cells, to approaches that will require development of novel tools, techniques, and innovative experimental design to allow increased resolution, sensitivity, and multiparameter measurements. Low-hanging fruit includes rigorous quantification of different stromal cell types and determining which ones express CXCR4, ckit, and other receptors responding to niche cues. Other important experiments are more complex, but clearly feasible with current tools and protocols. The cell type-specific Cre recombinase models established by Ding, Greenbaum, and others (Tables I and II) will no doubt be employed to several additional molecules proposed to regulate HSCs. These Cre/lox models can also clarify lineage relationships and turnover rates of different BM cell types.

In terms of new technologies, we see great value in further development of methods capable of measuring the three-dimensional architecture of vascular structures and the BM architecture, as by Nombela-Arrieta et al., and to employ these approaches to genetic-deletion models and conditions of haematopoietic stress. Highly sensitive approaches will be necessary to define protein gradients and the location of HSCs relative to such zones. Many conceptual and technical hurdles are shared with other organ systems, but the migratory nature of haematopoietic cells also poses some challenges unique to haematopoiesis. Clever strategies to track in vivo migration of cells have the potential to solve longstanding conundrums, with direct payoff towards stem-cell transplantation therapies.

Collectively, the recent findings reviewed here made major strides towards understanding extrinsic regulation of HSCs, with strong support for vascular regulation of HSC maintenance. It is important, however, to also consider the significant evidence for other cell types in HSC function, including adipocytes and OBCs (Naveiras et al., 2009; Krause et al., 2013). One possibility is that OBCs support HSCs indirectly by producing angiopoietin and other factors that act on vascular cells which, in turn, directly interact with HSCs. Cooperation of BM cells to create one unique HSC niche has not yet been distinguished from the possibility of different niches with different functions, but the notion that HSCs are regulated by a combination of complex factors is steadily growing.

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Conflict of interest

The authors declare that they have no conflict of interest.

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