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Cellular complexity of the bone marrow hematopoietic stem cell niche

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Abstract

The skeleton serves as the principal site for hematopoiesis in adult terrestrial vertebrates. The function of the hematopoietic system is to maintain homeostatic levels of all circulating blood cells, including myeloid cells, lymphoid cells, red blood cells, and platelets. This action requires the daily production of more than 500 billion blood cells every day. The vast majority of these cells are synthesized in the bone marrow, where they arise from a limited number of hematopoietic stem cells (HSCs) that are multipotent and capable of extensive self-renewal. These attributes of HSCs are best demonstrated by marrow transplantation, where even a single HSC can repopulate the entire hematopoietic system. HSCs are therefore adult stem cells capable of multilineage repopulation, poised between cell fate choices, which include quiescence, self-renewal, differentiation and apoptosis. While HSC fate choices are in part determined by multiple stochastic fluctuations of cell autonomous processes, according to the niche hypothesis, signals from the microenvironment are also likely to determine stem cell fate. While it had long been postulated that signals within the bone marrow could provide regulation of hematopoietic cells, it is only in the past decade that advances in flow cytometry and genetic models have allowed for a deeper understanding of microenvironmental regulation of HSCs. In this review, we will highlight the cellular regulatory components of the HSC niche.

Anatomic distribution of cell types in the bone marrow

In all vertebrates except fish, in which hematopoiesis occurs in the kidney, the bone marrow is the hematopoietic organ (Hartenstein 2006). The skeleton contains all cells of the osteolineage cells, from mesenchymal stem cells (MSCs) (also called skeletal stem cells (Bianco, Robey et al. 2010)), to chondrocytes, osteoprogenitors, osteoblasts and osteocytes. Osteoblasts form a layer, the endosteum, at the interface between the mineralized bone and the bone marrow contained within its center. At these endosteal sites, a population of F4/80+ macrophages (osteomacs) forms a canopy over mature osteoblasts at sites of bone formation (Chang, Raggatt et al. 2008). Arteriolar vessels, capillaries, and endothelium-bound venous sinuses branch throughout the bone marrow. Endothelial cells, macrophages, osteolineage, and stromal (also called reticular) cells that crisscross the space between vessels and endosteum form a three-dimensional scaffold that supports clusters of blood-forming cells as well as marrow adipose tissue (Fazeli, Horowitz et al. 2013), providing the complex marrow microenvironment that regulates hematopoiesis (Hartenstein 2006). HSC-derived cells that lose contact with their niche cells progress toward more differentiated stages, becoming

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committed progenitors and then precursors for lymphoid cells, red blood cells, thrombocytes, granulocyte/monocytes and granulocytes. These differentiating HSC progeny cells are then found nearer the center of the bone marrow, where they proliferate and form growing colonies of maturing blood cells. Once matured, blood cells cross the endothelium into the bloodstream. Immature lymphoid progenitors leave the bone marrow to populate the thymus and lymphoid organs, where they further differentiate (Hartenstein 2006).

The anatomic localization of HSCs in the bone marrow is controversial. Initial studies using transplanted labeled HSC-enriched cell populations suggested that HSCs preferentially localize to endosteal regions (Zhang, Niu et al. 2003; Wilson, Murphy et al. 2004; Xie, Yin et al. 2009). In contrast, in situ localization of HSCs using SLAM markers (CD150⁺ CD48⁻ CD41⁻ lineage⁻), suggests that the majority of HSCs are in contact with sinusoidal endothelium at bone-distant sites (Kiel, Yilmaz et al. 2005). HSCs are in direct contact with perivascular CXCL12-abundant reticular (CAR) cells (Sugiyama, Kohara et al. 2006) and nestin-GFP⁺ stromal cells (Mendez-Ferrer, Michurina et al. 2010), providing further support for a perivascular HSC localization. High resolution three-dimensional imaging of the vasculature in murine long bones provides a potential explanation for these divergent observations (Nombela-Arrieta, Pivarnik et al. 2013). Specifically, the endosteal region is highly vascular and most phenotypic HSCs are perivascular, whether localized to the endosteum or bone-distant sites.

The concept of the niche

HSC fate choices are determined in part by multiple stochastic fluctuations of cell autonomous processes (Cantor and Orkin 2001; Enver, Pera et al. 2009; Graf and Enver 2009). In addition, according to the niche hypothesis, signals from the microenvironment are also likely to determine stem cell fate. Schofield (Schofield 1978), in response to observations on the hematopoietic system, first proposed the concept of the niche, where specific niche cells establish close interactions with immature cells which can enforce stem cell behavior. This idea was supported by initial anatomical studies which demonstrated hierarchical distribution of hematopoietic cells in marrow cavities (Lord, Testa et al. 1975; Gong 1978). This anatomical compartmentalization is highlighted by more recent homing studies demonstrating the localization of labeled immature cells to endosteal sites, both postmortem and intravitaly (Nilsson, Dooner et al. 1997; Nilsson, Johnston et al. 2001; Lo Celso, Fleming et al. 2009; Xie, Yin et al. 2009). However, in spite of the instructive role of hematopoietic development in the inception of the niche hypothesis, it would take a quarter century before the role of the niche in HSC regulation could be proven in vivo, partially due to the complexity of the marrow microenvironment, which we outline below. Instead niche stem cell interactions would first be demonstrated in the drosophila gonad (Kai and Spradling 2003), and found to be important for adult stem cells in skin, brain, intestine as well as eventually bone marrow (Fuchs, Tumber et al. 2004; Losick, Morris et al. 2011).

Developmental migration of HSCs and homing to intramedullary sites

During development, HSCs are first identified in the aorta-gonad-mesonephron region (Medvinsky and Dzierzak 1996; Dzierzak and Speck 2008). These cells then migrate to the fetal liver and to fetal bone (Dzierzak 1999). Skeletal sites remain the sole physiologic regions of active hematopoiesis throughout adult life. At these sites, the enormous hematopoietic activity initiated by the HSCs results in the daily production of more than 500 billion blood cells every day (Fliedner 2002). The vast majority of these hematopoietic progeny cells is synthesized in the bone marrow, and arises from a limited number (1–5/10⁵ marrow cells) of HSCs. Proof-of-principle experiments have demonstrated that even a single HSC can repopulate the entire hematopoietic system (Osawa, Hanada et al. 1996). Direct

visualization suggests that immature hematopoietic cells are found in relatively fixed positions in the marrow (Suzuki, Ohneda et al. 2006; Lo Celso, Fleming et al. 2009). In addition, a subpopulation of HSCs is continuously mobilized through the circulation (Storb, Graham et al. 1977; Wright, Wagers et al. 2001; Wagers, Sherwood et al. 2002). Interestingly, cell autonomous motility contributes to egress of HSCs from the microenvironment (Lapid, Itkin et al. 2013). HSC mobilization is also dependent on fluctuating levels of microenvironmental CXCL12 (Katayama, Battista et al. 2006) and is regulated by circadian rhythms (Mendez-Ferrer, Lucas et al. 2008), at least in part enforced centrally through the sympathetic nervous system (SNS). The ability of HSCs to be mobilized and to home to the marrow through the action of the chemokine CXCL12 and its receptor CXCR4 is fundamental for the therapeutic use of HSCs for bone marrow transplantation. Therefore, as we will review, one of the research strategies to identify niche components has focused on cell populations capable of secreting CXCL12.

Non-hematopoietic HSC niche components

Osteolineage cells

Early anatomic studies elucidated the endosteal distribution of HSCs (Lord, Testa et al. 1975; Gong 1978). In addition, in vivo, long term HSCs tightly adhere to endosteal matrix (Haylock, Williams et al. 2007). Based on this localization of HSCs, cells of the osteoblastic lineage (to which we will refer to throughout this review as osteolineage cells) were the first cellular marrow component considered as the critical HSC niche constituent. In vitro, osteoblastic cells support HSC activity (Taichman and Emerson 1994; Taichman, Reilly et al. 1996). Loss of osteolineage cells disrupts hematopoiesis (Visnjic, Kalajzic et al. 2001; Visnjic, Kalajzic et al. 2004). Moreover, osteolineage cells produce many factors important for hematopoiesis (Marusic, Kalinowski et al. 1993; Taichman and Emerson 1994; Calvi, Adams et al. 2003), both secreted as well as cell bound (Taichman, Reilly et al. 1996; Arai, Hirao et al. 2004; Weber, Forsythe et al. 2006; Jung, Wang et al. 2007; Qian, Buza-Vidas et al. 2007; Yoshihara, Arai et al. 2007). Our laboratory and others provided initial in vivo evidence of phenotypic and functional HSC expansion through targeted activation of the osteoblastic cell lineage (Calvi, Adams et al. 2003; Adams, Martin et al. 2007; Mendez-Ferrer, Michurina et al. 2010; Bromberg, Frisch et al. 2012). In these murine models, osteolineage cells are activated either genetically, in a transgenic mouse model of constitutively active parathyroid hormone (PTH) signaling driven by the 2.3 kilobases fragment of the $\alpha 1(I)$ collagen gene promoter (Calvi, Sims et al. 2001), or by intermittent systemic administration of PTH(1–34) (Calvi, Adams et al. 2003; Bromberg, Frisch et al. 2012). Intermittent PTH treatment is bone anabolic (reviewed in (Goltzman 2008)). Consistent with a PTH-dependent effect on HSCs, patients with parathyroid adenomas and increased PTH levels have increased circulation of phenotypically-defined HSCs and endothelial progenitor cells (Ballen, Shpall et al. 2007; Brunner, Theiss et al. 2007). Simultaneously, Zhang et al. found that conditional inactivation of Bone Morphogenic Protein receptor 1A resulted in greater trabecular bone and expansion of osteolineage cells, with a similar increase in HSCs (Zhang, Niu et al. 2003). However, general expansion of osteoblastic cells is not sufficient to increase HSCs (Lymperi, Horwood et al. 2008). This observation is also supported by data on a murine model of fibrous dysplasia characterized by massive increases in trabecular bone which demonstrated reduced HSCs, lineage-specific defects in megakaryocyte and erythrocyte development and impaired hematopoietic recovery from myeloablative injury (Schepers, Hsiao et al. 2012). On the other hand, general disruption of osteolineage cells in a murine model of inflammatory arthritis is not sufficient to impair HSCs (Ma, Park et al. 2009). These data suggest that specific stages of osteoblastic differentiation may provide different HSC support and that multiple osteolineage cell populations are likely to play different roles in hematopoiesis. This concept is further supported by data suggesting that, while multiple

osteolineage subsets supported HSC long term reconstitution activity, some subsets mostly support HSC adhesion and homing, while others produce pro-HSC cytokines (Nakamura, Arai et al. 2010).

Multiple laboratories have contributed to an increased understanding of the diverse roles of osteolineage heterogeneity in support of HSCs by the microenvironment (Chitteti, Cheng et al. 2010; Chitteti, Cheng et al. 2010; Chitteti, Cheng et al. 2010; Nakamura, Arai et al. 2010; Cheng, Chitteti et al. 2011). The concept began developing that immature osteolineage cells are critical to HSC regulation. For example, data suggest that expression of Runx2, a transcription factor most highly expressed at early stages of osteoblast differentiation, appears to identify an osteoblastic population with greater HSC maintenance and enhancement potential (Chitteti, Cheng et al. 2010; Cheng, Chitteti et al. 2011). Conversely, when terminally differentiated osteoblastic cells or osteocytes are activated by constitutive PTH signaling, HSC frequency and function are unchanged, in spite of increased osteoblastic cells and trabecular bone (Calvi, Bromberg et al. 2012). In fact, agents that stimulate osteoblastic differentiation may be detrimental to HSC retention at the endosteum as evidenced by a shift in HSC localization to the vasculature (Yoon, Cho et al. 2012), (Xiao, Liu et al. 2009). Together these reports demonstrate heterogeneity in the osteoblastic cell pool with respect to its HSC-supportive properties, which appear to be dependent at least in part on osteoblastic differentiation stage.

One subset of osteolineage cells which has been controversial in its support of HSCs is the N-cadherin+ pool of osteolineage cells. Cadherins are calcium-dependent homotypic adhesion molecules that form adherens junctions. They are essential in fate specification of germline stem cells (Song, Zhu et al. 2002). Several reports implicate N-cadherin (N-CAD) in osteoblastic-HSC regulation (Zhang, Niu et al. 2003; Arai, Hirao et al. 2004; Wilson, Murphy et al. 2004). Moreover, knockdown of N-Cadherin in HSCs suppresses their long term engraftment (Hosokawa, Arai et al. 2010). Yet this work has been controversial (reviewed in (Levesque 2012)). *Ncad* expression increases with age in HSCs and in endosteal cells populations (Hosokawa, Arai et al. 2010), potentially explaining inconsistencies among various N-CAD studies. Our laboratory examined the bone and hematopoietic phenotypes of *Ncad* deletion from mature osteoblastic cells in both young and aged mice (Bromberg, Frisch et al. 2012). Loss of N-CAD corresponded to an age-dependent decrease in mineralized bone, suggestive of N-CAD involvement in terminal osteoblastic maturation, but we observed no change in HSC number or function at any age examined. In spite of speculation that the N-CAD+ osteoblastic population, which expands rapidly in response to injury (Dominici, Rasini et al. 2009), may be involved in HSC recovery from myeloablation, mice lacking osteoblastic N-CAD have no change in recovery from myeloablative injury (Bromberg, Frisch et al. 2012). Similarly, osteoblastic N-CAD was not required for the PTH-dependent HSC expansion (Bromberg, Frisch et al. 2012). These results are consistent with a companion report in which *Ncad* was deleted from immature OBs using an osterix-driven cre recombinase model (Greenbaum, Revollo et al. 2012). HSC number, cell cycle status, long-term repopulating activity, and self-renewal capacity were normal. Moreover, engraftment of wildtype cells into N-cadherin-deleted recipients was normal, as was G-CSF induced hematopoietic stem/progenitor cells (HSPCs) mobilization. Together, these data show that N-cadherin expression in osteoblast lineage cells is dispensable for HSC maintenance in mice.

While not directly required for HSC maintenance, N-CAD expression marks a stromal cell population that does affect HSCs. *Ncad* expression was found to increase up to 8.5 fold following stimulation with dimethyl prostaglandin E₂ (dmPGE₂) a synthetic analog of the inflammatory mediator prostaglandin E₂ (PGE₂) known to have effects on HSC survival and proliferation (North, Goessling et al. 2007; Frisch, Porter et al. 2009; Goessling, Allen et al.

2011; Porter, Georger et al. 2013). Furthermore, N-CAD identifies osteoprogenitor cell sources of HSC-active non-canonical Wnt ligands and inhibitors of canonical Wnt signaling at homeostasis (Sugimura, He et al. 2012). Specifically, disruption of non-canonical Wnt signaling through the cadherin adhesion molecule Flamingo and/or Frizzled8 using genetic models resulted in decreased frequencies and absolute numbers of HSCs, with increased HSC cycling and reduced frequency of quiescent HSCs in contact with N-CAD⁺ osteolineage cells (Sugimura, He et al. 2012). These studies underscore the role of non-canonical Wnt signaling in maintaining HSC quiescence at the endosteum.

Of note, strategies for hormonal osteolineage stimulation may not only provide translational therapy to manipulate the niche and therefore HSCs, but may also indicate additional niche components. For example, studies had demonstrated the role of T-cells in the anabolic action of PTH on the skeleton (Terauchi, Li et al. 2009; Tawfeek, Bedi et al. 2010; Bedi, Li et al. 2012). Based on these data, the same group went on to show that T cells mediate the PTH-dependent expansion of short-term HSCs through Wnt10b, resulting in Wnt signaling activation of both stroma and HSCs (Li, Adams et al. 2012).

Osteolineage cells may also represent a target for signals produced by malignancies involving the bone marrow (e.g. Multiple myeloma and leukemia), which may disrupt the normal HSC niche. For example, dkk1 and CCL3 production by multiple myeloma initiate not only osteoclastic activation, but also osteoblastic inhibition (Qiang, Chen et al. 2008; Fulciniti, Tassone et al. 2009; Vallet, Pozzi et al. 2011; Vallet, Pozzi et al. 2011). This concept was also demonstrated in a xenograft model of leukemia (Colmone, Amorim et al. 2008). Our laboratory recently demonstrated that leukemia disrupts the osteoblastic microenvironment, and identified CCL3 as a commonly secreted product of human myelogenous leukemia (Frisch, Ashton et al. 2012). In addition, the niche may serve as a target for malignant cells that metastasize in the marrow microenvironment. Work from Shiozawa et al. demonstrated in a xenograft model that human prostate cancer (PCa) cells directly compete with HSCs for occupancy of the mouse HSC niche (Shiozawa, Pedersen et al. 2011). In addition PCa cells could then be mobilized into the circulation using HSC mobilization protocols. Of note, these data do not discriminate between cellular components of the normal niche, therefore the occupancy of the HSC niche by cancer cells may not be restricted to the osteoblastic niche and likely occurs at any other anatomical sites in which a HSC microenvironment is established. Together, these data suggest that definition of the normal HSC niche may also have important therapeutic implication in pathologic conditions. Therefore, targeting the niche may provide a strategy in the treatment of leukemia or potentially other malignancies that affect the bone marrow.

Mesenchymal Stem Cells

Numerous experiments have demonstrated the important role of mesenchymal stem cells (MSCs), the multipotent stromal cells that give rise to the osteogenic lineage, as well as the adipocytes and chondrocytes, as active components of the HSC niche. This cell population has been difficult to define, in part because of its heterogeneity and the lack of consensus on its defining characteristics (adherence to plastic vs functional characteristics vs cell surface markers). Additional confusion is added by the use of the MSC abbreviation to designate preparations of human mesenchymal stromal precursor cells, which are now commercially available (Simmons and Torok-Storb 1991; Simmons and Torok-Storb 1991). The importance of standardizing the defining characteristics of MSCs was highlighted most recently by an excellent study, which identified a population of proliferative osteoblastic progenitors as MX-1 positive bone marrow stromal cells (Park, Spencer et al. 2012). This cell population could differentiate *in vitro* into osteolineage cells, adipocytes and chondrocytes, however, even in the setting of skeletal injury, this cell population is functionally restricted to an osteoblastic fate *in vivo* (Park, Spencer et al. 2012). In spite of

this caveat in reviewing the role of MSCs in the HSC niche, numerous data suggest an important role of MSC in HSC support, both in murine models and in humans. Co-transplantation of MSCs with HSCs improves donor engraftment in non-human primates (Masuda, Ageyama et al. 2009) and enhances murine HSC self-renewal (Ahn, Park et al. 2010). Self-renewing human osteoprogenitor cells found in the marrow in close proximity to sinusoids can form supportive HSC niches (Sacchetti, Funari et al. 2007). In addition, ex vivo studies demonstrated that preparations of human mesenchymal stromal cells expanded cord blood mononuclear cells (McNiece, Harrington et al. 2004; Robinson, Ng et al. 2006). The same research group went on to recently demonstrate that transplantation of human cord-blood cells expanded with mesenchymal cells could be performed safely (de Lima, McNiece et al. 2012), and that MSC-expanded cord blood, in combination with unmanipulated human cord blood, significantly improved engraftment, accelerating time to platelet and neutrophil recovery after transplantation in adults (de Lima, McNiece et al. 2012). Relevant to this line of research, Pinho and colleagues recently showed that PDGFR α and CD51 expression define a bone marrow stromal cell population in both mice and humans that is highly enriched for MSCs and can support HSPC expansion in vitro (Pinho, Lacombe et al. 2013).

Recently, our group and others focused on a critical signal for HSC homing to the marrow, CXCL12, to define the MSC subset responsible for HSC support. CXCL12 (stromal-derived factor-1, SDF-1) is a chemokine that is constitutively expressed by several bone marrow stromal cell populations, and it is known to play an essential role in regulating HSC quiescence (Nie, Han et al. 2008; Tzeng, Li et al. 2011), repopulating activity (Tzeng, Li et al. 2011), and retention in the bone marrow (Kawabata, Ujikawa et al. 1999; Peled, Petit et al. 1999; Ara, Itoi et al. 2003; Bonig, Priestley et al. 2004). Recently, Dr. Sean Morrison's and our group independently assessed the impact of the conditional deletion of *Cxcl12* from candidate niche cells on HSC maintenance (Ding and Morrison 2013; Greenbaum, Hsu et al. 2013). Deletion of *Cxcl12* using osterix-Cre or leptin receptor-Cre transgenes resulted in constitutive HSPC mobilization, but HSC function was largely intact. Osterix-Cre efficiently mediates recombination in CXCL12-abundant reticular (CAR) cells, osteoblasts, and osteocytes (Greenbaum, Hsu et al. 2013). Interestingly, leptin receptor-Cre also targets the majority of CAR cells but does not mediate recombination in osteoblasts (Ding, Saunders et al. 2012). These observations have several important implications. First, although CAR cells (defined as bone marrow stromal cells with very high CXCL12 expression) are reported to have both adipogenic and osteogenic capacity in vitro, only a small subset of leptin receptor-negative CAR cells contribute to osteoblast development in vivo. Second, CXCL12 expression from CAR cells, while essential for efficient retention of HSPCs in the bone marrow, is not required for HSC maintenance.

Both groups showed that deletion of *Cxcl12* using *Prx1-Cre* resulted in a significant loss of HSCs, long-term repopulating activity, and HSC quiescence. Like Osterix-Cre, *Prx1-Cre* targets CAR cells and osteoblasts but also targets platelet derived growth factor receptor- α ⁺ Sca⁺ (PaS) mesenchymal progenitors. A modest decrease in long-term repopulating activity (but not HSC quiescence) also was observed in mice with *Cxcl12* deleted from endothelial cells. Thus, mesenchymal progenitors and, to a lesser extent, endothelial cells, contribute to HSC maintenance. A prior study identified bone marrow stromal cells that express GFP under control of Nestin regulatory sequences as a key component of the stem cell niche (Mendez-Ferrer, Michurina et al. 2010). These Nestin-GFP⁺ stromal cells are enriched for colony-forming-unit-fibroblast (CFU-F) activity and express high levels of HSC maintenance genes, including Kit ligand and CXCL12. Of note, *Prx1*-targeted PaS cells have a much higher CFU-F frequency (more than 10%) compared with Nestin-GFP⁺ stromal cells (less than 1%), suggesting that *Prx1-Cre* targeted PaS are more highly enriched for multipotent mesenchymal progenitors (Greenbaum, Hsu et al. 2013). Surprisingly,

neither *Prx1-Cre* targeted PaS cells nor CAR cells express Nestin (Greenbaum, Hsu et al. 2013), and deletion of *Cxcl12* or Kit ligand using Nestin-*Cre* does not affect HSCs (Ding, Saunders et al. 2012; Ding and Morrison 2013). A potential explanation for these disparate observations is the possibility that the Nestin-GFP transgene results in aberrant expression of GFP that does not accurately reflect Nestin expression. We suggest that Nestin-GFP+ may mark a stromal cell population that includes multipotent mesenchymal progenitors and CAR cells (similar to *Prx1-Cre* targeted cells).

Endothelial and perivascular cells

Endothelial structures give rise to the first definitive HSCs during embryonal development (Chen, Yokomizo et al. 2009). Data also suggest a crucial role of the endothelium in adult HSC regulation. Phenotypic HSCs, identified by their expression of signalling lymphocytic activation molecule (SLAM) markers, localize preferentially to endothelial structures (Wright, Wagers et al. 2001; Kiel, Yilmaz et al. 2005; Kiel, Yilmaz et al. 2008). This is not surprising, since it has been long known that HSCs circulate and are spontaneously mobilized (Storb, Graham et al. 1977; Wright, Wagers et al. 2001), and the vasculature is required for trafficking of HSCs between marrow and the bloodstream. Moreover, endothelial cells secrete factors which expand immature hematopoietic cells *ex vivo*, and support HSCs after myeloablation (Chute, Muramoto et al. 2006; Butler, Nolan et al. 2010; Kobayashi, Butler et al. 2010). Specifically, functional heterogeneity of the marrow microenvironment has been described, where both sinusoids and arterioles are present, and regeneration of sinusoidal endothelial cells is required for hematopoietic recovery from myeloablation (Hooper, Butler et al. 2009). The role of endothelial cells in HSC proliferation is further highlighted by data in Winkler et al, which demonstrate that the endothelial specific cell adhesion molecule E-selectin induces HSC to proliferate *in vivo* (Winkler, Barbier et al. 2012). Moreover, endothelial cells contribute to HSC maintenance, as Ding et demonstrated that deletion of the Kit ligand gene specifically in endothelial cells results in loss of HSC (Ding, Saunders et al. 2012). Together these data show that endothelial cells contribute to HSC maintenance and proliferation *in vivo*.

Since HSCs preferentially localize near BM sinusoids (Kiel, Yilmaz et al. 2005), perivascular stromal cells have also been proposed as a niche for HSCs. These cells produce high levels of soluble HSC-supportive factors (Sugiyama, Kohara et al. 2006; Sacchetti, Funari et al. 2007). Data are now suggesting that, as is the case with osteolineage cells, the perivascular stromal population is heterogeneous, and comprises other candidate HSC niche entities including some Nestin-GFP+ mesenchymal cells (Mendez-Ferrer, Michurina et al. 2010), CXCL-12 (SDF-1)-abundant reticular cells (CAR cells) (Sugiyama, Kohara et al. 2006), and *Prx1-Cre* targeted mesenchymal progenitors (Greenbaum, Hsu et al. 2013). Therefore, genetic models have been instrumental in the definition of this cell population and its role in HSC regulation (Figure 1).

Another type of perivascular cell potentially involved in HSC regulation was identified as a critical source for Stem cell factor (SCF) or Kit Ligand, previously demonstrated to be required for HSC maintenance in its membrane-bound form (Barker 1994; Barker 1997). SCF was selectively deleted from hematopoietic, osteoblastic, Nestin-GFP-expressing stromal, endothelial or Leptin receptor (*Lepr*)-expressing perivascular stromal cell types (Ding, Saunders et al. 2012). Notably, HSC frequency and function were only impaired by the absence of SCF from Tie2+ endothelial and *Lepr*+ perivascular cells. These perivascular, *Scf*-expressing stromal cells lack *Nestin* expression but expressed high levels of *Cxcl12*, alkaline phosphatase, *Vcam1*, *Pdgfra* and *Pdgfrb* when compared to whole bone marrow cell preparations, confirming their mesenchymal and stromal cell composition, and implicating

niche-derived SCF requirement from Lepr⁺ perivascular cells as necessary for HSC maintenance in adult BM.

Neuronal and glial cells

Both neuronal as well as glial cells have been implicated in the modulation of HSC fates. Convincing data have suggested that G-CSF-mediated HSC mobilization is at least in part regulated by sympathetic nervous system (SNS) neurons (Katayama, Battista et al. 2006). SNS neurons coordinate the circadian oscillation of HSC numbers in the marrow and their mobilization to the bloodstream by regulating local production of CXCL12, as demonstrated by experiments in which ablation of SNS neurons resulted in loss of circadian controlled HSCs mobilization into the periphery (Mendez-Ferrer, Lucas et al. 2008). In addition, sympathectomy of one tibia in a mouse resulted in altered expression of CXCL12, while the sham operated contralateral tibia was unaffected (Mendez-Ferrer, Lucas et al. 2008).

An unexpected contribution of glial cells in HSC fate determination was discovered by examining the marrow source for transforming growth factor β (TGF- β). TGF β had previously been demonstrated to induce HSC quiescence ex vivo (Yamazaki, Iwama et al. 2009). While multiple marrow cells are potential sources of inactive TGF β , nonmyelinating Schwann cells are the major source of activated TGF- β in the bone marrow (Yamazaki, Ema et al. 2011). These glial cells are closely associated with HSCs, and produce numerous factors previously identified as playing a role in the HSC niche (Yamazaki, Ema et al. 2011). Ablation of this population in the bone marrow results in a loss of HSC dormancy and ultimately of HSC numbers (Barker 1997). Therefore, neuronal cells and glia have been recently implicated in HSC regulation in the bone marrow.

Adipocytes

Adipocytic cells represent a large portion of the adult marrow, especially in humans. In particular, an increase in the adipocytic marrow component has been associated with aging (Kirkland, Tchkonja et al. 2002; Rosen, Ackert-Bicknell et al. 2009), while it has been well established that the functional capacity of the hematopoietic system decreases with aging (Berkahn and Keating 2004). Moreover, age dependent changes have been demonstrated in both murine and human HSCs (Van Zant and Liang 2012), some of which could be induced by the microenvironment. In spite of these findings, initially adipocytes were considered as potentially supportive of HSCs. For example, the adipokine adiponectin is secreted by adipocyte, and its receptors are expressed by HSCs (DiMascio, Voermans et al. 2007). This adipocytic product has been demonstrated to increase proliferation of HSCs while retaining their repopulating potential (DiMascio, Voermans et al. 2007). Adiponectin however, is not solely produced by adipocytes in the marrow, but is also expressed by osteolineage cells (Berner, Lyngstadaas et al. 2004).

In contrast to these potentially beneficial effects of adipocytes on HSCs, adipocyte numbers have more recently been described as inversely related to numbers of HSCs in the marrow by comparing anatomically distinct regions of the skeleton that display varying levels of adiposity (Naveiras, Nardi et al. 2009). Additional studies demonstrated that loss of adipocytes in the marrow, either genetically or pharmacologically, resulted in enhanced engraftment of HSCs and improved hematopoietic recovery following myeloablative injury (Naveiras, Nardi et al. 2009). Therefore, a dominant inhibitory effect of adipocytes on HSCs has recently been postulated.

Hematopoietic HSC niche components

There is accumulating evidence that hematopoietic cells generate signals that indirectly modulate HSC function through regulation of stromal cells that comprise the stem cell

niche. In this section, we review recent data implicating osteoclasts, macrophages/monocytes, and neutrophils in the regulation of the stem cell niche.

Osteoclasts

Osteoclasts, by virtue of their close association with osteoblasts and proximity to the endosteal niche, have received considerable attention as a hematopoietic cell type that may modulate the stem cell niche. Genetic and pharmacologic approaches to modulate osteoclast function suggest that osteoclasts positively regulate HSCs. Inhibition of osteoclast activity by treatment with bisphosphonates is associated with a modest (1.5-fold) decrease in phenotypic HSCs (Flk2⁻ lineage⁻ Sca⁺ Kit⁺ cells) and a corresponding decrease in long-term repopulating activity (Lympieri, Ersek et al. 2011). A much more severe loss of HSCs is present in the bone marrow of *oc/oc* mice, which carry mutations of *Tirg* encoding for the $\alpha 3$ subunit of the vacuolar type ATPase complex. The *Tirg* mutations in *oc/oc* mice are associated with severe osteopetrosis due to impaired acidification and bone resorption at the ruffle border interface between osteoclasts and bone. Strikingly, there is a near complete absence of phenotypic HSCs (lineage⁻ Sca⁺ Kit⁺ cells) in the bone marrow of *oc/oc* mice (Mansour, Abou-Ezzi et al. 2012).

The mechanisms by which osteoclasts regulate HSCs are largely unknown. In *oc/oc* mice, the severe loss of bone marrow medullary space secondary to osteopetrosis likely contributes to the loss of HSCs. However, Mansour et al also reported a marked expansion of phenotypic mesenchymal progenitors (PDGFR α ⁺ Sca⁺ lineage⁻ cells) but a relative decrease in mature osteoblasts in *oc/oc* mice, raising the possibility that osteoclasts indirectly regulate HSCs by altering the stem cell niche (Mansour, Abou-Ezzi et al. 2012). Finally, osteoclasts may regulate HSC function through degradation of bone matrix, which results in the release of calcium ions and certain cytokines, such as TGF β , which can regulate HSC function (Yamazaki, Ema et al. 2011) (Adams, Chabner et al. 2006).

Several studies have examined the contribution of osteoclasts to HSC mobilization by granulocyte colony-stimulating factor (G-CSF). G-CSF treatment is associated with the mobilization of HSCs from the bone marrow to blood, and it is commonly used in the clinical setting to obtain sufficient donor HSCs from the blood for stem cell transplantation. Prior studies have established that G-CSF induces HSC mobilization primarily by suppressing osteoblasts and CXCL12 expression from bone marrow stromal cells (Petit, Szyper-Kravitz et al. 2002; Levesque, Hendy et al. 2003; Semerad, Christopher et al. 2005; Christopher, Liu et al. 2009). Thus, G-CSF induced HSC mobilization represents a useful tool to perturb and study the stem cell niche. Kollet and colleagues reported that activation of osteoclasts by injection of RANK ligand (RANKL) was associated with moderate HSPC mobilization (Kollet, Dar et al. 2006). Conversely, inhibition of osteoclasts, either genetically by knocking out PTPe or by injecting mice with calcitonin, blunts the mobilization response to G-CSF. The authors suggested that osteoclasts induce HSPC mobilization by release of the proteinase cathepsin K, which can, at least in vitro, cleave CXCL12. On the other hand, Miyamoto and colleagues showed using three different transgenic mouse models with impaired osteoclast activity (CSF-1, c-fos, or RANKL deficient mice) that HSPC mobilization, at baseline or after G-CSF treatment, is increased (Miyamoto, Yoshida et al. 2011). Consistent with this finding, several groups have reported increased osteoblast suppression and HSPC mobilization by G-CSF after bisphosphonate treatment (Winkler, Sims et al. 2010; Miyamoto, Yoshida et al. 2011). Thus, the preponderance of current evidence suggests that osteoclasts negatively regulate HSPC trafficking from the bone marrow but are dispensable for G-CSF induced HSPC mobilization.

Macrophages

There is considerable phenotypic, functional, and developmental heterogeneity in tissue macrophages (Yona, Kim et al. 2013). This extends to the bone marrow where several macrophages populations have been identified. Chang et al described an F480+ population of macrophages that are closely associated with osteoblasts and bone lining cells on endosteal and periosteal surfaces. Indeed, these cells, termed osteomacs, have been shown to form a canopy over mature osteoblasts at sites of bone formation under steady state conditions (Chang, Raggatt et al. 2008) or after bone injury (Alexander, Chang et al. 2011). Chow et al described a CD169+ population of macrophages (also F480+) that is associated with perivascular Nestin-GFP+ stromal cells (Chow, Lucas et al. 2011). Whether osteomacs and CD169+ macrophages represent distinct cell populations is unclear. Recently, Ludin et al reported the identification of a rare macrophage subset (representing less than 0.1% of total bone marrow cells) with high expression of α -smooth muscle actin (α -SMA) (Ludin, Itkin et al. 2012). These cells express high levels of cyclooxygenase-2 (COX-2) and may regulate HSC function through localized production of PGE2 (Ludin, Itkin et al. 2012). This population is also likely targeted by pharmacologic in vivo treatment with dmPGE2, which ameliorates sublethal irradiation-induced damage to the HSC pool by inhibiting apoptosis while preserving long term function (Porter, Georger et al. 2013). dmPGE2-mediated enhancement of hematopoietic recovery is likely to occur both through direct effects on HSPCs as well as changes to the microenvironment, where COX2 activity is increased and α -SMA+ macrophages are increased. In addition to macrophages, the bone marrow is a rich reservoir of other monocyte-lineage cells, including monocytes and myeloid dendritic cells.

There is accumulating evidence that macrophages in the bone play an essential role in regulating the stem cell niche and HSC function. In cultures of primary bone marrow stromal cells, the presence of macrophages significantly enhances the production of mature osteoblasts (Chang, Raggatt et al. 2008). Several experimental approaches have been employed to ablate monocytes/macrophages in vivo. MAFIA mice express a suicide fusion gene comprised of a FK506 binding domain and the cytoplasmic domain of Fas expressed under control of the c-fms promoter. Treatment of MAFIA mice with a chemical dimerizer induces Fas-mediated apoptosis in monocytes and macrophages. Ablation of monocytes/macrophages in this model is very efficient and is associated with marked suppression of osteoblasts (Chang, Raggatt et al. 2008) and HSPC mobilization into the blood (Winkler, Sims et al. 2010). Of note, macrophage ablation using the MAFIA mouse model is associated with considerable systemic inflammation, potentially indirectly contributing to HSPC mobilization. Treatment of mice with clodronate-loaded liposomes results in a more modest reduction in bone marrow monocytes/macrophages (80–90% reduction) and is not associated with obvious signs of systemic inflammation (Winkler, Sims et al. 2010; Chow, Lucas et al. 2011). Although the magnitude is reduced compared with MAFIA mice, monocyte-macrophage ablation with clodronate-loaded liposomes resulted in HSPC mobilization, osteoblast loss, and decreased expression of stem cell niche genes, including CXCL12, Kit ligand, and angiopoietin-1 (Winkler, Sims et al. 2010; Chow, Lucas et al. 2011). To further define the monocytic lineage cell population in the bone marrow that contributes to stem cell niche maintenance, Chow et al ablated CD169+ macrophages using transgenic mice that express the diphtheria toxin receptor under control of CD169 regulatory elements (Winkler, Sims et al. 2010; Chow, Lucas et al. 2011). They showed that ablation of CD169+ macrophages was associated with modest HSPC mobilization and decreased expression of CXCL12, Kit ligand, and angiopoietin-1 from Nestin-GFP+ bone marrow stromal cells. Interestingly, this same group recently showed that CD169+ macrophages play a key role in supporting erythropoiesis in the bone marrow (Chow, Huggins et al. 2013). Consistent with these findings, Westerterp et al recently showed that deletion of the ATP binding cassette transporters ABCA1 and ABCG1 in macrophages and/or myeloid dendritic

cells resulted in a loss of osteomacs and CD169+ macrophages in the bone marrow and was associated with modest HSPC mobilization and suppression of CXCL12 expression (Westerterp, Gourion-Arsiquaud et al. 2012).

Studies of G-CSF receptor deficient bone marrow chimeras established that G-CSF signaling in bone marrow stromal cells is not required for G-CSF induced stem cell niche suppression of HSPC mobilization (Liu, Poursine-Laurent et al. 2000). To evaluate the role of monocytic cells in G-CSF-induced HSPC mobilization, we generated transgenic mice in which expression of the G-CSFR is restricted to CD68+ monocytes and macrophages (Christopher, Rao et al. 2011). Treatment of these mice with G-CSF induces marked suppression of osteoblasts and CXCL12 expression and is associated with robust HSPC mobilization. Importantly, G-CSF treatment results in a marked loss of monocytes (Christopher, Rao et al. 2011) and osteomacs (Winkler, Sims et al. 2010) from the bone marrow.

Neutrophils

Previous studies suggested that neutrophils may contribute to G-CSF-induced HSPC mobilization by the release of specific proteinases, such as MMP9, cathepsin G, and neutrophil elastase, that degrade key stem cell niche molecules, including CXCL12 (Levesque, Takamatsu et al. 2001; Heissig, Hattori et al. 2002). However, we showed that G-CSF induced HSPC mobilization is normal in mice lacking these proteinases (Levesque, Liu et al. 2004). Moreover, we and others showed that G-CSF primarily suppresses CXCL12 at the transcriptional level (Petit, Szyper-Kravitz et al. 2002; Levesque, Hendy et al. 2003; Semerad, Christopher et al. 2005). Thus, the role of neutrophil proteinases in disrupting the stem cell niche during G-CSF treatment is questionable. As noted above, studies of CD68:G-CSF receptor transgenic mice show that G-CSF signaling in monocytic cells is sufficient to induce robust HPSC mobilization and CXCL12 suppression (Christopher, Rao et al. 2011). Moreover, these mice are severely neutropenic (due to the lack of G-CSF receptor expression on neutrophil lineage cells), suggesting that neutrophils are not required for G-CSF-induced HPSC mobilization. On the other hand, Singh et al recently reported that neutrophil depletion using an anti-LyG antibody modestly attenuated G-CSF-induced HSPC mobilization and osteoblast suppression. Together, these data suggest that neutrophils, while not required, may play a minor role to augment HSPC mobilization by G-CSF (Singh, Hu et al. 2012).

Model

Together, these data support a model in which monocytes/macrophages in the bone marrow provide signal(s) that are required for maintenance of stromal cells that comprise the stem cell niche (Figure 2). Specifically, monocytes/macrophages support osteoblast function and the expression of key stem cell niche genes, including CXCL12 and Kit ligand, from Nestin-GFP+ perivascular stromal cells. Although CD169+ macrophages clearly contribute to stem cell niche maintenance, ablation of these cells results in only modest HSPC mobilization and CXCL12 suppression (compared with MAFIA mice or CD68:G-CSF receptor mice). Thus, other monocyte/macrophage cell populations in the bone marrow also likely contribute to niche maintenance. Indeed, our preliminary data suggest that myeloid dendritic cells also generate signals that support CXCL12 production from bone marrow stromal cells (unpublished observations). G-CSF disrupts the stem cell niche and induces HPSC mobilization primarily by targeting monocytes/macrophages. In addition to resulting in a loss of monocytes/macrophages in the bone marrow, G-CSF suppresses the production of inflammatory cytokines from monocytes/macrophages, including tumor necrosis factor- α (TNF α) (Boneberg, Hareng et al. 2000). The signals generated by macrophages/monocytes that support stromal cells comprising the stem cell niche are currently unknown.

Physiologic regulation of the niche

So far we have highlighted different cellular components of the niche and their ability to support HSCs. However, these cell populations can also be influenced by physiologic stimuli, such as sympathetic innervation (Katayama, Battista et al. 2006), circadian rhythms (Mendez-Ferrer, Lucas et al. 2008) and hormonal signals (Calvi, Adams et al. 2003), which further add to the complexity of the niche. A localized physiologic modulator of the HSC niche of particular interest is hypoxia. Data suggest that hypoxia affects both niche cells as well as directly HSCs to consistently increase HSC quiescence. Data support a crucial role of hypoxia and hypoxia-inducible factor-1 (HIF-1) in bone development and turnover -as demonstrated by genetic deletion of *Hif1a* gene in osteoblasts affects bone formation (Wang, Wan et al. 2007). Stabilization of HIF in osteolineage cells expands HSCs, suggesting that hypoxic osteolineage cells are likely more supportive of HSC (Rankin, Wu et al. 2012). On the other hand, deletion of the *Hif1a* gene promotes HSC exit from G_0 and sensitizes HSCs to repetitive cycles of myelosuppression, while pharmacological stabilization of HIF-1 α protein increases HSC quiescence, demonstrating that HIF1 α is a critical regulator of HSC quiescence (Takubo, Goda et al. 2010; Forristal, Winkler et al. 2013). Prior studies had suggested that HSPCs with the ability to serially reconstitute are located in the endosteal region of the bone marrow, which is characterized by low perfusion and relative hypoxia (Levesque, Winkler et al. 2007; Winkler, Barbier et al. 2010). However, a recent study showed that the endosteal region is well vascularized (Nombela-Arrieta, Pivarnik et al. 2013). Moreover, the majority of HSPCs in the endosteal region are perivascular. Thus, HSPCs in the endosteal region are likely to be relatively well oxygenated. A potential explanation for this discrepancy is the observation that HSPCs display a hypoxic profile (defined by strong retention of pimonidazole and expression of HIF-1 α) regardless of their location in the bone marrow (Nombela-Arrieta, Pivarnik et al. 2013). Indeed, even HSPCs in the peripheral circulation display a hypoxic profile. Thus, intrinsic differences in metabolism rather than localization to a hypoxic microenvironment may define the hypoxic profile of HSCs.

Summary

Since the initial proposal of the niche hypothesis, there has been considerable progress in defining the cellular components that comprise the stem cell niche in the bone marrow. Current studies highlight the heterogeneity of the niche, with specific stromal cell populations producing signals that regulate specific aspects of HSC biology, such as quiescence and retention in the bone marrow. Mature hematopoietic cells, in particular macrophages, also contribute to HSC maintenance through regulation of stromal cells and possibly through direct effects on HSCs. The complexity of the stem niche allows for flexibility in HSC responses to environmental cues such as inflammation and provides a mechanism to tightly couple HSCs with bone metabolism. It also provides multiple potential therapeutic targets for manipulation to modulate HSC and their progeny. Indeed, by understanding the cellular and molecular components of the stem cell niche, we may one day be able to simply and safely manipulate HSCs in vivo on demand. Moreover, since the stem cell niche may provide signals that support certain malignancies, drugs that target the niche hold promise as a way to sensitize cancer cells to chemotherapy.

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Figure 1. Perivascular stem cell niche

The perivascular niche is comprised of endothelial cells and several, likely overlapping, mesenchymal stromal cell populations. These stromal cells provide key niche signals, such as CXCL12, kit ligand, and angiopoietin, that localize HSCs to the perivascular region and help maintain their quiescence and self-renewal capacity. Signals from the sympathetic nervous system (SNS) regulate HSCs, at least in part, by targeting these perivascular stromal cells.

Figure 2. Macrophage regulation of HSCs

Osteomacs (OM) and CD169⁺ macrophages (Mø) provide signals that contribute to osteoblast (Ob) maintenance and CXCL12 and kit ligand (Scf) expression from perivascular Nestin-GFP⁺ stromal cells. Recently, α-smooth muscle actin⁺ macrophages (αSM) have been identified that may regulate HSCs through local production of PGE₂. Osteoclasts (Oc) negatively regulate HSCs through unknown mechanisms.