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Establishment and Regulation of the HSC Niche: Roles of Osteoblastic and Vascular Compartments

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Abstract

Hematopoietic stem cells (HSC) are multi-potent cells that function to generate a lifelong supply of all blood cell types. During mammalian embryogenesis, sites of hematopoiesis change over the course of gestation: from extraembryonic yolk sac and placenta, to embryonic aorta-gonad-mesonephros region, fetal liver, and finally fetal bone marrow where HSC reside postnatally. These tissues provide microenvironments for de novo HSC formation, as well as HSC maturation and expansion. Within adult bone marrow, HSC self-renewal and differentiation are thought to be regulated by two major cellular components within their so-called niche: osteoblasts and vascular endothelial cells. This review focuses on HSC generation within, and migration to, different tissues during development, and also provides a summary of major regulatory factors provided by osteoblasts and vascular endothelial cells within the adult bone marrow niche.

Keywords

hematopoietic stem cells; HSC niche; hematopoiesis; vascular endothelial cells; osteoblasts

INTRODUCTION

In the early 1960s, it was discovered that adult mouse bone marrow contains a population of cells that can form myeloerythroid colonies in the spleen and rescue lethally irradiated mice in which the long-term hematopoietic repopulating activity had been ablated (Till and Mc, 1961; Becker et al., 1963). Using a combination of cell surface markers, Morrison and Weissman, (1994) were able to identify and isolate a near pure population of hematopoietic stem cells (HSC) from adult marrow that is responsible for multilineage blood cell generation throughout postnatal life.

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During adulthood, at any given time, ~75% of HSC are quiescent and arrested in the G₀ phase of cell cycle; ~8% enter the DNA synthesis (S) phase every day, self-renew and ultimately cause multilineage progenitors and differentiated blood cell types (Cheshier et al., 1999). Due to the limited life span of blood cells in circulation, HSC must be continually available to replace cells lost in the bloodstream. Such a demanding task would perhaps result in the exhaustion of HSC resources; however, there is a tightly regulated balance between HSC self-renewal and differentiation in vivo. The special microenvironment that modulates HSC behavior was first conceptualized by Schofield (Schofield, 1978), who paved the way for intense research on a now burgeoning field of study on stem cell niches. Within its niche, a stem cell stays quiescent, suspended from entering the cell cycle; however, depending on specific cues, it will either self-renew or differentiate and leave its niche. Even though our knowledge about HSC is more advanced than other adult stem cell types, there is still much to be discovered about the control of their replication and differentiation in vivo, and it is an immense challenge to recapitulate these regulatory processes outside the body. However, optimizing our ability to maintain HSC in an undifferentiated state in vitro for extended periods of time will broaden the application and efficiency of HSC transplantation protocols for the treatment of prevalent hematopoietic disorders. Thus, further delineating the microenvironmental factors that regulate HSC potential and fate will not only advance our understanding of their biology, but also provide insights needed for their clinical use.

In this review, we will summarize the events leading to HSC development during mouse embryogenesis, focusing on HSC interactions within their changing microenvironments (niches), as their generation and/or function moves from one tissue to another. We will also focus on the crucial extrinsic regulatory factors, including those provided by vascular endothelial cells, within their niche that modulate HSC potential and fate.

HSC FORMATION AND MIGRATION DURING DEVELOPMENT

Although much of what we know about mammalian hemato-vascular development comes from model organisms such as the mouse, which we will focus on herein, similar processes are known to occur during human development (Peault and Tavian, 2003). In the mouse, hematopoietic progenitors first arise in the extraembryonic yolk sac (YS) at E7.0–7.5 (Palis et al., 1999). The blood progenitors are thought to be in a primitive state with limited differentiation capacity, producing only erythroid and megakaryocytic cells (Tober et al., 2007), in a process termed primitive hematopoiesis. At E8.25, definitive hematopoiesis, generation of all blood cell lineages, begins in the YS. However, the true nature of the hematopoietic progenitors formed in the YS is controversial, because they cannot repopulate lethally irradiated adult recipients, despite their ability to engraft in neonates (Yoder and Hiatt, 1997). Adult repopulating HSC are first detected in the aorta gonad mesonephros (AGM) region of the embryo proper at E10.5 (Medvinsky and Dzierzak, 1996), although the major site of definitive hematopoiesis changes throughout gestation from AGM to fetal liver, and finally to fetal bone marrow (Fig. 1). The control of definitive hematopoiesis, as well as the role of vascular endothelium in this process within these sites, will be discussed herein.

Extraembryonic Yolk Sac

Of all the microenvironments that generate and/or support hematopoietic stem/progenitor cells throughout development, the extra-embryonic YS is among the simplest tissue, composed of mesodermal and endodermal germ layers.

There are two distinct waves of blood cell production within the murine YS (Fig. 2). Initially, at ~E7.0–7.5, the generation of primordial (unspecialized) endothelium and primitive erythroblasts occurs coincidentally within the mesoderm, forming structures referred to as blood islands (Palis et al., 1995). Since it is predominantly primitive erythroblasts produced at this early stage of development, this process is referred to as primitive hematopoiesis (Wong et al., 1986; Ferkowicz and Yoder, 2005).

The coincident emergence of the first endothelial and blood cells within the YS mesoderm is induced by soluble signals originating from the adjacent visceral endoderm. Endoderm-derived Indian hedgehog (IHH) and basic fibroblast growth factor (bFGF) (Faloon et al., 2000) have been shown to induce vascular endothelial growth factor receptor-2 (VEGFR2 or Flk1 or KDR) expression in underlying mesodermal cells that become responsive to VEGF-A, which is also produced by the endoderm (Breier et al., 1995). IHH also upregulates bone morphogenic protein-4 (BMP4) within mesodermal progenitors (Kelly and Hirschi, 2009) which, in turn, may contribute to VEGFR2 upregulation. Embryos deficient of VEGFR2 (*Flk1*^{-/-}), cannot respond to VEGF-A signaling and fail to form blood islands (Shalaby et al., 1995), as well as blood vessels. However, there are endothelial precursors formed, suggesting a role of VEGF-A signaling in endothelial cell survival, propagation and/or maturation, but not phenotypic induction. Hematopoietic failure is also observed in the *Flk1*^{-/-} mutants, which may be due to the lack of endothelial microenvironment to support blood production (Shalaby et al., 1995), although it is controversial as to whether erythroblasts are derived from endothelial cells during primitive hematopoiesis or independently fated (Kinder et al., 1999).

Definitive hematopoiesis begins within the YS at ~E8.25 (Cumano et al., 1996), concomitant with the onset of cardiac function and systemic circulation (Palis et al., 1999). The definitive myeloerythroid precursors that are formed are characterized by CD41 and C-Kit expression, and upon maturation begin to express CD45 (Mikkola et al., 2003). Hematopoietic cells also express cell surface proteins originally thought to be endothelial-specific, such as VE-cadherin (Kim et al., 2005), CD31 (Baumann et al., 2004), and endoglin (Chen et al., 2002). Moreover, genetic studies demonstrate that they also share regulatory pathways, since the disruption of specific common genes affects the development of both cell lineages (Sato, 1999; Schuh et al., 1999). This is not surprising, given that hematopoietic stem/progenitor cells within the YS are derived from a specialized subset of endothelium (Nadin et al., 2003), termed hemogenic endothelium, via a process regulated by retinoic acid signaling (Goldie et al., 2008). Thus, it is clear that the vasculature plays a critical role in the establishment and maintenance of definitive hematopoiesis during embryonic development.

Moore and Metcalf (1970) proposed that hematopoietic stem/progenitor cells initially generated within the YS serve as the source of both fetal and adult hematopoiesis. However, other groups have suggested that the earliest HSC with adult repopulating ability arise

within the embryonic AGM based on the observation that cells from the YS fail to reconstitute lethally irradiated adult mice (Cumano et al., 1995; Medvinsky and Dzierzak, 1996). These findings were later questioned by other studies which demonstrated that YS multilineage stem/progenitors are able to rescue lethally irradiated neonatal mice (Yoder and Hiatt, 1997; Yoder et al., 1997). Furthermore, when YS cells are cocultured with AGM-derived stromal cells, they acquire adult repopulating function (Matsuoka et al., 2001).

Samokhvalov and coworkers (2007) further tested this hypothesis by using the *Runx1* promoter in cell tracking studies. They confirmed that progenitors arise in the YS, migrate to the liver, and finally colonize the fetal bone marrow. Furthermore, removal of the YS tissue results in failure of development of hematopoietic populations in the liver. These studies indicate that YS hematopoietic stem/progenitor cells exhibit an intrinsic regulatory repertoire similar to adult HSC. However, to fully acquire their potential they require extrinsic signals that are absent in YS microenvironment, yet present in intraembryonic tissues such as AGM and liver.

Placenta

The placenta is another extra-embryonic organ, derived from trophoblast and mesoderm (Rossant and Cross, 2001) that demonstrates hematopoietic activity. Hematopoietic function of the placenta was proposed decades ago (Till and Mc, 1961), and more recent studies demonstrated in vitro hematopoietic progenitor activity of the placental origin at ~E8.5–9.0 (Alvarez-Silva et al., 2003), and adult repopulating capacity at ~E10.5 via in vivo transplantation studies (Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

The fact that systemic circulation is established ~E8.5 raised the question as to whether the multilineage stem/progenitor cells are autonomously generated within the placenta or migrate in from the YS. To demonstrate that HSC are generated within the placenta, *Ncx1* deficient mice were employed. In this model, embryos survive until E10.5, but systemic circulation is not established due to lack of cardiac contractile function; nonetheless, placental tissues were shown to generate HSC de novo in the absence of systemic blood circulation (Rhodes et al., 2008).

Since blood cells are derived from mesoderm during embryogenesis, chorionic and allantoic mesoderm are possible tissues of origin for placental HSC (Zeigler et al., 2006; Corbel et al., 2007). *Runx1-LacZ* reporter mice were used to localize HSC in the placenta, which are found predominantly near vessels of the chorioallantoic mesenchyme and the fetal labyrinth (Rhodes et al., 2008). Thus, similar to YS tissues, the vasculature in the placenta provides a unique microenvironment for HSC generation, expansion, and maturation. Interestingly, the HSC pool within the placenta is highest at E12.5–E13.5, when it contains 15 times more HSC than YS or embryo proper (Gekas et al., 2005), and the HSC number within the placenta dramatically decreases thereafter, coincident with HSC population of the fetal liver.

Embryonic Aorta-Gonad-Mesonephros

The AGM is formed from embryonic splanchnopleural mesoderm, in close association with definitive endoderm that derives from the epiblast during gastrulation (Tam and Behringer, 1997), and is the first intraembryonic region with demonstrated hematopoietic activity. At

E9.0 of mouse development, cells isolated from the AGM are capable of giving rise to B cells in irradiated mice (Godin et al., 1993). At E10.5, the AGM is known to harbor precursors with colony forming activity in the spleen (CFU-S) (Medvinsky et al., 1993), as well as multilineage hematopoietic stem/progenitor cells with self-renewing ability (Muller et al., 1994). The presence of HSC in the AGM is thought to be transient from E10.5 to E12.5, with little expansion or differentiation within this tissue (Muller et al., 1994).

The HSC in the AGM region divide more rapidly than those in the extra-embryonic tissue, which is consistent with its important role for embryonic hematopoiesis. As in the extraembryonic placenta, hematopoiesis is detected within the AGM after the onset of systemic circulation; therefore, it was unclear as to whether hematopoietic stem/progenitor cells are generated in situ, or migrate in from elsewhere via blood circulation. To address this issue, organ explant cultures were established and hematopoietic cells derived within the cultures were shown to be produced autonomously within AGM (Medvinsky and Dzierzak, 1996). Further studies have confirmed that HSC are generated in AGM *de novo*, and they are able to repopulate lethally irradiated adult recipient (Kumaravelu et al., 2002).

Observations from as early as the 1920s (Sabin, 1920) suggested that the hematopoietic cells generated within the AGM were budding from the ventral endothelium of the dorsal aorta. Earlier cell tracing studies tried to monitor this behavior by labeling endothelial cells in vivo via uptake of DiI-AcLDL. In one study, the avian embryo AGM vasculature was labeled with DiI-AcLDL before the emergence of blood cell clusters (CD45+), and within 24 hr, CD45+ DiI-AcLDL+ clusters were detected (Jaffredo et al., 1998). Similarly, Sugiyama et al. (2003) demonstrated the emergence of erythroid progenitors from DiI-AcLDL+ endothelial cells in the mouse AGM. *Runx1*-LacZ knock-in mice were also used to localize HSC in the AGM and track their origin (North et al., 2002). In E10–11 *Runx1*-LacZ knock-in mouse embryos, β -galactosidase positive cells are found in hematopoietic clusters budding into the lumen from the ventral aspect of the dorsal aorta. Further characterization using hematopoietic and endothelial markers indicate that HSC activity is not only in hematopoietic clusters (CD45+CD31+ and CD45+VEcad+) but also in endothelial cells (CD45–CD31+ and CD45–VEcad+), supporting the presence of hemogenic endothelium in the AGM.

Recently, multiple groups have conducted in vivo imaging studies in multiple model systems further confirming the generation of blood cells from aortic endothelium within the AGM. Real-time confocal imaging of *Sca-1*-GFP embryos in which Sca-1 is expressed in aortic endothelium, demonstrated that a subset of these cells causes blood cells (Boisset et al., 2010). In another study, Bertrand et al. (2010) generated *cmyb*: eGFP; *kdr*: mCherry double transgenic zebrafish and performed confocal time-lapse imaging. Their result confirmed hemogenic endothelium as the source of HSC in AGM. By utilizing *KDR*-GFP transgenic zebrafish, Kissa, and Herbomel (2010) demonstrated that this process does not involve cell division, but it occurs via endothelial-hematopoietic transition.

Attempts have been made to define the specialized microenvironment within the AGM that supports HSC generation, and confers adult repopulating potential in YS-derived cells (Matsuoka et al., 2001). Stromal cells within the AGM have been isolated and shown to

exhibit some phenotypic similarities to vascular smooth muscle cells and bone marrow mesenchymal stem cells (MSC), although they lack their full multilineage potential (Charbord et al., 2002; Durand et al., 2006). It is not surprising that cells that support definitive hematopoiesis in the AGM have properties similar to mural cells, since blood cells are generated from the endothelium (hemogenic endothelium). However, the specific cellular and/or molecular role that such cells play in regulating the generation and fate of multilineage hematopoietic stem/progenitor cells from the endothelium is not yet clear.

In contrast, it is clear that mesodermal-endodermal interactions are critical for the formation and function of the embryonic AGM region, just as in the extraembryonic YS. One of the earliest known regulators of hematopoiesis in the YS, hedgehog, also plays a role in the induction and growth of HSC within the AGM. Hedgehog is expressed by endodermal cells in AGM (Peeters et al., 2009) while endothelial cells (C-Kit⁻ CD34⁺) and hematopoietic stem/progenitor cells (C-Kit⁺ CD34⁺) express receptor and co-receptor, *Ptch1* and *Smo*, but no/low levels of *Ptch2*. Surrounding mesenchymal cells (C-Kit⁻, CD34⁻) within the AGM “niche” also express all three hedgehog receptor/transducer genes, as well as downstream targets *Gli1*, *Gli2*, and *Gli3* (Peeters et al., 2009); however, their specific contribution to the regulation of blood cell production from hemogenic endothelium in the AGM is not known.

Fetal Liver

Lineage tracing studies and tissue explant coculture experiments revealed that hepatic lineages differentiate from the foregut endoderm and the liver bud develops in the mouse embryo ~E8.0–9.0 (Tremblay and Zaret 2005; Gualdi et al., 1996). Multilineage hematopoietic stem/progenitor cells from the YS, AGM, and placenta migrate to the fetal liver ~E11 (Cumano and Godin, 2007). Within 24 hr, the number of HSC in the fetal liver increases from 3 to 66, and continues to double from E12.5 to E14.5, until it starts to decrease ~E15.5 (Morrison et al., 1995). This rapid expansion of HSC within the fetal liver suggests that this microenvironment provides mitogenic and self-renewal signals to HSC.

Fetal liver hepatic progenitors have been shown to promote HSC expansion via secretion of soluble effectors including angiopoietin-like 3, insulin-like growth factor-2 (IGF2), stem cell factor (SCF), and thrombopoietin (TPO) (Chou and Lodish, 2010). The fetal liver stroma not only provides a unique environment for HSC expansion but also impacts the differentiation (Mikkola et al., 2006) and maturation of HSC. Coculture of fetal YS HSC on fetal liver stroma cells promotes development of adult repopulating ability (Takeuchi et al., 2002). Fetal liver stromal cells exhibit epithelial to mesenchymal (EMT) behavior, and it is proposed that expansion and differentiation of fetal liver HSC is supported throughout EMT transition (Chagraoui et al., 2003).

Epithelial cells in fetal liver stroma express CD166 that promotes HSC adhesion and modulates HSC-stroma interactions (Cortes et al., 1999). Using a well-characterized fetal liver stroma cell line, AFT024, in a complex functional genomic approach, Hackney et al. (2002) performed the first molecular profiling of the fetal liver HSC niche to characterize stromal-derived signals that modulate HSC. Novel candidate signaling molecules were revealed (SCDB: <http://stromalcell.mssm.edu>), and previously known stem cell niche

signaling molecules, such as WNT, BMP, and Notch, were also verified to play a role in HSC regulation in this model.

It is interesting to note that stromal cells within fetal liver are phenotypically similar to MSC that reside within the adult bone marrow niche (Fromigue et al., 2008). Bone marrow-derived MSC are multipotent cells that can give rise to many cell types, including chondrocytes, osteoblasts, and adipocytes, and play an important role in the maintenance of HSC (Muguruma et al., 2006). The presence of MSC, per se, in the fetal liver has been shown; however, their functional role as a supportive component of the niche for fetal HSC is not well defined (Chagraoui et al., 2003). Thus, phenotypic and functional similarities among various support cells within multiple known HSC niches remain to be determined.

Fetal and Adult Bone Marrow

Fetal bone marrow develops late in embryogenesis, ~E14–E15 when bone tissue is just forming. Early in development, the endochondral bone structure is an avascular cartilage anlage differentiated from condensed mesenchyme. Upon vascularization of the skeletal primordium, cartilage hypertrophy occurs, followed by mineralization and osteogenesis (Mackie et al., 2008). The potential role of vascularization of bone tissues in establishing marrow and/or supporting HSC activity in fetal bone is yet to be determined. One limiting factor in understanding the fetal bone marrow HSC microenvironment, and the regulation thereof, is a lack of knowledge of the fetal bone marrow HSC phenotype, which is in sharp contrast to our understanding of the adult bone marrow HSC that has been extensively studied. Multiple strategies have been developed and adopted for the isolation of adult bone marrow HSC. One such strategy depends on the cellular expression of C-Kit, and Sca-1, in the absence of expression of hematopoietic lineage markers (Lin[−]), constituting the so-called KSL fraction of bone marrow cells that is thought to contain all HSC activity (Morrison and Weissman, 1994). To further purify HSC, per se, additional markers are needed, such as thymus cell antigen-1 (Thy1.1), SLAM F1 (CD150), FMS-like tyrosine kinase-3 (Flt3), interleukin receptor α (IL-7R α), and endoglin (CD105) (Kondo et al., 1997; Christensen and Weissman, 2001; Chen et al., 2002; Kiel et al., 2005). Another method to isolate adult marrow HSC involves the use of dye-exclusion properties, resulting in the sorting of a unique cell population referred to as the side population (SP; Goodell et al., 1996).

Although we do not yet understand the phenotype of fetal bone marrow HSC, per se, or relative to adult marrow HSC, we know that B-cell progenitors are detected in bone at ~E16 (Ogawa et al., 1988). HSC are thought to “home” to a fetal bone marrow environment from other embryonic sites of definitive hematopoiesis, homing to fetal bone marrow requires signaling via factors such as stromal derived factor-1 (SDF1) and its receptor-CXCR4 (Zou et al., 1998; Ara et al., 2003). In addition to soluble factors within marrow, osteoblasts are also thought to play a role in HSC maintenance and regulation based on the observation that HSC within adult marrow are localized near endosteal surfaces (Lord and Hendry, 1972; Patt and Maloney, 1972). Osteoblasts are differentiated from mesenchymal precursors in response to BMP signaling. Mice with conditional deletion of BMP receptor type IA (BMPRIA) exhibit increased numbers of spindle-shaped N-cadherin+CD45[−] osteoblastic

(SNO) cells, in their trabecular bone area. Parallel to this increase, HSC numbers also increase twofold, suggesting that SNO cells function as a supportive niche for HSC in adult bone marrow (Zhang et al., 2003). In another study that links HSC to osteoblasts, Calvi et al. (2003) utilized a transgenic mouse line with an activated PTH/PTHrP receptor (PPR) that is overexpressed in osteoblasts under collagen type I $\alpha 1$ promoter, resulting in an increase in osteoblasts and a parallel increase in HSC number.

In addition, Visnjic et al. (2004) took a different approach, using a collagen type I $\alpha 1$ promoter (Col2.3 Delta Thymidine Kinase) transgenic mice in which they induced ablation of osteoblasts via gancyclovir (GV) treatment. However, HSC number did not decrease as one would expect based on previous results. Instead, it took several weeks, during which time bone marrow cellularity was severely reduced, implying that other cellular components are involved in HSC maintenance.

The role of vascular endothelium in support of adult HSC has also been implicated (Kennedy et al., 1997; Huber et al., 2004). A combination of SLAM markers (CD150+CD48–CD244–) was used to label HSC and revealed that 60% of bone marrow HSC reside very close to sinusoidal endothelium, as compared to only 14% found attached to endosteum (Kiel et al., 2005; Kiel et al., 2007b). It is not clear whether the proposed endosteal and vascular niches are mutually exclusive; rather, it appears that each has a unique function in HSC regulation and that both are required to maintain HSC function.

It has been shown that sinusoidal endothelial cells are surrounded by CXCL12 secreting reticular cells and HSC are localized very close to these cells. Such reticular cells have also been found to close to endosteum (Sugiyama et al., 2006). Until recently, it has been widely accepted in the stem cell niche field that endosteal bone surfaces serve as a quiescent HSC niche, while the vascular niche functions as self-renewing HSC niche. However, more recent data suggest that the endosteum itself is highly vascularized and it is possible that this multicellular niche and its integrated signaling network more than likely modulates HSC behavior.

Of note, almost a third of HSC do not have contact with either endosteum or vascular endothelium, suggesting there are possibly other niche components supporting HSC function within marrow (Kiel et al., 2005). Frenette and coworkers (Katayama et al., 2006) discovered the possible role of bone marrow neuronal cells in HSC regulation. Ablation of noradrenergic neurons diminishes the HSC mobilization whereas $\beta 2$ adrenergic agonists enhanced their mobilization, which suggests the role of sympathetic nervous system in HSC localization (Katayama et al., 2006). Thus, we have much to learn about the cellular composition and function of the bone marrow HSC niche.

FETAL TO ADULT SWITCH: PHENOTYPIC AND FUNCTIONAL DIFFERENCES BETWEEN HSC POPULATIONS

HSC emergence and migration through different tissues during development suggests that HSC niches not only function to maintain HSC “stemness,” but also to facilitate HSC maturation that is required to sustain hematopoiesis postnatally, throughout mammalian

adult life. Throughout their journey in the embryo, HSC change both phenotypically and functionally. CD41 is the earliest marker expressed specifically by hematopoietic cells in the YS as early as E9.0 (Mikkola et al., 2003). CD45, pan-blood cell marker, is also expressed by hematopoietic cells in the YS (Goldie et al., 2008), AGM, and fetal liver (North et al., 2002). CD34 is another differentially expressed cell surface protein, which is expressed in fetal hematopoietic progenitors but downregulated as hematopoietic cells become more quiescent and in 7 week old mice CD34⁺ HSC start to emerge (Ogawa et al., 2001).

McKinney-Freeman et al. (2009) analyzed such differentially regulated cell surface markers using fetal and embryonic stem cell-derived HSC. Their results are consistent with the previous findings that CD41, CD45, and CD38 are differentially expressed by hematopoietic cells during development, and also support the idea that SLAM protein CD150 plays an important role in hematopoiesis. CD150 is not detected in E9.0 YS, E11.5 AGM, or E12.5 placenta HSC, but is expressed by fetal liver and adult bone marrow HSC (McKinney-Freeman et al., 2009). Furthermore, Mac-1, CD144, and AA4.1 are expressed by fetal liver, but not by adult bone marrow HSC (Morrison et al., 1995; Petrenko et al., 1999; Kim et al., 2005). Thus, fetal and postnatal HSC exhibit different phenotypes that likely reflect differences in origin, function, and regulation.

Unlike adult bone marrow HSC that are largely quiescent, 30% of fetal HSC are actively cycling. Quiescent and activated fetal HSC show differential cell surface protein expression. Quiescent HSC express CD38, which is then downregulated upon activation, concomitant with increased CD34 and Mac1 expression (Morrison et al., 1995; Rebel et al., 1996; Tajima et al., 2001).

Upon birth, most HSC are thought to be actively proliferating, but stop dividing and become quiescent within one week postnatally (Bowie et al., 2006). However, assessing HSC quiescence in vivo has proven to be difficult, and common in vivo labeling experiments utilizing bromodeoxyuridine (BrdU) incorporation into the newly synthesized DNA of replicating cells is unreliable for HSC labeling (Kiel et al., 2007a). Alternative methods, such as expression of Histone H2B–GFP fusion proteins, have been shown to label virtually all HSC and this technique, together with in vivo imaging, has enabled better monitoring of HSC behavior in vivo (Foudi et al., 2009). In fact, other recent progress in bioimaging has improved positional identification of HSC within marrow, revealing that they reside within two cell distances (10 μ m) from osteoblasts at the endosteal region (Kohler et al., 2009; Lo Celso et al., 2009; Xie et al., 2009).

BONE MARROW NICHE-HSC INTERACTIONS

HSC have to fulfill two critical tasks to function properly. First they have to maintain their number by self-renewing and second they must provide a constant blood supply by differentiating into all lineages when needed. While the embryo is growing, the requirement for blood cell types, in specific numbers, changes drastically to fulfill the metabolic requirements of the growing organism. This may be one reason why HSC develop within, and migrate through, different tissues during fetal development. It has been shown that the critical balance between self-renewal and differentiation of HSC is under tight control via

intrinsic and extrinsic factors that are modulated by their surrounding microenvironment. This microenvironment, or stem cell niche, consists of supporting cells (endothelial cells, osteoblasts, mesenchymal cells, reticular cells, etc.) and extracellular matrix. Within adult bone marrow, there are two main cellular components that have been demonstrated to maintain HSC function in vivo: osteoblasts and vascular endothelial cells (Fig. 3). Even though the majority of niche studies focuses on and favors the osteoblastic endosteal niche for maintaining HSC function in vivo, there is a growing body of data that support the critical role of bone marrow endothelial cells in the maintenance of HSC function, as well.

BONE MARROW OSTEOLASTIC NICHE

If there are specific niche cells that interact with and regulate HSC, their availability should be a determining factor in modulating HSC number within marrow. For osteoblasts, this was shown using genetic approaches to modulate their production within marrow. BMP signaling is known to promote osteoblast generation, and upon manipulation of BMP signaling via BMPRIA receptor, osteoblastic niche size was increased which, in turn, resulted in a ~2.4-fold increase in HSC number (Zhang et al., 2003). In addition, constitutively active PTH/PTHrP receptor (PPR), under control of an osteoblast-specific collagen promoter, also increases osteoblasts cell number, which results in ~2-fold expansion of HSC within marrow (Calvi et al., 2003). Parallel results are seen in wild type mice injected with parathyroid hormone (PTH) over 4 weeks (Calvi et al., 2003).

Although these studies indicate that osteoblasts play an important role(s) in regulating HSC, much work is needed to elucidate the molecular mechanism(s) by which osteoblasts modulate these effects. Various cell adhesion molecules and soluble factors have been proposed to mediate HSC and osteoblasts interactions within their endosteal niche. Among these, Notch, N-cadherin, stem cell factor (SCF), angiopoietin-1 (Ang-1), thrombopoietin (TPO), osteopontin (OPN), and WNT have been studied in detail and shown to play critical role in HSC regulation.

Notch

Notch receptor signaling between osteoblasts and HSC is required for HSC maintenance (Calvi et al., 2003). HSC attach to osteoblasts via interactions with Jagged-1, a Notch ligand (Calvi et al., 2003). Notch-Jagged-1 binding induces constitutive activation of parathyroid hormone receptor (PTHr), which results in increased HSC number. Notch signaling is thought to prevent HSC differentiation in favor of self-renewal which, in turn, leads to HSC expansion (Weber and Calvi, 2010). In further support of this idea, HSC exhibit active Notch signaling while interacting with osteoblasts in their niche; however, Notch signaling is downregulated in differentiated lineages (Duncan et al., 2005).

N-Cadherin

Both HSC and osteoblasts express N-cadherin, and bone marrow imaging studies suggest that spindle-shaped bone-lining osteoblasts (so-called SNO cells) communicate with HSC through N-cadherin interactions (Zhang et al., 2003). BrdU incorporation studies demonstrated that quiescent HSC with moderate N-cadherin reside close to osteoblasts

which have high N-cadherin expression (Haug et al., 2008). Furthermore, upregulation of N-cadherin on osteoblasts increases adherence of HSC on the endosteal surface, which is associated with HSC quiescence and diminished differentiation (Wilson et al., 2004).

The functions of both Notch and N-cadherin in HSC self-renewal and quiescence, respectively, are widely accepted in the HSC niche field; however, there are other reports that challenge their role as the major niche regulators. For example, when Notch1-deficient HSC is transplanted into a Jagged1-deficient bone marrow environment, they are still able to reconstitute lethally irradiated adult recipient mice (Mancini et al., 2005). These results suggest that there are other redundant Notch receptor and ligand partners that may participate in HSC self-renewal. In fact, one such combination, Notch/Delta-1/3/4, is required to maintain HSC in an undifferentiated state (Varnum-Finney et al., 2000; Duncan et al., 2005). Similarly, although there is a body of evidence suggesting N-cadherin regulates HSC maintenance, Kiel et al. (2007b) showed that N-cadherin, per se, is not necessary for HSC maintenance. A variety of molecular approaches, including qPCR, was used to evaluate N-cadherin expression in highly purified HSC, but its expression was not detected (Kiel et al., 2007b). In addition, HSC isolated from mice with conditionally deleted N-cadherin reconstitute lethally irradiated adult recipients, confirming the previous finding that N-cadherin is not required for homing and maintenance of HSC within the endosteal marrow niche (Kiel et al., 2009).

Stem Cell Factor

Early HSC characterization studies led to the discovery of a growth factor secreted by osteoblasts, called stem cell factor (SCF) that regulates HSC activity in vivo (Ikuta and Weissman, 1992; Miller et al., 1996; Miller et al., 1997) and self-renewal in vitro (Zandstra et al., 1997; Audet et al., 2002). HSC express a transmembrane receptor tyrosine kinase called stem cell factor receptor (C-Kit) that can bind to SCF, activating intracellular signaling important for HSC regulation.

Genes that produce SCF and C-Kit are referred to as the *Sl* and *W* loci, respectively. Mutations in the kinase domain of C-Kit result in reduced HSC activity (McCulloch et al., 1964). Mice carrying the *Sl* mutation, on the other hand, exhibit defects in the HSC niche (McCulloch et al., 1965), revealed by the fact that HSC from *Sl* mice could rescue lethally irradiated wild type recipients with a functional marrow niche (Barker, 1997). One of the downstream effectors of SCF, an adaptor protein referred to as *Lnk* regulates HSC self-renewal. *Lnk*^{-/-} mice exhibit increased numbers of HSC in vivo (Ema et al., 2005). HSC isolated from *Lnk*^{-/-} mice undergo more frequent self-renewal in vitro (Seita et al., 2007).

Angiopoietin-1

HSC express Tie2, a receptor tyrosine kinase that binds soluble effectors Ang-1 and -2 that are produced by osteoblasts (Arai et al., 2004). Tie-2 deficiency has no effect on fetal liver hematopoiesis and HSC migration to the bone marrow; however, *Tie2*^{-/-} HSC are depleted in bone marrow, suggesting that Ang-Tie-2 signaling may be required for bone marrow establishment or HSC quiescence (Puri and Bernstein, 2003). In fact, binding of Ang-1 to Tie-2 results in phosphorylation of Tie-2, which then activates the downstream

phosphatidylinositol 3-kinase (PI3-K)/Akt signaling pathway (Kim et al., 2000). PI3-K/Akt signaling activates cell survival pathways and cell cycle regulators including p21 that mediates growth arrest (Li et al., 2002). These anti-proliferation and antiapoptotic mechanisms may prevent HSC from metabolic exhaustion (Arai et al., 2004).

Thrombopoietin

Myeloproliferative Leukemia Virus Oncogene Receptor (Mpl) is a thrombopoietin (TPO) receptor that has higher expression in quiescent HSC relative to actively cycling HSC (Arai et al., 2009). Mpl-expressing HSC are thought to interact with TPO-expressing osteoblasts in the endosteal niche (Yoshihara et al., 2007), and interactions mediated through this signaling pathway are thought to maintain HSC quiescence (Arai et al., 2009). Mice deficient for either Mpl or TPO harbor far fewer HSC in the bone marrow than wild type littermates (Alexander et al., 1996; Kimura et al., 1998). In addition, administration of neutralizing Mpl antibodies disrupt HSC-niche interactions and result in decreased HSC numbers in vivo, whereas providing exogenous TPO results in transient increases in HSC number. These results suggest Mpl-TPO interactions play a role in HSC quiescence.

Osteopontin

Osteopontin (OPN) is an acidic glycoprotein secreted by osteoblasts, which regulates HSC via binding to integrin or CD44 receptors expressed on HSC. In vivo experiments in *OPN*^{-/-} mice demonstrate its role as a negative regulator of HSC proliferation, as there is an increase in total HSC number in an *OPN*^{-/-} microenvironment (Nilsson et al., 2005). Additionally, in the absence of OPN, there is abnormal HSC proliferation following parathyroid hormone administration (Stier et al., 2005). In the *OPN*^{-/-} microenvironment, there is also elevated stromal Jagged-1 and Ang-1 expression, which is associated with decreased HSC apoptosis. In contrast, medium supplemented with exogenous OPN bring HSC proliferation to a halt, providing further support for its antiproliferative effects on HSC (Nilsson et al., 2005). To further demonstrate this, *OPN*^{-/-} (and wild type) mice were fed BrdU for 4 weeks, during which time HSC from *OPN*^{-/-} mice incorporated BrdU, strongly suggesting a direct role of OPN in the maintenance of HSC quiescence via inhibition of cell cycle progression (Nilsson et al., 2005).

Wnt

Canonical Wnt signaling is active in adult bone marrow, presumably to promote self-renewal for the maintenance of an HSC reservoir (Reya et al., 2003). Supporting this hypothesis, retroviral induction of constitutive β -catenin signaling increases HSC number by 103-fold in long term cultures (Reya et al., 2003). In addition, in vitro treatment of HSC with recombinant Wnt3a leads to higher engraftment frequency upon transplantation, suggesting its role in HSC self-renewal (Willert et al., 2003).

Interestingly, these results obtained from in vitro studies are in conflict with in vivo experiments in which both conditional expression (Kirstetter et al., 2006) and deletion of β -catenin (Cobas et al., 2004) do not affect HSC self-renewal ability. Two other groups have also shown that constitutively active Wnt signaling inhibits HSC self-renewal, as well as differentiation in vivo (Kirstetter et al., 2006; Scheller et al., 2006). Such contradictory

results could be explained by involvement of redundant pathways in the absence of Wnt signaling, as well as crosstalk between Wnt signaling and other pathways in vivo.

In summary, the studies discussed above clearly reveal the role of osteoblasts, and effectors derived there from, in the regulation of HSC within the endosteal niche. However, none suggest that osteoblasts are the only cells within bone marrow that regulate HSC survival and fate. In fact, total ablation of osteoblasts within bone marrow has no effect on HSC number (Kiel et al., 2007b); thus, other cell types within the marrow niche must also play important roles in the regulation and maintenance of HSC.

BONE MARROW VASCULAR NICHE

Evidence during embryonic blood cell emergence, as well as later in adult bone marrow, collectively suggest that vascular endothelium also serves as an important component of the microenvironment necessary for the generation and maintenance of HSC. In fact, total elimination of the endosteal niche via local irradiation with radioisotope ^{89}Sr leads to a shift in hematopoiesis from the marrow to the spleen, which is a highly vascular extramedullary organ (Klassen et al., 1972). Furthermore, vascular endothelial cells give rise to HSC during embryonic development.

The current dogma in the adult hematopoietic biology field suggests that dormant, more quiescent HSC reside close to osteoblasts, whereas proliferating HSC are localized in close proximity to vascular endothelium. Although this is not definitely proven, phenotypically identical HSC (Lin-CD41-Sca1+C-KIT+CD48-CD150+ HSC) isolated from endosteal versus central regions of adult bone marrow exhibit different engraftment abilities (Grassinger et al., 2010). However, it is essential to keep in mind that vasculature is not compartmentalized to the central region of bone/marrow and, in fact, the endosteal region of bone is also vascularized. Therefore, the proposed osteoblast and vascular “niches” within marrow are not completely separable, and may function interdependently to generate and sustain HSC.

One rate limiting step in determining HSC localization in situ has been the need to use a complex set of phenotypic markers for their identification. However, Morrison’s group (Kim et al., 2006) was able to isolate HSC close to purity by using a triple SLAM marker combination (CD150+ CD48- CD41-). This scheme made it possible to label HSC in histological tissue sections, allowing further investigation of HSC colocalization relative to the osteoblasts and blood vessels. They found that 60% of HSC were in contact with sinusoidal endothelial cells, leading them to propose that sinusoidal vessels, specifically, serve as a vascular niche to regulate HSC function in bone marrow (Kiel et al., 2005). Although the role of vasculature has been shown to be important for HSC maintenance and regulation, the research elucidating the molecular mechanism of this interaction is still in its infancy. Here we will provide a summary of the major regulatory mechanisms proposed to mediate endothelial cell-HSC interactions, to date.

Cell-to-Cell Interactions

The role of endothelial cells, in general, in maintaining HSC has been demonstrated in vitro in adult and embryonic systems. Coculture of adult human bone marrow cells with human brain endothelial cells (HUBEC) increases their repopulating ability in SCID mice (Chute et al., 2002). Coculture of CD34-expressing human bone marrow cells with porcine microvascular endothelial cells also leads to ex vivo expansion of adult repopulating cells that successfully reconstitute SCID mice (Brandt et al., 1998). Primary endothelial cells (Tie-2-GFP, Flk-1+, and CD 41-) isolated from E9.5 YS and AGM also promote 9.4- and 11.4-fold increases, respectively, in hematopoietic progenitor production, as well as long-term repopulating ability (Li et al., 2003).

Interestingly, endothelial cells isolated from different tissues may possess differing abilities to regulate HSC. Tie2-GFP endothelial cells were isolated from nonhematopoietic adult mouse tissues, including brain, heart, lung, liver and kidney, and cocultured with adult HSC. The endothelial cells from the heart and liver maintained long-term repopulating ability; whereas, endothelial cells from the kidney were unsuccessful, even in the presence of hematopoietic growth factors (SCF, TPO, and IL6) (Li et al., 2004). Therefore, we have much to learn about the specific properties of vascular endothelial cells that generate and sustain HSC.

Vascular Cell Adhesion Molecules and Cytokines

The bone marrow vasculature is unique in terms of its cell adhesion molecule (CAM) expression. These specialized bone marrow endothelial cells have been shown to express E-selectin, P-selectin, VCAM1, and ICAM1, all of which are not expressed by other endothelial cells under homeostatic conditions, but only during inflammatory responses (Mazo et al., 1998). Focusing on calvarial flat bone, et al (2005) showed that the bone marrow vasculature contains unique regions that exhibit high expression of both E-selectin and SDF, both of which have been shown to mediate homing of circulating HSC. Whether the similar heterogeneous regions of the vasculature also exist in the long bone is not yet known.

SDF-1 and CXCL12 are among the most studied cytokines expressed by bone marrow endothelial cells (Sipkins et al., 2005), as well as stromal cells such as osteoblasts (Ponamaryov et al., 2000) and CXCL12-abundant reticular cells (CAR) (Sugiyama et al., 2006). Bone marrow stroma is relatively hypoxic and promotes constitutive expression of SDF-1 via its hypoxia-inducible factor 1 (*Hif-1*)-inducible promoter (Ceradini et al., 2004). SDF-1 and CXCL12 are thought to guide the migration and integrin-mediated binding of HSC, which express the CXCR4 receptor. SDF-1- and CXCR4-deficient mice exhibit hematopoietic failure in bone marrow, even though they have normal fetal liver hematopoiesis (Nagasawa et al., 1996; Zou et al., 1998; Ara et al., 2003).

Although a master regulator of angiogenesis, vascular endothelial growth factor (VEGF) signaling also regulates HSC homeostasis. There are two predominant receptor tyrosine kinases that mediate VEGF-A signaling: VEGFR1 (or Flt-1) and VEGFR2 (or Flk-1 or KDR). HSC, as well as endothelial cells, express both VEGF receptors (Kabrun et al., 1997;

Hattori et al., 2002). Gene deletion studies reveal the importance of VEGF-A signaling in both blood and vascular cells; VEGF-A- and VEGFR2-deficient mice die early in development, due to impaired hematopoietic and vascular development (Shalaby et al., 1995; Carmeliet et al., 1996; Ferrara et al., 1996).

HSC have also been shown to produce VEGF-A, suggesting possible regulation via autocrine signaling. In fact, blocking intracellular and extracellular components of VEGF-A signaling with neutralizing antibodies diminishes HSC colony formation (Gerber et al., 2002). Addition of VEGFR2 inhibitor to HSC in culture also blocks colony formation; however, neutralizing VEGF-A antibodies have no effect. These data suggest that intracellular autocrine VEGF-A signaling controls HSC behavior (Gerber et al., 2002).

Vascular endothelial cells also produce TGF- β and its growth inhibitory properties have been shown to control HSC quiescence in culture systems (Sitnicka et al., 1996; Garbe et al., 1997; Batard et al., 2000). Blocking TGF- β signaling with neutralizing antibodies causes HSC progenitors to enter cell cycle (Hatzfeld et al., 1991; Soma et al., 1996; Fortunel et al., 1998). The growth inhibitory role of TGF- β has also been demonstrated in vivo; TGF- β injection into mice results in decrease in HSC progenitor proliferation in bone marrow (Goey et al., 1989). In addition, human HSC with dominant-negative TGF- β RII exhibit increased proliferation and survival in vitro (Fan et al., 2002). These results suggest that TGF- β secreted by niche endothelial cells, or by HSC, plays an important role in HSC regulation (Kim and Letterio, 2003).

Other Regulatory Factors

As vascular endothelial cells gain more attention as a main cellular component of the HSC niche, we gain more insight about regulatory factors that they secrete, which potentially regulate HSC maintenance. Vascular endothelial-derived molecule adrenomedullin has been identified through gene ontology studies of human brain endothelial cells' (HUBEC) transcriptome and is suggested to play a role in promoting HSC progenitors in vitro (Chute et al., 2006). Zhang et al. (2008), using microarray approach, identified insulin-like growth factor binding protein-2 (IGFB2) and angiopoietin-like protein 5 (Angptl-5) specifically expressed by the mouse fetal liver derived endothelial cells, and showed that including these molecules in serum free media supplemented with SCF, TPO, and FGF1 increased human cord blood-derived HSC by 20-fold, compared to SCF, TPO, and FGF1 serum free media only. Furthermore, a recent genome wide expression analysis of HUBEC compared to non-brain human endothelial cells, revealed 13 upregulated genes that are likely to produce secreted proteins. Among these, *pleiotrophin* expression was found to be 25-times higher in HUBEC, compared to non-brain endothelial cells. The HSC progenitor (KSL) expresses the RPTP- β/ζ (pleiotropin receptor), further supporting the possible role of pleiotrophin in HSC regulation (Himburg et al., 2010).

In other studies, Butler et al (2010) investigated the functional role of sinusoidal vessels in HSC self-renewal using transgenic Notch reporter mice. When they impaired sinusoidal endothelial cell function via neutralizing antibodies for both VE-cadherin and VEGFR2, in vitro self-renewal as well as in vivo repopulating ability of HSC was diminished in parallel to downregulation of their Notch ligands. These results indicate that vascular endothelial

cells promote HSC self-renewal via VE-cadherin and VEGFR2 interactions. Vascular endothelial cell-derived SDF-1 and FGF4 have also been shown to mediate HSC progenitor interaction with the vascular niche and facilitate HSC differentiation (Avecilla et al., 2004). Taken together these data suggest that the balance between HSC self-renewal and differentiation is under the tight control of vascular endothelial cells in the HSC niche. Further studies need to be done to understand whether regulatory molecules identified in these studies similarly regulate HSC in the bone marrow niche.

Oxygen Gradient

One of the unique properties of bone marrow is its hypoxic microenvironment. Hypoxic zones have been proposed to give a long-term survival advantage to HSC by limiting their production of reactive oxygen species, which have deleterious and mutagenic effects on DNA stability. Quiescent cells also have lower metabolic requirements, enabling survival in a hypoxic state. HSC isolated from adult bone marrow using Hoechst dye exclusion (to isolate SP) and pimonidazole (PIM) staining (to identify hypoxic cells) revealed that the most primitive HSC, located at the tip of the SP profile, have the highest anti-PIM staining, suggesting that long-term HSC reside in a hypoxic environment (Parmar et al., 2007).

Whether the establishment of hypoxic zones in marrow is a result of the relative distance from vasculature or from the variation in local blood flow is not clear. PIM incorporation and BrdU labeling of cells within bone marrow revealed that the BrdU label-retaining cells in marrow localize near sinusoids that are thought to be relatively hypoxic due to low blood perfusion (Kubota et al., 2008) which, in turn, may allow higher local concentration of cytokines and growth hormone that regulates HSC function (Winkler et al., 2010).

CONCLUSIONS

The current knowledge on HSC phenotype and biology has enabled treatment of many hematological disorders. However, our understanding of the molecular mechanisms that regulate HSC maintenance within HSC niche is still lacking. Once outside their niche, “stemness” properties of HSC are lost in a short period of time; thus, limiting our ability to propagate HSC for cell therapies. Both osteoblasts and vascular endothelial cells play significant roles in regulating the delicate balance between HSC maintenance and differentiation. Evidence suggests that HSCs are distributed among different niches on the basis of their functional requirements provided by molecular signals from the niche they reside. Gaining a better understanding of the way niche maintains HSC in vivo is crucial to design novel in vitro HSC culture strategies to propagate them ex vivo for clinical uses to correct hematopoietic and vascular disorders.

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Figure 1.

Blood cells first emerge in the extraembryonic YS at ~E7.0. The placenta and embryonic AGM region exhibit de novo HSC generation potential at ~E10.5. At E11.0 HSC migrate into fetal liver where most HSC expansion takes place. Finally, HSC home to and reside within bone marrow at ~E16.5 onward.

Figure 2.

Primitive hematopoiesis, the generation of primitive erythroblasts, occurs within the extraembryonic YS. Definitive hematopoiesis begins within the YS at ~E8.25 and in AGM at ~E10.5, marked by the generation of adult repopulating HSC that Can differentiate into all mature blood cell lineages. EC: endothelial cells; BC: blood cells; SMC: smooth muscle cells.

Figure 3.

Self-renewal and differentiation properties of adult hematopoietic stem cells (HSC) are tightly regulated within their bone marrow niche. Osteoblasts and vascular endothelial cells are proposed to function as two main cellular components of the bone marrow niche. HSC interact with these cells types via indicated receptors and soluble effectors.