

The journey of developing hematopoietic stem cells

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Hematopoietic stem cells (HSCs) develop during embryogenesis in a complex process that involves multiple anatomical sites. Once HSC precursors have been specified from mesoderm, they have to mature into functional HSCs and undergo self-renewing divisions to generate a pool of HSCs. During this process, developing HSCs migrate through various embryonic niches, which provide signals for their establishment and the conservation of their self-renewal ability. These processes have to be recapitulated to generate HSCs from embryonic stem cells. Elucidating the interactions between developing HSCs and their niches should facilitate the generation and expansion of HSCs *in vitro* to exploit their clinical potential.

Introduction

The lifelong production of blood cells depends on hematopoietic stem cells (HSC) and their ability to self-renew and to differentiate into all blood lineages (Weissman, 2000). The original pool of HSCs is formed during embryogenesis in a complex developmental process that involves several anatomical sites (the yolk sac, the aorta-gonad-mesonephros region, the placenta and the fetal liver), after which HSCs colonize the bone marrow at birth (Figs 1, 2). During postnatal life, a steady state is established in which HSC pool size is maintained by the regulation of HSC self-renewal and differentiation. This is possible because the bone marrow contains specialized niches in which the multipotency of HSCs is conserved through cell divisions, while their progeny are directed towards lineage differentiation (Wilson and Trumpp, 2006). During homeostasis, most adult HSCs are quiescent and divide only rarely to maintain an appropriate quantity of differentiated blood cells and to renew the HSC pool (Cheshier et al., 1999). HSC pool maintenance and concomitant lineage differentiation are facilitated either by asymmetric self-renewal in which specific cell fate determinants are redistributed unequally to the two daughter cells; or via environmental asymmetry, in which one daughter cell leaves the niche that sustains HSC self-renewal and is then exposed to an environment that promotes lineage differentiation (Wilson and Trumpp, 2006). Interestingly, all LTR-HSCs (long-term reconstituting HSCs) in the adult bone marrow that can engraft a recipient upon transplantation are in G₀ phase (Passegue et al., 2005). Thus, although HSCs have to divide in order to self-renew, their cell division can only be safely completed within a correct niche; otherwise, their engraftment ability and survival is greatly challenged.

It has been a long-standing challenge to recapitulate *ex vivo* the correct micro-environment that supports HSC self-renewal. Cultured HSCs rapidly lose their ability to engraft and self-renew *in vivo*, which limits the options to maintain, expand or manipulate HSCs *in vitro* for therapeutic purposes. Likewise, attempts to use mouse or

human embryonic stem (ES) cells to generate HSCs that can permanently reconstitute the hematopoietic system of the recipient have not been successful, although differentiated hematopoietic cells can be generated with relative ease (Keller, 2005; Kyba and Daley, 2003; Wang et al., 2005a). So far, robust and sustained multi-lineage reconstitution of adult hematopoiesis from mouse ES cell-derived hematopoietic precursors has been obtained only by overexpressing the transcriptional regulators Hoxb4 and Cdx4 in mouse ES cells (Kyba et al., 2002; Wang et al., 2005b). Although these studies highlight important principles about the transcriptional programs that regulate key properties of definitive HSCs, a safer approach to generating HSCs for therapeutic applications would be to provide the appropriate signals from the environment where HSCs develop. In order to generate HSCs from ES cells, hematopoietic precursors have to go through the same developmental process as they do during embryogenesis. Thus, to learn how 'stemness' of HSCs can be established and maintained, it is crucial to define the cellular niches and key signals that support HSCs at each stage of development.

The challenge of fetal hematopoiesis is to generate differentiated blood cells that are immediately required for embryonic growth and development, and to establish concomitantly a stockpile of undifferentiated HSCs, even though the bone marrow and its specialized niches have not yet developed. Consequently, multiple anatomical sites participate in fetal hematopoiesis (Fig. 1). The shift of hematopoiesis from one location to another is required as the anatomy of the embryo changes during organogenesis. Furthermore, compartmentalizing fetal hematopoiesis into multiple sites might allow different inductive signals from the micro-environments to support the development of undifferentiated HSCs, while concomitantly generating mature blood cells in another location. The use of multiple fetal hematopoietic sites is common to many species, such as flies, amphibians, fish, birds, rodents and humans (Ciau-Uitz et al., 2000; Tavian and Peault, 2005b; Traver and Zon, 2002). To date, HSC development has been characterized in most detail in the mouse, which, in many respects, serves as a model for human hematopoiesis (Tavian and Peault, 2005a; Tavian and Peault, 2005b; Tavian and Peault, 2005c).

Embryonic hematopoiesis in mice starts after gastrulation, when a subset of specialized mesodermal precursor cells commit to becoming blood cells (Fig. 2). The first precursors migrate to the yolk sac to initiate embryonic red blood cell production, whereas definitive HSCs, which possibly originate from a different subset of mesodermal cells, develop in a different location (reviewed by Jaffredo et al., 2005). Importantly, the developing HSCs are required to complete a maturation process that permits their engraftment and survival in future hematopoietic niches. Furthermore, the initial HSC pool that emerges from hemogenic sites must expand to establish an adequate supply of HSCs for postnatal life. Consequently, fetal HSCs are largely cycling, and have to undergo symmetric cell divisions, in which both daughter cells retain self-renewal ability and multipotency, resulting in a net expansion of HSCs (Lessard et al., 2004). As fetal HSCs are markedly different from adult HSCs with respect to their cell cycle status and proliferative capacity, it is conceivable that different mechanisms control engraftment and self-renewal of HSCs during fetal and adult life.

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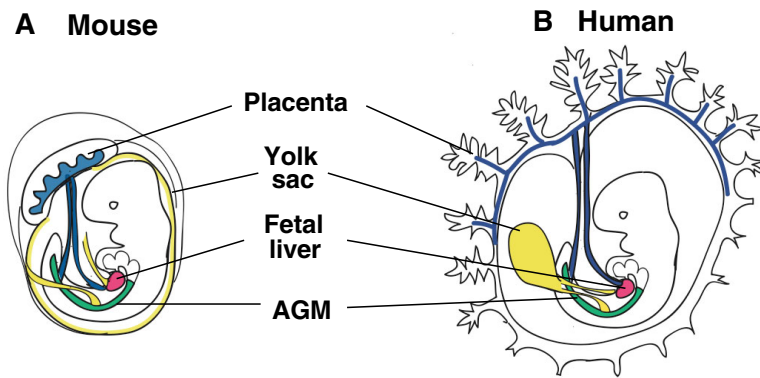


Fig. 1. Hematopoietic organs in mouse and human embryos. An illustration of (A) a mouse and (B) a human embryo, showing the hematopoietic organs at 11 days and 5 weeks of gestation, respectively (yellow, yolk sac; green, dorsal aorta; red, fetal liver; blue, umbilical vessels and fetal vasculature in the placenta). The hematopoietic function of the human placenta has not yet been experimentally proven. AGM, aorta-gonad-mesonephros region.

Multiple studies have documented that the AGM (aorta-gonad-mesonephros) region (see Figs 1, 2), which consists of the dorsal aorta, its surrounding mesenchyme and the urogenital ridges, is a source of definitive HSCs (Cumano et al., 1996; Godin and Cumano, 2002; Medvinsky and Dzierzak, 1996; Muller et al., 1994). Additionally, HSCs are formed in umbilical and vitelline arteries (de Bruijn et al., 2000). These HSCs probably colonize the developing fetal liver, which serves as the main organ for HSC expansion and differentiation until late fetal life, at which time bone marrow hematopoiesis is established. However, it has been questioned whether AGM-derived HSCs alone can supply the rapidly growing fetal liver HSC pool (Kumaravelu et al., 2002), or whether the yolk sac also supports the formation, maturation or expansion of HSCs later during development. Additional reservoirs for HSCs have been sought by mapping the anatomical distribution of HSCs in the embryo. Yet, no significant pools of HSCs have been found in the embryo proper (Kumaravelu et al., 2002). A novel perspective to HSC development came with the discovery that the murine placenta harbors a large pool of multipotential progenitors and HSCs during midgestation, indicating that the placenta has an important role in the establishment of HSCs (Alvarez-Silva et al., 2003; Gekas et al., 2005; Ottersbach and Dzierzak, 2005).

Despite the progress in identifying and quantifying HSCs in fetal organs, little is known about the origins of the different HSC pools and their relative contributions to adult hematopoiesis. It has been difficult to trace HSC precursors in mammalian embryos at earlier developmental stages when these cells express different surface markers compared with mature HSCs, and when they may not yet score in conventional transplantation assays. To facilitate the characterization of these developmental processes in the embryo, lineage- and developmental stage-specific mouse models have been generated (Gothert et al., 2005), and functional assays for immature HSC precursors have been improved (Table 1). Furthermore, our knowledge of the micro-environmental signals that support HSCs in the adult has improved rapidly, owing to gene-targeting studies that modify regulatory pathways that affect HSCs (Adams et al., 2006; Arai et al., 2004; Calvi et al., 2003; Wilson et al., 2004; Zhang et al., 2003). However, much work remains to be carried out in order to define the niches and signals that dictate specific stages of HSC development during fetal life.

This review follows the journey of developing HSCs through the fetal hematopoietic sites and focuses on the identification of the cells, their niches and the micro-environmental signals that are required to establish the pool of self-renewing HSCs for life.

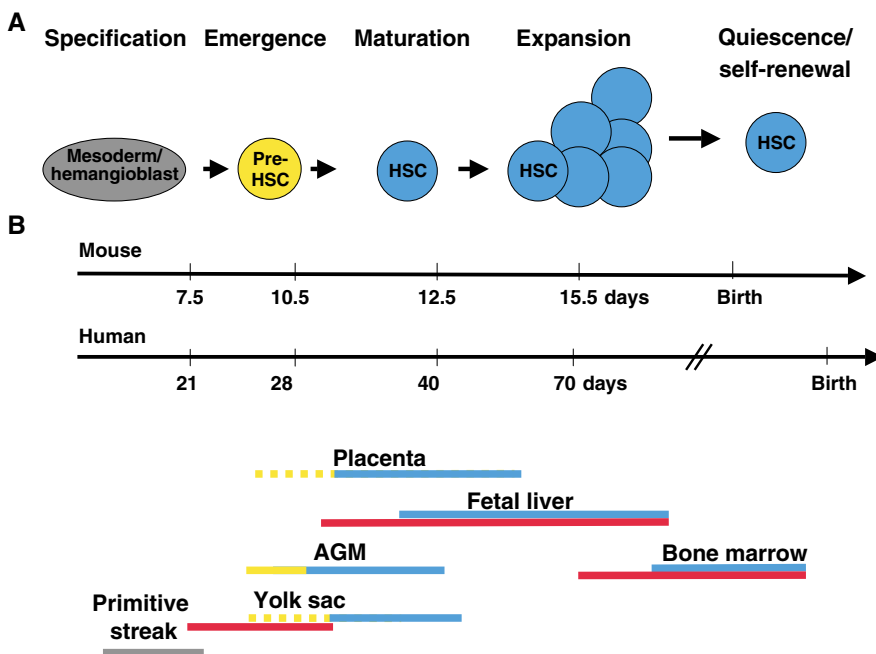


Fig. 2. Establishment of definitive hematopoietic stem cell (HSC) pools in mouse and human embryos.

(A) Hematopoietic development starts as specification of primitive streak mesoderm (gray) into hematopoietic and vascular fates. Nascent HSCs undergo a maturation process (blue) that allows them to engraft, survive and self-renew in future hematopoietic niches. Subsequently, fetal HSCs expand rapidly, after which a steady state is established in which HSCs reside in a relatively quiescent state in the bone marrow. (B) The ages at which mouse and human hematopoietic sites are active. Gray bars, mesoderm; red bars, active hematopoietic differentiation; yellow bars, HSC genesis; blue bars, presence of functional adult-type HSCs. Broken yellow bars for yolk sac and placenta indicate that de novo HSC genesis has not been experimentally proven.

Table 1. Identification of HSCs and their precursors during mouse ontogeny

Stage	Specification	Emergence	Maturation	Expansion	Steady state	Refs
Cell	Hemangioblast	Pre-HSC	HSC	HSC	HSC	
Location	Primitive streak?	Yolk sac? pSp/AGM Placenta?	Yolk sac? AGM Placenta	Placenta? Fetal liver	Bone marrow	Huber et al. (2004), Yoder et al. (1997a), Weissman et al. (1978), Medvinsky and Dzierzak (1996), Cumano et al. (1996, 2001), Gekas et al. (2005), Ottersbach and Dzierzak (2003)
Fetal age	E7.0-8.0	E8.5-10.5	E11.0-12.5	E11.5-16.5	Adult	
Markers	Brachyury ^{+/+} , Flk1 ⁺ , Scl ^{+/+}	Runx1 ⁺ , Cd41 ⁺ , Cd34 ⁺ , Cd45 ⁻ , VE- cadherin ⁺ , Sca1 ⁻ , Aa4.1 ⁺ , endomucin ⁺	Runx1 ⁺ , Cd41 ^{+/+} , Cd45 ⁺ , Cd34 ⁺ , VE- cadherin ⁺ , Sca1 ^{+/+} , Aa4.1 ⁺ , endomucin ⁺	Runx1 ⁺ , Cd45 ⁺ , Cd34 ⁺ , VE-cadherin ^{+/+} , endomucin ⁺ , Sca1 ⁺ , Mac1 ⁺ , Aa4.1 ⁺ , Cd150 ⁺ Cd48 ⁻	Runx1 ⁺ , Cd45 ⁺ , Sca1 ⁺ , Cd34 ⁻ , VE-cadherin ⁻ , endomucin ⁺ , Mac1 ⁻ , Aa4.1 ⁻ , Cd150 ⁺ Cd48 ⁻	Huber et al. (2004), Fehling et al. (2003), Chung et al. (2002), North et al. (2002, 2004), Mikkola et al. (2003), Ma et al. (2002), Bertrand et al. (2005), Matsubara et al. (2005), de Bruijn et al. (2002), Taoudi et al. (2005), Fraser et al. (2002), Kim et al. (2005), Kiel et al. (2005), Yilmaz et al. (2006), Ogawa et al. (2001), Jordan et al. (1990, 1995), Morrison et al. (1995), Rebel et al. (1996b)
Assay	Blast colony assay	Culture on OP9-stroma Explant culture Newborn transplantation <i>Rag2</i> ^{-/-} γ C ^{+/+} transplantation Transplantation to bone	Adult transplantation	Adult transplantation	Adult transplantation	Choi et al. (1998), Huber et al. (2004), Cumano et al. (1996, 2001), Medvinsky and Dzierzak (1996), Yoder et al. (1997a), Kumano et al. (2003), Matsubara et al. (2005)

Markers that change during ontogeny are displayed.

Abbreviations: AGM, aorta-gonad-mesonephros region; E, embryonic day; HSC, hematopoietic stem cell; Mac1, Cd11b; pSp, para-aortic splanchnopleurae; Rag2, recombination activating gene 2; Runx1, runt-related transcription factor 1; Sca1, Ly6a; VE-cadherin, vascular endothelial cadherin (cadherin 5).

The roadmap from mesoderm to hematopoietic fate

A long-standing hypothesis posits that hematopoietic cells originate from the hemangioblast, a mesodermal precursor cell that gives rise to blood and endothelial cells (Sabin, 1917). The existence of a clonal precursor that has both hematopoietic and vascular potential was first demonstrated *in vitro* by differentiating mouse ES cells (Choi et al., 1998; Kennedy et al., 1997). These cells identified a precursor that develops between days 2.5–4 of embryoid body (EB) differentiation and forms blast-like colonies (named blast colony forming cell, BL-CFC). These precursors give rise to primitive and definitive hematopoietic, endothelial and vascular smooth muscle cells. All BL-CFCs were found among the brachyury⁺ Flk1⁺ (Kdr⁺; kinase insert domain protein receptor) cells, indicating that they represent a specialized subset of mesoderm (Fehling et al., 2003) (Table 1), whereas subsequent commitment into hematopoietic fate is driven, and marked by, expression of Scl/Tal1 (T-cell acute lymphocytic leukemia 1) (Chung et al., 2002; D'Souza et al., 2005; Porcher et al., 1996; Shivdasani et al., 1995). Importantly, Flk1⁺ brachyury⁺ hemangioblasts were also found in the gastrulating mouse embryo (Huber et al., 2004); however, surprisingly few of the BL-CFCs that displayed both hematopoietic and vascular potential were found in the yolk sac. Instead, most of them were in the posterior primitive streak. Yet the ability to form primitive erythroid cells suggested that these hemangioblasts were precursors of yolk sac hematopoietic cells. These results imply that the first stages of

hematopoietic development take place before the cells migrated into the yolk sac, prior to the formation of the blood islands, which consist of developing primitive red cells and endothelial cells adjacent to visceral endoderm (Palis and Yoder, 2001). This model conflicts with a longstanding hypothesis that hemangioblasts reside in the yolk sac and form both hematopoietic and endothelial cells within a blood island. The migration of hematopoietic precursors from the primitive streak to yolk sac was shown to depend on vascular endothelial growth factor signaling through Flk1, as *Flk1*-knockout cells in chimeric mouse embryos accumulate in the amnion (Shalaby et al., 1997; Shalaby et al., 1995). Further studies have shown that specification of hematopoietic fate in the yolk sac depends on visceral endoderm and on signals from *Ihh* (Indian hedgehog) (Baron, 2001; Dyer et al., 2001) and *Bmp4* (bone morphogenetic protein 4) (Sadlon et al., 2004), demonstrating the importance of a proper micro-environment for hematopoietic commitment.

It remains to be shown whether all yolk sac hematopoietic cells and the definitive HSCs that emerge in the embryo, and possibly the placenta, originate from hemangioblasts. Indeed, multiple pathways might specify hematopoietic cells from mesoderm. The inherent differences between hematopoietic programs in the different sites, i.e. the ability of the yolk sac to generate primitive erythroid cells, and the delay in lymphoid potential, suggest that micro-environmental cues in sites of early hematopoietic specification may later dictate the ultimate output of the different hematopoietic

programs. The developmental stage at which the transient embryonic and definitive adult hematopoietic programs first diverge at the molecular level has yet to be defined.

The yolk sac as a source of embryonic blood cells

In mice, the yolk sac is essential for the development of the embryo as it provides the initial feto-maternal transport system before the placenta is formed, and is the source of the first blood cells (Ferkowicz and Yoder, 2005; Li et al., 2003; McGrath and Palis, 2005). It produces the first primitive erythroid cells that enter the circulation and the later definitive progenitors destined for the liver (reviewed by McGrath and Palis, 2005). Both hematopoietic progenitor populations originate within a well-defined region in the proximal yolk sac (Ferkowicz et al., 2003; McGrath et al., 2003). Primitive erythrocytes develop from precursors that are present in the yolk sac between E7.0 and 8.25, and express low levels of Cd41 (GpIIb, integrin α 2b; Itga2b – Mouse Genome Informatics). The hallmarks of the primitive erythroid lineage are retention of the nucleus while entering circulation, a large size, and the expression of both embryonic and adult globins (Ferkowicz et al., 2003; Palis et al., 1999). However, recent studies have shown that primitive erythrocytes enucleate in the embryonic circulation, concomitantly downregulating β H1-globin transcripts, while activating ϵ γ -globins and upregulating adult β -chains (Kingsley et al., 2006; Kingsley et al., 2004).

The definitive myeloerythroid progenitors develop in the mouse yolk sac slightly after primitive erythroid precursors (Cumano et al., 1996; Palis et al., 1999). These progenitors can be first identified by expression of Cd41 and Kit (receptor for stem cell factor), whereas

the pan-hematopoietic marker Cd45 (Ptpcr; protein tyrosine phosphatase receptor C) is turned on during their progressive maturation (Mikkola et al., 2003). As the yolk sac micro-environment does not support terminal differentiation into definitive blood cell lineages, these progenitors seed the fetal liver where they generate blood cells for the growing embryo. The definitive red cells that are derived from these precursors are smaller than primitive erythroid cells, express only adult globins and enucleate before entering the circulation.

Despite the abundant production of hematopoietic progenitors in the yolk sac, it is still unclear whether the yolk sac also generates HSCs. The main data arguing against this come from explant culture studies, which show that early yolk sac explants do not have the potential to generate adult reconstituting HSCs or lymphoid progeny (Cumano et al., 1996; Cumano et al., 2001; Medvinsky and Dzierzak, 1996) (Fig. 3). An alternative theory is that the yolk sac micro-environment becomes supportive for HSC development later on, as, by E12.5, yolk sac explant cultures can support HSC expansion in vitro (Kumaravelu et al., 2002). Other studies have shown that intrahepatic transplantation of yolk sac hematopoietic cells into conditioned newborn recipients or direct transplantation into the pre-circulation yolk sacs, permits engraftment of E9.5 or even E8.5 yolk sac cells, respectively (Weissman et al., 1978; Yoder et al., 1997a; Yoder et al., 1997b). Although these findings suggest that immaturity of the yolk sac hematopoietic cells accounts for their inability to survive in an adult environment, their relative contribution to adult hematopoiesis during normal development remains unclear. It is also possible that some of the yolk sac HSCs are derived from the vitelline artery or upper dorsal aorta, which are

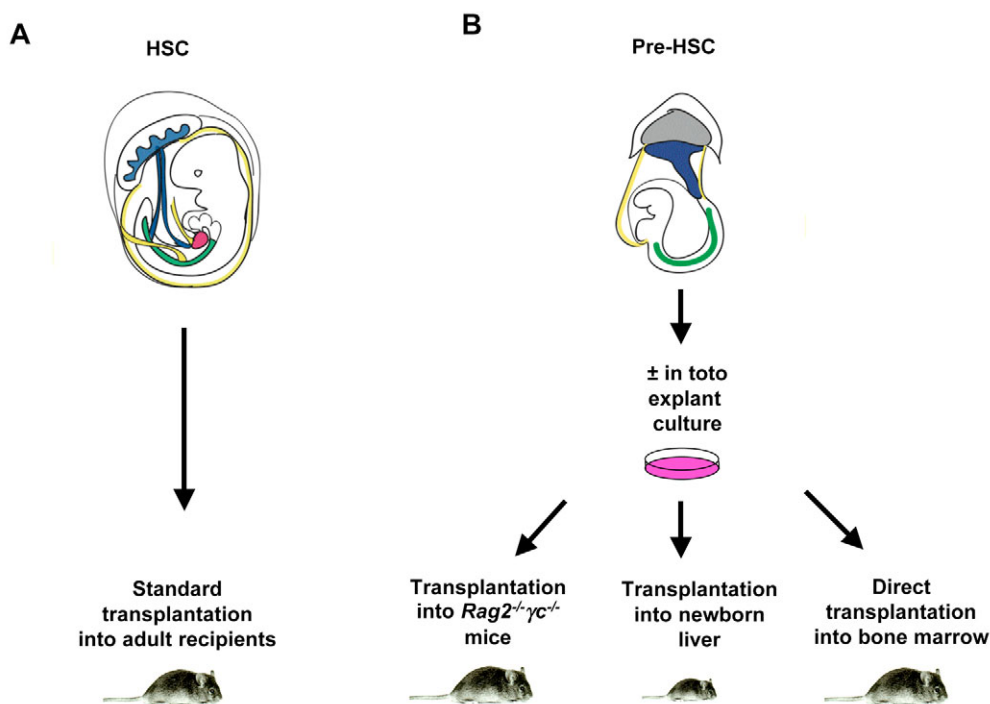


Fig. 3. Assays for fetal hematopoietic stem cell (HSC) development. (A) (Top panel) An E10.5-11 mouse embryo. HSCs can be reliably assayed only by transplanting them into myeloablated (e.g. irradiated) recipients (lower panel) and then documenting their ability to give rise to sustained (3-6 months) multilineage hematopoietic reconstitution. Functional adult-type HSCs that can achieve such reconstitution in this assay are found only in mouse embryos or extra-embryonic tissues after E10.5-11.5. (B) (Top panel) A mouse embryo at E9.0. HSC activity can be observed at a younger age, and higher hematopoietic chimerism from immature HSCs (pre-HSCs) can be obtained if more permissive recipients (e.g. newborn mice, *Rag2^{-/-}γC^{-/-}* mice, lower left) are used. Transplantation directly into the long bones of a recipient can also improve engraftment (lower right). Intermediate explant culture of pSP cells also allows detection of HSC activity at a younger age.

upstream of the yolk sac in circulation (de Bruijn et al., 2000). Nevertheless, as the yolk sac does not harbor a large quantity of HSCs, it is unlikely to support large-scale expansion of HSCs in vivo (Gekas et al., 2005).

Emergence of HSCs in the embryo proper

The first evidence that HSCs may develop in the embryo proper came from chick-quail chimera studies, in which HSCs derived from the embryo outcompeted yolk sac hematopoietic cells in their contribution to adult hematopoiesis (Lassila et al., 1978; Martin et al., 1979). Subsequently, intra-embryonic HSCs have been documented in multiple species (Jaffredo et al., 2005). In all species described, the intra-embryonic HSCs develop in close association with the ventral wall of the dorsal aorta, or in adjacent vitelline and umbilical arteries. This region, which is first called the para-aortic splanchnopleurae (pSP), and later becomes the aorta-gonad-mesonephros region (AGM), develops from the lateral plate mesoderm (LPM).

Hematopoietic progenitor cells appear in the pSP-region of mouse embryos by E8.5, yet functional HSCs that can reconstitute adult recipients are not found until E10.5-11.0 (Cumano et al., 1996; Godin and Cumano, 2002; Jaffredo et al., 2005; Medvinsky and Dzierzak, 1996; Muller et al., 1994) (Figs 2, 3; Table 1). Notably, at E10.5, the repopulation activity of AGM-derived HSCs in standard adult reconstitution assays is very low, whereas their transplantation into recombination activating gene 2 (*Rag2*)^{-/-} common γ chain (γ c)^{-/-} mouse recipients, which are deficient in B, T and natural killer cells, allows hematopoietic reconstitution at an earlier age and at a higher level (Cumano et al., 2001) (Fig. 3, Table 1). It was, thus, hypothesized that lack of major histocompatibility complex class I molecule expression in nascent HSCs may make them targets for natural killer cells. Furthermore, transplantation of E10.5 HSCs directly into the long bones improves hematopoietic reconstitution, suggesting that the homing and engraftment properties of young HSCs are poorly developed at this stage (Matsubara et al., 2005). When AGM cells are transplanted into conditioned newborn recipient mice, in which the liver is still an active hematopoietic organ, HSC activity could be detected at E9.5 (Kumano et al., 2003) (Fig. 3, Table 1), leading to the speculation that younger micro-environments are more supportive of fetal HSCs. Importantly, transplantable HSCs could be generated from the pSP region of pre-circulation embryos (E8.5) when the tissues were cultured as whole tissue explants, indicating that intra-embryonic HSCs develop in situ, rather than being imported via the circulation (Cumano et al., 2001) (Fig. 3, Table 1). Furthermore, stromal cells lines derived from the AGM and surrounding regions support HSCs in culture (Kusadasi et al., 2002; Oostendorp et al., 2002; Oostendorp et al., 2005). These data suggest that the pSP-AGM region is a source of HSCs, but then a maturation process is required to confer on them the engraftment and self-renewal abilities in the adult bone marrow micro-environment. As the phenotype of developing HSCs changes during this process, it is crucial to define the means to identify, localize and purify developing HSCs at all stages in order to ultimately define the molecular mechanisms of HSC maturation.

Markers of developing HSCs

Cd41 is the first and most specific surface marker that distinguishes cells that are committed to hematopoietic lineage from endothelial and other mesodermal cell types (Corbel and Salaun, 2002; Corbel et al., 2005; Ferkowicz et al., 2003; Mikkola et al., 2003; Mitjavila-Garcia et al., 2002) (Table 1). However, Cd41 is expressed only in nascent HSCs, whereas mature HSCs lose Cd41 expression, and

some lineage-committed progenitors and megakaryocytes maintain it (Bertrand et al., 2005; Kiel et al., 2005; Matsubara et al., 2005). Conversely, immature HSCs do not express the pan-hematopoietic marker Cd45, which appears by E11.5 (Matsubara et al., 2005). At this stage, Sca1 (Ly6a – Mouse Genome Informatics), a hallmark of adult type HSCs, is also upregulated (de Bruijn et al., 2002; Matsubara et al., 2005; Spangrude et al., 1988) (Table 1). Vascular endothelial cadherin (cadherin 5), a marker associated with endothelial cells, is expressed transiently during the emergence and maturation of HSCs (Fraser et al., 2002; Kim et al., 2005; Taoudi et al., 2005). Markers that are consistently expressed both in immature and mature fetal HSCs include Cd34, Cd31 (Pecam1), Kit and endomucin, although their expression is not restricted to hematopoietic cells (Baumann et al., 2004; Matsubara et al., 2005; Yoder et al., 1997a). A combination of signaling lymphocyte activation molecule (SLAM) family receptors (Cd150⁺Cd244⁺Cd48⁻), which have been previously associated with lymphocyte proliferation and activation, has been recently shown to mark HSCs from fetal liver stage through adult hematopoiesis, including mobilized and aging HSCs (Kiel et al., 2005; Kim et al., 2006; Yilmaz et al., 2006) (Table 1). Thus, SLAM markers introduce a code that may be more generally applicable for identifying HSCs. However, it has yet to be studied whether the same SLAM code already marks HSCs at their emergence.

As developing HSCs share specific characters with endothelial cells, such as surface marker expression and the ability to incorporate acetylated LDL (low density lipoprotein) (Sugiyama et al., 2003), it has been speculated that HSCs emerge from a subtype of endothelial cells, called hemogenic endothelium (Jaffredo et al., 2005). Furthermore, histological sections have revealed hematopoietic clusters budding from the ventral wall of the dorsal aorta and adjacent large vessels. Alternative hypotheses suggest that the intra-embryonic HSCs originate from the mesoderm/mesenchyme adjacent to the aorta (North et al., 2002) or from sub-aortic patches (Bertrand et al., 2005), and migrate through the endothelium into the lumen of vessels, concomitantly maturing to definitive HSCs. Indeed, individual cells in sub-aortic patches express surface markers that are characteristic of early hematopoietic precursors/immature HSCs [e.g. Cd41⁺, Kit⁺, Cd45⁻, Cd31⁺, Aa4.1⁺ (Cd93)], and display the potential to reconstitute sublethally irradiated *Rag2*^{-/-} γ c^{-/-} mouse recipients. These results imply that hematopoietic specification in the pSP/AGM region may occur before precursors cells contact the aortic endothelium.

Mice in which the *Runx1* (runt-related transcription factor 1) has been targeted (which encodes a transcription factor that is essential for definitive hematopoiesis) have been instrumental in defining and visualizing the genesis of HSCs (North et al., 1999; North et al., 2002; North et al., 2004; Okuda et al., 1996). *Runx1* is expressed in HSCs from the emergence of HSC precursors throughout ontogeny, affording the opportunity to identify candidate HSCs by, for example, studying *Runx1-lacZ* mouse embryos, in which *lacZ* has been targeted into the *Runx1* locus (Fig. 4). Interestingly, *Runx1-lacZ* cells localize both to the endothelium and mesenchyme adjacent to the large vessels. Although not all *Runx1*-expressing cells are likely to be HSCs, it has been suggested that the *Runx1*-expressing cells in aortic mesenchyme harbor precursors for HSCs that subsequently passage through the endothelium and are released into the circulation (Fig. 4). However, *Runx1* haploinsufficiency leads to changes in HSC kinetics and surface markers, such as abnormal HSC distribution and a delay in upregulating Cd45 expression (Cai et al., 2000; North et al., 2002). Thus, it remains to be shown whether the mesenchymal distribution of *Runx1-lacZ* cells

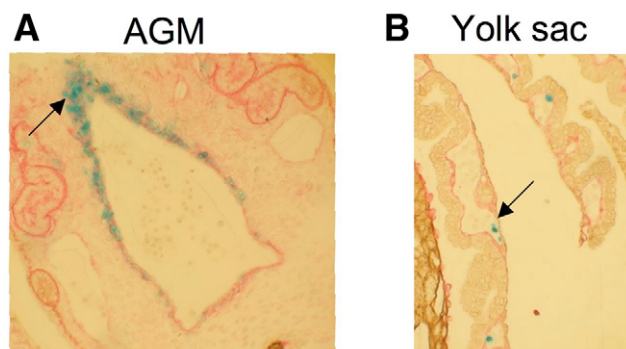


Fig. 4. Localization of developing hematopoietic stem cells (HSCs) in the mouse embryo. Immunohistochemistry of E10 *Runx1-lacZ* mouse embryo shows *Runx1-lacZ*-expressing cells (blue) are present in (A) the dorsal aorta and the adjacent mesenchyme (ventral, top left-hand corner), and (B) the yolk sac. Although not all *Runx1*-positive cells are HSCs, all developing HSCs and progenitors express *Runx1* and are therefore postulated to reside within the *Runx1*-expressing cell population. *Runx1-lacZ*, blue; laminin, red. Arrows indicate *Runx1-lacZ*-expressing cells. Images provided by H.K.A.M.

reflects normal HSC development. Interestingly, *Runx1-lacZ* knockout embryos, which die by E12.5 because of lack of definitive hematopoietic cells, also express *lacZ* in cells in the mesenchyme adjacent to the dorsal aorta from E10.5 onwards. This expression could mark the precursors that attempt to generate HSCs but are unable to do so in the absence of functional *Runx1* protein (North et al., 1999).

Zebrafish studies have established a link between Notch signaling and *Runx1* during HSC emergence, suggesting that Notch functions upstream of *Runx1*. In mice, *Notch1* is important for intra-embryonic definitive hematopoiesis, whereas yolk sac hematopoiesis is *Notch1* independent (Hadland et al., 2004; Kumano et al., 2003; Robert-Moreno et al., 2005). Notch signaling affects crucial cell fate decisions during fetal and adult hematopoiesis, influencing HSC self-renewal and lymphoid differentiation (Allman et al., 2002; Milner and Bigas, 1999; Ohishi et al., 2003). Importantly, the output of Notch signaling is linked to developmental timing and to the differentiation status of the target cell, demonstrating the complexity of the regulatory mechanisms that dictate the fate of HSCs.

Role of the placenta in establishing a definitive HSC pool

The placenta is essential for fetal development as it is responsible for fetomaternal exchange from midgestation and produces important hormones and cytokines (Cross et al., 2003; Rossant and Cross, 2001). Although the placenta has not been considered to be a hematopoietic organ, early reports suggested that the mouse placenta may exhibit hematopoietic activity (Dancis et al., 1977; Dancis et al., 1968; Melchers, 1979; Till and McCulloch, 1961). Studies in avian embryos have also revealed that the allantois, a meso-dermal appendage that functions as an excretory and respiratory outlet for the embryo, generates hematopoietic cells that, in quail-chick grafting studies, seed the bone marrow (Caprioli et al., 1998; Caprioli et al., 2001). In mice, the allantois forms the umbilical vessels and the mesodermal parts of the fetal placenta (Downs and Harmann, 1997). It develops after gastrulation as an extension of the posterior primitive streak and fuses with the chorionic plate by E8.5, whereafter the allantoic mesoderm interdigitates into the trophoblast

layer of the placenta giving rise to the fetal vasculature in the placental labyrinth. Within the labyrinth, fetal and maternal blood spaces come into close proximity, facilitating the exchange of gases and nutrients. Although the umbilical artery, which connects the dorsal aorta to the placenta, has been suspected to generate HSCs, HSC activity in the placenta has not been comprehensively studied until recently.

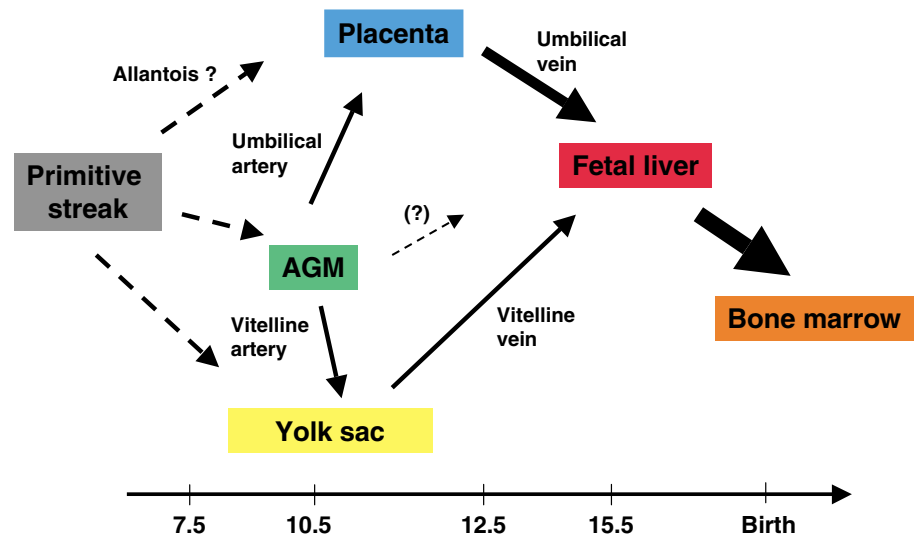
Hematopoiesis in the mouse placenta is evident from E9.0, when definitive multi-lineage progenitors appear (Alvarez-Silva et al., 2003), whereas mature HSCs are found 1.5–2.0 days later (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Placental HSCs are derived from fetal cells, and fulfil the most stringent functional criteria for adult type HSCs (Fig. 3). The early onset of HSC activity in the placenta, which is evident before HSCs are found in the fetal circulation or liver, suggests that placental HSCs may be generated in situ. If the placenta produces HSCs, a possible origin is the allantoic mesoderm. Although hematopoiesis has not yet been documented to occur in the early mouse allantois, this possibility needs to be investigated by assays that detect nascent hematopoietic precursors at an earlier stage of development than can conventional in vitro or in vivo hematopoietic assays (Fig. 3, Table 1). If allantoic mesoderm proves to be the source of placental HSCs, the placenta might generate HSCs by mechanisms similar to those in the pSP-AGM region, thereby extending the hematopoietic activity in the dorsal aorta and the large vessels to a larger anatomical area. Indeed, grafting experiments with genetically marked allantoises have shown that the allantoic mesoderm and the LPM that forms the AGM region have a similar developmental potential, in contrast to the paraxial mesoderm that gives rise to the somites (Downs and Harmann, 1997). However, vascularization of the allantois proceeds from the distal tip to the base, rather than by angiogenic sprouting from the dorsal aorta (Downs et al., 2004), supporting an independent origin of vascular cells, and possibly also of hematopoietic cells, from the allantoic mesoderm.

A striking feature of placental HSCs is the rapid expansion of the HSC pool between E11.5 and 12.5. As a result, the placenta harbors over 15-fold more HSCs than does the AGM region or the yolk sac, suggesting that the placenta may provide a unique micro-environment for HSC development (Gekas et al., 2005). The growth of the placental HSC pool may have various explanations, such as sustained production of HSCs in the placenta, symmetric self-renewal of placental HSCs or maturation of nascent HSC precursors into transplantable HSCs. Importantly, the expansion of the HSC pool during E11.5–12.5 was more pronounced than the relative expansion of the clonogenic progenitor pool, suggesting that the placenta micro-environment supports HSCs without concomitant differentiation into downstream progeny (Gekas et al., 2005).

It is also possible that HSCs originating from the AGM contribute to the expansion of the placental HSC pool. As the main vascular route by which the blood cells from the dorsal aorta circulate into the fetal liver goes through the umbilical vessels and the placenta (Sadler, 2006), this is also the most likely route for AGM-derived HSCs take (Fig. 5). Thus, it is possible that AGM HSCs are nurtured temporarily in the placental niches prior to seeding the fetal liver. Interestingly, placental HSC activity is increasing, HSCs accumulate in the fetal liver. After E13.5, however, the number of HSCs in the placenta decreases, while the liver HSC pool continues to expand (Gekas et al., 2005). As the fetal liver is directly downstream of the placenta in fetal circulation, the placenta may supply a major fraction of the HSCs that seed the liver (Fig. 5).

Fig. 5. Migratory and circulatory routes that connect fetal hematopoietic sites.

The primitive streak (gray) gives rise to the hemogenic mesoderm/hemangioblasts that migrate into the yolk sac (yellow), para-aortic splanchnopleurae (pSP)/aorta-gonad-mesonephros (AGM) region (green) and possibly through the allantois to the placenta (blue). Hematopoietic specification most probably occurs during the migratory process. The two main circulatory routes that connect fetal hematopoietic organs during midgestation are vitelline and umbilical circuits. The vitelline artery connects the upper dorsal aorta to the yolk sac, which connects to the fetal liver (red) via the vitelline vein. The umbilical artery connects the caudal part of dorsal aorta to the placenta, which connects to the liver via the umbilical vein. Although budding into the lumen and seeding through



circulation has been hypothesized as the main route by which nascent HSCs seed the fetal liver, direct migration of hematopoietic stem cells (HSCs) from the AGM to the fetal liver has not been formally excluded (indicated by '?'). Broken arrows indicate the migration of HSC precursors and unbroken arrows indicate circulation of HSCs through vasculature. Bone marrow (orange) is seeded by HSCs before birth. Larger black arrows indicate major HSC trafficking. The timing of these events is outlined by the timescale in embryonic days below.

Placental HSCs have a similar surface phenotype to fetal liver HSCs, both of which express Cd34, Kit and Sca1 at E12.5 (Gekas et al., 2005; Ma et al., 2002; Ottersbach and Dzierzak, 2005). However, despite the phenotypic similarity of placental and fetal liver HSCs, the midgestation placenta is not occupied by numerous single-lineage progenitors and definitive erythroid intermediates (as is the fetal liver, which actively promotes erythropoiesis) (Gekas et al., 2005). However, it remains possible that the placenta functions as a fetal lymphoid organ, as it has been shown that B-cell precursors appear in the mouse placenta before they are found in the fetal liver (Melchers, 1979). Cells that have the potential to generate B-lymphoid cells in plaque-forming assays were found in the placenta as early as E9.5; their number peaked at E12.5 and then declined, displaying very similar kinetics to those observed for placental HSCs (Gekas et al., 2005). It remains to be shown whether this reflects the presence of HSCs in the placenta, or whether differentiation into B-lymphoid cells occurs during their residence in the placenta.

Little is known about the cellular niches that support HSCs in the placenta, or about the signals involved in their maturation, expansion and mobilization. When the placental HSC pool grows, the placenta is hypoxic, consistent with the notion that HSCs develop in hypoxic environments, and the signals that drive vasculogenesis are shared with developing HSCs. Furthermore, many signaling molecules and cytokines that have been linked to HSC biology are expressed in the extra-embryonic ectoderm/placental trophoblasts or allantoic mesoderm/endothelial cells (Calvi et al., 2003; Clemens et al., 2001; Fujiwara et al., 2001; Hattori et al., 2002; Sibley et al., 2004; Zhang and Lodish, 2004). Further functional studies are required to identify the crucial niche cells and molecular cues that support HSC development in the placenta.

Fetal liver in supporting HSC expansion and differentiation

The fetal liver is the primary fetal hematopoietic organ and the main site of HSC expansion and differentiation. However, it does not produce HSCs *de novo*, but is believed to be seeded by circulating hematopoietic cells (Houssaint, 1981; Johnson and Moore, 1975). The first phase of fetal liver seeding initiates at E9.5-10.5, as the liver

rudiment becomes colonized by myeloerythroid progenitors that generate definitive erythroid cells. This first wave of hematopoietic seeding most probably derives from the yolk sac, which has prepared numerous definitive progenitor cells and establishes the first vascular connections to the fetal liver through vitelline vessels (Fig. 5).

The first HSCs appear in the fetal liver at E11.5. Although not experimentally proven, it is likely that majority of the HSCs colonizing the liver derive from the AGM and the placenta via the umbilical vessels, which is the second major vascular circuit that connects to the fetal liver (Fig. 5). After E12.5, the fetal liver becomes the main fetal organ where HSCs undergo expansion and differentiation. The number of HSCs reaches a maximum of ~1000 HSCs by E15.5-16.5, after which it reaches a plateau and starts to decline (Ema and Nakauchi, 2000; Gekas et al., 2005; Morrison et al., 1995). At all times, the fetal liver is rich in single-lineage progenitor cells, consistent with its important role in producing differentiated blood cells. As gestation proceeds, the main focus of hematopoietic differentiation changes. The early fetal liver is rich in CFU-Es (colony-forming unit erythroid) and proerythroblasts, reflecting active definitive erythropoiesis, whereas myeloid and lymphoid progenitors accumulate with developmental age. It is conceivable that the fetal liver microenvironment is modified during fetal development in order to meet the changing needs of lineage differentiation and HSC expansion. Conversely, the difference in fetal liver hematopoietic profile during mid- and late gestation may reflect the lifespan of the precursor cells that originally seed the organ, as mouse models that target transient fetal hematopoietic populations have shown that the early fetal liver is occupied by a progenitor population that may not contribute to adult hematopoiesis (Emambokus and Frampton, 2003; Li et al., 2005).

Little is known about the fetal liver niches that support HSC expansion and differentiation. Interestingly, the HSC pool expands rapidly in the fetal liver, whereas in the bone marrow, most HSCs are quiescent. Indeed, fetal liver HSCs are actively cycling, and outcompete adult bone marrow HSCs when transplanted into irradiated recipients (Harrison et al., 1997; Morrison et al., 1995; Rebel et al., 1996a), suggesting that inherent differences exist between fetal and adult HSCs. Conversely, the fetal liver microenvironment

may also provide signals that promote symmetric self-renewing divisions of the fetal HSC pool. Investigators have attempted to define the key components of the fetal liver micro-environment that support HSC expansion by establishing stroma cell lines from midgestation mouse liver cells (Hackney et al., 2002). By comparing the expression profiles of fetal liver-derived stroma cell lines with different abilities to support expansion of HSCs, multiple differentially expressed genes have been identified that probably play an important role in the *in vivo* fetal liver HSC niche. The functions of the individual differentially expressed genes in HSC biology have yet to be defined.

Another study showed that Cd3⁺Ter119(Ly76)⁻ cells in the liver can support HSC expansion in co-culture (Zhang and Lodish, 2004). Although the identity of the supportive cells is unknown, gene expression analyses identified Igf2 (insulin-like growth factor 2) and angiopoietin-like proteins as key molecules secreted by these cells (Zhang et al., 2006; Zhang and Lodish, 2004). When applied alone, or in combination, Igf2 and angiopoietin-like proteins support HSC expansion and/or survival in culture. It remains to be shown whether the fetal liver Cd3⁺ cell population and the secreted factors are unique to fetal liver microenvironment, or are used in HSC niches throughout ontogeny.

Establishing homeostasis in bone marrow HSC niches

The skeletal system develops during the third week of mouse gestation, concomitantly establishing a unique micro-environment for HSCs within the bone marrow. Skeletal development begins at E12.5 as mesenchymal condensations, in which mesenchymal cells first give rise to chondrocytes that create a cartilaginous framework for the skeleton (Olsen et al., 2000). Chondrocytes are later replaced by osteoblasts that generate calcified bone through endochondral ossification. Vascular invasion into the developing bones facilitates circulation through the bones, and the seeding of hematopoietic progenitor and stem cells. Clonogenic progenitor activity in the long bones starts at E15.5, whereas functional HSCs are found from E17.5 onwards (Christensen et al., 2004; Gekas et al., 2005). As HSCs can be found in circulation several days before, delay in HSC colonization implies that the early fetal bone marrow micro-environment is unable to attract HSCs and support their engraftment and self-renewal. Sdf1 (stromal cell-derived factor 1), a ligand for chemokine (C-X-C motif) receptor 4 (Cxcr4), is an important chemokine in bone marrow stromal cells that attracts HSCs to fetal bone marrow, whereas it is largely dispensable for the formation of the fetal liver HSC pool (Ara et al., 2003). Much remains to be carried out to elucidate how the switch from fetal liver to bone marrow hematopoiesis occurs.

Significant progress has been made in understanding the composition of HSCs niches in the adult bone marrow (reviewed by Wilson and Trumpp, 2006). Mice with reduced *Bmpr1a* (bone morphogenetic protein receptor 1) signaling, or constitutive expression of *Pth* (parathyroid hormone)/*Pth*-related protein receptor in the bone, exhibit increased osteoblast and HSC numbers, leading to the identification of osteoblasts as niche cells for HSCs (Calvi et al., 2003; Zhang et al., 2003). Furthermore, recent studies suggest HSCs recognize Ca²⁺ upon engraftment into the endosteal surface of bone, as *Casr* (calcium-sensing receptor) knockout mice fail to establish proper bone marrow hematopoiesis (Adams et al., 2006). This is despite the fact that the HSC pool in the mutant fetal liver develops normally, and the ability of HSCs to home initially to the bone marrow remains unaffected.

Another important mechanism that regulates HSC and niche interactions is angiopoietin signaling. Angiopoietin 1, which is secreted by the osteoblasts, interacts with its receptor, Tie2/Tek

(endothelial-specific receptor tyrosine kinase), expressed on the surface of HSCs, and promotes the adherence, quiescence and survival of HSCs (Arai et al., 2004). Homotypic interactions between N-cadherin on the surface of the osteoblasts and HSCs might also be important in anchoring HSCs to the endosteal surface (Arai et al., 2004). Further evidence of the importance of N-cadherin in niche interactions has come from studies of conditionally targeted *Myc* mouse knockouts (Wilson et al., 2004). Loss of *Myc* in bone marrow HSCs leads to a drastic expansion of the HSC pool, while differentiation is severely impaired, pinpointing a central role for *Myc* in regulating the balance between HSC self-renewal and differentiation. Loss of *Myc* is accompanied by the upregulation of N-cadherin and integrins on the surface of HSCs, indicating that these adhesion molecules are instrumental in anchoring the HSC to the niche, which prevents their differentiation (Wilson et al., 2004).

Improved HSC purification strategies have also facilitated the identification of HSC niches in greater detail. By localizing HSCs with a combination of SLAM-family markers (Cd150⁺Cd244⁻Cd48⁻Cd41⁻), another putative niche for adult HSCs has been identified in the sinusoidal endothelium of the bone marrow and spleen (Kiel et al., 2005; Yilmaz et al., 2006). Interestingly, HSCs were frequently located in the bone marrow in contact with endothelium, whereas only a fraction localized to the endosteum. Furthermore, in mice in which HSCs were mobilized from bone marrow by cyclophosphamide/G-CSF (granulocyte-colony stimulating factor) treatment (which leads to extramedullary hematopoiesis in the spleen), HSCs were mainly found in association with sinusoidal endothelium within parafollicular areas of the red pulp. No differences were evident in the cell cycle status between the HSCs that were associated with the endosteum or sinusoidal endothelium in the bone marrow. Further studies will be required to elucidate the specific role of the different adult HSC niches.

The shift from a proliferative fetal HSC to a quiescent conservatively self-renewing adult HSC is accompanied by distinct changes in surface marker expression. A characteristic feature of quiescent adult HSCs is the downregulation of Cd34, which occurs by 10 weeks of mouse development, probably reflecting the establishment of homeostatic steady-state in bone marrow hematopoiesis (Ogawa et al., 2001) (Table 1). Furthermore, changes in Cd34 expression are reversible, as Cd34⁻ HSCs may upregulate Cd34 expression upon activation after 5-fluorouracil (5-FU) treatment, or mobilization into peripheral circulation by G-CSF (Ogawa, 2002). Another pronounced difference is downregulation of specific lineage markers from adult HSCs. Although adult HSCs are defined by a lack of expression of lineage commitment markers, fetal HSCs can express selected lineage markers, such as the monocyte/macrophage marker *Mac1* (Cd11b), and B-cell marker *Aa4.1* (Jordan et al., 1995; Jordan et al., 1990; Morrison et al., 1995; Rebel et al., 1996b) (Table 1). It has yet to be determined whether these changes reflect the promiscuous expression of these markers during fetal development, or whether these molecules are differentially required in HSCs during fetal and adult life. HSCs also change their surface phenotype during culture, adding yet more complexity to their identification and purification (Zhang and Lodish, 2005).

Trafficking of HSCs during development and steady state

The development of an intact circulatory system is a prerequisite for the survival and growth of the embryo, and for the establishment of the definitive hematopoietic system. Before circulation is established, mesodermal precursors migrate from the primitive

streak to future hematopoietic sites, i.e. the yolk sac, the AGM and possibly the allantois/placenta (Fig. 5). The vascular system develops concomitantly, and once the heart starts to beat around E8.5, vitelline circulation is established, connecting the dorsal aorta, the yolk sac and the heart. Although this is considered to be the onset of circulation, mainly primitive red cells enter the bloodstream at this point. However, the definitive hematopoietic progenitors predominantly reside in the yolk sac until E9.25 (Palis et al., 1999), and become distributed freely within the vascular system only after E10.5 (McGrath et al., 2003). By this time, the vitelline vessels have penetrated through the liver rudiment, facilitating initial hematopoietic seeding of the fetal liver (Fig. 5).

The second major vascular route, the umbilical circuit, develops shortly thereafter. Umbilical vessels form from the allantoic mesoderm after chorioallantoic fusion, and connect the dorsal aorta to the placental labyrinthine vascular network and subsequently to the fetal liver (Fig. 5). The development of umbilical and labyrinthine vasculature is essential for embryonic development, as revealed by knockout mice that exhibit placental defects and subsequent embryonic lethality (Rossant and Cross, 2001). As the placenta is placed in a strategically favorable position between the dorsal aorta and the fetal liver, this positioning might ensure that HSCs, which may still be primitive in their adhesive properties, efficiently seed the next anatomical niche that supports their development. It is likely that the interactions between the developing HSCs and hematopoietic niches become more specialized towards the end of gestation, as hematopoietic niches are refined. Indeed, some mutant mouse strains, such as the *Casr* knockout mouse are less able to establish bone marrow hematopoiesis, whereas fetal liver hematopoiesis is relatively unaffected (Adams et al., 2006). However, the mechanisms that initially attract fetal HSCs to establish their residence in the fetal liver and promote their exit and seeding of the bone marrow remain unknown.

Although circulating blood harbors many hematopoietic progenitors from midgestation, definitive HSCs appear later in circulation than in the AGM, the placenta and the yolk sac, and are always rare compared with other circulating hematopoietic cells (Christensen et al., 2004; Gekas et al., 2005; Kumaravelu et al., 2002). Thus, although HSCs circulate during both fetal and adult hematopoiesis, and their ability to do so is essential for HSC pool establishment and maintenance, HSCs appear to reside most of the time in secure hematopoietic niches. Indeed, in the adult mouse, HSCs disappear from circulation 1-5 minutes after transplantation, demonstrating extremely rapid homing to target hematopoietic niches (Wright et al., 2001). Approximately 100 HSCs are in circulation during normal homeostatic conditions (Wright et al., 2001). Studies using parabiotic mice, where two individual animals are connected through circulatory systems, and using consecutive transplantations into unconditioned immunodeficient mice, suggest that a small number of HSC niches are freed constantly and can be filled by circulating HSCs, while the exiting HSCs establish residence in another anatomical location (Bhattacharya et al., 2006; Cao et al., 2004; Wright et al., 2001).

Concluding remarks

The importance of stem cell micro-environments has been only recently fully appreciated. Yet, much can be learned from the biology of HSCs by studying the niches that support them. Although many common signals may be used in fetal and adult HSC niches, distinct regulatory mechanisms exist that promote the de novo generation or expansion of HSCs during fetal life, or support the relative quiescence of HSCs during steady state

hematopoiesis in the adult. This is exemplified by differential requirements for transcription factors during specific stages of HSC development (Teitell and Mikkola, 2006), and by inherent differences between fetal and adult HSCs, as shown by their predisposition to undergo expansion or quiescence, and by their different surface marker profiles. These inherent differences should not be overlooked, but may instead be used to identify a subtype of HSCs that is more likely to retain self-renewal ability during therapeutic manipulations. The challenge is to dissect the hematopoietic niches in various anatomical locations into specific cellular and molecular components that direct each stage of HSC development, and translate that knowledge into the tools that can be used to culture or manipulate HSCs. A key factor that limits the availability of HSCs for bone marrow transplantation is the difficulty to sustain their self-renewal capacity and multipotency *ex vivo*. Understanding how these programs are established and maintained during development will be instrumental for developing better culture systems. Ultimately it should be possible to learn how to recapitulate the correct micro-environment for *ex vivo* expansion of HSCs derived from cord blood, adult bone marrow or mobilized peripheral blood, or even for the generation of long-term reconstituting HSCs from novel HSC sources, such as human ES cells (Lerou and Daley, 2005). These advances will ultimately improve HSC-based therapies for blood cell disorders such as hematopoietic malignancies, and inherited anemias and immunodeficiencies (Bordignon, 2006).

H.K.A.M.'s research was supported by the NIH and Harvard Stem Cell Institute Seed Grant.

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