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REVIEW

On the origin of hematopoietic stem cells: Progress and controversy

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Abstract Hematopoietic Stem Cells (HSCs) are responsible for the production and replenishment of all blood cell types during the entire life of an organism. Generated during embryonic development, HSCs transit through different anatomical niches where they will expand before colonizing in the bone marrow, where they will reside during adult life. Although the existence of HSCs has been known for more than fifty years and despite extensive research performed in different animal models, there is still uncertainty with respect to the precise origins of HSCs. We review the current knowledge on embryonic hematopoiesis and highlight the remaining questions regarding the anatomical and cellular identities of HSC precursors.

Contents

The concept of hematopoietic stem cells: More than a century ago

The development of cell biology research coincides with the advance of microscopes in the 19th century. It was finally possible to directly observe the various blood cell types and to

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witness their proliferation and differentiation (Mazzarello, 1999). On the basis of his observations, the German pathologist Franz Ernst Christian Neumann (1834–1918) concluded that the site of blood formation was the bone marrow. He also proposed the pioneer theory in which one cell might be at the origin of all blood cell lineages. The Russian scientist Alexander A. Maximow (1874–1928) also developed and introduced the theory of a common cell for the complete blood-building system or hematopoiesis, as well as the idea of a micro-environmental niche for these cells within the bone marrow (Maximow, 1909). The concept of Hematopoietic Stem Cells (HSCs), although very controversial at the time, was born and has led to the beginning of stem cell research (Ramalho-Santos and Willenbring, 2007).

Ernest A. McCulloch and James E. Till gave the first experimental proof of the stem cell theory by performing the transplantation of bone marrow cells into irradiated mice (Till and McCulloch, 1961; Becker et al., 1963). These cells gave rise to myeloid multilineage colonies in the spleen of transplanted animals, the number of colonies being proportional to the number of injected cells. Such experiments demonstrated the multilineage potential of single bone marrow cells (so-called CFU-S, Colony-Forming Unit in the Spleen) (Siminovitch et al., 1963). However, because these cells only have limited self-renewing capabilities, they are not considered to be true stem cells which must be both multipotent and self-renewing. E. Donnall Thomas performed the first successful stem cell transplantation on identical human twins in 1957 (Thomas et al., 1957). This has formally demonstrated that intravenous injection of bone marrow cells allows long-term repopulation with the production of new blood cells. The oncologist Georges Mathé also performed transplantations on Yugoslavian nuclear workers whose bone marrows were damaged by irradiation (Mathé et al., 1959). Later on, he successfully cured a patient with leukemia (pre-treated by irradiation) after allogenic bone marrow transplantation (Mathé et al., 1963). For nearly 50 years, such transplantations have been performed to treat patients with blood-related disorders. Adult HSCs can now be highly enriched with a combination of several surface markers. However, no unique HSC marker has so far been identified, as is the case for most stem cell categories. Thus, a functional test is required to prove that genuine HSCs are present in a cell population. *In vivo* transplantation is the gold standard experimental procedure to prove retrospectively, by analyzing the HSC progeny, that the cells are capable of multilineage differentiation in addition to self-renewal.

Adult bone marrow, cord blood and mobilized peripheral blood are sources of HSCs used in transplantation protocols in the case of many blood-related diseases, such as leukemia. However, the number of HSCs in these tissues remains low, which creates a major obstacle for both HSC use in clinical and fundamental research. Despite extensive studies and a continuously better understanding of the complicated intrinsic and extrinsic regulation of HSCs, it is still very difficult to reproduce *in vitro* conditions allowing efficient HSC expansion without inducing cell differentiation (Hofmeister et al., 2007). Embryonic Stem Cells (ESC) and induced Pluripotent Stem (iPS) cells can generate *in vitro* cells of the different hematopoietic lineages including erythrocytes, myelocytes and megakaryocytes (Sakamoto et al., 2010). However, the generation of new HSCs either from ESCs or iPS cells, although very promising, remains very limited to date (Kyba et al., 2002; Burt et al., 2004). Interestingly, it was recently shown that human fibroblasts derived from the skin could directly be reprogrammed to multilineage hematopoietic progenitors after ectopic expression of the single transcription factor Oct-4 (Szabo et al., 2010). Reprogrammation without the need to generate artificial pluripotent stem cells might be a promising direction in the future to generate HSCs *in vitro*.

Therefore, a major goal remains to understand the complex cellular and molecular network that controls HSC generation and expansion. In fact, HSCs that are mainly slow cycling (Wilson et al., 2008) and reside in the bone marrow during the entire life of an individual, are not generated there. They are produced and expanded during embryonic development before colonizing in the bone marrow prior to birth (Christensen et al., 2004). Thus, studying HSC development in the embryo is a powerful tool and a reliable model to decipher the complex characteristics and regulation of HSCs. We will describe in this review the ontogeny of the hematopoietic system in different animal models, the controversies concerning the cellular and anatomical origin of HSCs and the recent insights into the field, notably documented by *in vivo* confocal imaging approaches.

The journey of a hematopoietic stem cell: From embryo to adult

HSCs are at the foundation of the entire adult blood system. The multipotency property allows single HSCs to differentiate and proliferate into more committed progenitors and precursors that will then produce all mature cells from the erythroid, myeloid and lymphoid lineages. Given the fast turnover in mature blood cells, only HSCs, which are by definition able to self-renew while keeping their multilineage properties, can sustain blood production during the entire life of an organism.

Embryonic hematopoiesis has been studied in various animal models (e.g. amphibian, avian, rodent), but the focus of this chapter will mainly be on the mouse embryonic model. Adult HSCs are generated during embryonic development. The first hematopoietic cells produced in the embryo are differentiated cells that are most likely needed at the time for proper oxygenation and protection of the developing embryo. The initial site of hematopoietic production is the yolk sac (YS) (Moore and Metcalf, 1970; Palis et al., 1999; Ferkowicz and Yoder, 2005). Starting at embryonic day (E)7.25 of mouse development, this extra-embryonic tissue, composed of both visceral endoderm and mesoderm, transiently produces large nucleated erythrocytes termed erythroblasts (Fig. 1A) (Palis et al., 1999), as well as some macrophages and megakaryocytes (Xu et al., 2001). A layer of endothelial cells will surround these first blood cells to form specialized structures called blood islands (Ferkowicz and Yoder, 2005; Haar and Ackerman, 1971). Some evidence (reviewed later) led to the idea that both cell types (hematopoietic and endothelial) are generated from a common mesodermal precursor known as the hemangioblast (Ferkowicz and Yoder, 2005; Shalaby et al., 1995, 1997; Eichmann et al., 1997; Choi et al., 1998; Huber et al., 2004). Mature cells can easily be identified directly under the microscope by specific morphological criteria, or by analysis of surface marker expression using, for example, immunohistochemistry. On the other hand, hematopoietic progenitors

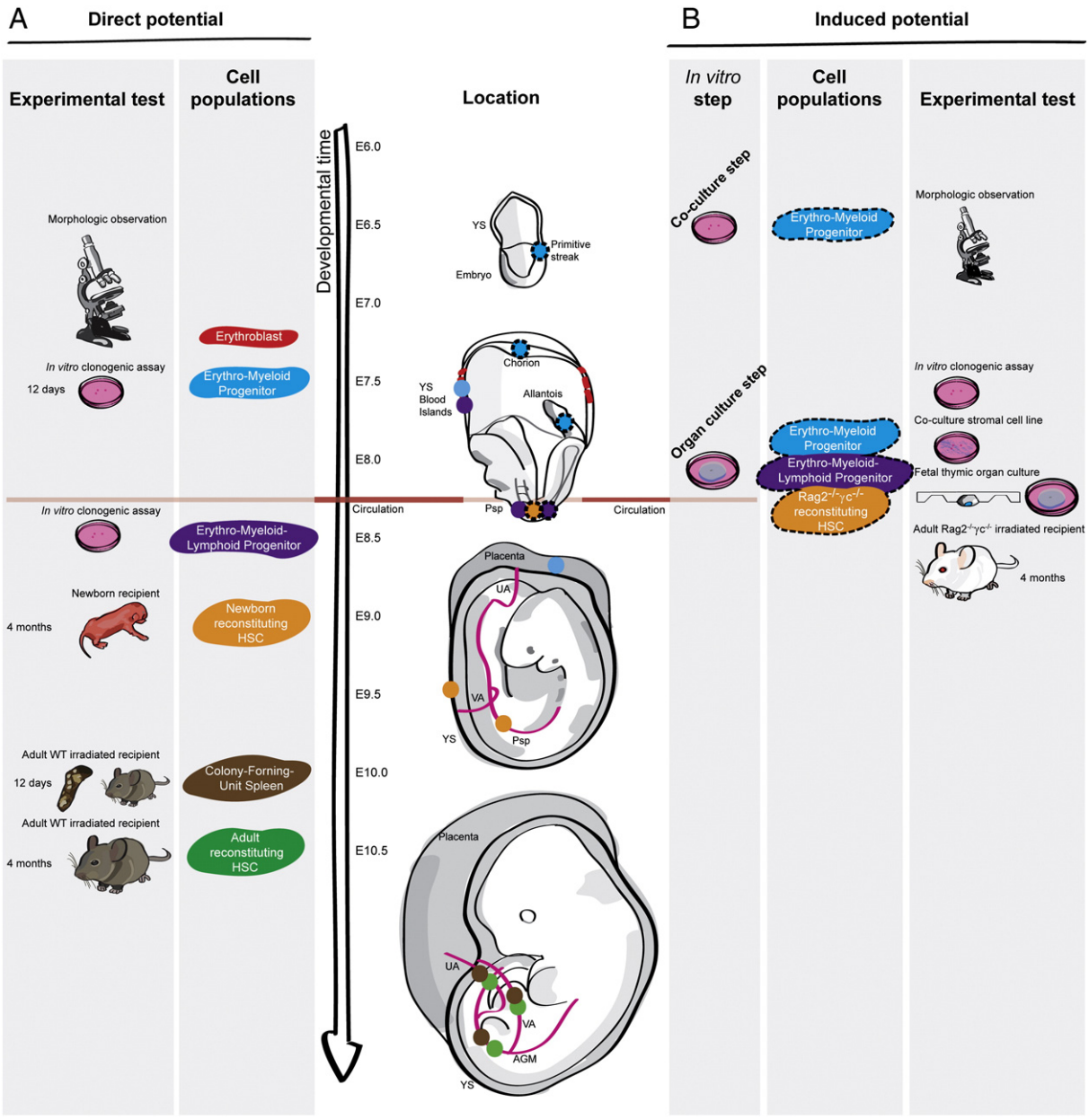

Figure 1 First sites of hematopoietic stem/progenitor cell appearance during mouse embryonic development. The scheme represents the early mouse embryonic development, from E6.0 until E10.5. Different types of hematopoietic cells have been found in different anatomical sites throughout development. However, it is important to note that the time point of the appearance of hematopoietic progenitor/stem cells and the site where they are detected differ according to the experimental approach used to reveal the hematopoietic potential. The hematopoietic potential of the embryonic tissues has been tested directly on freshly isolated tissues (A) or after an *in vitro* culture step (B) to reveal a hematopoietic potential undetectable on fresh tissues. The first time points of appearance of the different hematopoietic stem and progenitor cells are indicated in the different anatomical sites as well as the *in vitro* and *in vivo* assays used to identify the hematopoietic cells. HSC: hematopoietic stem cells, YS: yolk sac, UA: umbilical artery, VA: vitelline artery, P-Sp: para-aortic splanchnopleura, E: embryonic day post-coitus, WT: wild-type.

are identified retrospectively in a short-term *in vitro* assay (clonogenic assay). In such a culture system, different types of lineage-restricted progenitors (erythroid, granulocytic, macrophagic and/or megakaryocytic) can be identified and quantified for their ability to form colonies by looking at specific characteristics (e.g. composition, size, color). By using such assays, it was shown that beginning at E7.5-E8, erythroid-myeloid progenitors start to appear in the YS (Moore and Metcalf, 1970; Palis et al., 1999) and in the chorion and allantois (which later fuse to form the placenta and the umbilical cord) (Palis et al., 1999; McGrath et al., 2011; Alvarez-Silva et al., 2003; Corbel et al., 2007; Zeigler et al., 2006). In addition, it is possible to identify erythroid-myeloid cells in E6.75 embryos once the cells have been co-cultured on OP9 stromal cell lines (Furuta et al., 2006) (Fig. 1B). Slightly later, at E8.5, erythroid–myeloid–lymphoid progenitors are

found in the YS and the Para-aortic Splanchnopleura (P-Sp, region formed by the dorsal aorta, omphalomesenteric (or vitelline) artery, gut and splanchnopleura) (Godin et al., 1995). Indeed, B progenitors were identified after co-culture of the different tissues on S-17 stromal cell line, while T progenitors were identified in a fetal thymic organ culture assay (Fig. 1A). Culturing of YS and P-Sp before the establishment of circulation showed that these multipotent progenitors are of P-Sp origin (Fig. 1B) (Cumano et al., 1996). At E10, CFU-S are also detected in the YS and the intra-embryonic Aorta-Gonad-Mesonephros region (AGM, region corresponding to the earlier P-Sp) (Medvinsky et al., 1993) (Fig. 1A).

Adult HSCs can be highly purified (Purton and Scadden, 2007; Osawa et al., 1996). This is in contrast to the first HSCs found in the embryo, which have been more difficult to enrich thus far (Taoudi et al., 2005). Many markers currently used to isolate adult HSCs do not apply for embryonic HSCs. Moreover, surface marker expression on HSCs varies during development (Robin et al., 2011; McKinney-Freeman et al., 2009) and also between strain and species. For example, the SLAM marker CD150, which allows for a high enrichment of HSCs in the adult bone marrow (Kiel et al., 2005) and fetal liver (Kim et al., 2006) when used in combination with the classical marker combination LSK ($Lin^{-}Sca1^{+}ckit^{+}$), is not a marker of AGM HSCs (McKinney-Freeman et al., 2009). The lineage antibody panel (Lin) has been designed and used routinely to deplete mature cells from the erythroid, lymphoid and myeloid lineages (Muller-Sieburg et al., 1986). Such depletion does not apply to purifying embryonic HSCs because Mac-1 (CD11b), a marker classically expressed by macrophages/monocytes in adults, is also expressed by a fraction of AGM HSCs (Sanchez et al., 1996). Moreover, HSCs and endothelial cells share many surface markers, reflecting their close developmental relationship (Garcia-Porrero et al., 1998). The only reliable method used to identify HSCs is to perform a long-term *in vivo* assay where the multilineage repopulation and self-renewal abilities of the cells are tested after transplantation into primary and secondary adult wild-type irradiated recipients. Using this standard assay, HSCs were first detected in the AGM region starting at E10.5 (Fig. 1A) (Medvinsky and Dzierzak, 1996; Muller et al., 1994). More precisely, HSCs are restricted to the aorta, as shown by the subdissection of the AGM region to separate the aorta from the urogenital ridges before performing transplantation (de Bruijn et al., 2000). HSC activity in the AGM is transient and stops after E12. Interestingly, HSCs are also found at E10.5 in two other major vessels, the vitelline and umbilical arteries (de Bruijn et al., 2000) (Fig. 1A). Slightly later (E11–11.5), HSCs are also detected in other major highly vascularized hematopoietic sites: the YS, placenta and fetal liver (Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005; Gekas et al., 2005). Only very few HSCs (~4–11 cells) are present in the complete mouse conceptus at E11. By E12, the HSC number multiplies by at least 14 times, mainly in the placenta and fetal liver (Kumaravelu et al., 2002; Gekas et al., 2005). The liver becomes the main HSC reservoir at mid-gestation until the HSCs start to colonize the bone marrow at E17 (Christensen et al., 2004). HSCs behave differently in the embryo (compared to adult) as they transit through several anatomical sites or niches, and are actively self-renewing (Morrison et al., 1995; Bowie et al., 2006). The embryonic microenvironment that composes the successive niches is still poorly described, but it certainly influences the equilibrium between HSC self-renewal and differentiation.

In comparison, HSC niches in adult bone marrow are well described (Levesque and Winkler, 2011). Two types of niches, very close spatially, have been reported so far. In the endosteal niche, HSCs are in close contact to the endosteal bone surface where the main supportive cell type, the osteoprogenitor population, maintains HSCs in a quiescent/slow-cycling state (Calvi et al., 2003; Zhang et al., 2003; Raaijmakers et al., 2010; Lo Celso et al., 2009). In the second niche, HSCs are associated with the sinusoidal endothelium (Kiel et al., 2005), but it remains to be determined whether it represents a functional niche or only a transition site (Purton and Scadden, 2008). The use of *in vivo* and *ex vivo* time-lapse confocal microscopy has nicely shown that transplanted HSCs preferentially localize close to the endosteal bone surface while committed progenitors localize further away (Lo Celso et al., 2009; Xie et al., 2009). The most potent HSC niches are most likely hypoxic (Winkler et al., 2010). The well-defined medullar microenvironment of the niches maintains most HSCs in an immature and quiescent/slow-cycling state, the quiescence status being the hallmark of their long-term HSC properties (Wilson et al., 2008; Cheshier et al., 1999). A small pool of HSCs will eventually self-renew only a few times during the lifetime (Wilson et al., 2008). However, perturbation of homeostasis promotes their self-renewal more rapidly (Wilson et al., 2008). The mesenchymal stem cells (which have adipogenic, osteogenic and chondrogenic potential), that express the intermediate filament protein nestin, were recently described as a very important player in maintaining the function of the HSC niche (Mendez-Ferrer et al., 2010). Such cells produce SDF-1α (CXCL12) and SCF (Stem Cell Factor) that are important for HSC maintenance. These factors are part of a long and non-exhaustive list of intrinsic and extrinsic factors essential for HSC fate regulation. It includes transcription factors (e.g. SCL, Runx1, Cbfβ, Lmo2, GATA2), cell cycle regulators (e.g. p27kip1, p21cip1/waf1), hematopoietic cytokines (e.g. TPO, Flt3/Flk2 ligand, IL-3) and developmental regulators (e.g. BMP-4, Tie2/Angiopoietin-1, Wnt/β-catenin, TGF-β/p21, VCAM-1, Hedgehog, Notch/Jagged 1) (Zon, 2008). Nevertheless, no studies have yet pinpointed the exact molecular network architecture that distinguishes self-renewing from non-self-renewing hematopoietic cells.

Bone marrow niches, where the pool of HSCs is maintained constant in adults, are now well described. This is not the case for the successive niches where HSCs transit throughout development. Thus the study of hematopoietic cells and their various surrounding microenvironments during ontogeny has become very important in providing some of the missing pieces of the puzzle which explain how HSCs are generated and expanded without stem cell potential loss. To that end, it is first important to understand where they are generated. We will therefore next review the current knowledge about the anatomical origin of HSCs.

Intra- or extra-embryonic origin of hematopoietic stem cells: A long historical uncertainty

It has been impossible so far to ascertain the precise anatomical site(s) of HSC emergence in the mouse conceptus. Indeed, the blood is already circulating (starting at E8.25–8.5) (Ji et al., 2003) between extra- and intra-embryonic tissues at

the time when the first HSCs start to be detected (E10.5) (Fig. 1). Thus, it cannot be ruled out that HSCs emerge in one site and rapidly colonize another site via the blood circulation.

Lessons can be learned from experiments performed in the different mammalian and non-mammalian animal models available. In particular, the avian embryo is a very powerful model, which has been instructive since the beginning of research on developmental hematopoiesis. The *in ovo* development of avian embryos allows for easy access and manipulation, such as the injection of cells, the graft of tissues (prior blood circulation) or the *ex vivo* development of embryos cultured *in vitro* (Stern, 2005). In the late 1960s, Moore and Owen hypothesized that all HSCs were of YS origin because the transplantation of 7-day YS cells into 14-day-old irradiated chicken embryos led to the colonization of lymphoid organs (Moore and Owen, 1967). The hypothesis of a YS HSC origin was soon challenged when hetero-specific chimeras, composed of quail embryos grafted on chick YS, were generated before the start of circulation between the embryo and YS (Dieterlen-Lievre, 1975). Quail cells are easily recognizable from chicken cells by their dark nucleus, as well as by the use of species-specific antibodies. The analysis of chimeras revealed that all blood cells were of quail origin, thus demonstrating their intra-embryonic origin (ruling out their YS origin). Quail cells were indeed able to colonize the spleen and thymus rudiments, the bursa of Fabricius and bone marrow (Dieterlen-Lievre, 1975). The generation of chick–chick homo-specific chimeras (allowing the growth of the animal until adulthood) definitively confirmed the intra-embryonic origin of adult blood (Lassila et al., 1978). In the amphibian model, grafting strategies are also applicable due to the external development of the embryo. Blood cells in the *Xenopus* (frog) are found in two different compartments: the ventral blood island (VBI) and the dorsal lateral plate (DLP, mesodermal region containing the dorsal aorta). VBI is the mammalian YS equivalent and DLP is the AGM equivalent. When VBI is grafted in a host *Xenopus*, it contributes to the transient production of erythroid cells that last only during the embryonic stage (Turpen et al., 1981). In contrast, the graft of DLP leads to the durable production of both lymphoid and myeloid cells, proving the definitive hematopoietic potential of the DLP region (Turpen et al., 1981). Interestingly, VBI and DLP compartments derive from distinct blastomeres as early as the 32-cell embryo stage (Ciau-Uitz et al., 2000).

The remarkable grafting experiments performed in avian and amphibian models unambiguously showed the intra-embryonic origin of adult HSCs in both species. Such grafting strategies are not feasible so far in mammalian embryos due to their intra-uterine development. However, some trials have been made to determine the site of HSC origin. These experiments use tissues isolated before the onset of circulation to exclude any possible contamination by cells migrating from one site to another via the blood circulation. Pre-circulatory E8 YS cells injected into an E8 host YS cavity could provide (1 month after transplantation) CFU-S progenitors able to form spleen colonies when the cells were transplanted into secondary recipients (Weissman et al., 1978; Ueno and Weissman, 2010). Unfortunately, the intra-embryonic counterpart was not tested in that study. Alternatively, Samokhvalov et al. used a genetic labeling approach to mark, by using a tamoxifen inducible promoter, Runx1 expressing cells during a restricted time period of early embryonic development (E7.5–E8.5) (Samokhvalov et al., 2007). Runx1 is an essential transcription factor for the formation of HSCs (Okuda et al., 1996; Wang et al., 1996; Sasaki et al., 1996), but is also expressed by early hematopoietic cells located in the YS blood islands (Samokhvalov et al., 2007). Assuming that tamoxifen induced labeling is restricted to the aforementioned period of time, they were able to trace the fate of the YS Runx1 expressing cells and found that all blood lineages were labeled in adults (9–12 months old), including the phenotypically defined HSC population (Samokhvalov et al., 2007). Therefore, both studies argue in favor of a YS origin for at least part of the adult HSC population. Nevertheless, in another experiment, cultured pre-circulatory YS and P-Sp (E8) were tested for their ability to reconstitute adult irradiated immunodeficient mice ($Rag2^{-/-}\gamma c^{-/-}$ (Colucci et al., 1999)) (Cumano et al., 2001). Only the P-Sp region could provide long-term multilineage reconstitution in this type of immunodeficient recipients that were alymphoid and devoid of NK cell activity. Cultured YS cells could only provide short-term myeloid reconstitution. Although this experiment suggests that HSCs could have an entirely intra-embryonic origin, one could conclude that the *ex vivo* YS does not provide the appropriate environment for HSC precursors to mature into a transplantable cell type.

Thus, it is worth mentioning that none of these aforementioned studies undoubtedly provide a definitive answer concerning the potential origin of HSCs in the mouse embryo. To add to the complexity, it is possible, as shown in the *Xenopus* embryo (Turpen et al., 1997) and suggested in mouse (Matsuoka et al., 2001), that both YS and P-Sp have intrinsic HSC potential, but that the *in vivo* microenvironment would define the fate of HSC precursor cells. Despite uncertainty on the anatomical origin of HSCs, much work has been performed to understand what cell type developmentally precedes HSCs.

Cellular origin of hematopoietic stem cells: The hemogenic endothelium

The observation of both a temporal and spatial localization of blood and endothelial cells in the YS blood islands led to the concept of the hemangioblast, a common precursor for hematopoietic and endothelial cells (Sabin, 1920). Since then, there has been genetic and functional evidence for this concept. For example, Flk-1 deficient embryos lack blood and endothelium (Shalaby et al., 1995). Likewise, $Flk1^{-/-}$ ESCs fail to contribute to blood and endothelial lineages in chimeras, suggesting the existence of $Flk1^{+}$ hemangioblast cells (Shalaby et al., 1997). In chickens, mesodermal cells expressing the Flk1 homologue can differentiate clonally either into endothelial or hematopoietic lineages in the presence or absence of VEGF (Eichmann et al., 1997). Also, the blast colony-forming cells (BL-CFC), identified during mouse ESC differentiation, are able to generate cells of both endothelial and hematopoietic lineages (Choi et al., 1998). Such bipotential precursors, most likely corresponding to the hemangioblast, can be detected in gastrulating mouse embryos (Huber et al., 2004). They arise in the primitive streak before migrating to the YS, where they will differentiate *in vivo* into hematopoietic and endothelial cells (Huber et al., 2004; Vogeli et al., 2006).

The hemangioblast usually refers to the bipotential precursor at the origin of early YS hematopoiesis (Huber, 2010). However, a strikingly close connection between endothelial and hematopoietic cells can also be observed later on, in the main intra-embryonic vessels (the dorsal aorta and the umbilical and vitelline arteries) (Dantschakoff, 1909; Dieterlen-Lievre et al., 2006; Yokomizo and Dzierzak, 2010). There, hematopoietic cells are often seen grouped together in clusters of cells (Intra-Aortic Hematopoietic Clusters, IAHCs), tightly attached to the endothelial layer of the vessels. This close association between endothelium and hematopoietic cells has led to the hypothesis in the early 20th century that specialized endothelial cells, termed hemogenic, would have the ability to give rise to hematopoietic cells (Dantschakoff, 1909; Jordan, 1917). The presence of IAHCs has been described in many vertebrate species (Dieterlen-Lievre et al., 2006). Decades later, it was also shown by immunostaining on fixed embryo sections that IAHCs express both hematopoietic and endothelial markers, again emphasizing the close developmental relationship between the two cell lineages (Garcia-Porrero et al., 1998). IAHCs, due to their morphologic and phenotypic characteristics, are presumed to contain hematopoietic stem and progenitor cells (Dieterlen-Lievre and Martin, 1981). This is now, in part, confirmed since mice deficient for the transcription factor Runx1, that do not have any HSCs or progenitors, are also devoid of IAHCs (embryos die around E11.5) (Okuda et al., 1996; Wang et al., 1996; Cai et al., 2000; North et al., 1999). In addition, HSCs and IAHC cells co-express similar HSC markers (e.g. c-kit) and endothelial markers (e.g. CD31, VE-cadherin, CD34) at E11.5.

The hypothesized endothelial origin of IAHCs (and therefore of the putative HSCs) was first tested in the avian model. Pre-IAHC stage embryos received injections directly into the heart with Ac-DiI-LDL (endothelial cells specifically uptake this molecule) and were examined 24h later. Newly formed IAHCs were found to express the pan-hematopoietic marker CD45 while still retaining the Ac-DiI-LDL staining (Jaffredo et al., 1998, 2000). Such experiments therefore proved the endothelial origin of the newly formed hematopoietic cells. Such specialized endothelial cells capable of generating hematopoietic cells are referred to as hemogenic. Similar lineage tracing experiments were also performed in the mouse embryo and gave a similar conclusion (Sugiyama et al., 2003). The transition from endothelial cells to hematopoietic cells is now well known in the chicken embryo (Fig. 2). The ventral endothelial cells become thicker, start to express CD45 and form IAHCs. The endothelial floor is progressively replaced by endothelial cells of somitic origin that are no longer hemogenic (Pouget et al., 2006). Such a process regulates the aortic hematopoietic production both in time and space. In mice, an alternative hypothesis has been proposed based on the observation of a cell population located in the sub-aortic mesenchyme that expresses hematopoietic markers (Bertrand et al., 2005). According to this hypothesis, HSC precursors would migrate through the endothelium (without taking any characteristics of the endothelium) and bud into the lumen of the aorta to form IAHCs.

Thus, various experimental approaches have been undertaken to determine the exact cellular origin of HSCs in different animal models. Constant cell labeling and time-lapse imaging performed during mouse ESC cultures and BL-CFC development have shown that the hemangioblast generates hematopoietic cells through the formation of a hemogenic endothelial intermediate population (Lancrin et al., 2009; Eilken et al., 2009). Such cells are also present in gastrulating embryos. Another strategy has been to perform lineage tracing of endothelial cells *in vivo*, by marking these cells at a precise stage of development using Cre-mediated recombination driven by the VE-Cadherin (mouse) (Zovein et al., 2008; Oberlin et al., 2010) or KDR promoter (zebrafish) (Bertrand et al., 2010) (two genes expressed by endothelial cells). In these conditions, a portion of adult hematopoietic cells from all hematopoietic lineages were labeled indicating that their ancestors had undergone an endothelial step at an earlier stage during mouse and zebrafish development. Furthermore, conditional deletion of Runx1 in the endothelial compartment completely impaired HSC as well as IAHC formation (Chen et al., 2009). As a whole, these experiments give further support to the endothelial origin of adult HSCs.

It is now clear that adult HSCs are generated via an endothelial step at an earlier stage during embryonic development. However, the locations of the specialized hemogenic endothelial cells (which are so far undistinguishable from non-hemogenic endothelial cells) as well as insight into the mechanism leading to hematopoietic cell commitment remain unclear. In order to answer these questions, *in vivo* live imaging approaches have been used. Zebrafish embryos are transparent and are therefore a powerful tool for live microscopic observation. Three independent groups used confocal microscopy to look at the aortic region of KDR-GFP zebrafish embryos during different time points of embryonic development: 24.5–30 hours post fertilization (h.p.f.) (Lam et al., 2010), 30–38 h.p.f. (Bertrand et al., 2010) and 36–52 h.p.f. (Kissa and Herbomel, 2010). In all cases, the emergence of hematopoietic cells from ventral endothelial cells of the dorsal aorta was observed. This hematopoietic commitment coincided with CD41, Lmo2, c-myb or Runx1 expression (all being HSC markers). The cells bud in the sub-aortic mesenchyme and later access the circulation through the neighboring axial vein (it should be noted that IAHCs do not seem to form in the lumen of the embryonic aorta of zebrafish). The transition from endothelial cells to hematopoietic cells does not require cell division but rather cell shape transformations. Indeed, hemogenic endothelial cells bend toward the mesenchyme and round up, leading to detachment from the neighboring endothelial cells (Fig. 2). Interestingly, the knock-down of Runx1 leads to the death of the emerging cells (Kissa and Herbomel, 2010). The zebrafish model therefore clearly shows that hematopoietic stem/progenitor cells emerge from a polarized hemogenic endothelium located in the ventral part of the dorsal aorta.

Due to the *in utero* development and opacity of the mouse embryo, only few attempts have been made thus far to study the hematopoietic development in the mouse embryo by live imaging (Jones et al., 2002). Furthermore, the region of interest, the dorsal aorta, is located deeply within the opaque embryo. To bypass this problem and access the aorta, thick slices of non-fixed embryos have been used (Boisset et al., 2010). Alternatively, dorsal tissues were removed from the caudal part of the embryo. In both cases, such dissection procedures allow optical access to the aorta (in the anterior/posterior axis or from a dorsal perspective). After removal of the head and tail of the embryos, intra-aortic injection of an

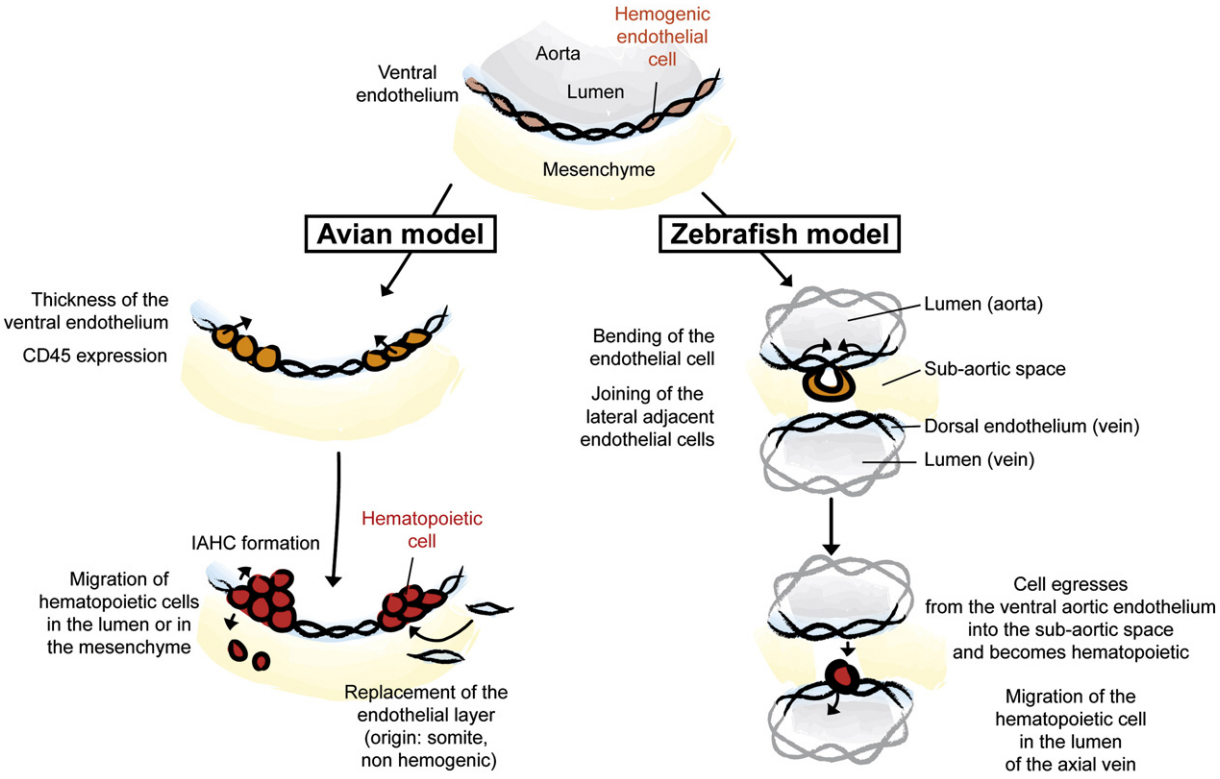

Figure 2 Formation of hematopoietic stem/progenitor cells in the aorta of the chicken and zebrafish embryos. Hematopoietic cells and intra-aortic hematopoietic clusters (IAHCs) are generated from hemogenic endothelial cells located in the ventral aspect of the aorta. It is important to note that the process leading to the transition from endothelial cells into hematopoietic cells differs between avian and amphibian models.

antibody directed against CD31 (an endothelial marker) conjugated to a fluorescent probe was performed. To trace the putative hemogenic endothelium, *Ly-6A (Sca-1)-GFP* transgenic embryos (Ma et al., 2002) were used since Ly-6A is expressed in some endothelial cells of the aorta as well as in all HSCs, but not in the surrounding mesenchyme (at E10.5) (de Bruijn et al., 2002). The hematopoietic commitment was imaged using *CD41-YFP* knock-in embryos (Zhang et al., 2007) since CD41 is so far the earliest marker indicative of hematopoietic commitment (Ferkowicz et al., 2003; Mikkola et al., 2003). During time-lapse imaging, it was possible to see cells from the $CD31^+$ ventral endothelium budding into the lumen of the aorta (Boisset et al., 2010). These cells either expressed Ly-6A-GFP or started to express CD41 as they emerged. They still expressed CD31 after emergence and co-expressed c-kit (a HSC marker) as shown after staining of the slices after confocal imaging. Altogether, hematopoietic stem/progenitor cells emerge from the mouse ventral aortic endothelium (Boisset et al., 2010; Boisset and Robin, 2010). However, it is not clear whether the endothelial to hematopoietic transition in the mouse embryo requires cell remodeling/bending or asymmetric division. Nevertheless, in the *in vitro* ESC model and *in vivo* in the zebrafish and chicken embryos, the transition is happening without cell division (Jaffredo et al., 1998; Eilken et al., 2009; Kissa and Herbomel, 2010). Also, it has been suggested that a hemangioblastic cell type might be present in the aortic region (He et al., 2010). It should be noted that a consensus definition for hemogenic endothelium and hemangioblast is lacking, despite the two referring to the close association between blood and endothelium. For instance, the hemangioblast is defined functionally by its ability to differentiate clonally into both hematopoietic and endothelial lineages. But it is not clear whether a hemogenic endothelial cell is also able to do so. If this is true, a hemogenic endothelial cell would then be a hemangioblast with the morphology or phenotype of endothelial cells (integrated into vessels). If not, then hemogenic endothelial cells would only give rise to blood cells and not to endothelium. Thus, more work needs to be carried out in order to clarify this issue.

Remaining questions

The presence of numerous IAHCs in the aorta raises many questions. (1) Although IAHCs are generally believed to be HSCs, there is an obvious incongruity between the low number of HSCs (<2 per aorta at E11.5) (as calculated by limiting dilution and transplantation into irradiated adult recipients) (Kumaravelu et al., 2002; Gekas et al., 2005; Robin et al., 2006) and the number of cells that make up the IAHCs (between 400 and 600 c-kit^+ cells at E11.5) (Yokomizo and Dzierzak, 2010). Thus, most IAHC cells are not genuine HSCs. (2) Another discrepancy concerns the temporal delay between the appearance of IAHCs (>E9.5) and HSCs (>E10.5) (Muller et al., 1994; Yokomizo and Dzierzak, 2010). (3) HSCs are restricted to the ventral part of the aorta (Taoudi and Medvinsky, 2007), whereas IAHCs are present all around the

aorta of the mouse embryo (Yokomizo and Dzierzak, 2010). It is important to note that all other species tested have IAHCs present strictly in the ventral part of the aorta. In addition, (4) the total number of HSCs present in the entire embryo increases from 11 to 152 HSCs in 24 h (from E11.5 to E12.5) (Gekas et al., 2005). Such an amplification would imply ~4 symmetrical divisions (one division every 6 h), which might be an improbable situation.

All the inconstancies concerning HSCs and IAHCs have led to the idea that an intermediate cell state exists between the hemogenic endothelium and the HSC state. These cells are referred to as pre-HSCs, HSC precursors or pre-definitive HSCs. No cells isolated from early embryonic stages (<E10.5) are able to reconstitute a wild-type adult irradiated recipient, the classical assay to identify HSCs. The first evidence of pre-HSCs came from experiments showing that YS cells or circulating blood cells from E9 mouse embryos could long-term reconstitute different *W (c-kit)* mutants when injected *in utero* into E11–E15 fetuses (Toles et al., 1989). Some of the pregnant dams even showed long-term chimerism. These cells have not yet been shown to reconstitute an adult irradiated recipient and thus, by definition, are not classically defined as HSCs, but are nevertheless capable of self-renewal: the main characteristic of HSCs (multilineage reconstitution was unfortunately not tested in these experiments) (Toles et al., 1989). It was also shown that E9 YS and P-Sp cells can reconstitute (long-term and multilineage) busulfan treated newborn recipients or unconditioned *W41/W41* neonatal mice (Yoder et al., 1997a, 1997b; Yoder and Hiatt, 1997; Peeters et al., 2005) (Fig. 1). The donor cells from the primary transplanted recipient could also reconstitute secondary recipients when injected into adults (Yoder and Hiatt, 1997). Thus, cells with HSC characteristics (pre-HSCs) exist before genuine adult-type HSCs. It has been suggested that pre-HSCs are unable to engraft an adult recipient because they lack maturity to niche in the adult microenvironment (Yoder et al., 1997a). For example, they might not express the required homing molecules or chemokine/growth factor receptors. In addition, MHC class I molecules are nearly not expressed by embryonic cells isolated before E10.5, thus becoming potential targets for the host adult NK cells (Cumano et al., 2001; Jaffe et al., 1990).

An attempt was made to establish the link between pre-HSCs and HSCs. *Ex vivo* culture of dissociated/reaggregated E11.5 AGM explants have been used as a tool to study the dynamics of HSC production (Taoudi et al., 2008). After 96 h of culture, a 150-fold HSC expansion was observed in the reaggregates. Because HSCs were mainly found in the slow-cycling population, such cell expansion would be better explained by maturation of pre-HSCs into HSCs, rather than cell division of a small pool of HSCs. Pre-HSCs appear to be restricted to the VE-Cadherin$^+$CD45$^-$ subpopulation at E10.5, which maturate into a VE-Cadherin$^+$CD45$^+$ subpopulation at E11.5 (Taoudi et al., 2008; Rybtsov et al., 2011). Given that IAHCs also express these markers at E11.5, it seems plausible that at least part of the IAHCs contain pre-HSCs. However, it must be remembered that AGM explants performed at early E10 do not provide any HSCs capable of adult reconstitution (Medvinsky and Dzierzak, 1996), although the aorta contains many IAHCs (Yokomizo and Dzierzak, 2010) and cells capable of newborn reconstitution during these early stages (Yoder et al., 1997a; Yoder and Hiatt, 1997). Thus, new approaches are required in order to determine the intermediate steps needed for hemogenic endothelial cells to generate fully potent HSCs.

An interesting remaining question is whether hemogenic endothelial cells are already committed to producing blood or whether any endothelial cell is capable of doing so depending on the influence of a singular environment. It is of interest to note that hemogenic endothelial cells found in different animal models are ventrally polarized. Indeed, transplantable mouse HSCs reside in the sub-dissected ventral part of the aorta while both dorsal and ventral parts contain hematopoietic progenitors (Taoudi and Medvinsky, 2007). Although there is a clear polarized location of HSCs in the aorta, the mechanisms behind it are unclear, but would give profound insight into how HSCs are generated. In the avian model, the embryonic aorta is formed by endothelial cells of mixed origins. The roof and sides of the aorta are formed by endothelial precursors originating from somitic mesoderm (Pardanaud et al., 1996). On the other hand, the floor of the aorta, capable of hematopoietic production, is formed by precursors migrating from the splanchnopleural mesoderm (Pardanaud et al., 1996). Since no clonal experiments were done, it is difficult to conclude on the precise nature of these precursors. They could be hemangioblasts (that would form the mature endothelium and hemogenic endothelium) or already be committed to hemogenic endothelial cell precursors (that would only integrate with the ventral aortic endothelium to generate hematopoietic cells).

Following the establishment of the aortic endothelium, the importance of environmental cues in the fate of specialized endothelial cells was highlighted. Quail somatopleural mesoderm placed in contact with endoderm tissues could thereafter integrate into the floor of the dorsal aorta (although initially lacking such potential) and form IAHCs. On the other hand, splanchnopleural mesoderm treated with ectoderm tissues had a lower potential to integrate into the floor of the aorta (Pardanaud and Dieterlen-Lievre, 1999). These experiments suggest that the different types of mesoderm have cells with hemogenic endothelial potential, but that only the splanchnopleural mesoderm instructs these cells to ultimately become hemogenic. In the mouse model, the aorta dissected along with ventral endoderm tissue (including the gut) provided a greater repopulation in irradiated adult recipients when compared to the aorta dissected along with dorsal ectoderm tissue (including the neural tube) (Peeters et al., 2009). Such results suggest that polarized factors determine hematopoietic fate. In this case, the next question is to determine which factors are implicated. In chicken embryos, growth factors such as VEGF, bFGF or TGFβ1 led to similar effects on quail somatopleural mesoderm, as with exposure to endoderm tissues (Pardanaud and Dieterlen-Lievre, 1999). On the other hand, growth factors like EGF and TGFα could mimic the effect of contact between splanchnopleural mesoderm and ectoderm (Pardanaud and Dieterlen-Lievre, 1999). In mice, the culture of AGM as explant in presence of hedgehog proteins during 3 days enhances the hematopoietic activity in the AGM (similarly to the explant culture of AGM plus gut) (Peeters et al., 2009). However, the reverse situation was observed when AGM dissected with endoderm (gut) tissue was incubated with blocking antibody targeting hedgehogs (similar to the explant culture of AGM plus dorsal tissue), at least in the early stage of HSC emergence (E10.5) (Peeters et al., 2009). Likewise, in zebrafish embryos, hedgehog seems to affect the stage before HSC emergence,

when the dorsal aorta is forming, which eventually affects definitive hematopoietic specification (Gering and Patient, 2005; Wilkinson et al., 2009). Bone Morphogenetic Protein (BMP) 4 is another inducer of the hematopoietic fate. Again in zebrafish, it was shown that BMP4 is required for HSC emergence, while not affecting the arterial specification, suggesting that BMP4 has a latter effect on HSC specification in comparison to Hedgehog. In mice, a mild increase in HSC activity was observed after the addition of BMP4 to AGM explant cultures whereas the addition of Gremlin, a BMP antagonist, greatly decreased it. Notably, BMP4 expression was predominant in the ventral part of the aorta in different cell populations, including the surrounding mesenchyme and cells of the IAHCs (Durand et al., 2007). IL-3, a well-known adult hematopoietic interleukin, is also a potent survival and amplification factor of mouse embryonic HSCs (Robin et al., 2006; Robin and Durand, 2010). Finally, another essential player for definitive hematopoiesis formation is Notch. However, it seems to be required mainly when HSCs emerge from the endothelium, either in zebrafish (Bertrand et al., 2010; Gering and Patient, 2005; Lee et al., 2009) or mice (Kumano et al., 2003; Robert-Moreno et al., 2005, 2008). Although it is clear that Hedgehogs, BMP4, IL-3 and Notch act to induce definitive hematopoietic formation, it remains to be shown which cell types produce and/or respond to these signals: mesodermic precursor, hemogenic endothelial cells and/or IAHC cells. It is of interest to note that although the hemogenic endothelium localization is strictly polarized in most models, this seems less stringent in the mouse embryo since IAHCs are also present, albeit in fewer number, in the roof of the dorsal aorta (Yokomizo and Dzierzak, 2010; Taoudi and Medvinsky, 2007).

Conclusion

Since the discovery of IAHCs in the early 20th century and of HSCs about 50 years later, a tremendous amount of work has been performed to understand their nature, origin and fate. The connection between HSCs and IAHCs has been established, as well as their direct endothelial origin. However, important long-standing questions are still lacking definitive answers. The formation of HSCs in the embryo is thus one of the most striking and thought-provoking models to study. From a stem cell biology perspective, where hierarchy is the gold standard, it is puzzling to observe that blood stem cells do not derive directly from a mesoderm undifferentiated blood precursor, but from a seemingly differentiated—or mature—endothelial population. Later, migration of HSCs through multiple niches results in a paradigm for the regulation of the newly acquired stem cell fate (i.e. the fragile balance between self-renewal, differentiation and quiescence). Given the profound interest in reprogramming differentiated cells to a stem cell state, it will definitely still be of interest to continue pursuing a greater understanding of how cells acquire self-renewing capabilities and regulate them in such a natural set of events, as it happens in the developing embryo.

Competing financial interests

The authors declare that they have no competing financial interests.

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