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# Embryonic origin of the adult hematopoietic system: advances and questions

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## Summary

Definitive hematopoietic stem cells (HSCs) lie at the foundation of the adult hematopoietic system and provide an organism throughout its life with all blood cell types. Several tissues demonstrate hematopoietic activity at early stages of embryonic development, but which tissue is the primary source of these important cells and what are the early embryonic ancestors of definitive HSCs? Here, we review recent advances in the field of HSC research that have shed light on such questions, while setting them into a historical context, and discuss key issues currently circulating in this field.


**Key words: AGM region, Yolk sac, Placenta, dHSC, Pre-dHSC**

## Introduction

The ontogeny of the vertebrate hematopoietic system is characterized by two waves: embryonic hematopoiesis, which generates transitory hematopoietic cell populations, such as primitive erythrocytes and some myeloid cells; and permanent, definitive hematopoiesis, which originates later in development from definitive hematopoietic stem cells (dHSC) and which gives rise to all the mature blood (erythroid, myeloid and lymphoid) lineages in the adult organism.

It was Francoise Dieterlen-Lievre in 1975 (Dieterlen-Lievre, 1975), who showed, using quail chick chimeric embryos, that the adult hematopoietic system originates inside the embryo proper: 'haemopoietic stem cells of the definitive blood cell series originate from a source other than the yolk-sac, and that this source must be intra-embryonic' (Dieterlen-Lievre, 1975). The use of the term 'definitive' in this context is clear, meaning 'fully formed and developed'. Thus, although from the mid 1990s the term 'definitive' has also been used to refer to any hematopoietic progenitor cells emerging during embryogenesis or during embryonic stem (ES) cell differentiation that can produce myeloid and adult-type enucleated erythroid cells, historically and more accurately, the term 'definitive HSC' (dHSC; see Glossary, Box 1) refers to a stem cell that gives rise to the true adult hematopoietic system. The term 'definitive HSC' is specific to developmental hematopoiesis because it refers to a stage in HSC development when an HSC acquires the ability to provide long-term hematopoiesis upon transplantation to wild-type irradiated adult recipients (see Box 2).

Investigating the developmental events that lead to the formation of a functioning hematopoietic system in an adult organism has been a challenging pursuit for several reasons: (1) in contrast to solid tissues, the cells of the hematopoietic system are scattered throughout the organism, and multiple sites of hematopoietic activity exist in the embryo, thus obscuring its anatomical boundaries; (2) dHSCs in the early embryo are very rare: there is approximately one dHSC per hematopoietic organ in the E11.5 mouse embryo prior to the onset of fetal liver hematopoiesis (Kumaravelu et al., 2002); (3) the embryo contains an unknown number of pre-dHSCs (see Glossary, Box 1), which contribute to the growing dHSC pool; and finally, (4) no markers are known to be exclusively expressed on dHSCs in the embryo – markers such as c-kit, CD34, Sca-1, Mac1, VE-cadherin and CD45, are shared with other cell types (North et al., 2002; Sanchez et al., 1996; Taoudi et al., 2005).

It has also been a challenge to derive dHSCs from ES cells ex vivo, an important goal for regenerative medicine. In order to achieve this aim, the events that lead to the development of the first definitive HSC during embryogenesis need to be identified. Although various transplantation and ex vivo maturation assays have been developed (see Box 2) and some progress has been made, the identification of pre-dHSCs in the mouse embryo and tracking of the origin of dHSCs to the earliest embryonic stages of vertebrate development remain challenging tasks.

Hematopoiesis research utilizes several vertebrate model organisms, including mice, zebrafish and chick, each of which has its own advantages. For example, the external development of avian and amphibian embryos has enabled important information to be obtained on the spatial origin of hematopoiesis through in vivo grafting, and zebrafish studies have facilitated the real-time imaging of hematopoietic development. In this review, we discuss recent advances in our understanding of the development of the definitive hematopoietic system (focusing predominantly on mouse research but also considering findings from other model species), which include the identification of a common progenitor for hematopoietic and endothelial cells, called the hemangioblast (see Glossary, Box 1), and new insights into the relationship between the physical forces of circulation and the genetic mechanisms of hematopoiesis. We also highlight existing controversies and discussions in the field. Given this focus, we do not discuss ES cell differentiation and certain aspects of hematopoietic development in non-mammalian vertebrates. We refer readers to other reviews on these topics (Bertrand and Traver, 2009; Cerdan and Bhatia, 2010; Chen and Zon, 2009; Ciau-Uitz et al., 2010a; Cumano and Godin, 2007; Dzierzak and Speck, 2008; Gering and Patient, 2008; Martinez-Agosto et al., 2007; Murry and Keller, 2008; Zon and Paik, 2010).

## Hematopoiesis in the embryo: an overview

Between E7.0 and E7.5 of mouse development, the extra-embryonic mesoderm ingresses through the posterior primitive streak and undergoes hematopoietic differentiation in the yolk sac (see Glossary, Box 1) (Silver and Palis, 1997). Although hematopoietic cells in the yolk sac were described in the late 19th century, it was

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**Box 1. Glossary**

**Aorta-gonad-mesonephros (AGM) region.** An embryonic tissue originating from the para-aortic splanchnopleura (P-Sp) and consisting of the dorsal aorta and urogenital ridges (UGR). It is involved in generating dHSCs prior to the onset of hematopoiesis in the fetal liver.

**Blast colonies.** Colonies of ES-cell- or embryo-derived cells of mesodermal origin (hemangioblasts) that can differentiate into hematopoietic, endothelial and smooth muscle cells.

**Blood island(s).** An isolated area of hematopoietic differentiation in the early yolk sac prior to formation of the vascular network.

**CFU-C, colony-forming units-culture.** Progenitor cells that can be stimulated in vitro to generate a colony of hematopoietic cells, which cannot self-renew, although some can be replated a limited number of times. CFU-Cs are classified according to the composition of the colonies they generate.

**CFU-S, colony forming units-splenic.** Immature progenitor cells that can form morphologically distinguishable colonies of myeloid cells in spleens of irradiated animals following transplantation. Some have a limited self-renewal capacity determined by secondary transplantation.

**Definitive hematopoietic stem cell (dHSC).** Rare cells in the embryo that can give rise to all lineages of an adult hematopoietic system. They are defined by their ability to reconstitute the hematopoietic system of irradiated wild-type animals on transplantation (see Box 2).

**Dorsal aorta.** A major arterial vessel in the embryo that is part of the AGM region.

**Erythromyeloid progenitors (EMPs).** Progenitor cells capable of producing erythrocytes and granulocytes/macrophages but not B- or T-lymphocytes.

**Hemangioblast.** A cell that can differentiate into both endothelial and hematopoietic lineages.

**Hematogenic endothelial cell.** An endothelial cell that can differentiate into a hematopoietic cell.

**High proliferation potential forming cell (HPP-CFC).** Myeloid progenitors with high proliferative potential that form large (≥5 mm) diameter colonies.

**Multipotent progenitors (MPP).** Progenitor cells that give rise both to lymphoid and erythromyeloid lineages but that cannot self-renew.

**OP9 culture system.** A culture technique that uses OP9 stromal cells derived from newborn mouse calvaria that can facilitate the efficient differentiation of embryonic cells into hematopoietic and endothelial lineages.

**Para-aortic splanchnopleura (P-Sp).** A caudal part of the E8.0 mouse embryo that contains the dorsal aorta, omphalomesenteric artery, gut and splanchnopleural lining of these tissues. Part of this later forms the AGM region.

**Pre-definitive hematopoietic stem cell (pre-dHSC).** A cell of the HSC lineage in the embryo that has hematopoietic identity that cannot yet repopulate wild-type adult irradiated recipients. Pre-dHSCs develop into dHSCs by acquiring characteristics of adult bone marrow HSCs (such as the capacity to self-renew, homing receptors, etc.).

**Rag2γc$^{-/-}$ mice.** A severely immunocompromised mouse mutant strain that lacks B, T and NK cells. They are used to detect some low level long-term repopulating cells that cannot engraft wild-type recipients.

**Urogenital ridges (UGR).** A part of the AGM region, which harbors the embryonic kidney rudiments and the genital ridges.

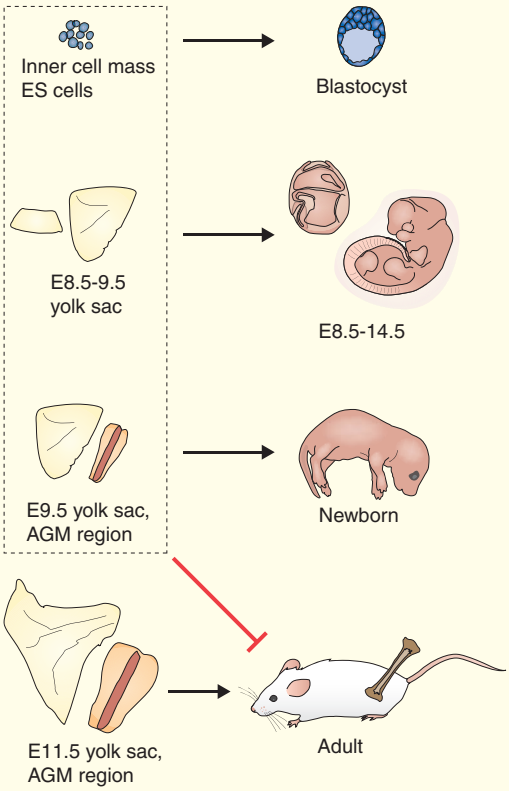
**Yolk sac.** An extra-embryonic organ, consisting of extra-embryonic endoderm and mesoderm, in which the first visible hematopoietic differentiation occurs. It is the source of large primitive nucleated erythrocytes that express embryonic hemoglobins and some early myeloid cells.

only with the development of in vitro assays that active hematopoiesis within the yolk sac was demonstrated experimentally (Moore and Metcalf, 1970). In these experiments, the yolk sac was shown to harbor in vitro clonogenic myeloid progenitors (called CFU-Cs, for colony forming units-culture) and, importantly, adult-type dHSCs and CFU-S (for colony forming units-splenic) (see Glossary, Box 1). However, a more stringent assay, in which irradiated recipient mice were used to prevent the regeneration of endogenous splenic colonies, showed that the yolk sac lacks CFU-S prior to E9.5 (Medvinsky et al., 1993) and subsequent studies showed lack of dHSCs prior to E11.5 (Medvinsky and Dzierzak, 1996; Muller et al., 1994). Thus, paradoxically, yolk sac hematopoiesis occurs in the absence of dHSCs. Further studies identified the presence of CFU-S in the aorta-gonad-mesonephros (AGM) region of the mouse embryo (see Glossary, Box 1), beginning at E9.5, in consistently higher numbers than in the yolk sac (Medvinsky et al., 1993). Also, lymphoid precursors appear inside the embryo earlier than they do in the yolk sac (Godin et al., 1993) although this conclusion has recently been challenged (Yoshimoto et al., 2011). Finally, the AGM region has been shown to be a powerful source of dHSC activity (Muller et al., 1994; Medvinsky and Dzierzak, 1996). These findings suggested that adult mammalian hematopoiesis has an intra-embryonic origin, in agreement with earlier observations made in avian embryos (Cormier and Dieterlen-Lievre, 1988; Dieterlen-Lievre, 1975), but they did not fully exclude the possibility that the yolk sac contributes to the adult hematopoietic system as embryonic ancestors of dHSCs were not detectable owing to the lack of appropriate assays. The identification of additional embryonic hematopoietic sites that harbor dHSCs has further complicated the picture of hematopoietic development (Fig. 1A,B) (Cumano and Godin, 2007; Dzierzak and Speck, 2008), and the exact role of the various embryonic HSC niches in the generation of the adult hematopoietic system in the mouse remains a debatable issue. As such, Fig. 1 takes into account conflicting views on this subject.

As mentioned above, studies of the development of the adult hematopoietic system are hampered by several factors. In the absence of unique markers, quantitative limiting dilution analysis, which is based on the transplantation of aliquots that contain very few dHSCs, has become an important tool that enables the anatomical description of dHSC development (Szilvassy et al., 1990). At the pre-liver stage of development, the E11.5 AGM region, placenta and yolk sac each contain approximately one dHSC and subsequent dHSC production occurs via maturation of pre-HSCs (Gekas et al., 2005; Kumaravelu et al., 2002). Colonization of the fetal liver by dHSCs at E12.5 coincides with their appearance in the embryonic circulation. Further expansion of dHSCs in the fetal liver probably occurs through proliferation and is mediated by such molecules as angiopoietin-like factors and Sox17 transcription factor (Kim et al., 2007; Zhang et al., 2006). β1 integrins are also essential for fetal liver colonization by dHSCs. β1 integrin-null HSCs fail to colonize the mouse liver and instead accumulate in the circulation (Hirsch et al., 1996; Potocnik et al., 2000). By contrast, α4 integrin$^{-/-}$ mouse embryos develop normal dHSCs both in the AGM region and in the yolk sac, and these dHSCs do colonize the liver although with reduced efficiency (Gribi et al., 2006). However, during fetal/neonatal stages, α4 integrin-null dHSCs progressively lose their capacity to complete differentiation (Arroyo et al., 1996; Arroyo et al., 1999).

Although involvement of the fetal liver and bone marrow in hematopoiesis varies between different vertebrate species, the initial sites of hematopoietic activity, such as the yolk sac and the

**Box 2. Functional analysis of HSC development**




A key characteristic of adult bone marrow hematopoietic stem cells (HSCs) is their capacity to engraft and provide a long-term supply of all types of blood cells to adult irradiated wild-type recipients upon intravenous injection. Whole-body irradiation is used to ablate hematopoietic cells of a host in order to clear the HSC niches to accept transplanted exogenous HSCs. A highly purified population of HSCs shows a remarkably high efficiency of long-term multilineage engraftment (>3.5 months) when individual cells were transplanted (Kiel et al., 2005).

Although somewhat artificial, the long-term repopulation assay employs the natural abilities of adult HSCs to home to bone marrow niches, to self-renew and to differentiate into all myeloid and lymphoid lineages. As such, the long-term multilineage engraftment remains a key in vivo functional test for the detection and characterization of HSCs. The absence of either of the above HSC properties results in the lack of long-term hematopoietic repopulation. Other hematopoietic progenitor cells can only repopulate an irradiated host for up to 6 weeks owing to their inability to self-renew.

Definitive (d)HSCs that possess a full set of adult HSC properties emerge only at a certain stage of development. The direct transplantation of even large numbers of embryonic cells prior to E10.0 into adult wild-type irradiated recipients does not produce long-term hematopoietic repopulation (Muller et al., 1994), shown by red blocking bar in the accompanying figure. Lack of engraftment means the absence of dHSCs, although their precursor pre-dHSCs must exist in the embryo.

As shown in the Box figure, other functional assays have been devised to detect pre-dHSCs that allow cells to mature either in vitro or in vivo. This figure shows how the hematopoietic potential of immature cells that precede dHSC specification, isolated from different sources or tissues (left), can be revealed by their transplantation into the embryo and into newborn recipients, which leads to their contribution to the hematopoietic system of the adult host. This is probably because embryonic and newborn microenvironments ensure the maturation of embryonic cells into dHSCs, which can then function in the adult bone marrow. Beginning from late E10-11, dHSCs mature in the embryo and hematopoietic tissues can successfully repopulate wild-type adult irradiated recipients.

AGM region, are largely conserved. Given this multiplicity of hematopoietic sites and a variety of hematopoietic progenitors in the embryo, as well as the highly migratory nature of hematopoietic cells, can the origin of dHSCs be pinned down?

## Embryonic and adult hematopoietic hierarchies: their relationship during development

A major puzzle in defining the embryonic origin of the mouse adult hematopoietic system comes from the observation that during embryogenesis members of the hematopoietic hierarchy appear in a reverse sequence compared with that of the adult hematopoietic hierarchy (Fig. 2). How does the production of differentiated hematopoietic cells and CFU-Cs occur in the absence of dHSCs? Two models that account for this sequence reversal can be proposed.

Model 1 suggests that the mouse embryo generates embryonic and adult (definitive) hematopoietic systems independently (Fig. 3A). The embryonic system emerges first, serves the short term needs of the developing embryo and exists transiently. The definitive (adult) hematopoietic system emerges independently through the specification of dHSCs, which ensure life-long hematopoiesis. Model 2 suggests a common origin for the embryonic and adult hematopoietic hierarchies (Fig. 3B). This model implies the existence of a common hematopoietic ancestor cell, which first generates a ‘wave’ of embryonic hematopoiesis and later produces dHSCs. This common hematopoietic ancestor is not detectable in standard transplantation assays until later when dHSC specification occurs.

The appearance of CFU-Cs prior to the specification of dHSCs fits both models (Fig. 3). In both models, dHSCs mature from pre-dHSCs by acquiring certain essential stem cell characteristics. Interestingly, the progressive enhancement of hematopoietic potential during both mouse and zebrafish development is observed at the progenitor level: the first wave of primitive erythrocytes is followed by the sequential appearance of more diverse progenitor waves (Palis et al., 1999; Bertrand et al., 2008; Chen and Zon, 2009). Thus, two alternative pathways might underlie the later appearance of dHSCs: (1) the first progenitors and dHSCs are not linearly linked and develop in independent hierarchies (Model 1); and (2) progenitors gradually develop ‘stemness’ and become dHSCs (Model 2). In this review, we avoid using the term ‘definitive progenitors’, nowadays often applied to any multipotent clonogenic progenitor in the embryo, owing to uncertainty as to which hierarchy (embryonic/transitory or definitive/adult) they belong.

How do these two models relate to the tissue organization of hematopoiesis in the embryo (Fig. 3)? Model 1 is typically associated with the concept of a dual origin of hematopoiesis and is currently broadly accepted. Specifically, the yolk sac and the AGM region (and perhaps placenta) are considered to represent the sites of embryonic and definitive hematopoiesis, respectively. Observations from non-mammalian vertebrates (avian, amphibian and zebrafish models) support this view (Ciau-Uitz et al., 2000; Dieterlen-Lievre, 1975; Dzierzak and Medvinsky, 2008). Model 2 is usually associated with the idea that the entire (embryonic and adult) hematopoiesis in the animal originates exclusively from the yolk sac, with the AGM region functioning as an intermediate ‘educational’ site for developing HSCs prior to their colonization of the liver (Samokhvalov et al., 2007).

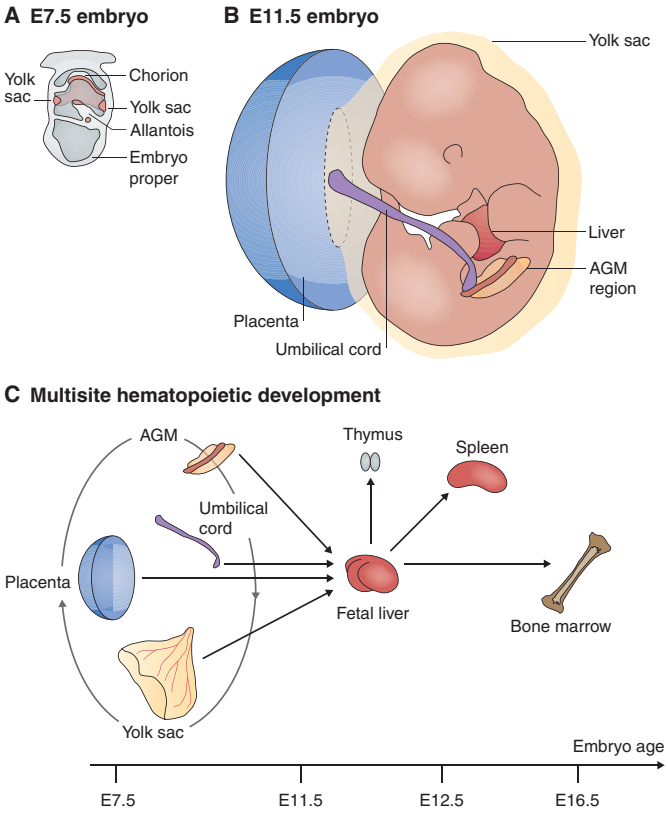



**Fig. 1. Continuity in embryonic hematopoietic development.** (**A**) A schematic of an E7.5 mouse embryo (anterior to the left), showing extraembryonic hematopoietic sites (yolk sac, chorion, allantois), which are marked by *Runx1* expression (pink). In the body, *Runx1* expression begins soon after E8.0 in the posterior region within the para-aortic splanchnopleura (P-Sp), in the dorsal aorta (not shown). The liver rudiment starts developing by the end of E9. (**B**) A schematic of an E11.5 mouse embryo (anterior uppermost), showing tissues that develop into embryonic hematopoietic stem cell (HSC) niches: the chorion contributes to the placenta; the allantois to the umbilical cord and partly to the placenta; the P-Sp develops into the aorta-gonad-mesonephros (AGM) region. The yolk sac expands and encompasses the embryo. (**C**) A model of multisite hematopoietic development, showing hematopoietic progenitors and definitive (d)HSCs from the sites shown in B colonizing the liver rudiment and each other (as shown by the circular arrow). Disagreement exists about which of these sites is the genuine source of dHSCs and the adult hematopoietic system. After expansion in the fetal liver, dHSCs colonize the bone marrow, spleen and thymus. In adulthood, the thymus and spleen are colonized by bone marrow progenitors (not shown).

Which biological features of the AGM region make it the prime candidate for being the site of the origin of the adult hematopoietic system? We discuss these features further below.

## The AGM region: an embryonic site of HSC activity

### Patterning and hematopoietic programming of the AGM region

The formation of the AGM region follows gastrulation and has been best described in non-mammalian vertebrates such as zebrafish and *Xenopus laevis*. In the amphibian embryo, the dorsal aorta and the ventral blood island (see Glossary, Box 1), which are analogous to the yolk sac, originate from different blastomeres, thus having distinct spatial origin (Ciau-Uitz et al., 2000). Shortly after gastrulation, laterally located mesodermal stripes (termed dorsolateral plate, DLP, in amphibians and posterior lateral mesoderm, PLM, in zebrafish) migrate towards the midline beneath the notochord and form the dorsal aorta/hematopoietic cells, cardinal veins and nephric ducts (see Fig. 4) (Fouquet et al., 1997; Gering et al., 1998). In the mouse embryo, genetic labeling has shown that hematopoietic clusters (discussed in more detail below) and the endothelium of the dorsal aorta originate from the lateral mesoderm (Zovein et al., 2010).

There are three key stages to the adult hematopoietic program in the AGM region: (1) DLP/PLM specification; (2) migration of cells from the DLP to the midline and the formation of the dorsal aorta; and (3) arterial specification and emergence of adult hematopoietic cells. The specification of DLP-derived cells into endothelial lineage cells, marked by Flk1 and Fli1, and into hematopoietic lineage, marked by Scl, Gata2 and Lmo2, occurs prior to the morphogenetic movement of DLPs to the midline (Dooley et al., 2005; Gering et al., 1998). Promiscuous endothelial-hematopoietic gene expression in the DLP is thought to indicate the presence of hemangioblasts. As shown in *Xenopus*, both expression of Scl and Gata2 in DLPs and medial migration of $Flk1^{+}$ DLP cells is regulated by Vegf (Ciau-Uitz et al., 2010b; Cleaver and Krieg, 1998), which itself is under control of Tel1 (ETV6) transcription factor (Ciau-Uitz et al., 2010b).

Once the cells of the DLP reach the midline, some of them express important hematopoietic transcription factors, such as Runx1. Whereas midline angioblast coalescence occurs in zebrafish embryos even when arterial and adult hematopoietic programs are suppressed, the subsequent morphogenesis of the aorta is intimately linked to arterial and adult hematopoietic development (Burns et al., 2005; Gering and Patient, 2005; Lawson et al., 2001). In zebrafish embryos, activation of both the arterial and adult hematopoietic programs is under the control of a signaling cascade that involves Hedgehog, Vegf and Notch. In this context, the Hairy-related transcription factor Hey2 acts downstream of Vegf and, unexpectedly, upstream of Notch (Rowlinson and Gering, 2010). Bmp4 is essential only for the hematopoietic program but not for arterial specification (Wilkinson et al., 2009). In the mouse, Bmp4 and Shh have been reported to expand dHSC numbers in the AGM region (Durand et al., 2007; Peeters et al., 2009).

### AGM region: a source of HSCs

The pivotal role of the AGM region in dHSC formation (Muller et al., 1994) became evident once it was shown to have the autonomous capacity to generate dHSCs (Medvinsky and Dzierzak, 1996). Explants of mouse E10.5 and E11.5 AGMs can initiate and expand dHSCs, respectively (Kumaravelu et al., 2002). In the presence of growth factors, including interleukin 3, endogenous dHSCs can undergo a 150-fold expansion in explanted AGMs, approximating the process in the fetal liver (Robin et al., 2006; Taoudi et al., 2008). To date, attempts to initiate or to expand dHSCs from other early hematopoietic tissues have not been successful (de Bruijn et al., 2000b; Ottersbach and Dzierzak, 2005).

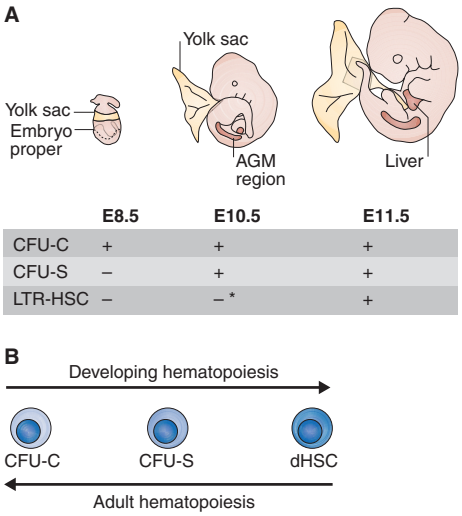
Prior to the establishment of the circulation, cultured explants of mouse E8.0 para-aortic splanchnopleura (P-Sp; see Glossary, Box 1) can develop in vitro multilineage progenitors, but yolk sac hematopoietic potential is limited to erythromyeloid lineages (Cumano et al., 1996), as is also seen in human embryo yolk sacs (Tavian et al., 2001). Cultured mouse E8.0 P-Sp explants can generate long-term lymphomyeloid cells, which can repopulate $Rag2\gamma c^{-/-}$ immunocompromised recipients (see Glossary, Box 1

and Box 2); yolk sac explants can generate only short-term myeloid engrafting cells in this assay (Cumano et al., 2001). Although P-Sp-derived repopulation is low level and the lineage relationship with high-level repopulating dHSCs remains to be elucidated, cumulative evidence suggests that the P-Sp/AGM region is a primary source of dHSCs in the embryo.

Transplantations of mouse E11.5 dorsal aorta and urogenital ridges (UGRs, see Glossary Box 1) into adult wild-type irradiated recipients have shown that dHSCs localize almost exclusively to the dorsal aorta (de Bruijn et al., 2000b; Taoudi and Medvinsky, 2007), which correlates with a lack of 'intra-aortic' clusters in cardinal veins. However, by E12.5, dHSCs are present in UGRs (de Bruijn et al., 2000b), and E11.5 UGR explants can also generate dHSCs and CFU-S, either from precursors that immigrate from the dorsal aorta or that form de novo (de Bruijn et al., 2000a; de Bruijn et al., 2000b). These data indicate that UGRs are integral functional parts of the AGM region.

## Dorsoventral polarization of dHSC development

Intra-aortic cell clusters, which are found in various species, express hematopoietic and endothelial markers and are usually attached to the ventral floor of the dorsal aorta (Jaffredo et al., 1998; Medvinsky et al., 1996; Smith and Glomski, 1982; Tavian et al., 1996; Yokomizo and Dzierzak, 2010). They are thought to derive from the endothelium as a result of the initiation of the hematopoietic program (see Fig. 5). In *Xenopus* and zebrafish, the endothelial floor and underlying mesenchyme of the newly formed dorsal aorta express key markers of hematopoietic specification: Scl, Runx1, Gata-2, cMyb and Ikaros (Ciau-Uitz et al., 2000; Gering and Patient, 2005). Runx1 shows a similar expression pattern in mammals (Azcoitia et al., 2005; North et al., 1999). Ventrally expressed Bmp4 in sub-aortic mesenchyme (Durand et al., 2007; Marshall et al., 2000; Wilkinson et al., 2009) and Shh in the developing gut might be responsible for dHSC specification in the floor of the dorsal aorta (Peeters et al., 2009). Although Hh is required for the initiation of the hematopoietic program, it later activates a non-hematogenic program in the roof of the zebrafish dorsal aorta (Wilkinson et al., 2009). Such opposite effects of hedgehog signaling on hematopoietic development are probably stage- and concentration-dependent. In addition, the distinct origin of the roof and floor of the dorsal aorta (from somitic and lateral mesoderm, respectively), as shown in avian embryos, is likely to contribute to dorsoventral asymmetry in HSC development (Pardanaud et al., 1996). The short-term HSC activity of the AGM region (Muller et al., 1994; Kumaravelu et al., 2002) might be explained by a gradual replacement of the ventral hematogenic endothelium with a non-hematogenic somite-derived endothelial lining (Pouget et al., 2006). Although mouse aortic floor and roof are equally enriched with CFU-Cs, only floor explants support the expansion of CFU-Cs (Taoudi and Medvinsky, 2007). Meanwhile, dHSCs are localized mainly to, and can be expanded ex vivo by, the aortic floor. In addition to hematopoietic budding inside the aortic lumen, the sub-endothelial migration of hematopoietic cells has also been observed (Jaffredo et al., 1998). In zebrafish, it has been recently shown that multipotent hematopoietic progenitors (MPP)/HSCs (see Glossary, Box 1) bud from the aortic floor and migrate ventrally, entering the circulation through the cardinal vein (Bertrand et al., 2010a; Kissa and Herbomel, 2010; Lam et al., 2010) (Fig. 4). Whether interstitial ventrolateral migration towards cardinal veins also occurs in the mouse embryo remains unclear.




| | E8.5 | E10.5 | E11.5 |
|---|---|---|---|
| CFU-C | + | + | + |
| CFU-S | – | + | + |
| LTR-HSC | – | – * | + |

**Fig. 2. Appearance of hematopoietic progenitors and HSCs in the mouse embryo.** (**A**) (Upper) Schematics of developing mouse embryos at stages E8.0, E10.0 and E11.0, showing sites of hematopoiesis: the yolk sac and embryonic liver (the placenta, also such a site, is not shown). (Lower) Time points at which hematopoietic progenitors appear. Early E8.0 embryos contain CFU-Cs that can be detected in vitro, but not CFU-S or dHSCs. CFU-S are first detectable by late E9-early E10. The first dHSC is reliably detected only by E11.0. Asterisk indicates that on very rare occasions HSCs can be detected. (**B**) The sequence in which hematopoietic progenitors and dHSCs appear during development at the time points shown in A. This sequence is opposite to that in the adult, in which CFU-C and CFU-S progenitors derive from dHSCs (as denoted by the lower arrow). CFU-C, colony-forming units culture; CFU-S, colony-forming units splenic; dHSC, definitive hematopoietic stem cell.

## Runx1 and Notch: key regulators of AGM hematopoiesis

The transcription factor Runx1 is a key regulator of dHSC development in various species (Burns et al., 2005; Kalev-Zylinska et al., 2002; Swiers et al., 2010). Although yolk sac erythropoiesis in *Runx1*-null mouse embryos is almost normal, the embryos lack clonogenic progenitors and dHSCs and die by E12.5 (Okuda et al., 1996; Yokomizo et al., 2008). Runx1 is expressed preferentially in hematopoietic clusters, in some endothelial cells and in the mesenchyme that underlies the endothelium, marking potential candidate progenitors of the developing HSC lineage (North et al., 1999). *Runx1* is expressed in AGM dHSCs as shown by transplantation (North et al., 2002; Nottingham et al., 2007). The lack of intra-aortic clusters in *Runx1*$^{-/-}$ embryos led to a key idea in this field: that *Runx1* regulates endothelial-hematopoietic transition in the dorsal aorta (Fig. 4B) (North et al., 1999; Yokomizo et al., 2001). Indeed, *Runx1* deficiency prevents the differentiation of hematopoietic cells from ES cell-derived hematogenic endothelium (Lancrin et al., 2009). Inhibition of Runx1 by morpholino in zebrafish blocks endothelial-hematopoietic transition in the dorsal aorta and results in the apoptosis of cells 'attempting' to undergo this process (Kissa and Herbomel, 2010). The role of Runx1 in the generation of mouse dHSCs from aortic endothelial cells is likely to be similar (Chen et al., 2009). Interestingly, hematopoietic activity in the mouse *Runx1*$^{-/-}$ AGM region can partly be rescued by overexpression of *Runx1*, suggesting that AGM hematopoiesis originates locally and is yolk sac independent (Goyama et al., 2004).

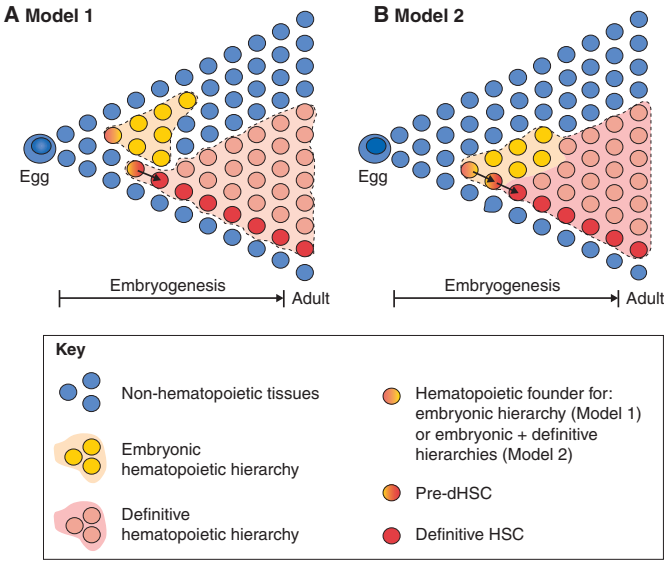



**Fig. 3. Models of the relationship between embryonic and adult hematopoietic hierarchies in mouse development.** (**A**) In Model 1, the embryonic and definitive (adult) hematopoietic hierarchies are two separate systems in which the embryonic hematopoietic hierarchy is a transitory cell population that emerges early during development and the definitive hematopoietic hierarchy emerges independently during later development from separately specified dHSCs, which develop from pre-dHSCs. (**B**) In Model 2, one hematopoietic system develops. Both embryonic and definitive hematopoietic hierarchies originate from a common hematopoietic founder cell. The embryonic hierarchy is an early transitory 'branch' of the hematopoietic system and the definitive hematopoietic hierarchy emerges later from dHSCs, which mature from an earlier intermediate hematopoietic cell (pre-dHSC). In both models, hematopoietic progenitors emerge prior to dHSC specification (in opposite orientation to the adult hematopoietic hierarchy, as outlined in Fig. 2). Neither model implies a single tissue is the origin of hematopoiesis. In principle, more than one tissue might contribute to embryonic and/or definitive hematopoietic hierarchies. dHSCs, definitive hematopoietic stem cell.

Notch signaling is also essential for dHSC development, as shown by the lack of contribution of *Notch1*$^{-/-}$ ES cells to the adult hematopoietic system of chimeric [wild type::*Notch1*$^{-/-}$] mice (Hadland et al., 2004) and by the failure of *Notch1*$^{-/-}$ E9.5 yolk sac and P-Sp cells injected into newborn recipients to contribute to adult hematopoiesis (Kumano et al., 2003). In contrast to *Runx1*$^{-/-}$ mutants, *Notch1*$^{-/-}$ embryos develop normal numbers of CFU-Cs in the yolk sac and very few in the body of the embryo (Hadland et al., 2004; Kumano et al., 2003). Suppression of Notch signaling in zebrafish also inhibits hematopoiesis in the AGM, but not in the yolk sac or posterior blood island (Bertrand et al., 2010b). Thus, Notch1 might be a specific developmental regulator of the true definitive hematopoietic hierarchy. Indeed, in the mouse E10.5 dorsal aorta, Notch1, as well as the Notch ligands Jag1 and Jag2 and its targets Hes1, Hrp1 (Hdgfl1 – Mouse Genome Informatics) and Hrp2 (Hdgfrp2 – Mouse Genome Informatics), are preferentially expressed in the ventral floor and in intra-aortic clusters (Robert-Moreno et al., 2005; Robert-Moreno et al., 2008). Notch1 directly regulates Gata2 (Robert-Moreno et al., 2005; Robert-Moreno et al., 2008), a key factor of HSC development, which in turn is involved in the regulation of Runx1 (Nottingham et al., 2007). Enforced expression of both *Gata2* and *Runx1* can partly rescue AGM hematopoiesis in *Notch1* and *Jagged 1* mutants (Nakagawa et al., 2006; Robert-Moreno et al., 2008), again suggesting an independent origin of AGM hematopoiesis in the mouse.

In addition to the absence of intra-aortic clusters, *Runx1* and *Notch* mouse mutants exhibit interesting morphological changes in the AGM region. *Runx1* deficiency causes abnormally excessive crowding of *Runx1*-null mesenchymal cells beneath the dorsal aorta (North et al., 1999). It is not clear whether this is because aortic hematogenic endothelial cells (see Glossary, Box 1) undergo mesenchymal instead of hematopoietic differentiation in the absence of Runx1, or whether the formation of hematogenic endothelium from the underlying mesenchyme is blocked, resulting in the accumulation of mesenchymal cells. Notch regulates multiple aspects of endothelial biology, including the specification of arterial and venous endothelium (Phng and Gerhardt, 2009). In the absence of the common Notch signaling transducer recombination signal-binding protein 1 for j-kappa (RBPJκ; also known as Rbpj or RBPjk) or of Jagged 1, stratification of the mouse aortic endothelium occurs, which might reflect a block in endothelial-hematopoietic transition (Robert-Moreno et al., 2005; Robert-Moreno et al., 2008). By contrast, deletion of Chicken ovalbumin upstream promoter transcription factor II (CoupTFII), an orphan receptor that suppresses Notch signaling, results in 'arteriorization' of the venous endothelium and emergence of hematopoietic clusters in cardinal veins (You et al., 2005), suggesting that arterial and HSC programs are intimately linked. Accordingly, in the aorta of Tel1 *Xenopus* morphants, the arterial program is silenced concurrently with the suppression of the HSC program (Ciau-Uitz et al., 2010b).

Interestingly, AGM hematopoiesis is sensitive to changes in dosage of some genes. A haploid dose of both Runx1 and Gata2 reduces production of dHSCs in the AGM region (Cai et al., 2000; Ling et al., 2004). Meanwhile, Runx1 haploinsufficiency results in an early appearance of dHSCs in the YS (Cai et al., 2000). Mechanisms underlying such spatial and temporal rearrangements in HSC development within the embryo are not clear, but may hold a clue to how dHSCs are specified in the embryo.

Although numerous data indicate that AGM hematopoiesis is self-autonomous and intrinsically regulated, other tissues are also involved in hematopoiesis, as we discuss below.

## Early embryonic hematopoiesis outside the AGM region

### Placenta: a source or reservoir?

Although it has been known that the fetal placenta harbors dHSCs (Dancis et al., 1977; Dancis et al., 1968), the possibility that the placenta is colonized by HSCs from the fetal liver obscured the significance of this finding. The chorionic portion of the mouse placenta expresses *Runx1* from an early stage of development (Fig. 1A), and when isolated prior to fusion with the allantois can generate hematopoietic cells in culture (Zeigler et al., 2006). E8.0-9.0 mouse placenta contains many hematopoietic progenitors, including replatable HPP-CFC (see Glossary, Box 1) (Alvarez-Silva et al., 2003). As shown by transplantation, dHSCs appear in the placenta concomitantly with the AGM region by E10.5-11.0 (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). In parallel with AGM activity, the placenta rapidly develops a substantial dHSC pool (13-50 dHSC by E12.5), which declines by E15.5, suggesting that dHSCs translocate to the liver (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). The human placenta is also a niche for dHSCs (Robin et al., 2009).

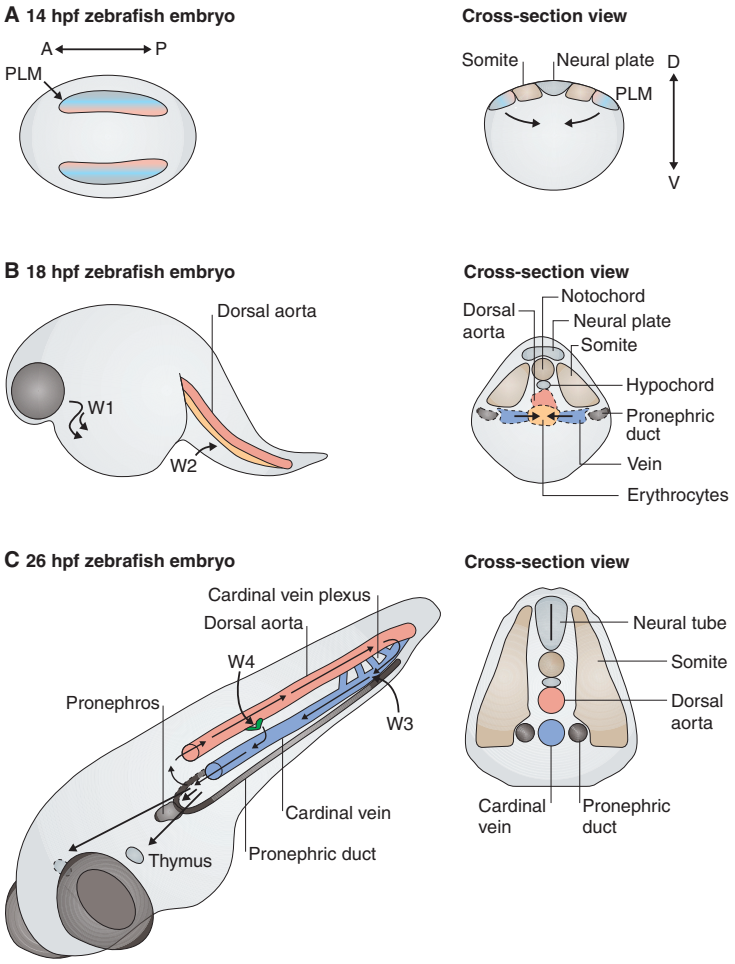



**Fig. 4. Zebrafish hematopoiesis occurs in four sequential waves.** (**A**) A 14 hours post fertilization (hpf) zebrafish embryo viewed from above (left) and in cross-section view (right), showing lateral stripes of posterior lateral mesoderm (PLM) that will form the dorsal aorta, cardinal vein, blood cells and the pronephros and its ducts after its migration towards the midline. The curved arrows show the two PLM stripes migrating to the midline. (**B**) An 18 hpf zebrafish embryo viewed from the side with dorsal uppermost (Left) and in cross-section view (right). At this stage, the wave of myeloid (pu.1$^+$/Scl$^+$) precursors, which originate in the anterior lateral mesoderm, migrate laterally across the yolk sac and mature into macrophages and granulocytes (see wave 1, W1). The migration of the PLM stripes towards the midline (as denoted by two black converging arrows in B, right) result in the differentiation of embryonic erythroid cells (denoted in pale orange shading) underneath the dorsal aortic angioblast cord (shown in pink) in wave 2 (W2). (**C**) A 26 hpf zebrafish embryo with cross-section view shown on the right. At this stage, erythromyeloid progenitors appear that lack lymphoid differentiation potential in the caudal hematopoietic tissue (also called posterior blood island and caudal vein plexus). These progenitors colonize the pronephros but not the thymus (wave 3, W3). W3 is followed by the emergence of MPP/HSCs from the floor of the dorsal aorta (wave 4, W4). MPP and HSC formation occurs through a process called endothelial-hematopoietic transition: the elongated endothelial cell (marked in green) starts expressing Runx1, c-Myb, CD41, bends and becomes round. It then migrates through the mesenchymal layer and enters the cardinal vein (thin curved arrow). Through the circulation, HSCs colonize organs of adult hematopoietic activity (pronephros and thymus), shown by straight bold arrows. The direction of circulation is shown by thin arrows within vessels. HSC, hematopoietic stem cell; MPP, multipotent progenitor.

In the placenta of the *Ncx1*-null mouse (*Ncx1* encodes a sodium-calcium exchanger membrane; also known as Slc8a1 – Mouse Genome Informatics) an active circulatory system is absent owing to heart beat failure. However, hematopoietic cell clusters similar to intra-aortic clusters still form, and it was proposed that the placenta generates dHSCs autonomously (Rhodes et al., 2008). *Ncx1*$^{-/-}$ placentas can generate lymphoid cells, however, attempts to confirm autonomous generation of dHSCs in explant cultures of the wild-type placenta have been unsuccessful (Robin et al., 2006). Whether the placenta initiates dHSC development or only supports maturation of exogenous dHSCs requires further investigation.

### Umbilical cord: what is its dHSC power?

The mammalian umbilical cord largely derives from the allantois, which, after gastrulation invades the exocoelomic cavity, reaches the ectoplacental cone and forms the chorio-allantoic fusion which contributes into placenta (Downs, 2002). Although the avian allantois is hematopoietically active (Caprioli et al., 1998), whether the mouse allantois is has been questioned (Downs et al., 1998). However, Runx1 was detected in the mouse allantois prior to fusion with the ectoplacental cone (Fig. 1A) and its hematopoietic potential was unveiled in culture (Corbel et al., 2007; Zeigler et al., 2006).

The mouse umbilical vessels develop Runx1-positive cell clusters similar to those observed in the dorsal aorta (Liakhovitskaia et al., 2009; North et al., 1999), and at E10.5 contain rare dHSC at frequencies comparable with the AGM region at this stage (~1 dHSC per 30 embryos) (de Bruijn et al., 2000b). As explant cultures of the umbilical cord fail to generate dHSC, the contribution of the allantois/umbilical cord to adult hematopoiesis remains unknown.

### dHSC generation: does the yolk sac have an active role?

E9.0-10.0 yolk sac cells can mature into dHSCs in newborn animals and can subsequently provide long-term contribution to adult hematopoiesis (Yoder et al., 1997a; Yoder et al., 1997b). Similar results have been achieved by the transplantation of E9.0 yolk sac cells into the embryo in utero (Toles et al., 1989). These cells are more numerous in the yolk sac than in the body and are CD34$^+$cKit$^+$ (Yoder et al., 1997a). CD34$^+$cKit$^+$ cells purified from the E10.5 AGM region can develop in culture into cells that can repopulate adult Rag2γc$^{-/-}$ at low levels (Bertrandet et al., 2005). What is the origin of these cells: the P-Sp or the yolk sac? The answer to this is not clear as these cells are not functionally detectable in younger E8.0 embryos. Earlier reports indicated that E8.0 yolk sac cells when injected transplacentally can mature and contribute to adult hematopoiesis (Weissman et al., 1977). However, the primary origin of these cells (yolk sac or P-Sp) remains unclear. An attempt to determine the contribution of the yolk sac to adult hematopoiesis has been made using induced genetic labeling of *Runx1*$^+$ cells (Samokhvalov et al., 2007). However, owing to the unclear duration of labeling, the presence of Runx1-expressing cells outside of the yolk sac and the very low contribution of labeled cells to adult hematopoiesis, it is difficult to draw firm conclusions from this study.

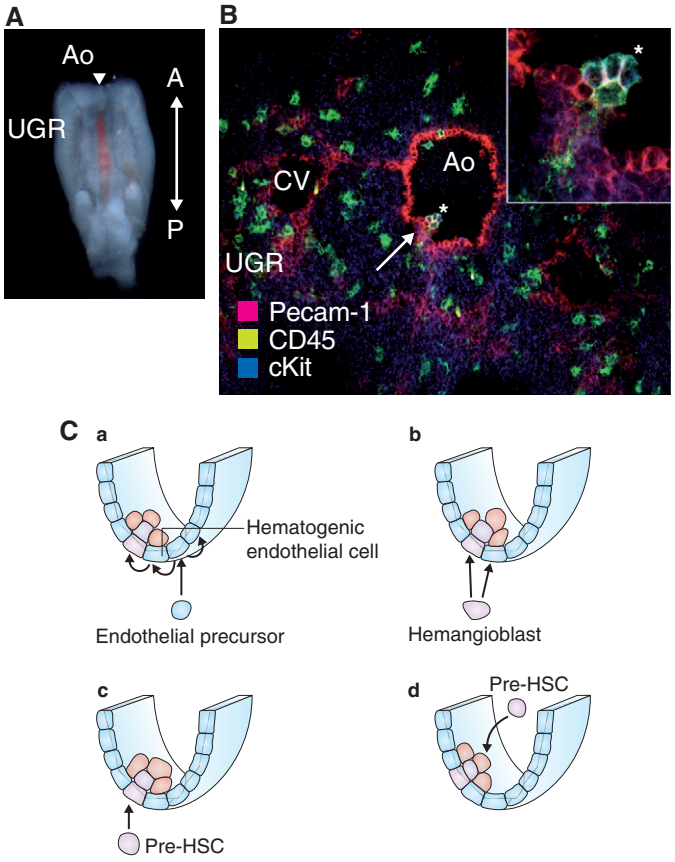



**Fig. 5. Possible mechanisms of intra-aortic cluster formation.** (**A**) A dissected E11.5 mouse AGM region, showing the dorsal aorta (Ao) and urogenital ridges (UGR). (**B**) Transverse section of E11.5 AGM region, showing the Ao and an intra-aortic cluster that is triple-positive for Pecam-1, CD45 and c-kit (magnified in the insert). Note the indentation (arrow), which suggests that active invagination of the endothelial layer is associated with cluster formation. Asterisk indicates intra-aortic cluster. CV, caudal vein. (**C**) A schematic cross-section of the ventral floor of a mouse Ao showing four possible origins of dHSCs. (**a**) An endothelial precursor gives rise to endothelial cells (blue), some of which become hematogenic and give rise to the definitive hematopoietic stem cells (dHSCs, pink) and more differentiated hematopoietic cells. A Pre-dHSC intermediate (purple) between the hematogenic endothelial cell and dHSC is shown integrated into the endothelial layer. (**b**) A hemangioblast (purple) gives rise separately to the dHSC and to non-hematopoietic (structural) endothelial cells. A pre-HSC intermediate may be integrated into the endothelial lining but in fact is not an endothelial cell. (**c**) Sub-endothelial pre-dHSC of non-endothelial origin matures into a dHSC. It is a fully hematopoietically committed cell and, in contrast to hemangioblasts, pre-HSCs do not generate endothelial cells. (**d**) A migrant pre-dHSC of non-endothelial origin arrives through circulation and integrates into the endothelial lining.

Although the pre-circulatory E8.0 mouse yolk sac is restricted to generating short-lived myeloid progeny (Cumano et al., 2001), by E11.5 it contains true multilineage dHSCs. Furthermore, by E12.5, when the expansion of dHSCs in the AGM has significantly diminished, the yolk sac acquires the capacity to expand dHSCs in culture (Kumaravelu et al., 2002). Although it is possible that the E12.5 yolk sac microenvironment becomes competent to expand dHSCs of exogenous origins, the initiation of dHSCs de novo by the yolk sac cannot be ruled out. dHSCs in heterozygous *Runx1*$^{+/-}$ embryos develop prematurely in the yolk sac by E10.5, indicating that this location, in principle, is not prohibitive for dHSC development (Cai et al., 2000).

Different sites of hematopoietic activity in the embryo are linked by the circulation, but is the role of the circulation in hematopoiesis only to facilitate cell trafficking? We discuss this further below.

## Circulation: its role in early hematopoietic development

### The effects of disrupted circulation on hematopoiesis

By the early E8.0 (approximately four somite pairs) stage of mouse development, the yolk sac vasculature connects to the embryo body and the heart starts beating. Because the primitive vascular system is not sufficiently developed and is densely packed with erythrocytes, massive movement of yolk sac erythroid cells into the body of the embryo is observed only by E10.0 (McGrath et al., 2003).

The role of the circulation in developing embryos has been studied in *Ncx1* mutants, which fail to initiate heart beating and die by E10.0 (Lux et al., 2008). *Ncx1*-null embryos develop normal numbers of erythrocytes and CFU-Cs in the yolk sac, but their body lacks these progenitors, indicating that AGM hematopoietic activity depends on colonization by yolk sac cells. Similar results were obtained with *Rac1* mutant embryos, in which cell migration is impaired (Ghiaur et al., 2008). Although the yolk sacs of these mutants develop normal numbers of CFU-Cs, intra-aortic clusters do not form and hematopoiesis in the liver rudiment is dramatically reduced (Ghiaur et al., 2008). These data indicate that the yolk sac has a role in the emergence of intra-embryonic hematopoiesis. It is clear that yolk sac-derived hematopoietic cells populate the body of the embryo, but to what degree does AGM hematopoiesis depend on this? Recently, a novel factor came into play that shed light on this issue.

### Shear stress and NO signaling in AGM hematopoiesis

Physical pressure created by blood flow in the vasculature has a profound effect on endothelial cell physiology and gene expression (Lehoux and Tedgui, 2003). Pulsating blood flow generated by the beating heart causes various physical effects on the endothelial lining. Of these, shear stress, which is generated by frictional force, has attracted particular attention. Endothelial cells can sense blood flow via mechanoreceptors and transduce these signals to the nucleus, to cause transcriptional and phenotypic changes (Garin and Berk, 2006). Nitric oxide (NO) signaling plays an important role in Vegf-induced angiogenesis (Ziche et al., 1997). Shear stress elevates NO signaling in endothelial cells (Niranjan et al., 1995); NO signaling is also involved in regulating hematopoiesis in the adult hematopoietic system (Krasnov et al., 2008; Michurina et al., 2004). The apparent relationship between vascular endothelium and hematopoiesis within the AGM region led to the proposal that biomechanical forces created by blood flow may induce the generation of hematopoietic cells from aortic endothelium via the activation of NO signaling (Adamo et al., 2009; North et al., 2009).

Indeed, under conditions that recreate shear stress in vitro, both ES cells and AGM-derived cells upregulate Runx1 and cMyb transcription factors and increase the production of CFU-Cs (Adamo et al., 2009). These effects can be suppressed by L-NAME, an inhibitor of NO signaling, but not by the inactive D-NAME stereoisomer. In vivo administration of L-NAME, but not of D-NAME, results in the absence of intra-aortic clusters and in a marked decrease of CFU-C, CFU-S and dHSC production in the AGM region of treated mouse embryos (Adamo et al., 2009; North

et al., 2009). Together with in vitro analyses, these experiments identified the NO pathway as a mediator of shear stress induction of intra-embryonic hematopoiesis in vivo. Analysis of the zebrafish mutant 'silent-heart' has also revealed that in the absence of blood flow, the number of *Runx1*$^{+}$ and CD41$^{+}$ cells in the embryonic dorsal aorta is significantly reduced (Murayama et al., 2006; North et al., 2009). This phenotype was rescued using S-nitroso-N-acetylpenicillamine (SNAP), the NO donor, providing evidence that shear stress acts through NO signaling to induce the formation of blood in the dorsal aorta. Further analysis using knockout mice indicated that NO synthase 3 (Nos3) plays a key role in inducing hematopoietic development in the AGM region (North et al., 2009). Thus, hematopoietic deficiency within the body of *Ncx1*$^{-/-}$ embryos is partly attributable to the lack of shear stress created by a heartbeat.

These experiments dissociated the roles of the yolk sac and the AGM region in the establishment of intra-embryonic hematopoiesis. They show that although hematopoietic progenitors generated in the yolk sac colonize the AGM region, hematopoiesis can also be independently induced in the AGM region by blood flow within the body. What is known about the cells that give rise to dHSCs in the embryo proper?

## Cellular origins of the hematopoietic system

### Concept of the hemangioblast

Similarities in the development of endothelial and hematopoietic lineages have long been observed by researchers in this field. We are not aware of any genes expressed in the endothelium that are not also expressed in a developing hematopoietic lineage. By contrast, hematopoietic cells express a number of lineage-specific markers that are absent from the endothelium.

### Hemangioblast or hematogenic endothelial cell?

Since the late 19th century, anatomical studies of the yolk sac in different species have suggested that blood islands are clonal structures. In histological sections, early blood islands appear as tight groups of undifferentiated cells that soon form an inner erythroid cluster surrounded by an outer endothelial lining. This morphology led to the hypothesis that bi-potent hemangioblasts might exist that give rise to both hematopoietic and endothelial cells (Maximow, 1909; Sabin, 1920). By E8.0, mouse blood islands undergo extensive remodeling resulting in the formation of an endothelial network filled with blood. However, on whole-mount preparations, it was noticed that the area of blood formation is a single belt-like structure that encircles the yolk sac (Ferkowicz and Yoder, 2005) (Fig. 6A). Rare endothelial cells do not compartmentalize this area into multiple individual blood islands, and it is only on sagittal sections that this belt-like structure looks like two 'classical' blood islands (Ueno and Weissman, 2006). This basic fact was overlooked for many years perhaps because the idea of the clonal origin of blood islands provides a good platform for explaining commonalities between the hematopoietic and endothelial programs. Classical images of discrete multiple blood islands existing in the avian yolk sac (Minko et al., 2003) also contributed to this misperception.

Although the clonal origin of the large non-compartmentalized blood island encircling the yolk sac is no longer a relevant issue, this does not negate the idea of the existence of a hemangioblast. Initially, such bi-potential cells were not identified: early avian yolk sac cells could produce in vitro either hematopoietic or endothelial, but not mixed, colonies (Eichmann et al., 1997); and orthotopic transplantations in mouse embryos revealed that hematopoietic and endothelial cells originate in different regions adjacent to the primitive streak (Kinder et al., 1999). It was an in vitro ES cell blast-colony assay that identified the hemangioblast (Choi et al., 1998; Keller, 2005; Kennedy et al., 2007). Equivalent cells have also been identified within the posterior primitive streak of the mouse embryo, which also give rise to smooth muscle cells (Huber et al., 2004), indicating that these cells are tri- rather than bi-potent. Interestingly, by the time the yolk sac is colonized by hemangioblasts, they have segregated into endothelial and hematopoietic lineages (Fig. 6B). Thus, the old idea of the

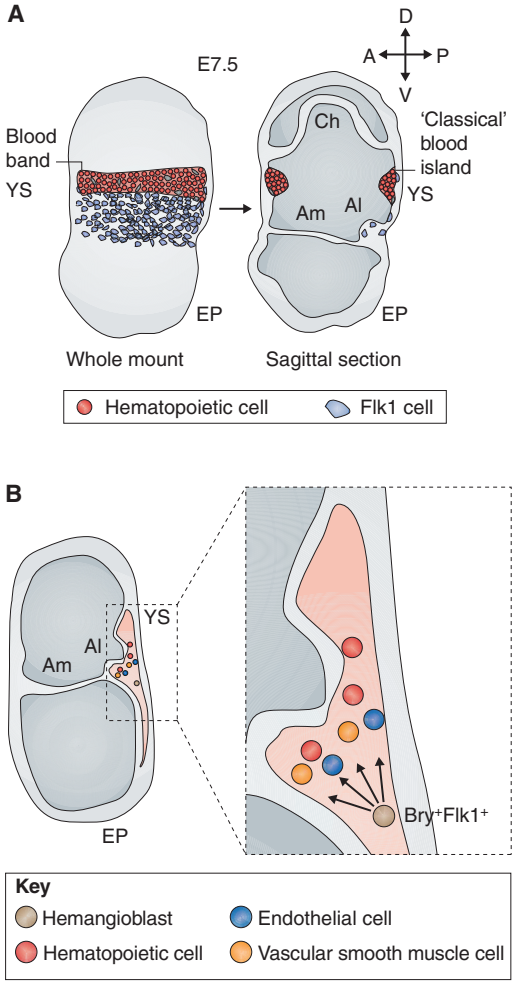



**Fig. 6. Development of yolk sac hematopoiesis.** (**A**) (Left) A schematic of a whole-mount preparation of an E7.5 mouse embryo, showing that hematopoietic (CD41$^{+}$) cells in the yolk sac (YS, red) are organized into a single belt-like structure that encircles the extra-embryonic portion of the embryo. Flk1$^{+}$ mesodermal and endothelial cells (blue) progress into the YS with delay. (Right) A schematic of a sagittal section through this embryo, which shows how isolated 'blood islands' are an artifact created by sectioning in this plane and that blood islands are not ensheathed in an endothelial covering by the late headfold stage (E8.0). (**B**) (Left) A schematic of a sagittal section through an E7.5 mouse embryo, showing that the segregation of the hematopoietic and endothelial lineages from the Bry$^{+}$Flk$^{+}$ hemangioblast occurs prior to the migration of hematopoietic cells to the YS (see magnified inset). The hemangioblast also generates vascular smooth muscle cells. The allantois, shown here, was not analyzed in the original paper (Huber et al., 2004). Al, allantois; Am, amnion; Ch, chorion; EP, embryo proper.

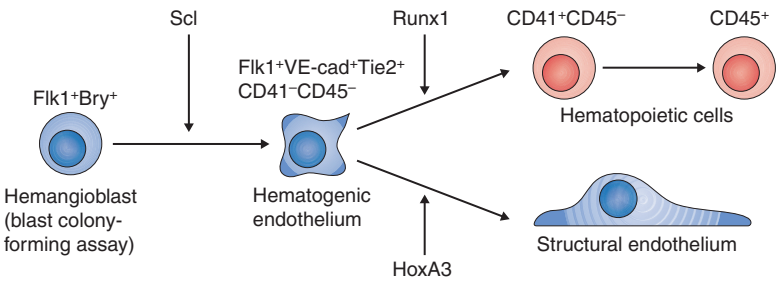



**Fig. 7. The hemangioblast to hematogenic endothelium lineage pathway.** A schematic showing how a hemangioblast transits through the hematogenic endothelium stage prior to generating hematopoietic cells. Hematogenic endothelium can generate both hematopoietic cells and structural endothelium. A blast colony-forming assay enables this developmental pathway to be replicated ex vivo. Useful markers for the identification of these stages are indicated. The formation of the hematogenic endothelium is Scl-dependent and that of hematopoietic cells is Runx1-dependent. HoxA3 antagonizes the hematopoietic program and maintains the endothelial characteristics of cells.

hemangioblast based on yolk sac blood island morphology has undergone a transformation and its location has shifted from the yolk sac to the embryo proper. Using a single-cell labeling technique, hemangioblasts have also been reported in developing zebrafish (Vogeli et al., 2006).

Given that the hemangioblast has been localized to the gastrulating posterior mesoderm (Huber et al., 2004), could it be that these cells are not just specialized tri-potential endothelial/hematopoietic/smooth muscle precursor cells but mesodermal cells with a broader differentiation potential? Some limitations of differentiation capacity of posterior mesoderm are known, for example, it does not form cardiomyocytes (Kattman et al., 2006). However, the full potential of these cells needs further investigation, either by developing an in vitro clonal assay broadly supporting various mesodermal lineages or by tracking their fates in vivo.

## Concept of hematogenic endothelium

Based on morphological observations of intra-aortic clusters, the idea has been proposed that hematopoietic cells differentiate from the endothelial lining of the dorsal aorta (reviewed by Dieterlen-Lievre et al., 2006). In ovo labeling of the chick dorsal aorta gave early indications that hematopoietic cells emerge from the aortic endothelium (Jaffredo et al., 1998). In other early experiments, ES cell-derived and embryo-derived endothelial VE-cad$^+$CD45$^-$ cells and hematopoietic VE-cad$^-$CD45$^+$ cells were sorted and functionally tested in vitro using an OP9 co-culture system (see Glossary, Box 1) (Nishikawa et al., 1998a; Nishikawa et al., 1998b). These studies showed that the efficiency with which myeloid and lymphoid cells could be generated from the endothelial fraction was much higher than it was from the hematopoietic fraction itself. A small fraction of hematogenic endothelial cells could also generate clonally both hematopoietic and endothelial cells. Hematopoietic differentiation from endothelial cells has also been tracked in live cultures (Eilken et al., 2009). In this study, endothelial colonies were derived clonally from mouse ES cells and their endothelial identity verified using immunophenotypic and morphological criteria. Shortly after the formation of the endothelial colony, some cells began to express CD41, an early marker of embryonic hematopoietic progenitor cells (Corbel and Salaun, 2002; Emambokus and Frampton, 2003; Ferkowicz et al., 2003; Mikkola et al., 2003), and then proliferated and formed CD41$^+$CD45$^+$ hematopoietic cells. Interestingly, some colonies did not entirely transform into hematopoietic cells and maintained an endothelial component, suggesting that the hematogenic endothelium can also contribute to the structural endothelium (Fig. 7).

The differentiation of hematopoietic cells from a hematogenic endothelium has been recently supported by the findings of two studies, in which the conversion of aortic endothelial cells into hematopoietic cells was demonstrated by live imaging in zebrafish embryos (Bertrand et al., 2010a; Kissa and Herbomel, 2010). A similar conclusion was also reached by another study from live imaging of slices of the mouse AGM region (Boisset et al., 2010).

As such, the concept of the hemangioblast and the hematogenic endothelium have both received experimental confirmation, and a recent report has reconciled these two pathways by proposing that the Flk1$^+$ hemangioblast first generates the hematogenic endothelium (Scl-dependent stage), which then produces hematopoietic cells (Runx1-dependent stage) (Fig. 7) (Lancrin et al., 2009).

What are the mechanisms that regulate the balance between endothelial and hematopoietic differentiation? It has been shown that HoxA3 expression in the early embryonic endothelium of the dorsal aorta restrains hematopoietic differentiation by suppressing key hematopoietic factors, including Runx1 and by maintaining the expression of endothelial-specific genes (Iacovino et al., 2011). Expression of Runx1 erases the endothelial program set by HoxA3 and induces hematopoietic differentiation. Accordingly, HoxA3-null mouse embryos show premature and increased formation of Runx1$^+$ cells in the dorsal aorta.

## Endothelial origin of the adult hematopoietic system

Although endothelial cells in the mouse embryo are capable of generating cells of the hematopoietic lineage, do they generate the first dHSCs that give rise to the entire adult hematopoietic system?

### Lineage tracing of endothelial progeny

Although dHSCs in the E11.5 AGM region have been shown to localize to the luminal surface of the dorsal aorta (de Bruijn et al., 2002), AGM dHSCs co-express both endothelial (VE-cadherin) and hematopoietic (CD45) markers (North et al., 2002; Taoudi et al., 2005) and, therefore, are not endothelial cells. Indeed, AGM VE-cad$^+$CD45$^+$ cells, which are enriched for hematopoietic progenitors and HSCs, lack endothelial potential (Taoudi et al., 2005). Over time, dHSCs progressively lose VE-cadherin expression; it completely disappears in bone marrow dHSCs while remaining present on some fetal liver dHSCs (Kim et al., 2005; Taoudi et al., 2005). A similar transient population of mixed endothelial-hematopoietic identity has also been described in the zebrafish (Bertrand et al., 2010a). These data suggest, but do not prove, that dHSCs in the mouse originate from the embryonic endothelium.

To clarify this issue, the progeny of VE-cad$^+$ cells were tracked by two research groups by either constitutive or induced VE-cadherin-specific Cre-mediated labeling during early mouse development (Chen et al., 2009; Zovein et al., 2008). In both cases, most adult blood cells were labeled, owing to

recombination either in the embryonic endothelium or in early dHSCs, which also express VE-cadherin. The first group proposed that this labeling pattern occurred because VE-cadherin is not transcribed in hematopoietic cells, but is a carry over of the protein produced in endothelial precursors (Zovein et al., 2008), a conclusion which in our view requires further confirmation. The other group showed that selective Cre-mediated deletion of *Runx1* in VE-cad$^+$ cells results in the ablation of clonogenic progenitors, intra-aortic clusters and HSCs, characteristic of *Runx1* knockout embryos (Chen et al., 2009). By contrast, in Vav-Cre deletor mice, in which Runx1 was specifically ablated in the hematopoietic lineage, CFU-Cs and dHSCs were still present, indicating that *Runx1* deficiency blocks the endothelial-hematopoietic transition but does not affect downstream events. Further support for this conclusion should come from an examination of whether the Vav-Cre induces recombination in the entire HSC lineage, including the VE-cad$^+$CD45$^+$ population, which contains both pre-dHSCs and dHSCs.

Live-imaging using transgenic reporter Flk1, cMyb, CD41 and Runx1 zebrafish embryos has also enabled MPP/HSCs that are undergoing the specific process of endothelial-hematopoietic transition, to be directly visualized together with their migration to their definitive locations in the kidney and thymus (Bertrand et al., 2010a; Kissa and Herbomel, 2010; Lam et al., 2010). The exiting of cells from the endothelium is so significant that it reduces the diameter of the dorsal aorta (Kissa and Herbomel, 2010).

## Identification of pre-dHSCs

How do dHSCs appear at the luminal surface of the dorsal aorta (de Bruijn et al., 2002)? Several scenarios can be considered, some of which imply that the HSC lineage only temporarily integrates with the endothelial layer (Fig. 5).

The AGM explant culture system has enabled dHSC development to be replicated in vivo (de Bruijn et al., 2000b; Medvinsky and Dzierzak, 1996) and has proven to be useful for the analysis of genetic mutations and growth factors in HSC development (Cai et al., 2000; Ling et al., 2004; Peeters et al., 2009; Robin et al., 2006). However, attempts to use the competent AGM environment to mature exogenous cells into dHSCs by injection into explants have as yet been unsuccessful (A.M. and E. Dzierzak, unpublished), which has precluded an accurate analysis of the roles of individual cell populations in dHSC development.

More recently, a dissociation-reaggregation technique has enabled this hurdle to be overcome (Sheridan et al., 2009; Taoudi et al., 2008). It has been known from classical embryological studies that dissociated embryonic rudiments after aggregation can partly reinstate spatial organization and specific cellular interactions (Grobstein, 1953; Gyevai et al., 1978). This appeared to be true also for three-dimensional organoids reaggregated from dissociated cells of the AGM region (Taoudi et al., 2008). This study showed that over four days of culture, E11.5 AGM reaggregates can expand the number of dHSCs by 150-fold. Two lines of evidence indicate that the AGM reaggregate culture replicates the maturation process occurring in the embryo. First, the expansion of dHSCs occurs only during the last 48 hours of culture. Secondly, label-retaining analysis shows that, in contrast to actively proliferating CFU-Cs, most dHSCs by the end of culture undergo no more than four or five cell divisions. These observations rule out the possibility that all 150 HSCs form through amplification of a sole pre-existing dHSC, and infer maturation from pre-dHSCs.

The incorporation of the dissociation/reaggregation step prior to culture enables cells of interest to be replaced with their GFP-labeled equivalents, an approach that has revealed that nearly all dHSCs generated in AGM reaggregates originate from the VE-cad$^+$CD45$^+$ pre-dHSCs (Taoudi et al., 2005). As the VE-cad$^+$CD45$^+$ immunophenotype is common to both pre-dHSCs and dHSCs, more specific cell markers need to be identified to physically separate these two cell types in the AGM region. Most VE-cad$^+$CD45$^+$ cells are organized in large intra-aortic clusters (Fig. 5), but some are also present in small clusters and as individual cells. Owing to their specific morphology, it is tempting to think that large clusters represent sites of dHSC maturation, although there is no direct evidence for this.

**A**

Definitive HSC numbers

| | E10 | E11 | E12 |
|---|---|---|---|
| AGM | ~0* | 1 | 3 |
| Yolk sac | 0 | 1 | 2 |
| Circulation | 0 | ~0 | 3 |
| Liver | 0 | 0.7 | 53 |
| Body | 0 | 0 | 6 |
| Placenta | 0 | 1 | 13-50 |
| **Total** | | **~4** | **80-115** |

**B**

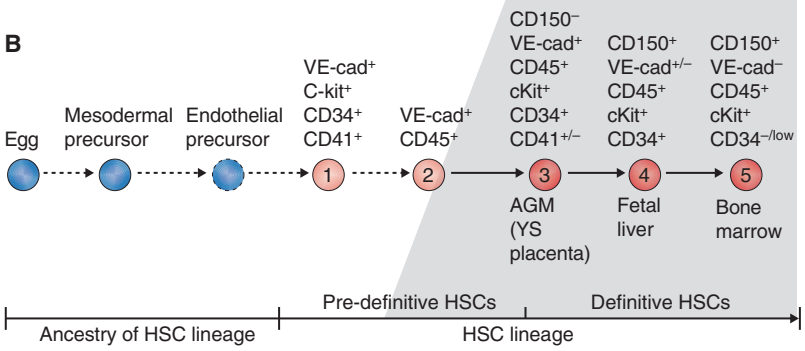



**Fig. 8. Developmental pathway leading to dHSC specification.** (**A**) The numbers of definitive hematopoietic stem cells (dHSCs) that can be found in various hematopoietic sites in the E10.0, E11.0 and E12.0 mouse embryo. dHSC emergence (as highlighted by the gray shading) is gradual, implying that dHSCs are recruited from more immature pre-dHSCs. Quantitative data for dHSC numbers are derived from previously published studies (Gekas et al., 2005; Kumaravelu et al., 2002; Ottersbach and Dzierzak, 2005). Asterisk indicates that on very rare occasions dHSCs can be detected. (**B**) A schematized pathway of dHSC specification; events are less certain prior to dHSC specification. Dotted lines represent presumptive transitions from one stage of lineage progression to another. (For phenotypic identity of individual stages, see Table S1 in the supplementary material.) (1) A pre-dHSC isolated from E9.0 and E10.0 embryos identified by transplantation into newborn recipients (Yoder et al., 1997a; Yoder et al., 1997b); (2) a pre-dHSC that matures into dHSCs within the AGM microenvironment as shown in vitro (Taoudi et al., 2008); (3-5) dHSCs that undergo some changes in their phenotype during development. Abbreviations: YS, yolk sac.

Similar to dHSCs in the AGM region, pre-dHSCs already express the CD45 hematopoietic marker and therefore cannot be considered to be endothelial. Furthermore, in E9.5 embryos, cells capable of maturing into dHSC upon injection into newborn recipients are CD41 and VE-cadherin positive (Ferkowicz et al., 2003; Fraser et al., 2002). Since CD41 marks megakaryocytes and all hematopoietic progenitors in the early embryo (Bertrand et al., 2005; Corbel and Salaun, 2002; Ferkowicz et al., 2003; Mikkola et al., 2003), these cells cannot be considered to be endothelial either. Although some CD41 cells express VE-cadherin, they lack endothelial potential (Hashimoto et al., 2007; Li et al., 2005). The role of VE-cadherin in pre-dHSCs might be in facilitating interactions with endothelial cells and mediating essential signaling (Carmeliet et al., 1999). Thus, although dHSCs could originate from the embryonic endothelium, the divergence of dHSC and endothelial lineages probably occurs prior to E9.5 (Fig. 5C). It has also been proposed that dHSCs originate from $CD45^{low/neg}$ cells that localize to sub-endothelial areas from which they transit via the endothelial lining of the dorsal aorta into the circulation prior to colonization of the liver (Fig. 5C) (Bertrand et al., 2005).

### Embryonic pathway to dHSC specification

In the absence of uniquely specific markers, the verification of the pathway that leads to the specification of dHSCs in the mouse can only be achieved using functional transplantation assays. Although dHSCs can function in an adult bone marrow microenvironment and can be tested by transplantations into adult recipients, their upstream ancestry must mature first in the embryo to become a dHSC. In utero and in 'newborn' transplantation assays have been developed to achieve this (Fig. 8).

Despite the current gaps in our knowledge, it is now possible to predict the embryonic pathway that leads to the specification of dHSCs. In our opinion the HSC lineage pathway begins from the point when either CD41 or CD45 hematopoietic markers are upregulated. At early stages of development, the HSC lineage is represented by pre-dHSCs, which have not yet acquired adult-repopulating capacity. Later, the capacity to engraft adult irradiated recipients develops and marks the emergence of dHSCs.

## Conclusions

Research in avian and amphibian embryos has revealed the presence of two independent transient (embryonic) and permanent (definitive) hematopoietic lineage hierarchies, and current data indicate that this is likely to also be the case for the mammalian embryo. The distinct genetic regulation of intrabody (AGM) and yolk sac hematopoiesis, as revealed in *Notch1* and *Ncx1* mutant studies, also supports this idea. Many data indicate that dHSCs arise locally from the P-Sp/AGM region; however, certain unanswered questions mean that final consensus on this issue has yet to be reached. For example, do the inductive interactions that lead to the step-wise maturation of HSCs require the ancestors of HSCs to migrate between tissues? This major issue requires further experimental analysis.

In early embryogenesis, hematopoietic cells originate from the hematogenic embryonic endothelium, which in turn arises from the hemangioblast. Although direct visualization provides convincing evidence for the endothelial origin of MPP/HSCs in zebrafish, in our view the origin of HSCs in the mouse is less certain, mainly owing to the expression of hematopoietic (CD41 and CD45) markers in early pre-dHSCs.

Unless some specific markers are found, it is going to be extremely difficult to track the origin of dHSCs from the pre-gastrulation mouse embryo. Studies aiming to recapitulate in vitro their development from early embryonic stages might provide important insights into this issue. For this, the identification of cells and growth factors involved in this process in vivo would be essential. Further development of Cre-based cell-tracking systems in vivo and the enhancement of visualization techniques might provide further insight into HSC development in the mouse. Studies of HSC development in non-mammalian vertebrates will also benefit from developing additional experimental approaches, such as a functional HSC self-renewal assay.

Apart from an academic interest, studies of the origin of dHSCs in the embryo have important practical implications. In the absence of enforced gene expression, the in vitro differentiation of ES cells into dHSCs remains a highly challenging task; this is a crucial obstacle to the application of pluripotent cell technology for clinical use. Development of in vitro systems that recapitulate cues from in vivo dHSC development might aid this task.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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**Table S1. Markers expressed in pre-HSCs and HSCs**

| | HSC source | | | | | |
|---|---|---|---|---|---|---|
| Marker | Newborn repopulating cell at E9.5 (population 1*) | Pre-dHSC AGM at E11.5 (population 2*) | dHSC AGM at E11.5-12.5 (population 3*) | dHSC placenta at E12.5 (population 3*) | dHSC fetal liver at E13.5-14.5 (population 4*) | dHSC adult bone marrow at ≥10 weeks (population 5*) |
| Lin† | | | | | – [1] | – [2] |
| Mac1 | | | +/– [3] | | + [4] | – [5] |
| CD48 | | | – [6] | – [6] | – [7] | – [8] |
| CD150 | | | – [6] | – [6] | + [7] | + [8] |
| CD201 | | | | | + [9] | + [10] |
| CD34 | + [11] | | + [3, 6] | + [6, 12] | +/– [3, 12] | – [13-15] |
| cKit | + [11] | | + [3, 16] | + [12] | + [3, 4, 12] | + [17] |
| Sca1 | | | +/– [16, 18] | + [19] | + [1, 4] | + [2] |
| Flk1 (Kdr) | | | +/– [16] | | – [16] | – [14] |
| Flt3 | | | | | + [1] | – [20, 21] |
| CD41 | + [22] | | +/– [6] | +/– [6] | –/low [7, 22] | –/low [8, 22] |
| CD31+ | + [23] | | + [16, 24] | | + [16, 23] | + [23, 25] |
| VE-Cadherin | + [26] | + [27] | + [16, 28] | | +/– [16, 28, 29] | – [28, 29] |
| CD45 | – [26] | + [27] | + [16, 28] | | + [9, 16, 28] | + [9, 16] |
| ESAM | | | | | + [30] | + [31] |

*As defined in Fig. 8B.
†Lin, lineage markers for adult bone marrow: CD3, CD4, CD8, B220, Mac1, Gr1, Ter119.
AGM, aorta-gonad-mesonephros region; dHSC, definitive hematopoietic stem cell; HSC, hematopoietic stem cell.


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