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Hematopoietic (stem) cell development: How divergent are the roads taken?

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Abstract

The development of the hematopoietic system during early embryonic stages occurs in spatially and temporally distinct waves. Hematopoietic stem cells (HSC), the most potent and self-renewing cells of this system, are produced in the final ‘definitive’ wave of hematopoietic cell generation. In contrast to HSCs in the adult, which differentiate via intermediate progenitor populations to produce functional blood cells, the generation of hematopoietic cells in the embryo prior to HSC generation occurs in the early waves by producing blood cells without intermediate progenitors (such as the ‘primitive’ hematopoietic cells). The lineage relationship between the early hematopoietic cells and the cells giving rise to HSCs, the genetic networks controlling their emergence, and the precise temporal determination of HSC fate remain topics of intense research and debate. This Review article discusses the current knowledge on the step-wise embryonic establishment of the adult hematopoietic system, examines the roles of pivotal intrinsic regulators in this process, and raises questions concerning the temporal onset of HSC fate determination.

Keywords

hematopoietic development; EHT; HSC; HPC; Gata2; Runx1; HSC fate; embryo; ES cell differentiation

Introduction

The adult hematopoietic system consists of a hierarchy of cells that progress from a stem cell state to the terminally differentiated cells of over ten blood cell lineages. While hematopoietic stem cells (HSC) are rare, long-lived and self-renewing, there are many intermediate progenitor cell types that in a stepwise manner, lose their multi-lineage and self-renewing potency before becoming mature functioning blood cells. Much is known about the adult hematopoietic hierarchy, but only recently do we begin to know the variety of hematopoietic cells in the embryo and to understand how they relate to the establishment of the adult hierarchy and HSCs. In this review we discuss the current knowledge on the embryonic development of the adult hematopoietic system focusing on endothelial-to-

hematopoietic cell transition (EHT), and on some of the pivotal transcriptional regulators and their targets involved in this process, and in the generation of HSCs.

Hematopoiesis is initiated by transient generation of primitive cells

Blood cells are one of the first differentiated cell/tissue lineages generated in the vertebrate embryo. Surprisingly, they are produced even before the circulation is established [1]. Transient waves of hematopoietic cell production are first initiated extra-embryonically in the yolk sac (YS) blood islands (Figure 1) [2] which are derived from mesodermal cells that migrate to the YS at neural plate stage. At embryonic day 7 (E7) the mesodermal aggregates generate the first blood cells [2, 3]. The emergence of blood cells in the YS is in close relationship to the appearance of endothelial cells that form the first vascular structures. This spatiotemporal association between the emergence of hematopoietic and endothelial cells has led to the hypothesis that they arise from a common bipotential ancestor, which is termed the hemangioblast [1].

The first genetic evidence supporting a common precursor for hematopoietic and endothelial lineages came from deletion of the *Flk1* receptor tyrosine kinase gene in the mouse. *Flk1* expression is detected as early as at E7 in the YS mesoderm [4]. Embryos lacking *Flk1* are not viable and interestingly, show a complete absence of mesodermal cell aggregates in the YS. It was concluded that *Flk1* is required for mesodermal cell migration to form YS blood islands and for making hematopoietic and endothelial cells [5] thus suggesting that a bipotential hemangioblast generates hematopoietic and endothelial cells. Intriguingly, lineage marking/tracing experiments have shown that there is little/no overlap in the mesodermal precursors that are forming the endothelial and hematopoietic cells in individual blood islands, suggesting a separation in fate early before migration to the YS [6].

Mouse embryonic stem (ES) cell hematopoietic differentiation studies facilitated the search for putative hemangioblast-like cells. ES cells are pluripotent cells derived from the inner cell mass of the blastocyst [7]. They are characterized by self-renewal ability and the capacity to recapitulate early embryonic development by differentiating into cell derivatives of all three embryonic germ-cell layers [8].

ES cells differentiated in hematopoietic culture conditions for 2.5 days generated blast colony forming progenitor cells (BL-CFC), that were able to give rise to both, hematopoietic and endothelial cells [9]. The BL-CFC (putative hemangioblast) represents a transient population that persists for a very short time in the differentiation culture. It expresses genes common to both hematopoietic and endothelial lineage, including *Flk1* [10]. More recently it has been shown that the BL-CFC have an additional differentiation potential to cardiomyocyte lineage [11] and thus, the physical isolation of the hemangioblast remains difficult. Nonetheless, to better understand embryonic hematopoiesis *in vitro* ES cell hematopoietic differentiation models have been widely used, as they recapitulate the early stages of hematopoietic cell development and differentiate to almost all hematopoietic lineages, thus facilitating biochemical analyses of transcription factors and other regulatory molecules involved in development.

The earliest blood cells detected in the embryo are primitive erythrocytes, macrophages and megakaryocytes

Blood cells that emerge in the first wave of hematopoietic cell generation are ‘primitive’ erythrocytes, macrophages and rare megakaryocyte progenitors [2, 12]. This developmental wave is categorized as ‘primitive’ due to the distinctive characteristics of the erythrocytes and erythrocyte colony forming unit cells (EryP-CFU-Cs). ‘Primitive’ red blood cells are nucleated and are 3 times larger than fetal and 6 times larger than adult erythrocytes [13, 14]. Moreover, they produce a developmentally distinct embryonic (β H1) globin, which is not detected in adult erythrocytes. ‘Primitive’ erythrocytes peak in numbers at E8.25 and disappear rapidly by E9 [2, 12]. The short developmental time of these cells resembles the transient nature of hemangioblast-like cells, thus supporting the hypothesis that they originate from a short-lived precursor.

Concurrently, rare macrophage progenitors are detected in the YS [2, 15]. ‘Primitive’ macrophages from this first YS hematopoietic wave (E7–7.5) are directly derived from the blood islands and do not go through a monocyte intermediate [16–18] that characterizes the macrophages generated from HSCs in the adult bone marrow. Once the bloodstream is established at E8.25–8.5 [19] the YS derived macrophages migrate to the developing tissues where they become ‘tissue resident’ macrophages expressing high levels of F4/80 macrophage surface marker. These include macrophages in the skin, microglia in the brain, Kupffer cells in the liver and Langerhans cells in the epidermis. Recent lineage tracing studies suggest that ‘tissue resident’ macrophages in the skin, liver and lung are replaced before birth by ‘monocyte derived’ macrophages generated in later waves of hematopoietic development [20]. In contrast, the labeled brain microglia cells are retained throughout adult life. Unique to these macrophages, as compared to those in the adult, are high F4/80 expression, *c-Myb* transcription factor independence and *PU.1* transcription factor dependence [20–23]. By E9.5, the quantitative abundance of phenotypic ‘primitive’ macrophages and megakaryocytes in the embryo further suggests that these cells are directly generated in the first hematopoietic wave and not from the later waves of hematopoietic progenitor and stem cell generation [15, 24].

The need for these early blood cells in the embryo before the circulation is established is puzzling. ‘Primitive’ erythrocytes may be necessary for providing the rapidly growing embryo with oxygen, macrophages for phagocytosis of cells during tissue remodeling and for lymphatic development but the role of megakaryocytes is uncertain, although they are closely associated with red blood cells.

Multipotent progenitors are generated in the YS during a second wave of blood cell generation

After the generation of ‘primitive’ erythrocytes, macrophages and megakaryocytes, another wave of hematopoietic cell production begins at E8.25 in the YS (Figure 1). It overlaps temporally with the first wave [2], but produces functionally more complex bipotential erythroid-myeloid progenitors (EMP). EMP cells express high levels of tyrosine receptor

kinase ckit (CD117) and CD41, and by E9.5 are positive for granulocyte-monocyte marker CD16/32 expression [24]. EMP-derived erythrocytes are distinguished from their earlier, 'primitive' counterpart by the expression of adult (β major) globin [2] and by undergoing enucleation. Thus, based in this complexity and the generation of adult-like cells this wave is termed 'definitive' [25]. However, $ckit^{hi}CD41^{+}CD16/32^{+}$ EMPs lack lymphoid cell potential, and are able to provide only short-term *in vivo* reconstitution, giving rise to mainly circulating red blood cells [24]. Hence, EMPs are distinct from HSCs.

Study of *Ncx1* null which lack circulation show that EMPs are generated in the YS and not in the embryo proper through E9.5 [24, 26]. They appear to emerge from $ckit^{+}$ cell clusters found in the venous and arterial vessels of the YS [27]. These cells then colonize the newly forming liver around late E9 [25] give rise to the large numbers of erythrocytes, macrophages, granulocytes and monocytes found before the establishment of a permanent hematopoietic system [20].

Other hematopoietic cells generated in the second wave are rare cells with lymphoid potential, B-1 B cell progenitors. They are detected at E8.5/9.5 in the YS and aorta [28–30]. Mast cells are also found in the YS from E9.5 onwards [31]. Taken together, this 'definitive' wave of hematopoietic cell generation yields more adult-like functionally competent blood cell types. Also, there is growing evidence that these cells may play an interactive role in promoting the third wave of hemogenesis and HSC generation [32–34].

HSCs and HPCs emerge by EHT

Adult type HSCs are defined by their robust ability to long-term repopulate all blood lineages upon transplantation into irradiated adult recipients. In the mouse embryo, the first adult HSCs appear and are autonomously generated in the aorta-gonad-mesonephros (AGM) region at E10.5 (Figure 1) [35, 36]. They are also found in the vitelline and umbilical arteries (VA, UA) and in the head [37, 38]. Shortly thereafter, HSCs are detected in the YS, placenta, circulation and fetal liver (FL) [35, 36, 39, 40]. Whereas the YS and placenta may be capable of autonomously generating HSCs, the FL serves only as a niche for the expansion of HSCs (and EMPs) made in the other tissues [39, 41, 42]. Just before birth HSCs migrate to the bone marrow where they reside throughout mammalian adult life in specialized niches [43].

HSCs are generated from a subset of embryonic endothelial cells that possess hemogenic potential - the hemogenic endothelial cells (Figure 2A) [44, 45]. They are detected at the time when clusters of hematopoietic cells appear on the ventral wall of the dorsal aorta. These intra-aortic hematopoietic cluster cells (IAHC) are $ckit^{+}$ and at E10.5, approximately 600 IAHCs (1–19 $ckit^{+}$ cells per cluster) were found along the length of the embryo by whole mount embryo imaging [46]. Figure 2B shows *Gata2* mouse model where *Gata2* marks the IAHCs. The clusters are also found in the YS vasculature (Figure 2C). Vital imaging of the mouse embryonic aorta at the time of HSC generation revealed the transition of morphologically flat endothelial cells to cells that bulge out of the vascular wall and form round hematopoietic cells in the lumen of the aorta. This process was visualized in *Ly6a* (*Sca1*) GFP fluorescent reporter transgenic embryos. GFP is expressed in all embryonic and

adult HSCs in the mouse [37, 38, 40, 47, 48] and hence, is an excellent reporter for observing the emergence of HSC. To visualize EHT in the aorta by confocal time-lapse imaging, thick sections of *Ly6aGFP*E10.5 embryos were stained with a combination of antibodies against hematopoietic and endothelial cell surface markers [49]. Hemogenic endothelial cells that give rise to HSCs could be distinguished from other aortic endothelial cells by the expression of GFP. Rare GFP⁺ckit⁺CD41⁺ cells were observed bulging into the lumen of the aorta directly from GFP⁺CD31⁺ ventral aortic endothelial cells, thus facilitating tracking single cells as they transition from an endothelial cell to a HSC/HPC. This process is generally known as the endothelial-to-hematopoietic transition (EHT).

EHT has also been imaged in zebrafish embryos, however the process is slightly different than that observed in mouse embryos. The hemogenic endothelial cells in zebrafish bulge abluentially, and emerge as hematopoietic cells in the interstitial region between the aorta and axial vein. Moreover, multi-cell clusters do not form. Emerging zebrafish HS/PCs are marked by *c-Myb* expression [50]. Vital time-lapse imaging of compound transgenic *c-Myb-GFP:Kdr1(Flk1* endothelial marker)-*mCherry* zebrafish embryos demonstrates that hematopoietic cells acquiring CD41 expression emerge directly from endothelium in the ventral side of the dorsal aorta [51–53]. They move quickly to extravasate into the lumen of the axial vein where they enter the circulation. They are next found to enter specific niches in caudal hematopoietic tissue, the equivalent of the mouse fetal liver [54].

EHT has also been recapitulated *in vitro* and detected by time-lapse imaging of ES cell hematopoietic differentiation cultures. ES cell-derived cells expressing the endothelial marker Tie2 and ckit, when exposed to hematopoietic culture conditions, give rise to CD41⁺ hematopoietic cells that down-regulate Tie2 [55, 56]. Together these data provide *in vitro* and *in vivo* morphological and phenotypical evidence of HSC/HPC emergence via EHT – a process that is conserved in human ESCs [57] and all vertebrate embryos [44] thus far examined.

Pivotal regulators of EHT and HSC generation

Several hematopoietic transcription factors have been found to play an essential role in the generation of ‘definitive’ hematopoietic cells in the mouse embryo. As two of the most frequently studied, *Gata2* and *Runx1* are the focus of this section. The importance of these factors in the process of HS/PC generation was first highlighted by the creation of germline knockout mice. *Gata2* and *Runx1* homozygous deletions resulted in embryonic lethality at E10.5 and E12.5 respectively, accompanied by severe fetal liver anemia [58, 59]. Functional studies revealed that although *Runx1*^{-/-} mice make ‘primitive’ hematopoietic cells, they completely lack ‘definitive’ hematopoietic progenitors in the YS and fetal liver and importantly, no HSCs are generated in the AGM [59, 60]. Similarly, the *Gata2*^{-/-} embryos are defective for ‘definitive’ hematopoiesis, as demonstrated by greatly reduced progenitor numbers [58, 61]. Recently it was demonstrated by using a *Gata2Venus* reporter mouse model that *Gata2* is expressed in all functional HSCs, and in most HPCs [62]. *In vitro* hematopoietic differentiation experiments with *Gata2*^{-/-} and *Runx1*^{-/-} ES cells show that they retain the ability to undergo ‘primitive’ erythroid differentiation, however, at reduced levels. ‘Definitive’ hematopoietic progenitor generation is profoundly impaired. Analysis of

ES cell-generated *Gata2*^{-/-} and *Runx1*^{-/-} chimeric mice revealed a lack of knockout cell contribution to any of the hematopoietic organs [58, 59]. Thus, *Gata2* and *Runx1* play pivotal roles in hematopoietic development, affecting mainly the ‘definitive’ stage in which HPCs and HSCs are generated.

The temporal and spatial expression patterns of *Runx1* and *Gata2* in the embryo (as determined by *in situ* hybridization, immunostaining and/or knockin/transgenic reporters) support their important cell-intrinsic role in HSC and HPC generation. These factors are expressed at E8.0 in the YS, which at that time is the main site of hematopoietic cell (EMP) generation [62–65]. Slightly thereafter, from E8.5 to E11.5 *Runx1* marks the endothelial cells on the ventral side of the aorta, umbilical and vitelline arteries, placenta and head [38, 63, 66, 67]. Although *Gata2* is expressed in the endothelial cells lining the aorta already at E8.5, the frequency of cells expressing *Gata2* increases in the AGM and FL concurrent with the emergence of IAHC and the first HSCs [62]. It is also expressed in the vitelline and umbilical arteries and the placenta. Moreover, both *Gata2* and *Runx1* are expressed in IAHCs in the embryonic arteries and all such hematopoietic clusters are absent in the aortae and other major arteries of *Gata2*^{-/-} and *Runx1*^{-/-} embryos [63, 68–71].

The continuum of expression during the transition from endothelial cells to hematopoietic cluster cells in static images of the aorta implicates these factors in the process of EHT. Indeed, conditional deletion of *Gata2* and *Runx1* in hemogenic endothelium marked by *vascular endothelial-cadherin* (*Vec*) expression or *Tie2* expression demonstrates that these factors are essential in the hemogenic endothelial cells for the formation of hematopoietic clusters and importantly, for the generation of functional HPCs and HSCs [47, 71, 72]. Moreover, *Runx1* is required for HSC generation between E10.5 and E11.5, as shown by tamoxifen induced deletion in *Vec* expressing cells [73]. The vital imaging of *Runx1* morphant zebrafish embryos provided an interesting insight into its role. In the absence of *Runx1*, aortic endothelial cells undergo sudden death as they attempt transition to hematopoietic cells, thus suggesting that *Runx1* is required during EHT for the survival of emerging hematopoietic cells [51].

To test whether *Runx1* and *Gata2* are required in hematopoietic cells after they are generated in the mouse embryo, conditional deletion was performed in cells marked by *Vav* expression. Whereas *Runx1* is not required [70], *Gata2* continues to be essential in the HSCs after they are made [71]. Therefore, *Gata2* and *Runx1* are pivotal to HSC and HPC emergence in EHT during embryonic development, but are differentially required as hematopoietic development proceeds.

***Gata2* and *Runx1* levels are strictly controlled in EHT**

It is of importance to note that HSC and HPC development is highly dependent on the levels of *Runx1* and *Gata2* expression. *Gata2*^{+/-} embryos have profoundly reduced numbers of AGM HSCs, HPCs, and IAHCs. The bone marrow of *Gata2*^{+/-} adult mice contains normal quantities of HSCs, but these are qualitatively impaired, as observed in competitive transplantation assays [61, 71, 74]. Overexpression of *Gata2* also results in abnormal hematopoiesis: it reduces bone marrow colony forming unit-cell (CFU-C) and colony

forming unit-spleen (CFU-S) activity and results in a failure of multilineage reconstitution [75]. Hematopoietic differentiation of ES cells overexpressing *Gata2* suggests that abnormally high *Gata2* expression blocks T and B cell generation, resulting in myeloid-biased cell production [76, 77]. Thus, *Gata2* expression levels are likely to be involved in controlling cell fate decisions. Recent transcriptome analysis of placental cells suggests that *Gata2* is continuously expressed in hemogenic and hematopoietic progenitors, but downregulated during commitment to blood lineages [78]. Also, *Gata2* expression is downregulated during ES cell-derived hemangioblast differentiation into blast cells [79], thus indicating that levels of *Gata2* may play a role in HSC and HPC expansion and potency.

Runx1 also functions in a dose dependent manner. *Runx1*^{+/-} embryos generate fewer HPCs and HSCs [60, 80–82]. Fascinatingly, *Runx1*^{+/-} embryos experience a temporal shift in the emergence of HSCs. HSCs are detected earlier than normal: at E10 in the AGM and YS, and HSC activity is prematurely terminated in the E11 AGM [60]. The E10.5/11.5 aorta in *Runx1*^{+/-} embryos has fewer IAHCs, suggesting that *Runx1* haploinsufficiency reduces HSC generation, maintenance and/or proliferation. Thus, normal diploid levels of *Runx1* are essential during development for the timely emergence of HSCs and HPCs.

***Gata2* and *Runx1* function synergistically to regulate their downstream targets**

Although deletion of a single *Gata2* or *Runx1* allele disrupts HSC and HPC development, it does not result in embryonic lethality [58, 60, 63]. Strikingly, the analysis of *Gata2*^{+/-}:*Runx1*^{+/-} compound embryos showed a trend towards fewer hematopoietic progenitors and the absence of double haploinsufficient offspring due to embryonic lethality [83]. These data suggest that *Gata2* and *Runx1* function together in the same cells to control the expression of hematopoietic genes involved in HSC and progenitor cell generation.

Further evidence for combinatorial function of *Gata2* and *Runx1* comes from an extensive ChIP-seq and bioinformatics analysis revealing interaction complexes between a heptad of hematopoietic cell specific transcription factors that includes *Runx1* and *Gata2*. The vast majority of heptad bound promoter and enhancer regions of hematopoietic genes contain a GATA consensus binding sequence. Only approximately 40% of them contain a Runx consensus binding motif, suggesting that Runx1 recruitment to the regulatory elements within the complex is mediated by *Gata2* [83]. Combinatorial interactions within the heptad complex result in hematopoietic cell type-specific chromatin binding and downstream gene expression. How exactly the complex functions in cell fate specification, is yet unknown. Whether the factors act sequentially or all at the same time, whether they regulate each other and how individual factor levels affect complex formation is a matter of debate.

RNA sequencing of endothelial cells, hemogenic endothelial cells, HPCs and HSCs in the AGM show that heptad transcription factor expression is increasing during EHT, and is accompanied by transcriptional activation of several downstream target genes. One such target gene is the *G-protein coupled receptor 56* (*Gpr56*) that is significantly upregulated (38-fold) in HSCs as compared with hemogenic endothelial cells [84]. Notably, *Gpr56* expression is downregulated as a result of *Gata2* (regulatory element) deletion, which is

accompanied with severe disruption of hematopoiesis and embryonic lethality [75]. Moreover, ChIP experiments reveal direct binding of *Gata2* to the *Gpr56* +37 enhancer, [84] [85] thus indicating that *Gpr56* is a direct target of *Gata2*. The precise function of *Gpr56* in hematopoiesis is as yet unknown. It has been suggested to play a role in the maintenance of self-renewal [84], and it is essential for HSC repopulation potential in mice [86]. In zebrafish, *Gpr56* is required for the emergence of hematopoietic cells in the dorsal aorta [84] thereby supporting its functional involvement in EHT.

Another means by which the heptad complex may regulate HSC and HPC emergence is by inducing a stepwise expression of transcriptional suppressors *Gfi1* and *Gfi1b* – the direct targets of *Runx1*. The expression of these transcription factors in hemogenic endothelium is pivotal for the normal EHT transition. Time-lapse imaging of *Gfi1*⁺ cells show that they acquire *Gfi1b* expression in the IAHCs followed by upregulation of *ckit* and CD41 expression, indicating hematopoietic commitment. *Gfi1:Gfi1b* double knockout embryos lack IAHCs, and *ckit*⁺ cells stay embedded in the endothelial lining of the dorsal aorta sustaining their endothelial program (*Vec* and *Tie2* expression). Thus, *Runx1* together with *Gfi1* factors promote EHT by suppressing endothelial program, thereby allowing hematopoietic cells to emerge [87–89].

Do HSCs establish their fate prior to or during EHT?

Vital imaging demonstrates that HSCs and HPCs are generated by morphological transdifferentiation of specialized endothelial cells, and genetic tracing studies show that functional HSCs/HPCs descend from cells expressing endothelial markers. But when is hematopoietic fate, and more precisely, when is HSC fate established? Current research interests are addressing the issue of whether HSC fate and function is determined in the endothelium during EHT, or primed earlier or later in development.

A *Runx1+23 enhancer GFP (+23GFP)* reporter mouse was used to explore this issue. *Runx1* expression in vast majority of mouse hematopoietic stem and progenitor cells and aortic endothelial cells is controlled by a *Runx1 +23* enhancer, thus the *+23GFP* mouse model allows specific isolation of hemogenic endothelial cells [90, 91]. Transcription analysis (Fluidigm) with a panel of endothelial and hematopoietic genes demonstrated that at E8.5 the *+23GFP* expressing aortic hemogenic endothelium is distinguished from *+23GFP* negative endothelium by higher expression of hematopoietic regulators such as *Meis1*, *Gata2*, *Gata3* and *SCL*. Single cell transcriptome analysis showed in approximately 50% of *+23GFP* hemogenic endothelial cells that higher *Meis1* expression is accompanied by downregulation of the endothelial marker *Etv2*, thus arguing for hematopoietic fate establishment earlier than previously recognized [91]. At a later developmental time (at E10.5) in the *Ly6a GFP* model, the transcriptome of the aortic hemogenic endothelial fraction (CD31⁺*ckit*⁻GFP⁺) showed differences to the endothelial fraction (CD31⁺*ckit*⁻GFP⁻), with heptad transcription factor and Notch gene expression increased [84]. Few indications of hematopoietic gene expression were found in the hemogenic endothelial fraction as compared to the HPC/HSC fraction (CD31⁺*ckit*⁺GFP⁺). However, these experiments were performed with populations of sorted cells and await single cell transcriptomic analysis. Importantly, the expression of the heptad factors is the first and

pivotal step directing a hematopoietic program, and as such Runx1+23GFP is an excellent indicator showing that the hemogenic and hematopoietic programs are established already in a subset of endothelial cells at the beginning stage of 'definitive' hematopoietic cell development.

If hematopoietic and HSC commitment occurs earlier than functional HSCs emerge, the aortic endothelium may harbor immature cells that in the proper microenvironment are able to mature into functional HSCs. To test this, an OP9 stromal cell co-aggregation culture was established that facilitates the *ex vivo* maturation of hematopoietic/endothelial cells obtained by multi-surface marker phenotypic sorting [92]. Using this approach it was shown that E9.5 dorsal aorta contains a VEC⁺CD41⁺CD45⁻CD43⁻ cell population (termed pro-HSC) that lacks repopulating activity in direct *in vivo* transplantation assays. However, when co-aggregated with OP9 and *ex vivo* cultured for 7 days, this population is able to reconstitute the hematopoietic system of the recipient [93]. These pro-HSCs are almost devoid of endothelial cells and it is thought that they may represent a stage directly downstream of the Runx1+23GFP⁺ hemogenic endothelium present in the E8.5 AGM [91].

Also, the E10.5 and E11.5 AGM is thought to contain immature HSCs – this VEC⁺CD41^{low}CD45⁻CD43⁺ population (termed pre-HSC) up-regulates CD45 expression when co-aggregated with OP9 and mature into functional, repopulating HSCs [92]. Interestingly, it has been proposed that the pre-HSCs may be generated independently of *Runx1*, as a developmental block is not observed before the transition of CD41⁺ cells to CD45⁺ in *Runx1* deficient mice [94]. Cells with a pre-HSC phenotype are present also in the E11.5 YS and FL, but they are not able to mature into engrafting HSCs. Thus, functional pre-HSCs are thought to be present mainly in the AGM region and in the extraembryonic arteries [92, 93, 95, 96].

These data propose that definitive HSCs may be primed for a hematopoietic gene expression program very early in development, making the precise temporal onset of the HSC program debatable. However, it should be taken into account that *ex vivo* manipulations, such as stromal cell and explant (co-)cultures, consequently introduce new variables into the model, that might not be present in *in vivo*. Advances in *in vivo* lineage and vital imaging tracing tools and single cell transcriptomics will assist in further investigations of such cells under more physiologic conditions representative of the *in vivo* embryonic milieu.

Recent studies have suggested that mouse embryonic head produces adult HSCs and HPCs independently from other hematopoietic organs and circulation. Lineage tracing experiments show that embryonic head-derived HSC progeny contribute to the adult HSC population [38]. However, to date, it has not been demonstrated that the head HSCs are emerging via an EHT in a similar manner to those in AGM. Moreover, the head vasculature lacks IAHCs [66, 97]. Also, head HSCs do not seem to go through the putative pre-HSC state since no/few pre-HSCs have been reported in the head as demonstrated by OP9 co-aggregation culture of E11.5 head region [98]. These studies suggest that there may be an alternative way by which functional HSCs are generated, and could include generation in different spatial and temporal frameworks, and different regulatory programs and networks. Defining such mechanisms could contribute to answering questions currently arising in the field of

hematopoiesis: for instance, such information may clarify the source of heterogeneity among HSCs – BMP-activated and BMP-non-activated, myeloid or lymphoid biased [99, 100]? Also, it may provide insight into why there are many more HPCs in the IAHCs than HSCs [96], and explain the source of the large cohort of FL HSCs that appears within 24 hours following the generation of the first HSCs in the AGM and the rapid decrease in the pre-HSC numbers in the AGM [98].

Concluding remarks

The adult hematopoietic system is established through the progressive generation of hematopoietic cells with increasing functional complexity, culminating in the *de novo* generation of long-lived self-renewing HSCs that provide the adult organism with all functional blood cells. Although the precise temporal onset of expression and of the HSC program and initiation of function is debatable, it is certain that it is directed by the expression of a small set of pivotal hematopoietic transcription factors. In combination these factors create a highly complex network that, depending on the time and levels of expression, drive the determination of hematopoietic progenitors and stem cell fate. It remains a future challenge to determine all the players in this process, to examine all the precursors to HSCs, as well as to cells along the process of endothelial-to-hematopoietic transition on the single cell level to converge transcriptomics with cell biology and function. This would ultimately enable the recapitulation of physiologic HSC development *in vitro* for the *de novo* production of transplantable HSCs for therapeutic strategies.

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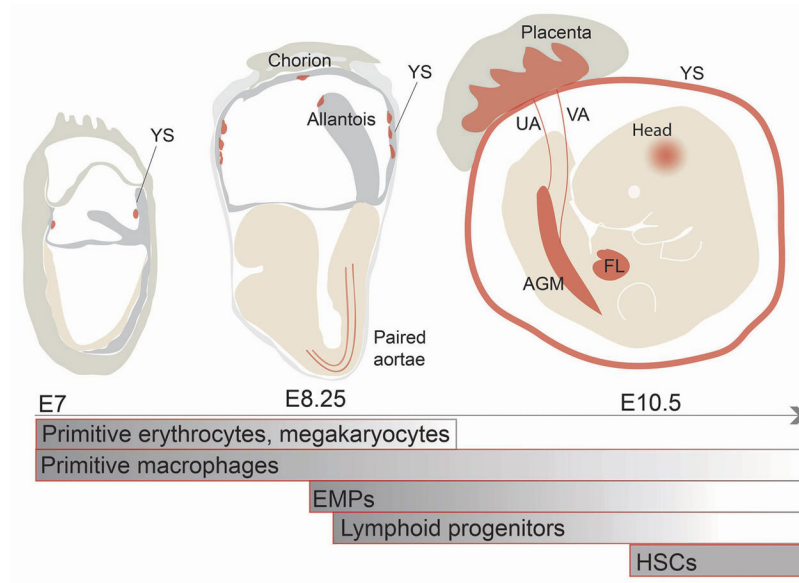


Figure 1. Sites and times of blood cell generation in the mouse embryo

Blood generation in the mouse embryo starts in the blood islands of extra embryonic yolk sac (YS) at embryonic day 7 (E7) with a transient wave of ‘primitive’ erythrocyte, megakaryocyte and macrophage production. The erythrocytes and megakaryocytes of that stage are short lived and disappear by E9. Primitive macrophages are hypothesized to be the source of tissue resident macrophages in the adult brain. The second wave of blood generation gives rise to bipotential erythroid-myeloid progenitors (EMPs) that emerge in the YS from E8.25. Shortly thereafter, lymphoid potential is detected. The paired dorsal aortae contain lymphoid potential as do the allantois/chorion. In the third hematopoietic wave, long-lived transplantable hematopoietic stem cells (HSCs) are generated beginning at E10.5 in the aorta-gonad-mesonephros (AGM) region. HSCs are also detected in the vitelline (VA) and umbilical (UA) arteries, YS, placenta and in embryonic head. HSCs and EMPs migrate to the fetal liver (FL) where they expand and reside before migrating to the bone marrow niches.

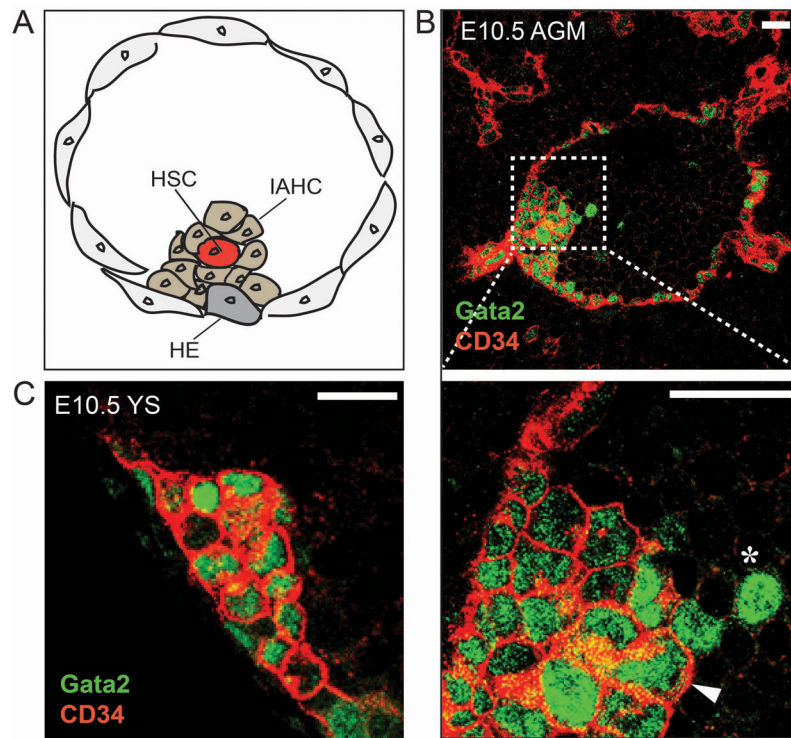


Figure 2. Emergence of HSCs in the AGM and in the YS

(A) A subset of aortic endothelial cells, the hemogenic endothelial (HE) cells transdifferentiate to form intra-aortic hematopoietic cluster cells (IAHC) and hematopoietic stem cells (HSC). A schematic diagram of a transverse section through an E10.5 mouse aorta indicates HE, IAHC and emerging HSC. (B) Transverse section through an E10.5 *Gata2Venus* mouse embryo. *Gata2* is one of the pivotal transcription factors expressed in HE, IAHC and emerging HSCs. Thus, *Gata2Venus* reporter can be exploited to visualize the emergence of HSCs. Section of an E10.5 aorta-gonad-mesonephros (AGM) shows *Gata2* (green) in most of the $CD34^+$ (red) IAHC (arrowhead) and some endothelial cells. *Gata2* is also detected in a few round $CD34^-$ cells (asterisk) that are closely associated with IAHC suggesting that they arose from clusters. (C) $CD34^+Gata2^+$ clusters are also found in the YS vasculature. Size bars=20 μ m.