



THE UNIVERSITY *of* EDINBURGH

Edinburgh Research Explorer

Hematopoietic (stem) cell development-how divergent are the roads taken?

Citation for published version:

Kauts, M-L, Vink, CS & Dzierzak, E 2016, 'Hematopoietic (stem) cell development-how divergent are the roads taken?', *FEBS Letters*. <https://doi.org/10.1002/1873-3468.12372>

Digital Object Identifier (DOI):

[10.1002/1873-3468.12372](https://doi.org/10.1002/1873-3468.12372)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

FEBS Letters

Publisher Rights Statement:

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made

General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact openaccess@ed.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Hematopoietic (stem) cell development—how divergent are the roads taken?

Mari-Liis Kauts^{1,2}, Chris S. Vink^{1,2} and Elaine Dzierzak^{1,2}

1 Centre for Inflammation Research, Queens Medical Research Institute, University of Edinburgh, UK

2 Department of Cell Biology, Erasmus MC Stem Cell Institute, Erasmus Medical Center, Rotterdam, The Netherlands

Correspondence

E. Dzierzak, Centre for Inflammation Research, Queens Medical Research Institute, University of Edinburgh, 47 Little France Crescent, Edinburgh EH16 4TJ, UK
Fax: +31 10 7044743
Tel: +31 10 7043169
E-mail: elaine.dzierzak@ed.ac.uk

(Received 11 July 2016, revised 11 August 2016, accepted 15 August 2016)

doi:10.1002/1873-3468.12372

Edited by Wilhelm Just

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: embryo; endothelial-to-hematopoietic cell transition; ES cell differentiation; Gata2; hematopoietic development; hematopoietic stem cells; hematopoietic progenitor cells; HSC fate; Runx1

The adult hematopoietic system consists of a hierarchy of cells that progress from a stem cell state to the terminally differentiated cells of over 10 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.

Abbreviations

AGM, aorta-gonad-mesonephros; BL-CFC, blast colony-forming progenitor cells; CFU-C, colony-forming unit-cell; CFU-S, colony-forming unit-spleen; EHT, endothelial-to-hematopoietic cell transition; EMP, erythroid–myeloid progenitors; ES, embryonic stem; FL, fetal liver; *Gpr56*, *G protein-coupled receptor 56*; HE, hemogenic endothelial; HSC, hematopoietic stem cells; IAHC, intra-aortic hematopoietic cluster cells; UA, umbilical arteries; VA, vitelline arteries; *Vec*, *vascular endothelial-cadherin*; YS, yolk sac.

Surprisingly, they are produced even before the circulation is established [1]. Transient waves of hematopoietic cell production are first initiated extraembryonically in the yolk sac (YS) blood islands (Fig. 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 with 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 segregation 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].

Embryonic stem 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

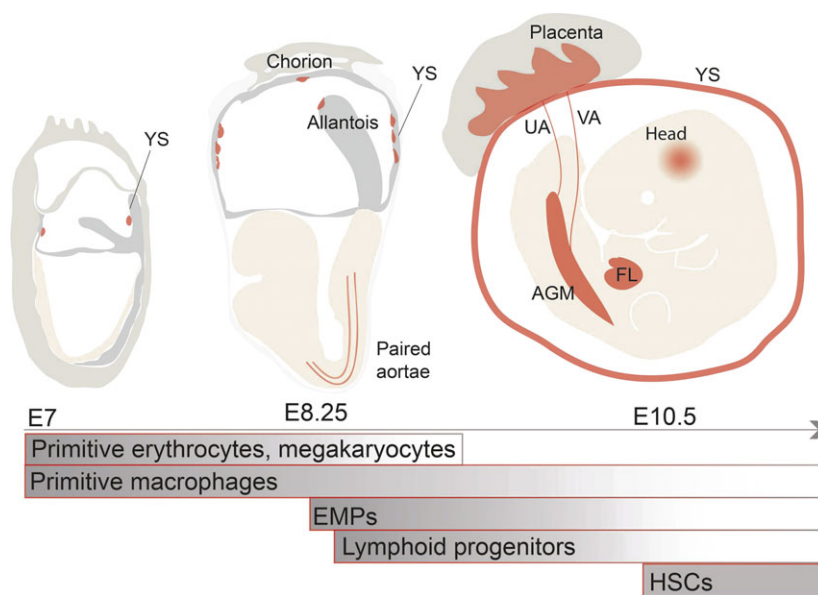


Fig. 1. Sites and times of blood cell generation in the mouse embryo. Blood generation in the mouse embryo starts in the blood islands of extraembryonic yolk sac (YS) at embryonic day 7 (E7) with a transient wave of 'primitive' erythrocyte, megakaryocyte and macrophage production. The erythrocytes and 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.

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 three times larger than fetal and six 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, Kupfer 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 *PUI* 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 (HPC) 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 (Fig. 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

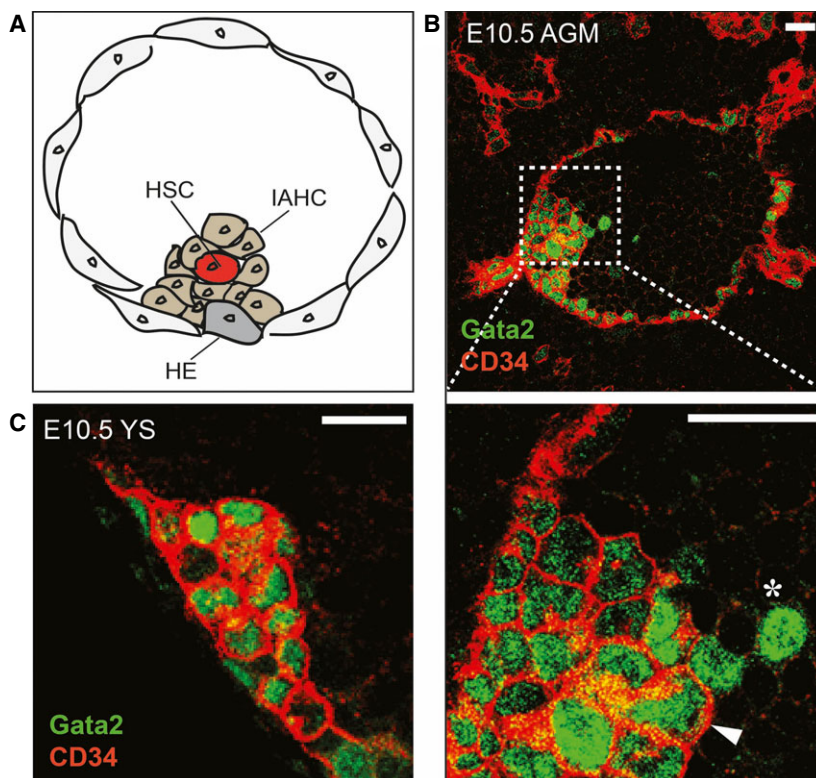


Fig. 2. Emergence of HSCs in the AGM and in the YS. (A) A subset of aortic endothelial cells, the hemogenic endothelial (HE) cells transdifferentiate to form intra-aortic hematopoietic cluster cells (IAHC) and hematopoietic stem cells (HSC). A schematic diagram of a transverse section through an E10.5 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 μm.

‘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 repopulate long term 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 (Fig. 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]. Although 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].

Hematopoietic stem cells are generated from a subset of embryonic endothelial cells that possess hemogenic potential—the hemogenic endothelial cells

(Fig. 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 (Fig. 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 the tracking of single cells as they transition from an endothelial cell to a HSC/HPC. This process is generally known as the EHT.

Endothelial-to-hematopoietic cell transition 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 abluenally, and emerge as hematopoietic cells in the interstitial region between the aorta and axial vein. Moreover, multicell 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].

Endothelial-to-hematopoietic cell transition 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 downregulate 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. Although *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 toward 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 by 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 coaggregation culture was established that facilitates the *ex vivo* maturation of hematopoietic/endothelial cells obtained by multisurface 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 coaggregated 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) upregulates *CD45* expression when coaggregated 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 coaggregation 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-nonactivated, 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 h 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.

Acknowledgements

The authors acknowledge funding support from the Landsteiner Society for Bloodtransfusion Research (1109), ZonMM – Netherlands Scientific Research Council – TOP (103127), and National Institutes of Health – NIDDK (R37 DK 54077). The authors acknowledge the grant support of the Landsteiner Society for Blood Research (LSBR 1344-1) and the NIH NIDDKK (R37 DK 054077).

References

- 1 Palis J, Chan RJ, Koniski A, Patel R, Starr M and Yoder MC (2001) Spatial and temporal emergence of high proliferative potential hematopoietic precursors during murine embryogenesis. *Proc Natl Acad Sci USA* **98**, 4528–4533.
- 2 Palis J, Robertson S, Kennedy M, Wall C and Keller G (1999) Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* **126**, 5073–5084.
- 3 Haar JL and Ackerman GA (1971) A phase and electron microscopic study of vasculogenesis and erythropoiesis in the yolk sac of the mouse. *Anat Rec* **170**, 199–223.
- 4 Yamaguchi TP, Dumont DJ, Conlon RA, Breitman ML and Rossant J (1993) flk-1, an flt-related receptor tyrosine kinase is an early marker for endothelial cell precursors. *Development* **118**, 489–498.
- 5 Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML and Schuh AC (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62–66.
- 6 Ueno H and Weissman IL (2006) Clonal analysis of mouse development reveals a polyclonal origin for yolk sac blood islands. *Dev Cell* **11**, 519–533.
- 7 Evans MJ and Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.

- 8 Bradley A, Evans M, Kaufman MH and Robertson E (1984) Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**, 255–256.
- 9 Choi K, Kennedy M, Kazarov A, Papadimitriou JC and Keller G (1998) A common precursor for hematopoietic and endothelial cells. *Development* **125**, 725–732.
- 10 Kennedy M, Firpo M, Choi K, Wall C, Robertson S, Kabrun N and Keller G (1997) A common precursor for primitive erythropoiesis and definitive haematopoiesis. *Nature* **386**, 488–493.
- 11 Kouskoff V, Lacaud G, Schwantz S, Fehling HJ and Keller G (2005) Sequential development of hematopoietic and cardiac mesoderm during embryonic stem cell differentiation. *Proc Natl Acad Sci USA* **102**, 13170–13175.
- 12 Tober J, Koniski A, McGrath KE, Vemishetti R, Emerson R, de Mesy-Bentley KK, Waugh R and Palis J (2007) The megakaryocyte lineage originates from hemangioblast precursors and is an integral component both of primitive and of definitive hematopoiesis. *Blood* **109**, 1433–1441.
- 13 Kingsley PD, Malik J, Emerson RL, Bushnell TP, McGrath KE, Bloedorn LA, Bulger M and Palis J (2006) “Maturation” globin switching in primary primitive erythroid cells. *Blood* **107**, 1665–1672.
- 14 Kingsley PD, Malik J, Fantauzzo KA and Palis J (2004) Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. *Blood* **104**, 19–25.
- 15 Bertrand JY, Jalil A, Klaine M, Jung S, Cumano A and Godin I (2005) Three pathways to mature macrophages in the early mouse yolk sac. *Blood* **106**, 3004–3011.
- 16 Naito M, Umeda S, Yamamoto T, Moriyama H, Umezu H, Hasegawa G, Usuda H, Shultz LD and Takahashi K (1996) Development, differentiation, and phenotypic heterogeneity of murine tissue macrophages. *J Leukoc Biol* **59**, 133–138.
- 17 Naito M, Takahashi K and Nishikawa S (1990) Development, differentiation, and maturation of macrophages in the fetal mouse liver. *J Leukoc Biol* **48**, 27–37.
- 18 Takahashi K, Yamamura F and Naito M (1989) Differentiation, maturation, and proliferation of macrophages in the mouse yolk sac: a light-microscopic, enzyme-cytochemical, immunohistochemical, and ultrastructural study. *J Leukoc Biol* **45**, 87–96.
- 19 McGrath KE, Koniski AD, Malik J and Palis J (2003) Circulation is established in a stepwise pattern in the mammalian embryo. *Blood* **101**, 1669–1676.
- 20 Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, Garner H, Trouillet C, de Bruijn MF, Geissmann F *et al.* (2015) Tissue-resident macrophages originate from yolk-sac-derived erythromyeloid progenitors. *Nature* **518**, 547–551.
- 21 Kierdorf K, Erny D, Goldmann T, Sander V, Schulz C, Perdiguero EG, Wieghofer P, Heinrich A, Riemke P, Holscher C *et al.* (2013) Microglia emerge from erythromyeloid precursors via Pu.1- and Irf8-dependent pathways. *Nat Neurosci* **16**, 273–280.
- 22 Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu B, Jacobsen SE, Pollard JW *et al.* (2012) A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*, **336**, 86–90.
- 23 Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, Beaudin AE, Lum J, Low I, Forsberg EC *et al.* (2015) C-Myb(+) erythro-myeloid progenitor-derived fetal monocytes give rise to adult tissue-resident macrophages. *Immunity* **42**, 665–678.
- 24 McGrath KE, Frame JM, Fegan KH, Bowen JR, Conway SJ, Catherman SC, Kingsley PD, Koniski AD and Palis J (2015) Distinct sources of hematopoietic progenitors emerge before HSCs and provide functional blood cells in the mammalian embryo. *Cell Rep* **11**, 1892–1904.
- 25 Frame JM, McGrath KE and Palis J (2013) Erythro-myeloid progenitors: “definitive” hematopoiesis in the conceptus prior to the emergence of hematopoietic stem cells. *Blood Cells Mol Dis* **51**, 220–225.
- 26 Lux CT, Yoshimoto M, McGrath K, Conway SJ, Palis J and Yoder MC (2008) All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. *Blood* **111**, 3435–3438.
- 27 Frame JM, Fegan KH, Conway SJ, McGrath KE and Palis J (2016) Definitive hematopoiesis in the yolk sac emerges from Wnt-responsive hemogenic endothelium independently of circulation and arterial identity. *Stem Cells* **34**, 431–444.
- 28 Yokota T, Huang J, Tavian M, Nagai Y, Hirose J, Zuniga-Pflucker JC, Peault B and Kincade PW (2006) Tracing the first waves of lymphopoiesis in mice. *Development* **133**, 2041–2051.
- 29 Yoshimoto M, Porayette P, Glosson NL, Conway SJ, Carlesso N, Cardoso AA, Kaplan MH and Yoder MC (2012) Autonomous murine T-cell progenitor production in the extra-embryonic yolk sac before HSC emergence. *Blood* **119**, 5706–5714.
- 30 Godin I, Dieterlen-Lievre F and Cumano A (1995) Emergence of multipotent hemopoietic cells in the yolk sac and paraaortic splanchnopleura in mouse embryos, beginning at 8.5 days postcoitus. *Proc Natl Acad Sci USA* **92**, 773–777.
- 31 Sonoda T, Hayashi C and Kitamura Y (1983) Presence of mast cell precursors in the yolk sac of mice. *Dev Biol* **97**, 89–94.

- 32 Travnickova J, Tran Chau V, Julien E, Mateos-Langerak J, Gonzalez C, Lelievre E, Lutfalla G, Tavian M and Kissa K (2015) Primitive macrophages control HSPC mobilization and definitive haematopoiesis. *Nat Commun* **6**, 6227.
- 33 Li Y, Esain V, Teng L, Xu J, Kwan W, Frost IM, Yzaguirre AD, Cai X, Cortes M, Maijenburg MW *et al.* (2014) Inflammatory signaling regulates embryonic hematopoietic stem and progenitor cell production. *Genes Dev* **28**, 2597–2612.
- 34 Espin-Palazon R, Stachura DL, Campbell CA, Garcia-Moreno D, Del Cid N, Kim AD, Candel S, Meseguer J, Mulero V and Traver D (2014) Proinflammatory signaling regulates hematopoietic stem cell emergence. *Cell* **159**, 1070–1085.
- 35 Muller AM, Medvinsky A, Strouboulis J, Grosveld F and Dzierzak E (1994) Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1**, 291–301.
- 36 Medvinsky A and Dzierzak E (1996) Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* **86**, 897–906.
- 37 de Bruijn MF, Speck NA, Peeters MC and Dzierzak E (2000) Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J* **19**, 2465–2474.
- 38 Li Z, Lan Y, He W, Chen D, Wang J, Zhou F, Wang Y, Sun H, Chen X, Xu C *et al.* (2012) Mouse embryonic head as a site for hematopoietic stem cell development. *Cell Stem Cell* **11**, 663–675.
- 39 Gekas C, Dieterlen-Lievre F, Orkin SH and Mikkola HK (2005) The placenta is a niche for hematopoietic stem cells. *Dev Cell* **8**, 365–375.
- 40 Ottersbach K and Dzierzak E (2005) The murine placenta contains hematopoietic stem cells within the vascular labyrinth region. *Dev Cell* **8**, 377–387.
- 41 Ema H and Nakauchi H (2000) Expansion of hematopoietic stem cells in the developing liver of a mouse embryo. *Blood* **95**, 2284–2288.
- 42 Kumaravelu P, Hook L, Morrison AM, Ure J, Zhao S, Zuyev S, Ansell J and Medvinsky A (2002) Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* **129**, 4891–4899.
- 43 Mendelson A and Frenette PS (2014) Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med* **20**, 833–846.
- 44 Jaffredo T, Nottingham W, Liddiard K, Bollerot K, Pouget C and de Bruijn M (2005) From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp Hematol* **33**, 1029–1040.
- 45 Dzierzak E and Speck NA (2008) Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol* **9**, 129–136.
- 46 Yokomizo T and Dzierzak E (2010) Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development* **137**, 3651–3661.
- 47 Chen MJ, Li Y, De Obaldia ME, Yang Q, Yzaguirre AD, Yamada-Inagawa T, Vink CS, Bhandoola A, Dzierzak E and Speck NA (2011) Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell* **9**, 541–552.
- 48 Ma X, de Bruijn M, Robin C, Peeters M, Kong ASJ, de Wit T, Snoijs C and Dzierzak E (2002) Expression of the Ly-6A (Sca-1) lacZ transgene in mouse haematopoietic stem cells and embryos. *Br J Haematol* **116**, 401–408.
- 49 Boisset JC, van Cappellen W, Andrieu-Soler C, Galjart N, Dzierzak E and Robin C (2010) In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature* **464**, 116–120.
- 50 Jing L and Zon LI (2011) Zebrafish as a model for normal and malignant hematopoiesis. *Dis Model Mech* **4**, 433–438.
- 51 Kissa K and Herbomel P (2010) Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature* **464**, 112–115.
- 52 Bertrand JY, Kim AD, Teng S and Traver D (2008) CD41+ cmyb+ precursors colonize the zebrafish pronephros by a novel migration route to initiate adult hematopoiesis. *Development* **135**, 1853–1862.
- 53 Bertrand JY, Chi NC, Santoso B, Teng S, Stainier DY and Traver D (2010) Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature* **464**, 108–111.
- 54 Tamplin OJ, Durand EM, Carr LA, Childs SJ, Hagedorn EJ, Li P, Yzaguirre AD, Speck NA and Zon LI (2015) Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell* **160**, 241–252.
- 55 Lancrin C, Sroczynska P, Stephenson C, Allen T, Kouskoff V and Lacaud G (2009) The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature* **457**, 892–895.
- 56 Eilken HM, Nishikawa S and Schroeder T (2009) Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* **457**, 896–900.
- 57 Rafi S, Kloss CC, Butler JM, Ginsberg M, Gars E, Lis R, Zhan Q, Josipovic P, Ding BS, Xiang J *et al.* (2013) Human ESC-derived hemogenic endothelial cells undergo distinct waves of endothelial to hematopoietic transition. *Blood* **121**, 770–780.
- 58 Tsai FY, Keller G, Kuo FC, Weiss M, Chen J, Rosenblatt M, Alt FW and Orkin SH (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226.

- 59 Okuda T, van Deursen J, Hiebert SW, Grosveld G and Downing JR (1996) AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell* **84**, 321–330.
- 60 Cai Z, de Bruijn M, Ma X, Dortland B, Luteijn T, Downing RJ and Dzierzak E (2000) Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity* **13**, 423–431.
- 61 Ling KW, Ottersbach K, van Hamburg JP, Oziemlak A, Tsai FY, Orkin SH, Ploemacher R, Hendriks RW and Dzierzak E (2004) GATA-2 plays two functionally distinct roles during the ontogeny of hematopoietic stem cells. *J Exp Med* **200**, 871–882.
- 62 Kaimakis P, de Pater E, Eich C, Solaimani Kartalaei P, Kauts ML, Vink CS, van der Linden R, Jaegle M, Yokomizo T, Meijer D *et al.* (2016) Functional and molecular characterization of mouse Gata2-independent hematopoietic progenitors. *Blood* **127**, 1426–1437.
- 63 North T, Gu TL, Stacy T, Wang Q, Howard L, Binder M, Marin-Padilla M and Speck NA (1999) Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* **126**, 2563–2575.
- 64 Minegishi N, Suzuki N, Yokomizo T, Pan X, Fujimoto T, Takahashi S, Hara T, Miyajima A, Nishikawa S and Yamamoto M (2003) Expression and domain-specific function of GATA-2 during differentiation of the hematopoietic precursor cells in midgestation mouse embryos. *Blood* **102**, 896–905.
- 65 Robert-Moreno A, Espinosa L, de la Pompa JL and Bigas A (2005) RBPj κ -dependent Notch function regulates Gata2 and is essential for the formation of intra-embryonic hematopoietic cells. *Development* **132**, 1117–1126.
- 66 Iizuka K, Yokomizo T, Watanabe N, Tanaka Y, Osato M, Takaku T and Komatsu N (2016) Lack of phenotypical and morphological evidences of endothelial to hematopoietic transition in the murine embryonic head during hematopoietic stem cell emergence. *PLoS One* **11**, e0156427.
- 67 Rhodes KE, Gekas C, Wang Y, Lux CT, Francis CS, Chan DN, Conway S, Orkin SH, Yoder MC and Mikkola HK (2008) The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell* **2**, 252–263.
- 68 Nardelli J, Thiesson D, Fujiwara Y, Tsai FY and Orkin SH (1999) Expression and genetic interaction of transcription factors GATA-2 and GATA-3 during development of the mouse central nervous system. *Dev Biol* **210**, 305–321.
- 69 Pimanda JE, Ottersbach K, Knezevic K, Kinston S, Chan WY, Wilson NK, Landry JR, Wood AD, Kolb-Kokocinski A, Green AR *et al.* (2007) Gata2, Fli1, and Scl form a recursively wired gene-regulatory circuit during early hematopoietic development. *Proc Natl Acad Sci USA* **104**, 17692–17697.
- 70 Chen MJ, Yokomizo T, Zeigler BM, Dzierzak E and Speck NA (2009) Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature* **457**, 887–891.
- 71 de Pater E, Kaimakis P, Vink CS, Yokomizo T, Yamada-Inagawa T, van der Linden R, Kartalaei PS, Camper SA, Speck N and Dzierzak E (2013) Gata2 is required for HSC generation and survival. *J Exp Med* **210**, 2843–2850.
- 72 Zovein AC, Hofmann JJ, Lynch M, French WJ, Turlo KA, Yang Y, Becker MS, Zanetta L, Dejana E, Gasson JC *et al.* (2008) Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell* **3**, 625–636.
- 73 Tober J, Yzaguirre AD, Piwarzyk E and Speck NA (2013) Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development* **140**, 3765–3776.
- 74 Rodrigues NP, Janzen V, Forkert R, Dombkowski DM, Boyd AS, Orkin SH, Enver T, Vyas P and Scadden DT (2005) Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis. *Blood* **106**, 477–484.
- 75 Gao X, Johnson KD, Chang YI, Boyer ME, Dewey CN, Zhang J and Bresnick EH (2013) Gata2 cis-element is required for hematopoietic stem cell generation in the mammalian embryo. *J Exp Med* **210**, 2833–2842.
- 76 Nandakumar SK, Johnson K, Throm SL, Pestina TI, Neale G and Persons DA (2015) Low-level GATA2 overexpression promotes myeloid progenitor self-renewal and blocks lymphoid differentiation in mice. *Exp Hematol* **43**, 565–577.
- 77 Ikonomi P, Rivera CE, Riordan M, Washington G, Schechter AN and Noguchi CT (2000) Overexpression of GATA-2 inhibits erythroid and promotes megakaryocyte differentiation. *Exp Hematol* **28**, 1423–1431.
- 78 Pereira CF, Chang B, Gomes A, Bernitz J, Papatsenko D, Niu X, Swiers G, Azzoni E, de Bruijn MF, Schaniel C *et al.* (2016) Hematopoietic reprogramming in vitro informs in vivo identification of hemogenic precursors to definitive hematopoietic stem cells. *Dev Cell* **36**, 525–539.
- 79 Lugus JJ, Chung YS, Mills JC, Kim SI, Grass J, Kyba M, Doherty JM, Bresnick EH and Choi K (2007) GATA2 functions at multiple steps in hemangioblast development and differentiation. *Development* **134**, 393–405.
- 80 Wang Q, Stacy T, Miller JD, Lewis AF, Gu TL, Huang X, Bushweller JH, Bories JC, Alt FW, Ryan G *et al.* (1996) The CBF β subunit is essential for

- CBFalpha2 (AML1) function in vivo. *Cell* **87**, 697–708.
- 81 Mukoyama Y, Chiba N, Hara T, Okada H, Ito Y, Kanamaru R, Miyajima A, Satake M and Watanabe T (2000) The AML1 transcription factor functions to develop and maintain hematogenic precursor cells in the embryonic aorta-gonad-mesonephros region. *Dev Biol* **220**, 27–36.
 - 82 Robin C, Ottersbach K, Durand C, Peeters M, Vanes L, Tybulewicz V and Dzierzak E (2006) An unexpected role for IL-3 in the embryonic development of hematopoietic stem cells. *Dev Cell* **11**, 171–180.
 - 83 Wilson NK, Foster SD, Wang X, Knezevic K, Schutte J, Kaimakis P, Chilarska PM, Kinston S, Ouwehand WH, Dzierzak E *et al.* (2010) Combinatorial transcriptional control in blood stem/progenitor cells: genome-wide analysis of ten major transcriptional regulators. *Cell Stem Cell* **7**, 532–544.
 - 84 Solaimani Kartalaei P, Yamada-Inagawa T, Vink CS, de Pater E, van der Linden R, Marks-Bluth J, van der Sloot A, van den Hout M, Yokomizo T, van Schaick-Solerno ML *et al.* (2015) Whole-transcriptome analysis of endothelial to hematopoietic stem cell transition reveals a requirement for Gpr56 in HSC generation. *J Exp Med* **212**, 93–106.
 - 85 Chacon D, Beck D, Perera D, Wong JW and Pimanda JE (2014) BloodChIP: a database of comparative genome-wide transcription factor binding profiles in human blood cells. *Nucleic Acids Res* **42** (Database issue), D172–D177.
 - 86 Holmfeldt P, Ganuza M, Marathe H, He B, Hall T, Kang G, Moen J, Pardieck J, Saulsbury AC, Cico A *et al.* (2016) Functional screen identifies regulators of murine hematopoietic stem cell repopulation. *J Exp Med* **213**, 433–449.
 - 87 Lancrin C, Mazan M, Stefanska M, Patel R, Lichtinger M, Costa G, Vargel O, Wilson NK, Moroy T, Bonifer C *et al.* (2012) GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood* **120**, 314–322.
 - 88 Thambyrajah R, Mazan M, Patel R, Moignard V, Stefanska M, Marinopoulou E, Li Y, Lancrin C, Clapes T, Moroy T *et al.* (2016) GFI1 proteins orchestrate the emergence of haematopoietic stem cells through recruitment of LSD1. *Nat Cell Biol* **18**, 21–32.
 - 89 Moignard V, Macaulay IC, Swiers G, Buettner F, Schutte J, Calero-Nieto FJ, Kinston S, Joshi A, Hannah R, Theis FJ *et al.* (2013) Characterization of transcriptional networks in blood stem and progenitor cells using high-throughput single-cell gene expression analysis. *Nat Cell Biol* **15**, 363–372.
 - 90 Nottingham WT, Jarratt A, Burgess M, Speck CL, Cheng JF, Prabhakar S, Rubin EM, Li PS, Sloane-Stanley J, Kong ASJ *et al.* (2007) Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood* **110**, 4188–4197.
 - 91 Swiers G, Baumann C, O'Rourke J, Giannoulatou E, Taylor S, Joshi A, Moignard V, Pina C, Bee T, Kokkaliaris KD *et al.* (2013) Early dynamic fate changes in haemogenic endothelium characterized at the single-cell level. *Nat Commun* **4**, 2924.
 - 92 Rybtsov S, Sobiesiak M, Taoudi S, Souilhol C, Senserrich J, Liakhovitskaia A, Ivanovs A, Frampton J, Zhao S and Medvinsky A (2011) Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med* **208**, 1305–1315.
 - 93 Rybtsov S, Batsivari A, Bilotkach K, Paruzina D, Senserrich J, Nerushev O and Medvinsky A (2014) Tracing the origin of the HSC hierarchy reveals an SCF-dependent, IL-3-independent CD43(-) embryonic precursor. *Stem Cell Reports* **3**, 489–501.
 - 94 Liakhovitskaia A, Rybtsov S, Smith T, Batsivari A, Rybtsova N, Rode C, de Bruijn M, Buchholz F, Gordon-Keylock S, Zhao S *et al.* (2014) Runx1 is required for progression of CD41+ embryonic precursors into HSCs but not prior to this. *Development* **141**, 3319–3323.
 - 95 Gordon-Keylock S, Sobiesiak M, Rybtsov S, Moore K and Medvinsky A (2013) Mouse extraembryonic arterial vessels harbor precursors capable of maturing into definitive HSCs. *Blood* **122**, 2338–2345.
 - 96 Boisset JC, Clapes T, Klaus A, Papazian N, Onderwater J, Mommaas-Kienhuis M, Cupedo T and Robin C (2015) Progressive maturation toward hematopoietic stem cells in the mouse embryo aorta. *Blood* **125**, 465–469.
 - 97 Li Z, Vink CS, Mariani SA and Dzierzak E (2016) Subregional localization and characterization of Ly6aGFP-expressing hematopoietic cells in the mouse embryonic head. *Dev Biol* **416**, 34–41.
 - 98 Rybtsov S, Ivanovs A, Zhao S and Medvinsky A (2016) Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. *Development* **143**, 1284–1289.
 - 99 Crisan M, Kartalaei PS, Vink CS, Yamada-Inagawa T, Bollerot K, van IJcken W, van der Linden R, de Sousa Lopes SM, Monteiro R, Mummery C *et al.* (2015) BMP signalling differentially regulates distinct haematopoietic stem cell types. *Nat Commun* **6**, 8040.
 - 100 Yamamoto R, Morita Y, Oechara J, Hamanaka S, Onodera M, Rudolph KL, Ema H and Nakauchi H (2013) Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**, 1112–1126.