Published in final edited form as:

Blood Cells Mol Dis. 2013 December ; 51(4): 206–212. doi:10.1016/j.bcmd.2013.09.005.

A short history of hemogenic endothelium

Gemma Swiers, Christina Rode, Emanuele Azzoni, and Marella F. T. R. de Bruijn

MRC Molecular Haematology Unit, MRC Weatherall Institute of Medicine, John Radcliffe Hospital, Radcliffe Department of Medicine, University of Oxford, Oxford, OX3 9DS, UK.

Abstract

Definitive hematopoietic cells are generated de novo during ontogeny from a specialized subset of endothelium, the so-called hemogenic endothelium. In this review we give a brief overview of the identification of hemogenic endothelium, explore its links with the HSC lineage, and summarize recent insights into the nature of hemogenic endothelium and the microenvironmental and intrinsic regulators contributing to its transition into blood. Ultimately, a better understanding of the processes controlling the transition of endothelium into blood will advance the generation and expansion of hematopoietic stem cells for therapeutic purposes.

Introduction

The cellular and molecular processes underlying the initial generation of hematopoietic stem cells (HSCs) during ontogeny have been intensively studied. Indeed, the current interest in stem cell-based therapies has emphasized the importance of understanding how tissue-specific stem cells are specified in development. The hematopoietic system has been a paradigm for this. In the mouse, hematopoiesis occurs asynchronously at different sites in the embryo (Figure 1). The first wave of hematopoiesis commences at E7.5 in the blood islands of the yolk sac with the generation of primitive erythrocyte progenitors [1-3]. These cells supply the growing embryo with oxygen. Primitive erythrocytes are thought to derive directly from a mesodermal precursor, and not from a bona fide HSC as the latter are not detectable in the conceptus at this early stage [4, 5]. Shortly after the formation of primitive erythrocytes, multi-potent and lineage-restricted hematopoietic progenitor cells that display adult-type morphology are generated asynchronously in different hematopoietic sites of the embryo (reviewed in [6, 7]), including the yolk sac, para-aortic splanchnopleura (PAS)/aorta-gonad-mesonephros (AGM) region, vitelline and umbilical arteries, placenta, and most recently recognized, the heart [8]. These progenitors are generally considered transient in nature as also they emerge in the absence of adult HSC activity, though some specific lymphoid and myeloid cells may persist into adulthood [9-11]. It is the yolk sac that provides most, if not all, of the hematopoietic progenitor cells that seed the fetal liver at E10. These progenitors differentiate into the first definitive-type erythro-myeloid cells that circulate in the embryo [12], and were recently shown to be critical to the midgestation survival of the embryo [13]. Finally, definitive HSCs capable of founding the adult

hematopoietic system and responsible for the life-long production of all blood cells are first and autonomously generated in the wall of the dorsal aorta in the AGM region, starting from E10.5 [4, 5, 14-18]. The transient progenitors and long-term HSCs have long been proposed to originate from a specialized subset of endothelium, so-called hemogenic endothelium (reviewed in [7, 19-21]). However, it was not until recently, through the application of live single cell imaging, that the endothelial origin of blood gained wider support. Alternative models do exist and are illustrated in [7]. In the present review, we provide a brief history of hemogenic endothelium highlighting recent advances in our understanding of the nature and origin of hemogenic endothelium, and the mechanisms underlying the endothelial-to-hematopoietic transition leading to the formation of the adult hematopoietic system.

Identification of hemogenic endothelium

The idea that blood cells developed from a hemogenic endothelium was proposed around 100 years ago and was based on microscopic observations of developing embryos and represented in beautiful drawings [22-24]. The first *in vivo* experimental support came much later, from lineage tracing of endothelium in the chick embryo [25, 26]. More recently, lineage-tracing studies in which Cre recombinase was expressed under the regulatory control of the endothelial marker VE-Cadherin (VECadh) lent further support to this model [27, 28]. Arguably, the strongest support for an endothelial origin of hematopoietic cells came from the recent time-lapse imaging studies that visualized the transition of endothelium into blood in real-time, both *in vitro* [29] and *in/ex vivo* [30-33]. The process of endothelial cells taking on a hematopoietic fate has been termed the endothelial-to-hematopoietic transition (EHT), and involves the bending out and rounding up of an endothelial cell and its subsequent detachment from the vascular wall and formation of a free-moving hematopoietic cell [30]. These imaging and lineage tracing studies provided compelling support for a large body of *in vitro* and *ex vivo* data that had earlier supported an endothelial origin of blood; for a detailed overview of these data we refer the reader to the recent reviews by [7, 34, 35].

The term hemogenic endothelium is often applied rather broadly to include all endothelial cells in sites of hematopoietic stem and/or progenitor cell emergence, as there are no unique cell surface markers that allow for the phenotypic identification and isolation of hemogenic endothelium. However, based on expression of the transcription factor Runx1 (discussed below) only a subset of the endothelium in these sites is believed to be hemogenic [36]. Functionally, hemogenic endothelium has been defined as displaying an endothelial phenotype and morphology and having the capacity to form hematopoietic offspring and endothelial tubules/sheets in culture [37, 38]. The overlap in cell surface marker expression on (hemogenic) endothelial and hematopoietic cells has further confounded the identification and isolation of hemogenic endothelium. Both endothelial cells and hematopoietic stem and progenitor cells (HSPCs) express the “endothelial” markers CD31 (Pecam), VECadherin (VE-Cadh; Cdh5) [37] and Flk-1 (kdr) [39]. Combination of VE-Cadh or CD31 with the hematopoietic markers CD41 [40-43], CD45 [44] and Ter119 [45] has been used to separate endothelial from hematopoietic cells (e.g. [38, 46-48]), and other identification strategies have also been employed e.g. [49] [50]. It is important to note that different studies have varied in the stringency with which hematopoietic progenitors were excluded. For example, some did not exclude CD41⁺ hematopoietic progenitor cells, which

would have affected the hematopoietic output of the “hemogenic endothelial” population in culture.

The core-binding factor-family transcription factor Runx1 marks just a subset of the endothelium in hemogenic sites (reviewed in [51]). In the hemogenic arteries (dorsal aorta, vitelline and umbilical) endothelial Runx1 expression precedes the emergence of hematopoietic cells, which has been interpreted as Runx1 marking the hemogenic endothelium. In the absence of Runx1, hemogenic endothelium is still made but no definitive hematopoietic cells are formed [51-56]. Deletion of Runx1 in VE-Cadherin⁺ cells indicated that Runx1 is critically required in this population [28]. Although Runx1 expression continues in the emerging HSPCs and their offspring, spatiotemporal differences in the requirement for Runx1 and other members of the core-binding factor family were reported for these cells [28, 57]. To obtain a transgenic Runx1 reporter that would allow for the positive identification of hemogenic endothelium, we had initiated studies into the transcriptional regulation of Runx1 and recently identified the *Runx1* +23 enhancer. This enhancer mediates reporter gene expression specifically to sites of HSPC emergence and colonization, but not to other areas of Runx1 expression later in development. In enhancer-reporter transgenic mice, hematopoietic cells are marked throughout development [48, 58]. In addition, we recently showed that this +23 enhancer-reporter transgene marks all hemogenic endothelium in the embryo, thus providing us with a unique marker to isolate hemogenic endothelium for detailed functional and molecular studies. Interestingly, analyses of +23-marked endothelium at the clonal level showed that specification of the hemogenic endothelium to the hematopoietic lineage occurs one to two days earlier in development than previously anticipated (Swiers et al., submitted), guiding studies aimed at identifying the signals that initiate this process to earlier time points.

Linking hemogenic endothelium and the HSC lineage

HSCs start to emerge in the dorsal aorta after E10.5 of mouse gestation [4, 5, 14, 17]. The live imaging of the emergence of hematopoietic cells in the dorsal aorta of the mouse and zebrafish, and in ES cell differentiation cultures has been extrapolated to include an endothelial origin for HSCs. Although attractive, this has not been formally demonstrated. Indeed, this would be technically challenging, given the rarity of fully functional HSCs in the dorsal aorta (at any given time there is approximately one definitive HSC in the E11.5 mouse dorsal aorta [7, 17]). Approaching this issue from the HSC as opposed to the endothelial end, Medvinsky and coworkers recently identified two distinct cell types that are part of the HSC lineage which they termed type I and type II pre-HSC [59, 60]. They showed that type I pre-HSCs give rise to definitive HSCs via the type II pre-HSC intermediate. Type I and II pre-HSCs have a VE-Cadherin⁺CD41^{lo}CD45⁻ and VE-Cadherin⁺CD41^{lo}CD45⁺ phenotype, respectively, and lacked the capacity to form endothelial tubules and sheets in culture. Dye labeling studies showed that these pre-HSCs were localized not only to the luminal aspect of the dorsal aorta, i.e. the endothelial wall and/or hematopoietic clusters where HSCs are found, but also to the subluminal wall. Whether the pre-HSCs subluminal to the dorsal aorta are associated with, for example, the remodeling vasculature of the vitelline artery (another site that harbors type I pre-HSCs and HSCs [14, 61], or result from an abluminal EHT of aortic endothelium remains to be established [59].

Two inferences can be made from these observations. First, if HSCs indeed originate from hemogenic endothelium, they do so via at least two non-endothelial cellular intermediates, and second, the segregation between the endothelial and HSC lineage occurs prior to E10.5 [59].

Hemogenic activity – an inherent property of all young endothelium?

Recent studies on the generation of blood from endothelium have mostly focused on the dorsal aorta, the site where adult HSCs are first generated. However, hematopoietic cell clusters that protrude from the endothelium have been observed not only here, but also in other hemogenic sites, including the vitelline and umbilical arteries, yolk sac, and placenta [23, 26, 61-63]. Lineage tracing indicates that EHT also occurs in those tissues [27, 28, 64]. All this brings to mind the suggestion made 100 years ago, that hemogenic activity is an inherent property of all young, undifferentiated embryonic endothelium [22, 23, 62]. In this respect, two recent mouse studies give food for thought. Studies by Li and colleagues suggested that the head vasculature is a source of definitive HSCs [47]. Using *in vitro* and transplantation assays, hematopoietic potential was found to reside among Tie2 expressing cells isolated from the head. However, these experiments did not address whether this potential arises *de novo* at this site or is due to e.g. cells in circulation. To explore this, the authors performed lineage tracing using a SP-A-Cre transgenic mouse line. In this mouse line, cre recombinase activity was previously reported in blood vessels in the brain, alveolar cells in the lung and the stomach epithelium [65]. When crossed with a ROSA-eYFP mouse line, eYFP-positive cells were seen to contribute to postnatal hematopoiesis, suggestive of the *in situ* emergence of hematopoietic cells in the head region. This report is in stark contrast to several other studies in mouse and human embryos, which could not find any HSC activity over background levels in the embryonic head [5, 17, 66]. Additional experiments will be needed to clarify these conflicting reports, including analyses of the SP-A-Cre line to provide robust information on the Cre expression pattern.

Another recent study uncovered the hemogenic activity of early cardiac endothelium in the mouse [8], using *Ncx1*^{-/-} embryos that lack a heartbeat [67]. This precludes the distribution of yolk sac-derived blood cells over the rest of the embryo and thus allows for the analysis of hemogenic activity of other sites. In these *Ncx1*^{-/-} embryos, hematopoietic cells were found in the E8.5-E9.5 endocardium, suggesting they originate here. These hematopoietic cells resembled the transient population of erythromyeloid progenitors also found in the yolk sac [13], indicating their transient nature. In this the cardiac endothelium bears reminiscence of the anterior hemangioblast-derived cells in zebrafish [68] and *Drosophila* [69].

Distinct types of hemogenic endothelium?

Is the choice to generate a hematopoietic progenitor or stem cell already made at the level of the hemogenic endothelium? In other words, are there different types of hemogenic endothelium for transient progenitor cells and HSCs? A transgenic rescue study of CBFβ null embryos suggests this may be the case. CBFβ is the heterodimeric partner of Runx1. Deletion of CBFβ essentially phenocopies the Runx1 null defect: embryos die around midgestation with fetal liver anemia and central nervous system hemorrhages [53]. CBFβ

function is somewhat less sensitive to variations in expression levels than Runx1 [70] making it an attractive factor for transgenic rescue approaches of Runx1/CBF β function. In this study, Chen and colleagues reintroduced a GFP-CBF β fusion protein on a CBF β deficient background, under the regulatory control of two different tissue-specific expression cassettes. Using the Tie2 cassette, GFP-CBF β was expressed in yolk sac endothelium as well as endothelial cells of the dorsal aorta, vitelline and umbilical arteries and placenta, resulting in the rescue of erythro-myeloid cells (EMPs), but, surprisingly, not HSCs. Conversely, expression of GFP-CBF β under the control of the Ly6a regulatory cassette did result in rescue of HSCs, but not EMPs. Ly6a-GFP expression was not detected in the yolk sac or intra-embryonic endothelium prior to E9.5, indicating that the hemogenic endothelium of the yolk sac may be different to the intra-embryonic hemogenic endothelium able to generate HSCs [13]. If there are different hemogenic endothelia, this will have important implications in the design of therapeutic strategies/*in vitro* differentiation cultures aimed at generating HSCs.

The microenvironment supporting EHT

The precise nature of the molecular mechanisms that underlie the transition of endothelial into hematopoietic cells has yet to be fully understood. Insights into intrinsic regulators have come from genetic and lineage tracing studies, described below. In addition, live cell imaging of the dorsal aorta demonstrated that EHT is accompanied by dramatic changes in cell morphology, with cells initially maintaining contact with their neighbors to ensure continuity of the endothelial cell layer [30-32], giving cues to the molecular pathways likely to be involved in this transition. Sheer stress caused by blood flow was shown to affect the development of the hematopoietic system, at least in part through nitric oxide signaling which induces Runx1 expression in the endothelium [71, 72] and reviewed in [21]. The role of the microenvironment in HSC generation has just begun to be touched upon. Elegant dissection studies in the mouse identified the ventral wall of the dorsal aorta as the HSC niche [18], opening the way for more detailed analysis of the cellular and signalling components in this region. The chick embryo has long been instrumental in this type of study. Recently, the Jaffredo group employed intricate dissections and labelling studies to reveal a requirement for the ventral sub-aortic mesoderm for the induction of Runx1 expression and the formation of intra-aortic clusters, independent of aortic specification [73].

What are the cells in the ventral wall of the dorsal aorta that confer support for the developing HSCs? Apart from structural endothelial cells, the budding hematopoietic cells are in close vicinity of vascular smooth muscle cells and peri-vascular mesenchyme, the latter extending into the gut mesentery [74] (Figure 2a). Interestingly, there is extensive Runx1 expression in the abluminal cell layers of the dorsal aorta ([36]; Figure 2b). This has occasionally been interpreted as indicating the presence of hematopoietic cells or their direct precursors. However, the expression of smooth muscle actin (SMA) in these cells argues that the majority of the peri-aortic Runx1⁺ cells are smooth muscle ([75]; Figure 2b), though a very small number may be the recently identified pre-HSCs [59]. In the absence of Runx1 there is a compaction of the cell layers ventral to the dorsal aorta [36, 48], suggesting that Runx1 plays a role in the migration/organization of the smooth muscle cell layer.

Interestingly, Runx1 affects the expression of a negative regulator of hematopoiesis, Dlk1 in the AGM smooth muscle cells [75]. The full contribution of smooth muscle cells to AGM hematopoiesis remains to be elucidated. Recently, another negative regulator acting at the onset of hematopoiesis was identified in studies in zebrafish and ES cells, namely the thrombin receptor F2r that acts through the RhoA/ROCK pathway. Inhibition or activation of F2r signalling increased or decreased the frequency of EHT in the zebrafish embryo, respectively [76]. Whether F2r is involved in mammalian EHT has yet to be determined. The sympathetic nervous system developing adjacent to the dorsal aorta has also been shown to play an important role in HSC development. Analysis of Gata3 null mice showed that these mice have a defect in HSC specification, which is non-cell autonomous and due to the lack of Gata3 in the sympathetic ganglia underlying the dorsal aorta. Indeed, the hematopoietic defect could be rescued *ex vivo* in explant AGM cultures by the addition of catecholamines [77]. For detailed overviews of the cellular components and critical signalling pathways such as BMP, Notch and Wnt involved in the AGM microenvironment we refer the reader to the reviews by Jaffredo et al. [78] and Bigas et al. [79] in this issue of BCMD.

In conclusion, the microenvironment that regulates EHT and provides a niche for the nascent HSCs harbours a complicated network of signals coming from different tissues. It will be important to integrate findings from different model organisms to discern common themes. The ultimate challenge will be to link the signals from the cells that surround and constitute the microenvironment of (prospective) hemogenic endothelial cells to the intrinsic regulators that are required for the initiation, development, and termination of EHT.

Intrinsic regulators of EHT

Runx1

Given its pivotal role in EHT a large amount of research has focused on the role of the transcription factor Runx1. The first insights of these studies showed that Runx1 represses the endothelial program whilst activating the hematopoietic program (reviewed in [51]). More recently, the Runx1 target genes Gfi1/Gfi1b, but not Pu.1 (Sfp1) or c-myb, were shown to down-regulate expression of endothelial genes in Runx1 null hemogenic endothelium derived from ES cells [80]. In addition, the Gfi1/Gfi1b transduced hemogenic endothelial cells initiated morphological changes akin to the formation of hematopoietic progeny, forming round cells that express CD41. However, they were unable to generate hematopoietic colonies *in vitro*, something CD41⁺ cells isolated from wild type ES cell cultures or embryos readily do. In complementary experiments, the loss of both Gfi1 and Gfi1b resulted in the generation of CD41 expressing adherent cells that maintained endothelial marker expression, which could only generate clonogenic progeny upon disaggregation [80]. Altogether these data indicate that the Runx1 target genes, Gfi1 and Gfi1b may be in part responsible for the repression of endothelial gene expression associated with the early steps of EHT.

At the molecular level, Runx1 was shown to mediate the formation of a hematopoietic-specific transcription factor binding profile at the onset of hematopoiesis. Using chip-sequencing, the binding pattern of three key hematopoietic transcription factors (Fli1, SCL

and C/EBP α) within the hemogenic endothelium prior to Runx1 expression was compared with their binding profile after Runx1 induction. After Runx1 induction, the binding of these factors was more similar to that observed in hematopoietic cells than in the hemogenic endothelium prior to Runx1 expression. Together these data implicate Runx1 in the re-organisation of transcription factor binding during EHT [81]. The mechanism by which Runx1 mediates the re-organisation of transcription factor binding is currently unknown.

HoxA3

The onset of hemogenic potential of the endothelium appears to be associated with a balance of Runx1 and HoxA3 expression within the endothelium of the dorsal aorta [82]. HoxA3 downregulation within the aortic endothelium correlates with the onset of Runx1 in these cells and re-expression of HoxA3 in nascent hematopoietic progenitors reactivates and maintains an endothelial program [82]. Sox17, another marker of the hemogenic endothelium, has also been implicated downstream of HoxA3 [82, 83].

Sox17

The transcription factor Sox17 was recently identified in human and mouse ES cell differentiation cultures as being important for the regulation of EHT. Sox17 is expressed and critically required for the generation of fetal (and neonatal) HSCs but not adult HSCs [84]. Sox17 is also expressed in the endothelium of the midgestation AGM and deletion of Sox17 using VE-Cadherin-Cre or Tie2-Cre mice resulted in embryonic lethality [83, 84]. Overexpression of Sox17 has been implicated in the expansion of a cell population that down-regulates endothelial markers and is able to generate hematopoietic cells, properties attributable with hemogenic endothelium [83, 85]. This is in contrast with an earlier report documenting an inhibitory, and apoptotic effect of Sox17 overexpression on hematopoietic development in mES cell differentiation cultures [86].

SCL

The transcription factor SCL is critically required for establishing hemogenic endothelium [50, 87-89]. Importantly, SCL is not required once the hemogenic endothelium has formed, placing it upstream of Runx1 [28, 50, 90]. Recently, loss of SCL was shown to initiate a cardiac-related transcriptional program in the yolk sac, indicating that SCL expression represses the cardiac program [89]. Conditional deletion of SCL indicated that this occurs early in development, possibly already in the hematopoietic-fated SCL expressing mesoderm.

Concluding remarks

Although progress has been made in our understanding of the cellular changes and gene regulatory mechanisms underlying EHT there is still much to learn. This is reflected in the difficulty to generate bona fide HSCs de novo from ES/iPS cells [50, 85, 91-93], or through direct reprogramming [94, 95]. This indicates that critical components of EHT, responsible for regulating the hematopoietic nature of these cells are missing in these culture systems and indicate the importance of understanding the cellular, molecular and niche requirements for EHT in vivo. In the past, studies in the embryo have been hampered by the paucity of

cells that can be obtained. Recent developments in the fields of single cell imaging and expression profiling now allow for the detailed analysis of single cells undergoing EHT in the embryo. These exciting new technologies are expected to contribute to a better understanding of how EHT is initiated and regulated, and how HSCs are generated, with the ultimate aim to harness the power of stem cells for regenerative medicine.

Abbreviations

AGM	aorta-gonad mesonephros
EHT	endothelial-to-hematopoietic transition
E	embryonic day
HSC	hematopoietic stem cell
PAS	para-aortic splanchnopleura

References

1. Palis J, et al. Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development*. 1999; 126(22):5073–84. [PubMed: 10529424]
2. Ferkowicz MJ, Yoder MC. Blood island formation: longstanding observations and modern interpretations. *Exp Hematol*. 2005; 33(9):1041–7. [PubMed: 16140152]
3. McGrath K, Palis J. Ontogeny of erythropoiesis in the mammalian embryo. *Curr Top Dev Biol*. 2008; 82:1–22. [PubMed: 18282515]
4. Medvinsky A, Dzierzak E. Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell*. 1996; 86(6):897–906. [PubMed: 8808625]
5. Muller AM, et al. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity*. 1994; 1(4):291–301. [PubMed: 7889417]
6. Dzierzak E, Speck NA. Of lineage and legacy: the development of mammalian hematopoietic stem cells. *Nat Immunol*. 2008; 9(2):129–36. [PubMed: 18204427]
7. Medvinsky A, Rybtsov S, Taoudi S. Embryonic origin of the adult hematopoietic system: advances and questions. *Development*. 2011; 138(6):1017–31. [PubMed: 21343360]
8. Nakano H, et al. Haemogenic endocardium contributes to transient definitive haematopoiesis. *Nat Commun*. 2013; 4:1564. [PubMed: 23463007]
9. Yoshimoto M, et al. Embryonic day 9 yolk sac and intra-embryonic hemogenic endothelium independently generate a B-1 and marginal zone progenitor lacking B-2 potential. *Proc Natl Acad Sci U S A*. 2011; 108(4):1468–73. [PubMed: 21209332]
10. Yoshimoto M, et al. Autonomous murine T cell progenitor production in the extra-embryonic yolk sac prior to HSC emergence. *Blood*. 2012
11. Schulz C, et al. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. *Science*. 2012; 336(6077):86–90. [PubMed: 22442384]
12. Lux CT, et al. All primitive and definitive hematopoietic progenitor cells emerging before E10 in the mouse embryo are products of the yolk sac. *Blood*. 2008; 111(7):3435–8. [PubMed: 17932251]
13. Chen MJ, et al. Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. *Cell Stem Cell*. 2011; 9(6):541–52. [PubMed: 22136929]
14. de Bruijn MF, et al. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. *EMBO J*. 2000; 19(11):2465–74. [PubMed: 10835345]
15. de Bruijn MF, et al. Hematopoietic stem cells localize to the endothelial cell layer in the midgestation mouse aorta. *Immunity*. 2002; 16(5):673–83. [PubMed: 12049719]
16. North TE, et al. Runx1 expression marks long-term repopulating hematopoietic stem cells in the midgestation mouse embryo. *Immunity*. 2002; 16(5):661–72. [PubMed: 12049718]

17. Kumaravelu P, et al. 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*. 2002; 129(21):4891–9. [PubMed: 12397098]
18. Taoudi S, Medvinsky A. Functional identification of the hematopoietic stem cell niche in the ventral domain of the embryonic dorsal aorta. *Proc Natl Acad Sci U S A*. 2007; 104(22):9399–403. [PubMed: 17517650]
19. Dieterlen-Lievre F. Hematopoiesis: progenitors and their genetic program. *Curr Biol*. 1998; 8(20):R727–30. [PubMed: 9778520]
20. Jaffredo T, et al. From hemangioblast to hematopoietic stem cell: an endothelial connection? *Exp Hematol*. 2005; 33(9):1029–40. [PubMed: 16140151]
21. Adamo L, Garcia-Cardena G. The vascular origin of hematopoietic cells. *Dev Biol*. 2011; 362(1):1–10. [PubMed: 21945862]
22. Jordan HE. Evidence of hemogenic capacity of endothelium. *Anat. Rec*. 1916; 10:417–420.
23. Emmel VE. The cell clusters in the dorsal aorta of mammalian embryos. *Am. J. Anat*. 1916; 19(3):401–421.
24. Sabin FR. Studies on the origin of blood vessels and of red corpuscles as seen in the living blastoderm of the chick during the second day of incubation. *Contributions to Embryology*. 1920; 9:213–262.
25. Jaffredo T, et al. Tracing the progeny of the aortic hemangioblast in the avian embryo. *Dev Biol*. 2000; 224(2):204–14. [PubMed: 10926760]
26. Jaffredo T, et al. Intraaortic hemopoietic cells are derived from endothelial cells during ontogeny. *Development*. 1998; 125(22):4575–83. [PubMed: 9778515]
27. Zovein AC, et al. Fate tracing reveals the endothelial origin of hematopoietic stem cells. *Cell Stem Cell*. 2008; 3(6):625–36. [PubMed: 19041779]
28. Chen MJ, et al. Runx1 is required for the endothelial to haematopoietic cell transition but not thereafter. *Nature*. 2009; 457(7231):887–91. [PubMed: 19129762]
29. Eilken HM, Nishikawa S, Schroeder T. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature*. 2009; 457(7231):896–900. [PubMed: 19212410]
30. Kissa K, Herbomel P. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*. 2010; 464(7285):112–5. [PubMed: 20154732]
31. Bertrand JY, et al. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*. 2010; 464(7285):108–11. [PubMed: 20154733]
32. Boisset JC, et al. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*. 2010; 464(7285):116–20. [PubMed: 20154729]
33. Lam EY, et al. Live imaging of Runx1 expression in the dorsal aorta tracks the emergence of blood progenitors from endothelial cells. *Blood*. 2010; 116(6):909–14. [PubMed: 20453160]
34. Antas VI, et al. Hemogenic endothelium: A vessel for blood production. *Int J Biochem Cell Biol*. 2012; 45(3):692–695. [PubMed: 23270729]
35. Zape JP, Zovein AC. Hemogenic endothelium: origins, regulation, and implications for vascular biology. *Semin Cell Dev Biol*. 2011; 22(9):1036–47. [PubMed: 22001113]
36. North T, et al. Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development*. 1999; 126(11):2563–75. [PubMed: 10226014]
37. Nishikawa SI, et al. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. *Development*. 1998; 125(9):1747–57. [PubMed: 9521912]
38. Nishikawa SI, et al. In vitro generation of lymphohematopoietic cells from endothelial cells purified from murine embryos. *Immunity*. 1998; 8(6):761–9. [PubMed: 9655490]
39. Shalaby F, et al. A requirement for Flk1 in primitive and definitive hematopoiesis and vasculogenesis. *Cell*. 1997; 89(6):981–90. [PubMed: 9200616]
40. Corbel C, Salaun J. AlphaIIb integrin expression during development of the murine hemopoietic system. *Dev Biol*. 2002; 243(2):301–11. [PubMed: 11884039]

41. Emambokus NR, Frampton J. The glycoprotein IIb molecule is expressed on early murine hematopoietic progenitors and regulates their numbers in sites of hematopoiesis. *Immunity*. 2003; 19(1):33–45. [PubMed: 12871637]
42. Ferkowicz MJ, et al. CD41 expression defines the onset of primitive and definitive hematopoiesis in the murine embryo. *Development*. 2003; 130(18):4393–403. [PubMed: 12900455]
43. Mikkola HK, et al. Expression of CD41 marks the initiation of definitive hematopoiesis in the mouse embryo. *Blood*. 2003; 101(2):508–16. [PubMed: 12393529]
44. Ledbetter JA, Herzenberg LA. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 1979; 47:63–90. [PubMed: 398327]
45. Kina T, et al. The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *Br J Haematol.* 2000; 109(2):280–7. [PubMed: 10848813]
46. Fraser ST, et al. Definitive hematopoietic commitment within the embryonic vascular endothelial-cadherin(+) population. *Exp Hematol.* 2002; 30(9):1070–8. [PubMed: 12225799]
47. Li Z, et al. Mouse embryonic head as a site for hematopoietic stem cell development. *Cell Stem Cell.* 2012; 11(5):663–75. [PubMed: 23122290]
48. Bee T, et al. Nonredundant roles for Runx1 alternative promoters reflect their activity at discrete stages of developmental hematopoiesis. *Blood*. 2010; 115(15):3042–50. [PubMed: 20139099]
49. Goldie LC, et al. Cell signaling directing the formation and function of hemogenic endothelium during murine embryogenesis. *Blood*. 2008; 112(8):3194–204. [PubMed: 18684862]
50. Lancrin C, et al. The haemangioblast generates haematopoietic cells through a haemogenic endothelium stage. *Nature*. 2009; 457(7231):892–5. [PubMed: 19182774]
51. Swiers G, de Bruijn M, Speck NA. Hematopoietic stem cell emergence in the conceptus and the role of Runx1. *Int J Dev Biol.* 2010; 54(6-7):1151–63. [PubMed: 20711992]
52. Okuda T, et al. AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis. *Cell*. 1996; 84:321–330. [PubMed: 8565077]
53. Wang Q, et al. Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc. Natl. Acad. Sci. USA.* 1996; 93:3444–3449. [PubMed: 8622955]
54. Cai Z, et al. Haploinsufficiency of AML1 affects the temporal and spatial generation of hematopoietic stem cells in the mouse embryo. *Immunity*. 2000; 13(4):423–31. [PubMed: 11070161]
55. Lacaud G, et al. Runx1 is essential for hematopoietic commitment at the hemangioblast stage of development in vitro. *Blood*. 2002; 100:458–466. [PubMed: 12091336]
56. Yokomizo T, et al. Runx1 is involved in primitive erythropoiesis in the mouse. *Blood*. 2008; 111(8):4075–80. [PubMed: 18250229]
57. Tober J, et al. Distinct temporal requirements for Runx1 in hematopoietic progenitors and stem cells. *Development*. 2013; 140(18):3765–76. [PubMed: 23924635]
58. Nottingham WT, et al. Runx1-mediated hematopoietic stem-cell emergence is controlled by a Gata/Ets/SCL-regulated enhancer. *Blood*. 2007; 110(13):4188–97. [PubMed: 17823307]
59. Rybtsov S, et al. Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J Exp Med.* 2011; 208(6):1305–15. [PubMed: 21624936]
60. Taoudi S, et al. Extensive hematopoietic stem cell generation in the AGM region via maturation of VE-cadherin+CD45+ pre-definitive HSCs. *Cell Stem Cell.* 2008; 3(1):99–108. [PubMed: 18593562]
61. Gordon-Keylock S, et al. Mouse extra-embryonic arterial vessels harbor precursors capable of maturing into definitive HSCs. *Blood*. 2013
62. Jordan HE. Aortic cell clusters in vertebrate embryos. *Proc Natl Acad Sci U S A.* 1917; 3(3):149–156. [PubMed: 16576205]
63. Yokomizo T, Dzierzak E. Three-dimensional cartography of hematopoietic clusters in the vasculature of whole mouse embryos. *Development*. 2010; 137(21):3651–61. [PubMed: 20876651]

64. Rhodes KE, et al. The emergence of hematopoietic stem cells is initiated in the placental vasculature in the absence of circulation. *Cell Stem Cell*. 2008; 2(3):252–63. [PubMed: 18371450]
65. Meng F, et al. Surfactant protein A promoter directs the expression of Cre recombinase in brain microvascular endothelial cells of transgenic mice. *Matrix Biol*. 2007; 26(1):54–7. [PubMed: 17059883]
66. Ivanovs A, et al. Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. *J Exp Med*. 2011; 208(12):2417–27. [PubMed: 22042975]
67. Koushik SV, et al. Targeted inactivation of the sodium-calcium exchanger (Ncx1) results in the lack of a heartbeat and abnormal myofibrillar organization. *FASEB J*. 2001; 15(7):1209–11. [PubMed: 11344090]
68. Peterkin T, Gibson A, Patient R. Common genetic control of haemangioblast and cardiac development in zebrafish. *Development*. 2009; 136(9):1465–74. [PubMed: 19297410]
69. Holz A, et al. The two origins of hemocytes in *Drosophila*. *Development*. 2003; 130(20):4955–62. [PubMed: 12930778]
70. Matheny CJ, et al. Disease mutations in RUNX1 and RUNX2 create nonfunctional, dominant-negative, or hypomorphic alleles. *EMBO J*. 2007; 26(4):1163–75. [PubMed: 17290219]
71. Adamo L, et al. Biomechanical forces promote embryonic haematopoiesis. *Nature*. 2009; 459(7250):1131–5. [PubMed: 19440194]
72. North TE, et al. Hematopoietic stem cell development is dependent on blood flow. *Cell*. 2009; 137(4):736–48. [PubMed: 19450519]
73. Richard C, et al. Endothelio-Mesenchymal Interaction Controls runx1 Expression and Modulates the notch Pathway to Initiate Aortic Hematopoiesis. *Dev Cell*. 2013; 24(6):600–11. [PubMed: 23537631]
74. Peeters M, et al. Ventral embryonic tissues and Hedgehog proteins induce early AGM hematopoietic stem cell development. *Development*. 2009; 136(15):2613–21. [PubMed: 19570846]
75. Mirshekar-Syahkal B, et al. Dlk1 is a negative regulator of emerging hematopoietic stem and progenitor cells. *Haematologica*. 2013; 98(2):163–71. [PubMed: 22801971]
76. Yue R, et al. Thrombin receptor regulates hematopoiesis and endothelial-to-hematopoietic transition. *Dev Cell*. 2012; 22(5):1092–100. [PubMed: 22521721]
77. Fitch SR, et al. Signaling from the sympathetic nervous system regulates hematopoietic stem cell emergence during embryogenesis. *Cell Stem Cell*. 2012; 11(4):554–66. [PubMed: 23040481]
78. Jaffredo T, et al. Dorso-ventral contributions in the formation of the embryonic aorta and the control of aortic hematopoiesis. *Blood Cells Mol Dis*. 2013
79. Bigas A, Guiu J, Gama-Norton L. Notch and Wnt signaling in the emergence of hematopoietic stem cells. *Blood Cells Mol Dis*. 2013
80. Lancrin C, et al. GFI1 and GFI1B control the loss of endothelial identity of hemogenic endothelium during hematopoietic commitment. *Blood*. 2012; 120(2):314–22. [PubMed: 22668850]
81. Lichtinger M, et al. RUNX1 reshapes the epigenetic landscape at the onset of haematopoiesis. *Embo J*. 2012; 31(22):4318–33. [PubMed: 23064151]
82. Iacovino M, et al. HoxA3 is an apical regulator of haemogenic endothelium. *Nat Cell Biol*. 2011; 13(1):72–8. [PubMed: 21170035]
83. Clarke RL, et al. The expression of Sox17 identifies and regulates haemogenic endothelium. *Nat Cell Biol*. 2013; 15(5):502–10. [PubMed: 23604320]
84. Kim I, Saunders TL, Morrison SJ. Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell*. 2007; 130(3):470–83. [PubMed: 17655922]
85. Nakajima-Takagi Y, et al. Role of SOX17 in hematopoietic development from human embryonic stem cells. *Blood*. 2013; 121(3):447–58. [PubMed: 23169777]
86. Serrano AG, et al. Contrasting effects of Sox17- and Sox18-sustained expression at the onset of blood specification. *Blood*. 2010; 115(19):3895–8. [PubMed: 20228271]
87. Porcher C, et al. The T cell leukemia oncoprotein SCL/tal-1 is essential for development of all hematopoietic lineages. *Cell*. 1996; 86(1):47–57. [PubMed: 8689686]

88. Schlaeger TM, et al. Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development. *Blood*. 2005; 105(10): 3871–4. [PubMed: 15677556]
89. Van Handel B, et al. Scl represses cardiomyogenesis in prospective hemogenic endothelium and endocardium. *Cell*. 2012; 150(3):590–605. [PubMed: 22863011]
90. Endoh M, et al. SCL/tal-1-dependent process determines a competence to select the definitive hematopoietic lineage prior to endothelial differentiation. *Embo J*. 2002; 21(24):6700–8. [PubMed: 12485991]
91. Choi KD, et al. Identification of the hemogenic endothelial progenitor and its direct precursor in human pluripotent stem cell differentiation cultures. *Cell Rep*. 2012; 2(3):553–67. [PubMed: 22981233]
92. Kennedy M, et al. T lymphocyte potential marks the emergence of definitive hematopoietic progenitors in human pluripotent stem cell differentiation cultures. *Cell Rep*. 2012; 2(6):1722–35. [PubMed: 23219550]
93. Ledran MH, et al. Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. *Cell Stem Cell*. 2008; 3(1):85–98. [PubMed: 18593561]
94. Szabo E, et al. Direct conversion of human fibroblasts to multilineage blood progenitors. *Nature*. 2010; 468(7323):521–6. [PubMed: 21057492]
95. Pereira CF, et al. Induction of a Hemogenic Program in Mouse Fibroblasts. *Cell Stem Cell*. 2013

Figure 1. Hematopoietic cell generation occurs asynchronously during development

A During development hematopoietic cell generation occurs in three separate, but overlapping waves. The first wave is transient, initiating around embryonic day (E) 7 in the yolk sac blood islands producing primitive erythrocytes and macrophages. The second wave is also transient, initiating around E8.5 and produces multi-potent and lineage-restricted hematopoietic cells with an adult-like morphology. The third wave is responsible for the generation of the definitive HSC from E10.5 in development.

B Schematic of the mouse embryo at the timepoints highlighted as important for hematopoietic cell generation in (A). The tissues responsible for hematopoietic cell

generation are labelled; see main text for detailed description. Al = allantois, YS = yolk sac, YS BI = yolk sac blood island, PSp = para-aortic splanchnopleura, Ht = heart endothelium, AGM = Aorta-gonad-mesonephros, Hd = head vasculature, VA = vitelline artery, UA = umbilical artery.

Figure 2. Hematopoietic cell generation in the E10.5 AGM region

A Schematic of a cross section through an E10.5 AGM with the ventral wall of the dorsal aorta boxed and magnified to reveal the different cell types. Clusters of hematopoietic cells (red) bud out from the hemogenic endothelium (pink) of the dorsal aorta. Non-hemogenic endothelium is labelled in light grey. Surrounding the endothelium is a layer of smooth muscle cells (mid grey) and underlying that are mesenchymal cells (dark grey).

B Expression of Runx1, the smooth muscle marker SMA (smooth muscle actin), and the endothelial marker CD31 in the E10.5 dorsal aorta. Sections were immunolabeled with the

appropriate antibodies, and images pseudo-coloured. Runx1 expression can be detected in endothelial cells (asterisk), hematopoietic cluster cells (arrow) and the abluminal cell layers of the dorsal aorta, here identified as smooth muscle (arrowhead). Runx1 (blue; Alexa Fluor 594), SMA (red; Cy3) and CD31 (green; Alexa Fluor 488), nuclear stain (Dapi; white). Merged channel image with nuclear stain (i) and without nuclear stain (ii). Single channel images for Runx1 (iii), CD31 (iv) and SMA (v) are shown for clarity. Scale bar is 50 μ m. Image was taken on a Zeiss LSM780 inverted microscope, processed using ZEN lite 2011, adobe photoshop and illustrator.