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## Of lineage and legacy – the development of mammalian hematopoietic stem cells

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### Abstract

The hematopoietic system is one of the first complex tissues to develop in the mammalian conceptus. Of particular interest within the field of developmental hematopoiesis is the origin of adult bone marrow hematopoietic stem cells. Tracing their origin is complicated because blood is a mobile tissue, and because hematopoietic cells emerge from multiple embryonic sites. The origin of the adult mammalian blood system remains a topic of lively discussion and intense research. Interest is also focused on developmental signals that induce the adult hematopoietic stem cell program, as these may prove useful for generating and expanding these clinically important cells *ex vivo*. This review presents a historical overview of, and the most recent data on the developmental origins of hematopoiesis.

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Defining the embryonic origins of specific cell lineages is important for understanding how tissues of the adult organism develop. The signaling events that induce the molecular programs governing lineage-specific fate decisions in embryonic cells provide insight into the complexity of lineage relationships, cell diversity, and ultimately tissue function in the adult. The process of blood cell development in the mammalian conceptus is particularly complex, as it occurs in multiple sites that are separated both temporarily and spatially. Furthermore, unlike stationary tissues, cells of the hematopoietic system circulate, and thus their ancestry and the distinct characteristics associated with their site of origin are confounded by the natural mobility of the system. Recent studies have begun to reveal the lineage relationships between, and molecular programs controlling hematopoietic cell emergence in the conceptus and the legacy of the cells emerging from distinct anatomic sites. This review focuses on the embryonic origins of the hematopoietic system, and the environments and molecules affecting the development of adult mammalian hematopoietic stem cells.

### Sites and cells: Where does it start?

The conceptus consists of embryonic tissues that will ultimately become part of the fetus, and extra-embryonic tissues that support fetal development. It has been long recognized that the first blood cells in the vertebrate conceptus appear in the extra-embryonic yolk sac concomitant with the developing vasculature. The yolk sac of early chick embryos was shown by histological studies to harbor the first visible hematopoietic cells, primitive erythrocytes<sup>1</sup>. The close physical association of primitive erythrocytes and their synchronous appearance with endothelial cells led to the postulate of a common mesodermal precursor for these two lineages coined the hemangioblast<sup>2</sup>. Studies utilizing the *in vitro* differentiation of totipotent mouse

embryonic stem cells (ES) produced the first functional evidence for mammalian hemangioblasts<sup>3,4</sup>, and later analyses of early stage mouse conceptuses revealed presumptive hemangioblasts expressing both the mesodermal marker Brachyury and fetal liver kinase 1 (Flk1) in the posterior region of the primitive streak to the yolk sac<sup>5</sup>. These hemangioblasts migrate to the yolk sac, at which point they become committed endothelial and hematopoietic progenitors (Fig 1a (left panel)), several of which contribute to the formation of each blood island<sup>6,7</sup>. Thus, during mammalian embryonic development, the earliest cohort of mesodermal cells emigrating from the primitive streak take on endothelial and hematopoietic fate prior to blood island formation, and give rise to primitive red blood cells and some of the yolk sac vasculature. The remainder of the yolk sac vasculature is derived from angioblasts that also emerge from the posterior primitive streak and do not contribute to blood<sup>8</sup>.

It was suggested in the 1970s that cells of the yolk sac were the source of the hematopoietic system in the adult mammal and that yolk sac cells emigrate to the fetal liver and thereafter to the bone marrow where they reside throughout adulthood<sup>9,10</sup>. However, tissue grafting approaches conclusively demonstrated that the yolk sac was not the source of adult blood in non-mammalian vertebrates<sup>11,12</sup> (Fig 1b, c). Inter- and intra-species grafting of avian embryo body and yolk sac (and more recently the allantois), prior to the emergence of blood cells and the onset of circulation, showed that the adult hematopoietic system originates from cells within the body of the embryo and from the extra-embryonic allantois, and not from the yolk sac<sup>11,13,14</sup> (Fig 1b). Likewise, orthotopic grafting of the ventral blood island (VBI; yolk sac equivalent) and dorsal lateral plate (DLP; intrabody region) in amphibian embryos revealed that the DLP is the source of adult hematopoietic cells<sup>12</sup>. Molecular marking experiments in xenopus embryos at the early cleavage stages demonstrated that the separation of presumptive VBI and DLP mesoderm occurs very early<sup>15</sup> (Fig 1c). The progeny of the C3 blastomere of regularly cleaving embryos contributes to intraembryonic (DLP) hematopoiesis while completely distinct blastomeres contribute to the anterior (C1 & D1) and posterior (D4) VBI. Separation at this early stage implies that the progeny of these cells experience spatially and temporally distinct signalling events during gastrulation and embryonic patterning. Indeed, the three blood compartments in xenopus (anterior VBI, posterior VBI, DLP) are specified from mesoderm that encounters different levels of the bone morphogenic protein (BMP)<sup>16</sup>. In addition, the morphogen fibroblast growth factor (FGF) differentially affects the timing of *Scl* and *Runx1* expression, both of which encode pivotal transcription factors that specify hematopoietic fate in the anterior versus posterior VBI<sup>17</sup>. Interestingly, by grafting VBI or DLP cells to the reciprocal site in the amphibian embryo, the prospective hematopoietic cells can be reprogrammed to the alternate adult or primitive hematopoietic fate. However, this is possible only within an early developmental window of time<sup>18</sup>, after which point primitive and adult states are thought to be epigenetically fixed.

Evidence for the separation of presumptive mesoderm in the mouse epiblast was provided through single cell marking experiments. By labelling prospective mesodermal cells at early primitive streak stages (E6.5/E7), cells from the posterior primitive streak were found to contribute to extraembryonic hematopoietic tissue, i.e. yolk sac and allantois<sup>19</sup> (Fig 1a, left panel), and not to the body of the embryo. These results were confirmed using an orthotopic grafting method<sup>8</sup> which further showed that at mid-streak stages, the mesoderm derivatives within the rostral embryo arise from epiblast cells that ingress through the anterior primitive streak prior to the cells that give rise to axial, paraxial and lateral (blood forming) mesoderm of the anterior trunk (Fig 1a, right panel). Lastly, the mesoderm emigrating from more caudal regions of the streak forms the paraxial and lateral mesoderm of the remaining trunk regions<sup>8</sup> (Fig 1a, right panel). Interestingly, the entire epiblast of the early- and mid-streak stage embryos contains hematogenic potential, but that potential thereafter becomes restricted to the trunk and posterior region of the embryo<sup>20</sup>. Thus, in remarkable similarity to the mesodermal fate map of the chick<sup>8</sup>, presumptive extraembryonic blood-forming mesoderm in the mouse

conceptus is the first to emerge from the primitive streak during gastrulation, followed by the intraembryonic mesoderm in a rostral to caudal sequence.

## From primitive erythrocytes to adult HSCs

*In vivo* transplantation assays in mammals have provided great insight into the specific cells that can generate a complete functional adult hematopoietic system. Hematopoietic stem cells (HSCs) are at the foundation of the adult blood differentiation hierarchy, and provide continuous hematopoietic cell production throughout life. Transplantations of cells from different regions of the embryonic day 8 (E8) to E12 mouse conceptus demonstrated that HSCs conferring complete, long term, multilineage, high level hematopoietic repopulation of irradiated adult recipient mice appear only beginning at E10.5 in the aorta-gonad-mesonephros (AGM) region of the embryo body, and in the vitelline and umbilical arteries<sup>21-23</sup> (Fig. 2, right panel). The appearance of HSCs three days after primitive erythrocytes are generated in the yolk sac (Table 1· Fig. 3) makes it unlikely that they share a recent common ancestry. These adult-repopulating HSCs (which are as potent as adult bone marrow HSCs) are autonomously generated in the AGM, as shown by explant cultures<sup>21</sup> and are located in the ventral aspect of the dorsal aorta<sup>24-26</sup>. Slightly thereafter, HSCs are found in other tissues; the placenta, yolk sac, and liver<sup>21, 22, 27, 28</sup>. The liver does not generate hematopoietic cells *de novo* but is instead colonized beginning at late E9 by hematopoietic cells made in other tissues<sup>29, 30</sup> (Fig. 3). The identification of the yolk sac and placenta as *de novo* generators of HSCs is precluded by the circulation (which is established at the 4-6 somite pair stage, approximately E8.25-8.5<sup>31</sup>), which easily distributes these cells throughout the conceptus. However, quantitative spatial and temporal analyses of HSCs suggest that the yolk sac<sup>32</sup> and placenta<sup>27, 28</sup> do contribute to the HSC pool in the liver either through expansion of pre-existing HSCs or by *de novo* generation of HSCs. It has been shown that there are more HSCs in the fetal liver than can be accounted for through HSC generation in the AGM alone<sup>32</sup>. The additive production of HSCs by AGM, yolk sac and placenta<sup>27</sup> most likely is responsible for the high numbers of fetal liver HSCs, although the liver itself may expand these cells<sup>33</sup>. Thus, within a span of three days, the mouse conceptus generates at least two very distinct and unrelated classes of functional hematopoietic cells – primitive erythrocytes at E7.5 and definitive adult repopulating HSCs at E10.5.

Other classes of hematopoietic cells are also generated/detected in the mouse conceptus between the time primitive erythrocytes and HSCs appear, including myeloid progenitors, lymphoid-myeloid (multipotent) progenitors, CFU-S and neonatal repopulating HSCs (Table 1). At E8.25, following the first wave of primitive erythropoiesis and before the circulation is established, myeloid progenitors are detected in the yolk sac and are thus generated in that site<sup>34, 35</sup>. After the circulation is established myeloid progenitors are also found in the trunk region<sup>36</sup>. However, prior to circulation, both the yolk sac and pSp (para-aortic splanchnopleura; prospective AGM region) contain cells with the potential to become myeloid progenitors as revealed in explant cultures<sup>37</sup>. Similar cultures of pre-circulation allantoises<sup>38, 39</sup> also revealed cells with myeloid potential. By E9, the placenta contains an abundance of myeloid progenitors<sup>40</sup>. More potent myeloid progenitors capable of forming colonies on the spleen following injection into irradiated mice (CFU-S progenitors) are present in both the yolk sac and the AGM beginning at E9<sup>41</sup>. Quantitative analyses show more CFU-S in the AGM than in the YS, suggesting that the AGM generates these cells<sup>21</sup>. More recently, studies of two mutant mice, VE-cadherin (*Cdh5*<sup>-/-</sup>) and *Ncx1*<sup>-/-</sup>, have provided strong *in vivo* evidence for the *de novo* production of definitive myeloid progenitors in the yolk sac. Conceptuses deficient for these genes do not establish a circulation between the yolk sac and embryo body; in *Cdh5*<sup>-/-</sup> conceptuses there is no vascular connection, while in *Ncx1*<sup>-/-</sup> conceptuses the vitelline vessels are intact but there is no heartbeat to promote the circulation. E9.5 *Cdh5*<sup>-/-</sup> conceptuses contained similar numbers of myeloid progenitors in the yolk sac as wild-type

conceptuses, although macrophage and mixed colony-forming progenitors were decreased in number<sup>42</sup>. In the *Ncx1*<sup>-/-</sup> conceptus, the numbers of myeloid progenitors of all types in the yolk sac were found to be equivalent to the cumulative number of progenitors in the *Ncx1*<sup>+/+</sup> conceptus in all anatomic sites<sup>43</sup>. No progenitors were found in the *Ncx1*<sup>-/-</sup> pSp suggesting that the yolk sac normally generates all of these progenitors and distributes them to the pSp and liver. Alternatively, it is possible that the *Ncx1*-deficient conceptuses, which lack hemodynamic stress, do not produce the proper signals to induce myeloid progenitor formation in the pSp<sup>44</sup>. Notwithstanding, it is clear that several types of definitive myeloid progenitors are generated *de novo* in the yolk sac. It would be interesting to examine the allantois in such circulation defective embryos for the *de novo* generation of myeloid progenitors.

A progenitor with more complex lymphoid-myeloid potential is found prior to the onset of circulation in the E8 pSp-AGM of the embryo following explant culture<sup>37</sup> (Table 1). When transplanted into irradiated immunodeficient recipients, this lymphoid-myeloid progenitor contributes to low-level, long-term, multilineage hematopoietic repopulation<sup>45</sup>. Yolk sac explants, on the other hand do not contain such cells until the circulation is established, suggesting that cells with lymphoid-myeloid potential are generated *de novo* in the pSp-AGM and could theoretically emigrate to the yolk sac. Similarly, in explant cultures with human yolk sac and pSp-AGM tissues isolated prior to the onset of circulation only the pSp-AGM tissue was found to contain cells with lymphoid-myeloid potential<sup>46</sup>.

A potent neonatal engrafting HSC was identified in the E9 mouse yolk sac and AGM. Both tissues contain c-kit<sup>+</sup>CD34<sup>+</sup> cells that, when injected directly in the liver of neonatal recipient mice, can yield high amounts of multilineage engraftment<sup>47</sup>. The yolk sac contains quantitatively more of these cells than the AGM. In contrast to E10.5 AGM HSCs (also c-kit<sup>+</sup>CD34<sup>+</sup>), which fully engraft adult recipients, the E9 c-kit<sup>+</sup>CD34<sup>+</sup> neonatal repopulating cells are incapable of engraftment when injected directly into adult mice<sup>47</sup>. Hence, there are at least five broad classes of hematopoietic cells in the mammalian conceptus as defined by activity in *in vitro* clonogenic or transplantation assays; primitive, pro-definitive (myeloid progenitors), meso-definitive (lymphoid-myeloid progenitors), meta-definitive (neonatal repopulating HSCs) and adult-definitive (adult repopulating HSCs) (Fig. 2). These classes of cells appear to be generated independently of each other and are generated in distinct hematopoietic sites.

## Direct precursors to definitive hematopoietic cells

The “definitive” classes of hematopoietic progenitor-stem cells illustrated (Fig. 2) are thought to arise through a slightly different process than the primitive erythroid progenitors. Discrete subsets of vascular endothelial cells appear to exhibit hemogenic potential during development<sup>1</sup>. In many species, histological and immuno staining studies have shown hematopoietic clusters tightly adherent to the ventral endothelium of the dorsal aorta, plus the umbilical and umbilical arteries (reviewed in<sup>48</sup>). These hematopoietic clusters appear to be emerging from the endothelium during the time the first definitive HSCs are detected. Metabolic lineage tracing (AcLDL-DiI) or retroviral labelling of endothelial cells prior to hematopoietic cell appearance performed in chick embryos has confirmed the endothelial-hematopoietic lineage relationship of aortic hematopoietic clusters<sup>49</sup>. One day after labelling of endothelial cells, AcLDL-DiI<sup>+</sup>CD45<sup>+</sup> hematopoietic cells were found in the lumen adhering to the ventral aspect of the aorta. Slightly later, the underlying aortic mesenchyme also contained AcLDL-DiI<sup>+</sup>CD45<sup>+</sup> cells, suggesting that labelled endothelial cells ingressed into the tissue. Clonal retroviral marking of the avian embryonic vasculature provided additional data confirming the fate commitment of an endothelial cell to the hematopoietic lineage<sup>50</sup>. Similar attempts have been made in *ex utero* cultured mouse embryos to establish lineage relationships between endothelial cells and hematopoietic cells. Twelve hours following intracardiac injection of

AcLDL-DiI into E10 mouse embryos, marked definitive erythroid cells were found in the circulation<sup>51</sup>. Taken together, these data strongly indicate an endothelial origin for definitive hematopoietic cells.

In the mid-gestation mouse embryo, the phenotypic profile of adult HSCs and the spatial localization of these cells in the AGM have been useful in providing information about their direct precursors. Indeed, all HSCs in the AGM are CD45<sup>+</sup><sup>25</sup>, Ly-6A (Sca-1) GFP<sup>+</sup><sup>26</sup>, c-kit<sup>+</sup>CD34<sup>+</sup><sup>52</sup>, Runx1<sup>+</sup><sup>25</sup>, SCL<sup>+</sup><sup>53</sup> and Gata2<sup>+</sup><sup>54, 55</sup>. These markers (with the exception of CD45) are also expressed by some or all endothelial cells in the ventral aspect of the dorsal aorta at E10/11. Most or all AGM HSCs express cell surface VE-cadherin<sup>25, 56</sup>, which is typically thought of as an endothelial marker. Whether the “hemogenic” endothelium provides full endothelial function in the mid-gestation mouse aorta or if instead it is comprised of cells from the underlying mesenchyme that temporarily assume endothelial characteristics on their way to forming hematopoietic clusters is a matter of conjecture and/or semantics.

Some studies have suggested that HSCs are generated from mesenchyme located directly underneath endothelial cells in the ventral aspect of the dorsal aorta<sup>25</sup>, or in discrete patches ventral-lateral to the dorsal aorta (sub-aortic patches)<sup>57</sup>. Runx1 is expressed in mesenchymal cells underlying the ventral aspect of the dorsal aorta, and Runx1<sup>+</sup> cells isolated from Runx1 haploinsufficient embryos based on a mesenchymal phenotype (CD45<sup>-</sup>, CD31<sup>-</sup> and VE-cadherin<sup>-</sup>) were found to possess HSC activity<sup>25</sup>. However, CD45<sup>-</sup>, CD31<sup>-</sup> and VE-cadherin<sup>-</sup> cells similarly isolated from wild-type embryos did not contain HSCs<sup>25</sup>. Transplantation data showed that cells from the subaortic patches (CD45<sup>-</sup>ckit<sup>+</sup>AA4.1<sup>+</sup>) have some repopulating activity in immunodeficient adult recipients (0.4-1.9% engraftment)<sup>57</sup>, however they are not as potent as the Runx1<sup>+</sup> or Ly-6A(Sca-1)GFP<sup>+</sup> aortic endothelial/cluster HSCs that provide up to 100% engraftment of irradiated adult recipients<sup>25, 26</sup>. The hematopoietic cells localized in the subaortic patches may be precursors to the fully potent HSCs found in the aortic endothelial hematopoietic clusters, or may represent differentiated progeny of hemogenic endothelium that has ingressed (as in the chick embryo) into this site, or may be an unrelated population of hematopoietic cells. Taken together, these mouse data strongly indicate that the direct precursors of HSCs are predominantly “hemogenic endothelial” cells. In addition, the vascular endothelium of the human embryo has blood-forming potential<sup>58</sup>.

It appears that cells in the aortic hematopoietic clusters are not homogeneous. For example, only some cells in chick aortic clusters are CD41<sup>+</sup><sup>59</sup>. Also, the Ly-6A GFP transgene marker associated with mouse aortic HSCs is expressed by only some cells in the aortic clusters and endothelium<sup>28, 60</sup>. This could indicate that some emergent Ly-6A-GFP<sup>+</sup> HSCs are undergoing differentiation (becoming GFP<sup>-</sup>) while residing in the cluster, or that adjacent Ly-6A GFP<sup>-</sup> hemogenic endothelium is contributing to Ly-6A GFP<sup>-</sup> hematopoietic cells in the clusters. In contrast to the strict ventral localization of hematopoietic clusters in the chick aorta, recent studies in the mouse have identified hematopoietic clusters on both the ventral and dorsal aspects of the dorsal aorta<sup>24</sup>. Functional studies indicate that definitive hematopoietic progenitors reside on both aspects of the aorta, but only the ventral aspect contains fully potent HSCs<sup>24</sup>. Interestingly, the chick aortic endothelium has dual origins: the dorsal aspect is derived from paraxial (somitic) mesoderm and the ventral aspect from splanchnic mesoderm<sup>61</sup>. Recent data<sup>62</sup> demonstrate that chick somitic endothelial cells replace the splanchnopleural hemogenic endothelium in the floor of the aorta once the hematopoietic cluster phase has ended. In the mouse, a small contribution of somitic-derived endothelial cells is also found in the aorta but in the lateral aspect<sup>63</sup>. To facilitate studies of the genetic programs that regulate hematopoietic specification, it will be important to establish the specific mesodermal origins of the hemogenic versus non-hemogenic endothelium in the mouse conceptus.

## Extrinsic factors and master regulators

Prospective hematopoietic cells are specified through sets of intrinsic master regulators (transcription factors) and influenced by morphogens and factors emanating from the surrounding cellular environment; i.e. developing adjacent germ cell layers and tissues. The *de novo* generation of murine hematopoietic cells in the bilaminar yolk sac (endoderm and mesoderm), the chorio-allantoic placenta (mesoderm and trophoblast) and the more complex AGM region (dorsal ectoderm, mesoderm and ventral endoderm) suggests distinct interactions and/or genetic programs are operative in each of these sites. Yet, the genetic program leading to hematopoietic specification should overlap to some degree between the distinct anatomical territories.

Interactions between endoderm and prospective hematopoietic mesoderm are necessary for hemogenic induction in the chick embryo. Blood island generation occurs only when the mesothelial and endoderm germ layers are cultured together – when cultured separately no primitive erythroblasts form<sup>64-66</sup>. Similarly, somitic mesoderm, which normally only contributes to endothelium in the dorsal aspect of the dorsal aorta and not to the ventral endothelium or hematopoietic clusters, could be reprogrammed to assume the latter fates following transient exposure to endoderm prior to grafting<sup>67</sup>. Several signalling molecules, including VEGF, bFGF, and TGF $\beta$ 1 could substitute for this endodermal signal<sup>67</sup>.

Studies in mouse conceptuses showed that contact with visceral endoderm is necessary for primitive hematopoiesis in yolk sac explants, and exposure to endoderm could respecify prospective neurectoderm to assume a hematopoietic fate<sup>68, 69</sup>. This endoderm signal can be replaced *in vitro* by heparin-acrylic beads soaked in Indian Hedgehog (Ihh). Ihh is normally produced by the visceral endoderm, and this expression pattern, together with the explant data, suggests that hedgehog signaling is essential for primitive erythropoiesis<sup>69</sup>. However, deletion of Ihh or its receptor Smoothed (Smo) in mice does not eliminate primitive erythropoiesis in the yolk sac, although it does profoundly affect yolk sac vascularization<sup>70</sup>.

Hedgehog signaling is essential for hematopoiesis in the zebrafish equivalent of the AGM. Hedgehog is situated at the beginning of a signaling cascade that includes the downstream effectors VEGF, Notch, GATA-2 and Runx1 and culminates in the formation of blood cells in the dorsal aorta<sup>71</sup>. VEGF, together with factors identified in the chick (bFGF, TGF $\beta$  and BMP4) are generally thought of as ventralizing factors, while dorsalizing factors that antagonize hematopoietic induction include EGF and TGF $\alpha$ <sup>67</sup>. Most of the ventralizing factors also appear to play a role in hematopoiesis in the mouse. ES cell differentiation cultures and gene-targeting studies have revealed a role for the FGF, TGF and the VEGF/Flk-1 signaling axes in vasculogenesis and hematopoiesis<sup>3, 72-74</sup>. VEGF is expressed by the yolk sac endoderm while Flk-1 is expressed by the mesoderm, and both are expressed intraembryonically as well<sup>75, 76</sup>. BMP signaling is also important for initiating the blood program in the mouse conceptus. *Bmp4*<sup>-/-</sup> embryos generally die around the gastrulation stage, and those that do survive exhibit profound decreases in yolk sac mesoderm and erythropoiesis<sup>74</sup>. Addition of BMP4 to ES cell differentiation cultures<sup>77</sup> and presumptive anterior head fold explants induces hematopoietic cell formation<sup>20</sup>. BMP4 also increases the number of HSCs in AGM explants<sup>60</sup>. Interestingly, BMP4 is localized in the mesenchyme underlying aortic clusters in the mouse<sup>60</sup> and human<sup>78</sup> embryo. Importantly, ventralizing factors control the expression of pivotal hematopoietic transcription factors such as SCL and GATA-1 that are important in hematopoiesis (reviewed in<sup>79</sup>).

Notch1 signaling is selectively important for AGM but not yolk sac hematopoiesis. Mutations that affect Notch signaling in zebrafish eliminate Runx1 expression and hematopoietic cluster formation in the AGM<sup>71, 80</sup>. Notch1-deficient mouse conceptuses die at E10 and contain

almost normal numbers of yolk sac primitive erythroid and erythroid-myeloid progenitors, but have no AGM hematopoiesis or HSCs<sup>81</sup>. Notch1, Notch4, and their ligands Delta-like 4, Jagged 1 and Jagged 2 are expressed in endothelial cells lining the dorsal aorta<sup>82</sup>. Overexpression of Runx1 in Notch signaling mutants in both zebrafish and mice will restore AGM hematopoiesis, indicating that Runx1 is genetically downstream of Notch<sup>80, 83</sup>.

Mice lacking the transcription factor GATA-2 suffer from severely impaired primitive erythropoiesis and a complete lack of other committed progenitors and HSCs and die at E10.5<sup>84</sup>. GATA-2 is expressed in the aortic endothelium<sup>55</sup> and is thought to affect the expansion of the hemogenic population emerging from these cells<sup>54</sup>. Interestingly, GATA-2 haploinsufficiency profoundly decreases the number of AGM HSCs, but yolk sac HSCs are only slightly affected. Runx1, another pivotal transcription factor for definitive hematopoiesis, is expressed ventrally in the mesenchyme, endothelium and hematopoietic clusters of the dorsal aorta<sup>25, 85</sup>. Runx1-deficient conceptuses have essentially normal primitive erythropoiesis but have no myeloid or lymphoid-myeloid progenitors of any sort, and no AGM HSCs<sup>86-88</sup>. The Ets-family transcription factor PU.1, which is required for definitive hematopoiesis, is a critical downstream target of Runx1<sup>89-92</sup>. Haploinsufficiency of Runx1 leads to an increase in AGM HSCs when these are directly isolated from the embryo and transplanted into irradiated adult mice<sup>25</sup>. However, when hematopoietic tissues of *Runx1*<sup>+/-</sup> conceptuses are first cultured as explants and then transplanted, they display interesting differential responses to Runx1 haploinsufficiency. HSCs were profoundly decreased in AGM explants but were increased in both yolk sac and placenta, suggesting that different regulatory networks, downstream targets, interacting molecules, or altered developmental timing are operative in these tissues<sup>93</sup>. Since transcription factors work in complexes and act at several points in hematopoietic development, the interplay between specific transcription factors is an important aspect in hematopoietic specification. The finding that hematopoietic stem cell-specific enhancers of *Scl* and *Runx1* can bind multiprotein complexes containing GATA and Ets<sup>94</sup> and GATA, Ets and SCL factors<sup>95</sup>, respectively, suggests a higher order of regulatory complexity. In addition, prostaglandin E2 and IL-1, which are normally associated with the regulation of inflammation molecules, also affect hematopoiesis in the zebrafish and mouse AGM<sup>96</sup> (Orelia, personal communication). Thus, an understanding of how the master regulators are controlled, and are fine-tuned with respect to their levels in different hematopoietic subpopulations and sites, will provide insight into the genetic network that governs hematopoietic emergence in the conceptus. By analogy to the ES cell program<sup>97</sup>, it is likely that just a small set of factors (Runx1, GATA, Ets and SCL) establishes hematopoietic cell identity in the conceptus.

## Tracing cells to secondary hematopoietic territories

Once the various hematopoietic progenitors and HSCs emerge from their anatomically distinct sites, they are thought to enter the circulation and colonize the fetal liver (Fig. 3). In zebrafish, CD41<sup>+</sup> hematopoietic cells enter the circulation by intravasation via the posterior cardinal veins<sup>98</sup>. The mode of entry of mammalian progenitors and HSCs into the circulation is presumed to occur through release of cells from hematopoietic clusters into the artery or yolk sac vasculature, but this has not been directly demonstrated. In the mouse, the liver rudiment is colonized from late E9 onwards, and later the spleen and thymus are seeded either directly or from the fetal liver<sup>99, 100</sup>. While it seems peculiar that the embryo first produces hematopoietic cells without the full potency of an adult HSC, these early classes of cells that are limited in potency and life span may provide maturation signals to the rudiments of the secondary hematopoietic territories. Indeed in the chick, several waves of early lymphocyte-like cells enter the thymus at receptive times and promote thymic growth<sup>101</sup>. Similarly, mouse genetic models have demonstrated interactive dialogues between early lymphocytes entering the developing thymus and thymic epithelium<sup>102</sup>. Thus, in addition to the well-known function of primitive erythrocytes in gaseous exchange, definitive myeloid and lymphoid-



myeloid progenitors may function to stimulate the growth of secondary hemato-lymphoid territories.

Attempts have been made to lineage trace cells in the conceptus that give rise to the permanent hematopoietic system in the adult mouse. In lieu of the *ex utero* studies that can be performed on non-mammalian vertebrates, lineage tracing in the mouse has relied on Cre-lox recombination marking technology together with tissue specific and inducible transgenes<sup>103</sup>. Several transcriptional control elements have been used to direct the expression of Cre recombinase to hematopoietic cells in the conceptus, including CD41, which is expressed in a subset of definitive hematopoietic progenitors<sup>34, 104</sup>; SCL, which is expressed in endothelial cells and definitive HSCs<sup>53</sup>; and Runx1 which is expressed in all definitive hematopoietic cells, hemogenic endothelium, and some mesenchymal cells<sup>25</sup>. All three genes are also expressed in the mesodermal precursors of the primitive erythrocytes. Rosa26 reporter strains were used to detect the cells in which Cre was active, and the progeny of those cells.

CD41-Cre marked a high percentage of lymphoid and myeloid cells in the fetus, but only 5% of adult bone marrow cells<sup>105</sup>. Therefore, although CD41 marks the majority of progenitors in the yolk sac<sup>34, 104</sup> most adult hematopoietic cells do not transit through a CD41-expressing precursor. The Scl- and Runx1-marking experiments utilized a tamoxifen-inducible Cre (ERT) to control the temporal window of active recombination<sup>106, 107</sup>. Activation of Scl-CreERT pregnant females at E10 and E11 of gestation marked approximately 10% of the cells in the adult BM, indicating that the progeny of SCL expressing cells at midgestation contribute to adult hematopoiesis. In the experiments utilizing Runx1-CreERT, pregnant females were injected with tamoxifen between E7.5 to E10.5<sup>107</sup>, and depending on the day of injection, varying numbers of marked cells were found in the adult BM. The authors' conclude that the adult hematopoietic cells were derived from yolk sac cells, since Runx1 is highly expressed in the yolk sac at the time of tamoxifen injection. However, others have found Runx1 expressing cells at the base of the allantois and in the pSp within 0.5 to 1 day following the onset of Runx1 expression in the yolk sac<sup>39, 95</sup>. The inability to precisely stage embryos *in utero*, together with the uncertain kinetics of CreERT activity and recombination, makes it difficult to discern from what anatomical site the marked cells were derived. Additionally, the Runx1-CreERT allele creates a Runx1 haploinsufficiency, known to affect both the temporal and spatial appearance of HSCs in the conceptus<sup>86</sup>. Hence, while the data confirm that Runx1 expressing cells from the early conceptus contribute to hematopoiesis in the adult, improvements in directing Cre recombination to specific sites of hematopoietic cell emergence will be necessary to unambiguously identify the anatomic origins of adult HSCs.

## Summary

Hematopoietic development in the mammalian conceptus occurs in several mesodermal lineages, as defined by cells emigrating from the primitive streak to three distinct hemogenic tissues; the yolk sac, pSp/AGM and chorio-allantoic placenta. At least five distinct classes of hematopoietic activities have been described, with an increasingly progressive generation of more complex hematopoietic activities that culminate in the *de novo* generation of adult repopulating HSCs. The direct precursors to hematopoietic cells in the conceptus are hemangioblasts and the hemogenic endothelium of the major embryonic vasculature. The genetic program directing hematopoietic fate determination involves known developmental signalling pathways that converge on the directed expression of a small set of pivotal hematopoietic transcription factors. It is the balance (levels and timing) of expression of these factors in the different embryonic tissues that drive the fate determination and emergence of hematopoietic progenitors and HSCs. And while the specific lineages of cells contributing to the mammalian adult hematopoietic system are in question, the legacy of Runx1 and SCL expressing embryonic cells in the adult is certain. The future challenge is to determine the

precise balance of factors necessary to direct HSC fate *ex vivo* in highly expandable and accessible populations of cells, such as embryonic (stem) cells or other somatic cells. This knowledge and insight gained from study of HSC emergence in vertebrate embryos will ultimately be directed towards the *de novo* production of fully potent transplantable HSCs for cellular and molecular clinical therapies of human blood-related genetic diseases and leukemias.

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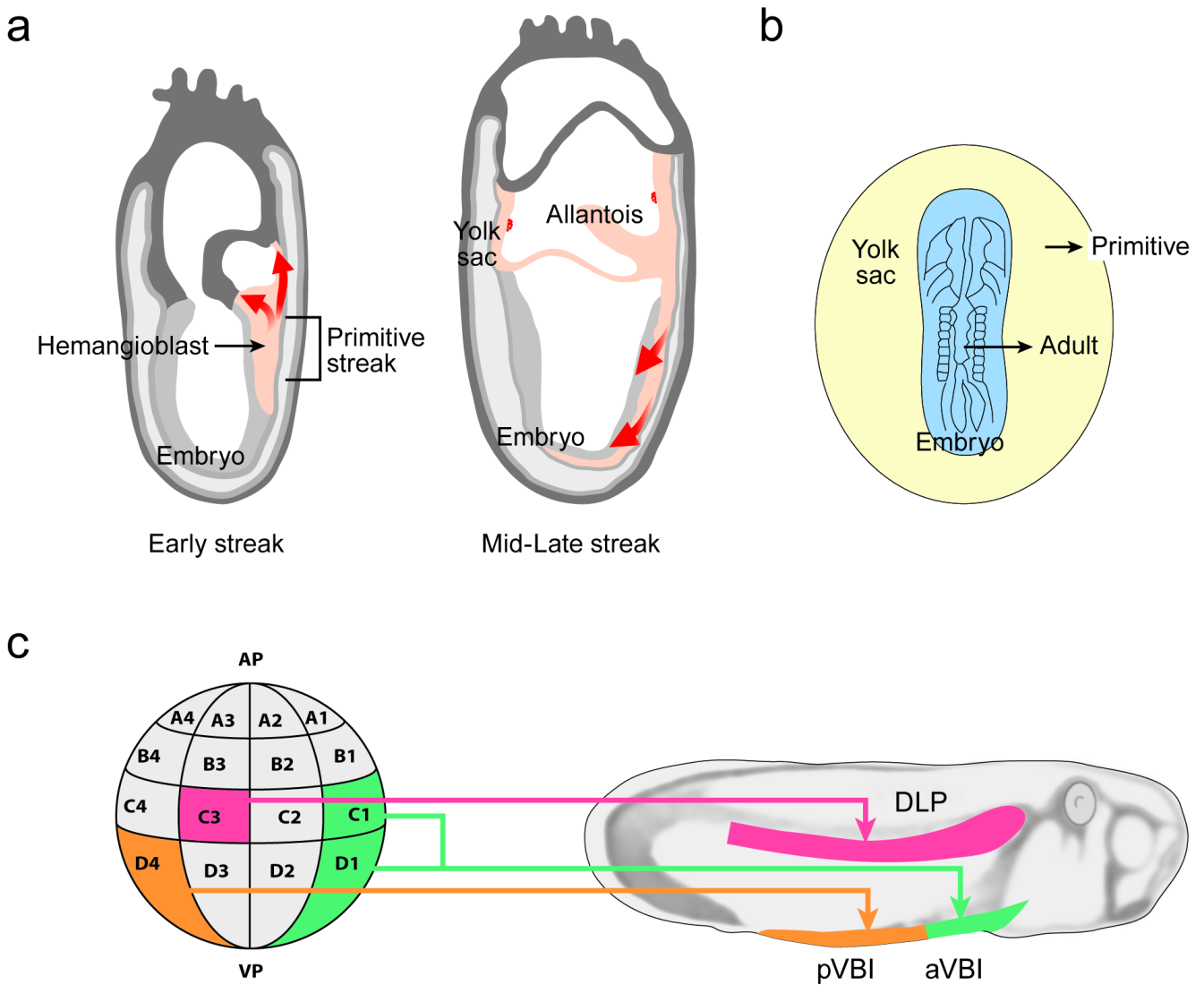
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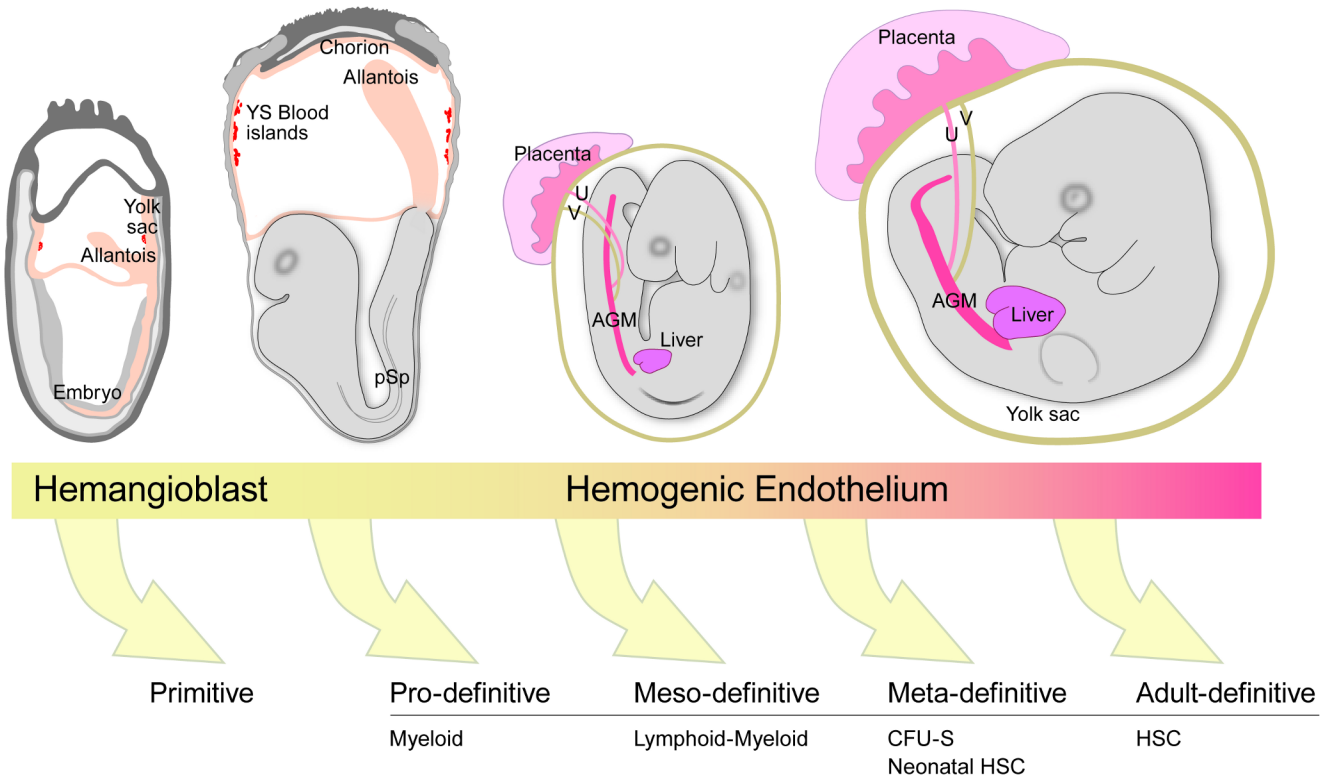
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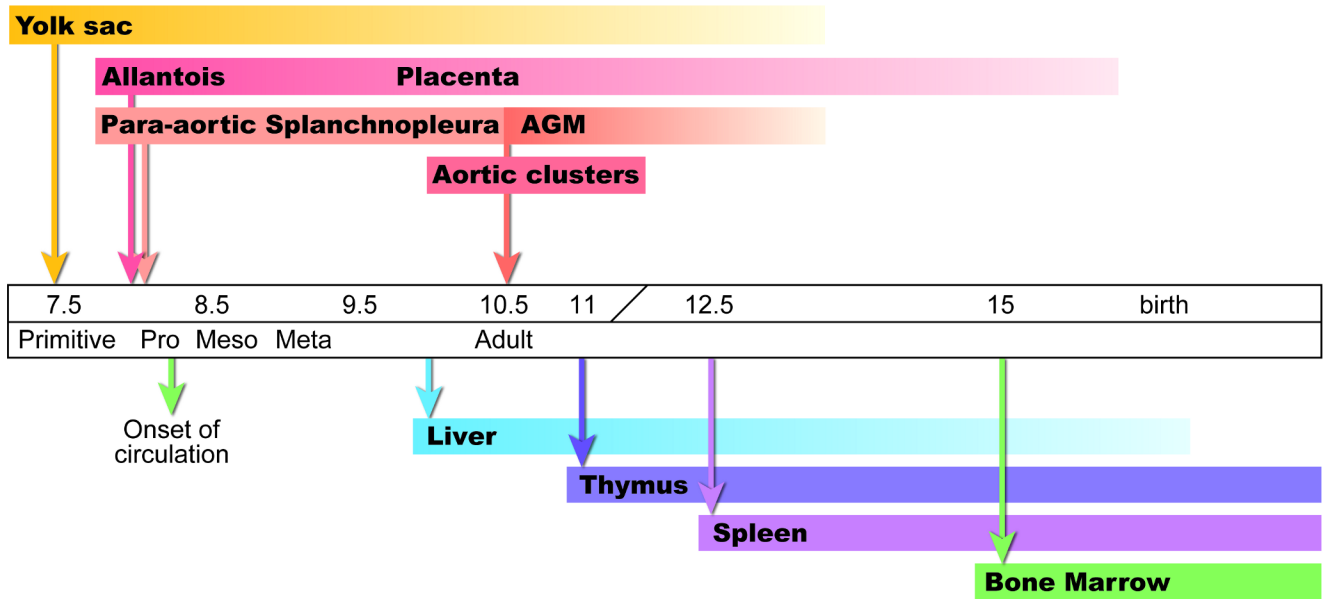
**Figure 1.**

Vertebrate hematopoietic development. **(a)** Mesodermal migration during the early streak (left panel) and mid/late streak stage (right panel) in the mouse conceptus. In the early streak stage, mesoderm emerging from the primitive streak forms the extraembryonic yolk sac and slightly later, the allantois. At the mid/late streak stage, mesoderm emerging from the anterior primitive streak forms the axial, paraxial and lateral mesoderm in the rostral region of the embryo. The mesoderm from the posterior primitive streak forms the paraxial and lateral mesoderm of the remaining trunk region of the embryo. The red arrows depict the emigration of mesoderm after egressing from the primitive streak. **(b)** Inter- and intra-species grafting in early pre-circulation avian embryos (for example a quail embryo body grafted onto a chick yolk sac) revealed the origins of the adult blood in the embryo body and not the yolk sac. **(c)** Amphibian embryos at the 32 cell stage (left) were genetically marked and the progeny traced to larval stages (right). The C3 blastomere gives rise to the DLP (dorsal lateral plate) mesoderm and more specifically to the dorsal aorta and the hematopoietic clusters within the lumen. The D4 blastomere gives rise to the pVBI (posterior ventral blood island) and the C1 + D1 blastomeres to the aVBI (anterior ventral blood island). Drawings adapted from <sup>11, 15</sup>; and the Edinburgh mouse atlas website (<http://genex.hgu.mrc.ac.uk/>).





**Figure 2.** Mouse conceptuses at E (embryonic day) 7.5, 8.25, 9.0 and 10.5 (respectively from left to right). At least five classes of hematopoietic cells (as defined by function) are progressively generated in the mouse conceptus as indicated by the arrows. The primitive class is derived from hemangioblasts and the pro, meso, meta and adult classes are thought to be derived from specialized vascular cells called hemogenic endothelium. Some of these cells are derived from distinct mesodermal lineages emigrating from the primitive streak (see Fig. 1). The E7.5 and E8.25 conceptuses show the outgrowing allantois that will fuse with the chorion to form the placenta. The circulation is established at E8.25-8.5. The E9 embryo has turned and is enveloped in the yolk sac. Colonization of the liver by hematopoietic progenitors begins at late E9. The E10.5 conceptus contains hematopoietic clusters in the dorsal aorta within the AGM regions, the vitelline (V) and umbilical (U) arteries and the first adult HSCs are found in these vessels. Drawings adapted from the Edinburgh mouse atlas website (<http://genex.hgu.mrc.ac.uk/>).



**Figure 3.** Time line of hematopoietic events in the mouse conceptus. The arrows above the time line indicate the onset of specific hematopoietic cell generation and/or appearance and the arrows below indicate the earliest time of colonization of the secondary hematopoietic territories.

**Table 1**  
**Spatial and temporal appearance of hematopoietic potential/cells in the mouse conceptus**

	Developmental time	Site	Functional activity	Conditions	Reference
Primitive	E7.5	Yolk sac <sup>a</sup>	Primitive erythroid	-	35
	E7.5/8 (0-4sp)	Yolk sac <sup>b</sup>	Primitive erythroid	<i>Nex1</i> <sup>-/-</sup>	43
Pro-definitive	E7.5/8 (EHF-2sp)	Allantois <sup>a</sup>	Erythroid-myeloid progenitor	Explant pre-culture	39
	E7.5/8 (0-7sp)	Allantois <sup>a</sup>	Erythroid-myeloid progenitor	Explant pre-culture	38
	E8.25	Yolk sac <sup>a</sup>	Erythroid-myeloid progenitor	-	35
	E8.25	Yolk sac <sup>b</sup>	Erythroid-myeloid progenitor	<i>Nex1</i> <sup>-/-</sup>	43
	E9.5	Yolk sac <sup>b</sup>	Erythroid-myeloid progenitor	<i>Vegf</i> <sup>-/-</sup>	42
Meso-definitive	E9.0	Placenta	Erythroid-myeloid progenitor	-	40
	E7.5/8 (0-8sp)	PSp <sup>a</sup>	Erythroid-myeloid-lymphoid progenitor	Explant pre-culture	37
	E7.5/8 (0-5sp)	PSp <sup>a</sup>	Multipotent low level repopulating progenitor	Explant pre-culture	45
Meta-definitive	E9.0	YS <sup>c</sup>	Neonatal repopulating HSC	-	47
	E9.0	AGM <sup>c</sup>	Neonatal repopulating HSC	-	47
	E9.0	YS <sup>c</sup>	CFU-S	-	41
	E9.0	AGM <sup>c*</sup>	CFU-S	-	41
	E10.5	AGM <sup>d*</sup>	Adult repopulating HSC	-	21, 22, 32
Adult-definitive	E10.5	Umbilical and Vitelline Vessels <sup>d</sup>	Adult repopulating HSC	-	23
	E10.5/11	Placenta	Adult repopulating HSC	-	27, 28

These data are from experiments in which hematopoietic activities in specific tissues were examined

<sup>a</sup> before the onset of circulation between the extraembryonic and intraembryonic tissues at E8.25;

<sup>b</sup> in conceptuses that lack circulation between these sites due to a genetic mutation;

<sup>c</sup> in midgestation conceptuses, although the time earliest time or first site of appearance has not yet been determined;

<sup>d</sup> in midgestation conceptuses identifying the first site in which the specific hematopoietic activity appears.

\* Autonomous expansion of CFU-S and HSCs in explant culture of these tissues suggests *de novo* generation.