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The Biochemistry of Hematopoietic Stem Cell Development

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Abstract

Background—The cornerstone of the adult hematopoietic system and clinical treatments for blood-related disease is the cohort of hematopoietic stem cells (HSC) that is harbored in the adult bone marrow microenvironment. Interestingly, this cohort of HSCs is generated only during a short window of developmental time. In mammalian embryos, hematopoietic progenitor and HSC generation occurs within several extra- and intra-embryonic microenvironments, most notably from ‘hemogenic’ endothelial cells lining the major vasculature. HSCs are made through a remarkable transdifferentiation of endothelial cells to a hematopoietic fate that is long-lived and self-renewable. Recent studies are beginning to provide an understanding of the biochemical signaling pathways and transcription factors/complexes that promote their generation.

Scope of the review—The focus of this review is on the biochemistry behind the generation of these potent long-lived self-renewing stem cells of the blood system. Both the intrinsic (master transcription factors) and extrinsic regulators (morphogens and growth factors) that affect the generation, maintenance and expansion of HSCs in the embryo will be discussed.

Major conclusions—The generation of HSCs is a stepwise process involving many developmental signaling pathways, morphogens and cytokines. Pivotal hematopoietic transcription factors are required for their generation. Interestingly, whereas these factors are necessary for HSC generation, their expression in adult bone marrow HSCs is oftentimes not required. Thus, the biochemistry and molecular regulation of HSC development in the embryo is overlapping, but differs significantly from the regulation of HSCs in the adult.

General significance—HSC numbers for clinical use are limiting, and despite much research into the molecular basis of HSC regulation in the adult bone marrow, no panel of growth factors, interleukins and/or morphogens has been found to sufficiently increase the number of these important stem cells. An understanding of the biochemistry of HSC generation in the developing embryo provides important new knowledge on how these complex stem cells are made, sustained and expanded in the embryo to give rise to the complete adult hematopoietic system, thus stimulating novel strategies for producing increased numbers of clinically useful HSCs.

Keywords

hematopoietic stem cells; AGM; development; transcription factors; signaling pathways

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1. Ontogeny of hematopoietic stem cells

1.1. Multiple waves of *de novo* hematopoietic generation in the embryo

To understand the biochemistry behind HSC development, the cells that make up the vertebrate embryo blood system need some introduction. Blood cell specification occurs at least three separate times in the mammalian embryo – resulting in three *de novo* waves of hematopoietic cell production (reviewed in [1]). While it seems strange for embryos to establish the hematopoietic system multiple times, this in fact is a recurrent theme during ontogeny. For example, the mouse excretory system is generated first as the transient pronephric kidney, a secondary transient mesonephric kidney and finally as a third long-lived metanephric kidney that functions throughout adult life. The three distinct wave-like generations of the hematopoietic system provide a means by which the embryo can be temporarily supplied with rapidly produced hematopoietic cells, while generating a highly complex adult hematopoietic system with long-lived self-renewing hematopoietic stem cells (HSC) at its foundation. Hematopoiesis in the embryo occurs in several tissues that include the yolk sac, aorta-gonad-mesonephros (AGM) region, placenta and liver (Figure 1A).

The first wave of blood generation produces short-lived primitive erythrocytes that are necessary to carry oxygen through the rapidly growing conceptus and also primitive macrophages and megakaryocytes. Primitive erythrocytes are generated from aggregates of mesodermal precursors or ‘hemangioblasts’, in the yolk sac blood islands. Described over 100 years ago, the overlapping ontogenic appearance of both erythroid and endothelial cells indicates a common mesodermal precursor with at least bi-lineage potential [2-3]. This is further supported by the overlap in genetic programs for the two lineages (i.e. expression of Flk-1 (KDR), Scl (Tal1) and CD34) and the lack of both lineages in embryos deficient for some of these genes [4-6]. Surprisingly, hemangioblasts *in vivo* are localized not in the yolk sac but in the posterior primitive streak [7]. As they migrate to the yolk sac they begin their commitment to endothelial and hematopoietic progenitors, with several of these cells contributing to the formation of each blood island [8]. The first wave of primitive hematopoietic cell generation begins at embryonic day (E)7.5 in the mouse conceptus and is highly conserved across vertebrate species, including man (at 16-20 days of gestation [9]).

In the mouse embryo the second wave of hematopoietic cell generation begins at E8/8.5, and overlaps with the first wave [10]. Definitive hematopoietic progenitors are *de novo* generated and some clusters of hematopoietic cells begin to appear in the major vasculature at E9.5. These hematopoietic progenitors are functionally more complex than primitive progenitors – they have multilineage potential (producing erythroid, myeloid and/or lymphoid cells), but they are not long-lived or self-renewing HSCs. *De novo* definitive progenitor generation occurs in the yolk sac, chorio-allantoic/placenta and the intraembryonic region around the aorta, as revealed by mouse embryo explant cultures and the *Ncx1*^{-/-} mouse model (embryos lack circulation due to no heartbeat [11-12] (reviewed in [13])). Thus, ‘definitive hematopoietic progenitors’ constitute the second wave of hematopoietic specification.

The third wave of hematopoietic cell specification provides for the generation of adult type HSCs. Grafting studies in avian embryos provided unequivocal proof that the adult blood system is not derived from the yolk sac, but instead from an intraembryonic source of cells localizing to the dorsal aorta (reviewed in [1, 13]). Clusters of hematopoietic cells consistently found on the ventral wall of the dorsal aorta and major arteries of the chick embryo, led to the proposition that HSCs for the adult hematopoietic system arise from vascular endothelial cells (Figure 1B). Work in the mouse embryo showed that the first adult-type HSCs are generated in the intrabody AGM region (Figure 1A and B). These transplantable HSCs (as potent as adult bone marrow HSCs) are generated beginning at

E10.5 and are thought to be contained within the vascular clusters within the aorta and vitelline and umbilical arteries [14-16]. The real-time generation of hematopoietic cells from 'hemogenic endothelial cells' lining the aorta (Figure 1C) has been demonstrated by vital confocal imaging in the mouse and zebrafish embryo [17-19]. The third wave of hematopoietic cell (HSC) generation is what generates the long-lived self-renewing HSCs that migrate, colonize and reside in the bone marrow throughout adult life.

1.2. Hemogenic endothelium as a source of definitive hematopoietic progenitors and HSCs

The generation of definitive hematopoietic progenitors (wave 2) and HSCs (wave 3) parallels the appearance of vascular hematopoietic clusters in the aorta, vitelline and umbilical arteries (Figure 1C). Histologic/immunostained sections through the midgestation embryo AGM region show that 'hemogenic' endothelial cells express some hematopoietic markers and some hematopoietic cluster cells express endothelial markers [20]. Cluster numbers peak at E10.5, when HSCs first appear. However, not all hematopoietic cells in the clusters are HSCs and not all clusters contain HSCs. There are many more cluster cells in the aorta than there are HSCs at this time point. Genetic studies using Cre-Lox recombination methods for deletion of pivotal intrinsic regulatory molecules show that HSC generation occurs only during a short window of developmental time [21-22]. It is unclear as yet whether all HSCs/cluster cells emerge from hemogenic endothelium, whether larger clusters form by proliferation of the emerging cell or through the recruitment of circulating cells. Recently, it was suggested that already hematopoietic committed cells (perhaps coming from the yolk sac, circulation [23] or other areas [24]) could mature to HSCs as they integrate into the appropriate microenvironment [24].

1.3 Molecular programming of HSC generation is distinct from programming of transient waves of hematopoietic cell generation

What makes the generation of HSCs so special as compared to earlier waves of blood generation? Each wave produces differentiated hematopoietic cells that circulate and move through the embryo to perform specific functions that are not so different from the differentiated hematopoietic cells arising from HSCs. Is it the microenvironment that directs the differences in cell potency, or is it the intrinsic program of the cells as they take on hematopoietic fate? Or is it both? These questions are presently driving investigations on the field of developmental hematopoiesis. The description of the complete genetic program of the nascent HSC and its direct precursor cell, the hemogenic endothelium, would open new possibilities and provide clues as to how to induce existing adult endothelial cells to become 'hemogenic' and thus, direct the programming of HSCs.

Indeed, the notion of different genetic requirements for the emergence of hematopoietic activities in specific embryonic sites and times has been revealed through targeted mutagenesis in mice. Differential requirements for the Runx1, Gata2, Gata3 transcription factors in fetal liver and AGM as compared to primitive yolk sac hematopoiesis has been shown (reviewed in [1, 13, 25]). Growth factor signaling, for example the receptor tyrosine kinase c-kit with its ligand SCF, is known to play roles during AGM and fetal liver stages, in activation of pathways that control hematopoietic cell survival, proliferation and/or differentiation (see review by Sharma and colleagues [26]). In contrast, the mutation of growth factor receptor Flk-1/VEGF ligand and the transcription factor Tal-1/SCL, results in the much earlier impairment at the embryonic yolk sac hematopoietic stage. Thus, the genetic programs of embryonic and adult hematopoietic cells appear to be somewhat overlapping during stages determining hematopoietic fate, but become unique as more complex properties are required in cells destined to become part of the adult hematopoietic system and may later become redundant. The molecular program guiding hematopoietic fate is initiated by extrinsic signals (growth factors and morphogens) coming from the

surrounding microenvironment in the distinct stages of development and these signaling pathways activate the expression of the pivotal transcription factors (Figure 2).

2. Intrinsic regulators of hematopoietic stem cell generation

Discoveries of three intrinsic regulators of HSC specification came from the genetic deletion experiments in the mouse. Pivotal hematopoietic transcription factors *Scf*, *Gata2* and *Runx1* were initially identified through chromosomal translocations and dysregulated expression in leukemic cells. Germ line deletion of each gene leads to midgestation embryonic lethality and profound anemia. Interestingly, these factors appear to work in complexes that bind to regulatory regions in a large overlapping set of hematopoietic specific genes.

2.1 SCL

The *Scf* (*Scf/TAL-1/TCL-5*) transcription factor is characterized by a basic helix-loop-helix domain (bHLH). As a member of the bHLH protein family, it acts through the HLH domain in a heterodimerization complex with E-proteins that recognize specific DNA sequences called Ebox motifs (G/ACANNTGG/A) [27-28]. Whereas *Scf* is most commonly known for its activity in acute T-cell leukemias (reviewed by [29]), the importance of *Scf* in normal hematopoiesis is still under research, using approaches that include germline and conditional deletion of the transcription factor in embryonic stem (ES) cells and mice.

Scf germline deficient mice show early embryonic lethality at E9.5, with an absence of hematopoiesis. This extensive anemic phenotype is also thought to be the cause of death of these embryos. No primitive or definitive hematopoietic cells are produced, yolk sac vasculature is abnormal and no vitelline vessels are formed [30-32]. *Scf* is required for hematopoietic specification in ventral mesoderm or 'hemangioblast' generation (wave 1). Its requirement for the subsequent 2nd and 3rd waves of mouse hematopoietic development is as yet unknown. The contribution of *Scf*^{-/-} ES cells to the formation of mouse chimeras has also been tested. *Scf*^{-/-} ES cells do not contribute to the formation of hematopoietic tissues, whereas they contribute to other various tissues. Further studies in this chimeric context proved *Scf* to be essential for lymphoid development [33]. An *in vitro* approach has been followed to test the hematopoietic potential of *Scf*^{-/-} ES cells. These cells show a complete lack of myeloid and erythroid hematopoiesis, an effect that can be rescued by retroviral introduction of *Scf* cDNA in these cells or by tamoxifen inducible *Scf* expression [31, 33-34].

Scf is normally expressed in the developing embryo in the hematopoietic, endothelial and neural tissues. Expression in endothelial cells and hematopoietic tissues is of particular interest because of the developmental connection of these two cell lineages (hemangioblast, hemogenic endothelium) [35-36]. Several studies demonstrate the specific role of *Scf* in the proper development of both endothelial and hematopoietic cell lineages (reviewed by Lecuyer and Hoang 2004) [27] and suggest that it functions at the interface between vascular endothelial and hematopoietic lineages. Lancrin et al. found that *Scf*^{-/-} Flk1⁺ ES-derived cells were unable to generate blast colonies and also that there was a complete lack of cells expressing hematopoietic markers c-kit and CD41, indicating that *Scf* is critical for the generation of the hemogenic endothelial population. Further studies by this group places its role in hematopoietic specification before the *Runx1* requirement [37] (see below). Differentiation studies in Zebrafish embryos and in ES cells show that *Scf* is a regulator of mesodermal patterning [38-39]. *Scf* serves as an indirect negative regulator of cardiogenesis, and this function is independent of its later role in hematopoietic differentiation [40-41]. Thus, *Scf* plays an important role in the presumptive hematopoietic progenitor/stem cell mesoderm during embryonic development [41-43].

To define the effects of *Scl* deletion in adult mice, conditional knock out approaches have been taken, circumventing the embryonic lethality resulting from its germline deletion. When deleted in the adult, lineage specific effects of *Scl* deficiency are found in erythroid cells and megakaryocytes, but not other myeloid lineage cells. Interestingly, *Scl* participates in a “core transcriptional network” (together with *Gata1* and *Klf1*) to regulate erythroid lineage differentiation[44]. It has been shown to be a direct regulator of a large group (>300) of erythroid specific genes, such as *Eklf1*, *Gata1*, *glycophorin A*, *c-kit* and *globin* [45]. Another study has revealed that *Scl*, through the transcriptional regulation of the cell cycle controller *p21*, regulates megakaryopoiesis. Importantly, although HSCs (and multipotent progenitors) express *Scl*, they do not rely on it for their ability to self-renew, differentiate and long-term reconstitute the blood system of irradiated adult recipients [43]. The apparent lack of effect of *Scl* deletion on adult HSCs is due to its redundancy with *Ly11*[46]. Recent findings indicate that the levels of *Scl* expression control adult HSC quiescence by restricting the G₁ entry of dormant HSCs [47].

2.2 Gata2

The *Gata2* transcription factor which plays a role during the development of hematopoietic progenitor and stem cells is a member of the *Gata* family of factors. The 6 evolutionarily conserved proteins - *Gata1* to *Gata6* - recognize and bind the A/TGATAA/G DNA sequence from which they take their name, and have two highly conserved zinc finger (ZnF) domains [48]. *Gata1*, 2 and 3 are relevant to the hematopoietic system – *Gata1* for the erythroid lineage, *Gata2* for hematopoietic stem and progenitor cells, and *Gata3* for T lymphocytes.

The expression pattern of *Gata2* in the mouse embryo provides some information on its importance in the hematopoietic development. It is highly expressed in hematopoietic cells of the fetal liver, placenta and in endothelial cells including the endothelium of the dorsal aorta from which HSCs emerge and intra-aortic hematopoietic cluster cells[49-52]. Moreover, high levels of *Gata2* mRNA are detected in cell populations enriched for HSCs [53]. It is also expressed in the CNS and fetal heart,

The importance of *Gata2* in the process of HSC generation was first highlighted by the creation of *Gata2* deficient mice. *Gata2*^{-/-} embryos exhibit embryonic lethality between E10-E10.5 characterized by severe anemia [54]. Primitive hematopoietic progenitor numbers are decreased. Definitive hematopoietic progenitors are most profoundly affected and no HSCs are produced[55]. The rescue of this lethal *Gata2*^{-/-} phenotype by functional complementation was performed using a YAC clone bearing the murine *Gata2* genomic locus [56]. Further experiments performed with *Gata2*^{-/-} ES cells differentiated into EBs under specific hematopoietic conditions, examined the hematopoietic progenitor compartment. The greatly reduced number of definitive hematopoietic progenitors and their poor expansion capacity lead to the conclusion that *Gata2* is required for proliferation and survival of early hematopoietic cells [57]. Analysis of ES cells chimeric mice reveal a lack of contribution of *Gata2*^{-/-} cells to any hematopoietic tissue [54]. Interestingly, *Gata2*^{+/-} embryos are greatly reduced in AGM HSC numbers as shown by transplantation experiments [55]. In these studies the number of Ly6A-GFP cells, in which the HSC population can be found, is decreased 10 fold. However, by the time such animals reach adulthood the number of HSCs is normal but they are qualitatively impaired, as shown in competitive transplantation assays. Thus, *Gata2* plays several distinct roles during development - HSC production, expansion and potency.

Putative genome wide target genes of *Gata2* have been described recently in the hematopoietic progenitor cell line HPC-7. In genes important for hematopoiesis, *Gata2* binding sites overlap with those of other transcriptional factors (such as *Scl*, *Runx1*, *Lmo2*, *Lyl1*, *Fli-1* and *Erg*), implying the collaboration of these factors in a “heptameric”

transcription factor complex [58]. Another study in bone marrow progenitor cell populations suggests that Gata2 and Scl function in combination with Ldb1, another transcriptional factor essential for hematopoiesis [59].

Although, the *Gata2*^{-/-} mouse studies provide unequivocal proof that *Gata2* is one of the major key players in hematopoiesis, the precise function of this protein is still uncertain. Retroviral driven *Gata2* overexpression in adult bone marrow hematopoietic progenitor cells blocks their ability to contribute to hematopoiesis as well as their ability to expand [60]. *Gata2/ER* enforced expression in FDCP cells (hematopoietic progenitor cell model) and in BA/F3 cells (IL-3 dependent hematopoietic progenitor cell line) triggers differentiation to monocyte and granulocyte cell fate, while it blocks self-renewal potential [61]. These results reveal varying roles for Gata2 in multipotent hematopoietic progenitors and suggest that Gata2 levels make the difference. Particularly in embryonic development, the diploid dose of Gata2 is required to make the normal number of HSCs. The ability to reach the threshold level of Gata2 expression is probabilistic in the haploid context, thus many fewer HSCs are generated. Recently, *GATA2* haploinsufficiency has been found to underly some human immunodeficiency syndromes [62]. Additionally, the effect of Gata2 in the growth suppression of hematopoietic progenitor cells may be explained by the formation of different complexes with other transcription factors that are expressed during later developmental stages [63]. Despite progress in understanding the function of Gata2, its exact role in HSC generation still needs further investigation.

2.3 Runx1

Runx1 (*AML1*, *Cbfa*) belongs to the family of the DNA binding proteins that include *Runx2* and *Runx3* that have high homology with the *Drosophila* gene *runt* (reviewed in [64]). Runx1 binds directly to DNA sequence TGT/CGGT through its Runt homology domain (RHD), which is a 118 amino acids domain centrally located in the protein. Runx1 heterodimerizes with Cbfb to form the core binding factor (CBF). This interaction increases its affinity for the DNA consensus sequence [65-66].

The high frequency of chromosomal rearrangements in the *Runx1* (*AML*) locus in acute myeloid leukemia (AML) cells was one of the reasons that made *Runx1* an interesting subject for intense study in the blood system (reviewed by [67]). *Runx1* binds to the regulatory elements (enhancer core motifs) of several genes and provides for tissue specific gene expression of molecules known to be important for hematopoiesis. Some of these genes encode GM-CSF, IL-3 and CSF1 receptor [68].

The importance of Runx1 in the development of the hematopoietic system was first revealed in *Runx1* deficient mice. Deletion of this transcription factor results in severe hematopoietic defects that lead to embryonic lethality at E12.5. In contrast the early normal production of the primitive hematopoietic system, the most obvious effect of *Runx1* deficiency is the complete absence of definitive hematopoiesis in the yolk sac (YS) and the fetal liver (FL) at midgestation. The AGM region was found to contain no HSCs. Lack of contribution to the hematopoietic tissues by *Runx1*^{-/-} cells was also observed in chimeric mice made with *Runx1*^{-/-} ES cells. EB differentiation of *Runx1*^{-/-} ES cells and analysis for the presence of hematopoietic progenitors confirmed the lack of definitive hematopoiesis [65, 68-69]. Interestingly, deficiency of core binding factor β (Cbfb), the partner of Runx1 in the core binding factor complex (CBF), results in the same phenotype as Runx1 deficiency [65, 70-71].

The temporal and spatial expression pattern of *Runx1* argues for its importance in the generation of the HSCs and progenitor cells. *Runx1* expression localizes to the endothelial cells of the yolk sac, the vitelline and umbilical arteries, the placenta and most importantly

the ventral wall of the dorsal aorta [12, 16, 72]. It is expressed in the aorta just before the emergence of the HSCs and the progenitor cells, implicating *Runx1* in the hematopoietic fate process in hemogenic endothelial cells. Evidence to support this theory showed that *Runx1*^{-/-} embryos lack hematopoietic clusters and *ex vivo* cultures of *Runx1*^{-/-} endothelial cells possess no hematopoietic potential [16, 73]. A more recent study [21] confirmed the specific role of *Runx1* in the generation of hematopoietic cells from the hemogenic endothelial cells. Specific deletion of *Runx1* in VE-cadherin (VEC) expressing endothelial cells (VEC-Cre mediated *Runx1* excision) resulted in lack of HSC and progenitor generation, phenocopying the hematopoietic defects found in the germline deleted embryos. Interestingly, the specific deletion of the gene in a hematopoietic cell context (Vav-cre mediated *Runx1* excision) showed that *Runx1* is no longer required after HSCs are generated. Thus, *Runx1* plays an important role in the endothelial to hematopoietic transition process by which definitive progenitors and HSCs are generated. However, although it is expressed in HSCs after they are generated, *Runx1* function is no longer required.

Similarly to *Gata2*, haploinsufficiency of *Runx1* has notable effects on HSC generation. A haploid dose of the transcriptional factor leads to only half the number of HSCs in the AGM region [74]. It also results in the slightly earlier appearance of HSCs in the normal sites of hematopoiesis in the embryo [75]. Haploinsufficiency and trisomy of *Runx1* (on chromosome 21) in man predisposes the individual and/or results in hematopoietic dysfunction (leukemia and Down's Syndrome). Thus, proper regulated control of transcription factor levels in HSCs is an important but often less studied aspect. An interesting experiment to test whether *Gata2* and *Runx1* function in the same cells (precursors, hemogenic endothelial, hematopoietic cells) was performed. Since adult *Runx1*^{+/-} and *Gata2*^{+/-} mice are viable and present with a relatively normal hematopoietic blood profile, matings were established to generate double heterozygous mice. Interestingly, no *Runx1*^{+/-}:*Gata2*^{+/-} mice were born. However, *Runx1*^{+/-}:*Gata2*^{+/-} embryos could be harvested and fetal livers were found to contain fewer hematopoietic progenitors than single heterozygous mutants [58]. These findings, together with chromatin immunoprecipitation studies in hematopoietic cell lines, strongly suggest that *Runx1* and *Gata2* act in concert to control expression of hematopoietic genes involved in HSC and progenitor cell generation.

3. Extrinsic regulators of hematopoietic stem cell generation

The embryonic microenvironment surrounding the major vasculature – the dorsal aorta, vitelline and umbilical arteries – has been a major focus of study to identify and characterize the extrinsic regulators involved in the generation of HSCs. Signaling pathways such as TGFβ, BMP, Hedgehog, Wnt and Notch control hematopoiesis in the embryo. Since HSCs emerge from the ventral endothelium of the aorta, it is thought that the underlying mesenchymal cells produce signaling molecules that impact directly or indirectly on hemogenic endothelial and emerging hematopoietic cells. Indeed, the midline expression of Hh in the gut and the ventral expression of BMP4 in the mouse embryo suggest that these factors play an *in vivo* physiologic role in hematopoietic specification and generation in the aorta. In Zebrafish, BMP4 affects *Runx1* and *Gata2* expression [76] and in the adult mouse, the BMP pathway controls the number of HSCs by regulating the size of the niche [77]. Moreover, the Hedgehog (Hh) signaling pathway has also been implicated in Zebrafish hematopoietic development through its induction of VEGF production, subsequent Notch expression and thereafter, Notch mediated expression of *Gata2* in the aorta [78]. In the adult mouse, several independent groups have demonstrated that Hh does not regulate HSCs (reviewed by [79]). In contrast, HSCs have been found to be controlled in a dose dependent manner by the Wnt signaling pathway [80]. At early developmental times, the Wnt/β-catenin signaling pathway is required for the transition between hemogenic endothelium to HSC in

the AGM region of the mouse embryo, but it is not required for their maintenance[81]. This pathway also plays a role in the specification of HSC in the Zebrafish AGM[82]. We describe here some of the known factors and signaling pathways, including FGF, VEGF, Notch, TGF β , Hh and BMP4, that although they have been studied individually, they presumably converge in the same cohort of cells to affect hematopoietic ontogeny in the mammalian embryo.

3.1 Hedgehog

The Hedgehog signaling pathway is critical during development of the major organ systems of vertebrates and acts by controlling proliferation, cell survival, migration and differentiation in a time and dose dependent manner [83]. Endoderm-mesoderm interactions are essential for hematopoietic induction in the developing mouse conceptus, with Hh being produced by endodermal cells. Visceral endoderm and Hh signals have been shown to divert the fate of prospective neuroectoderm to hematopoietic fate [84]. In zebrafish, Hh induces VEGF expression in the somites that leads to the migration of lateral mesodermal cells to the midline [85]. Gene targeting studies show that Hh is required for murine YS hematopoiesis/angiogenesis. *Ihh* expression is found in the endoderm layer of the YS, and Gli1 (intracellular transducer of Hh pathway) expressing cells are found in the mesoderm beginning at E6.5 [86]. In parallel, *VEGF*, a downstream target of Hh, is expressed in YS endoderm. The *Flk-1* gene, encoding the receptor of VEGF, is expressed in the mesoderm and in the embryo body and placenta in the vasculature [5, 87]. Despite its important role in vasculogenesis, only 50% of *Ihh*^{-/-} embryos do not have any hematological defects at E11. However, they become anemic and have a much smaller fetal liver (FL) at E13.5. It is during this developmental stage when definitive hematopoietic cells are established and maintained in the fetal liver microenvironment [88]. This study, as well as studies in zebrafish, indicate that the Hh signaling pathway controls definitive but not primitive hematopoiesis [78, 88]. Moreover, during *Drosophila* development, Hh signaling molecules instruct cells within the hematopoietic niche to maintain hematopoietic progenitors in a precursor state [89]. We examined the effects of the ventrally located gut tissue, which is Hh producing, on the generation of the definitive HSC in the AGM region of mouse embryo. Gut tissue specifically induced HSC activity in early E10 AGM, as did exogenously added Hh [90]. Thus, Hh signaling is early player in AGM HSC generation although other pathways may compensate in its absence.

3.2 BMP

Hh and BMP signaling act in a polarizing manner on adult blood formation in the Zebrafish aorta, with Hh expression in the notochord (dorsal) and BMP expressed ventrally [91]. Deficiencies in the intracellular transducers of the BMP signaling pathway, Smad1 or Smad5, results in the failure of the embryo to generate definitive hematopoietic progenitors [92]. *In vitro*, BMP4 plays a major role in regulating hematopoietic differentiation in murine and human embryonic stem cells [93-94]. At high concentration, BMP4 mediated signaling acts to maintain the proliferation of HSCs derived from human umbilical cord blood rather than promoting their differentiation [95]. In mouse, we found *BMP4* mRNA and protein expressed in the ventral mesenchyme underlying the E10/11 mouse aorta. Ly6A-GFP⁺ hemogenic endothelial cells, HSCs and hematopoietic clusters are in close association with these mesenchymal cells and express BMP receptors, suggesting the direct action of BMP4 on these cells [96]. Addition of BMP4 to E11 AGM explants enhances HSC numbers, while Gremlin, a BMP antagonist suppresses HSCs. Our recent data show that BMP4 is indispensable for HSC growth in AGM explants cultured under serum-free conditions (Crisan, M, unpublished observations). This is consistent with the data showing that *Smad5* deficient mice die between E9.5 and E11.5 with variable phenotypes affecting amnion, gut, heart, neural tube and the YS where the absence of a well organised vasculature is observed

[97] Indeed, immunostainings with an anti-phospho (P)-Smad1/5/8 antibody (BMP4/2 specific Smads) show P-Smad expression in ventral aortic endothelium and hematopoietic cells (unpublished results, A. Zwijsen, Leuven, BE; P ten Dijke, Leiden, NL), suggesting that the pathway is highly active in the E11 AGM. Our most recent preliminary data in *BRE-GFP* (BMP Response Element) transgenic mice show GFP⁺ cells in the vascular hematopoietic clusters and aortic endothelium at E11, and that transplantable AGM HSCs are BMP activated. However, the canonical BMP signaling pathway is not required for hematopoietic stem and progenitor cells in either the adult bone marrow or fetal liver suggesting that other pathways may regulate HSC development [98]. In the same time, *Gli1*^{-/-} mice show increased long-term HSC engraftment [99]. This is consistent with the effect of cyclopamine, a Hh antagonist, on hES cells where blood development was observed to be BMP-dependent [100]. Thus, BMP and Hh pathways may converge to control definitive hematopoiesis during mouse ontogeny.

3.3 FGF

FGFs regulate cellular proliferation, survival, migration, and differentiation. It is important for mesoderm induction during gastrulation followed by the dorso-ventral patterning of the mesoderm [101-104] FGF is also acting as a inducer of neovascularization and neoangiogenesis [105-106] and is involved in promoting proliferation and maintenance of hematopoietic progenitors and stem cells [107]. It is unclear whether FGF is required during hematopoietic ontogeny. Studies show that FGF positively regulates hematopoietic stem and progenitor cells [108-110] although recent data demonstrate that FGF-1 alone was unable to support hematopoietic progenitor proliferation *in vitro* [111]. However, in combination with SCF, TPO and IGF-2, a cytokine cocktail so-called STIF medium defined by Zhang, FGF-1 efficiently supported the *in vitro* amplification of murine and human HSCs [112]. Hematopoietic stem and progenitor expansion was further increased in the presence of mesenchymal stromal cells, most likely due to the synergic effects of the growth factor cocktail containing FGF-1 and cell-cell contacts between the cells [111].

Using mice overexpressing constitutively active FGFR2 (under control of the Tie-2 promoter), Shigematsu et al. demonstrate that FGFR2 signaling facilitates donor hematopoietic engraftment in a transplantation scenario and that this is due to its anti-apoptotic effects [113]. Finally, high FGF activity negatively regulates the formation of primitive blood in chick embryo and promotes endothelial cell differentiation. Opposing results are obtained when FGFR2 is blocked [114]. The negative influence of FGF signaling was also reported in *Xenopus* embryos through downregulation of Gata2 transcription factor, a key hematopoietic target gene of BMP4 [115]. Cooperation between FGF and other signaling pathways also occurs in other cells such as osteoblasts, which serve as the hematopoietic niche for adult HSPC (reviewed by [116]). Altogether, these studies demonstrate that FGF acts not only directly through hematopoietic stem and progenitor cells, but also acts indirectly through their niche.

3.4 VEGF

VEGFA is well known angiogenic factor and acts mainly through VEGFR2, also named Flk-1 or KDR. Complete deficiency and haploinsufficiency of VEGF during murine development causes embryonic lethality between E9.5 and E10 due to an absence of angiogenesis and hematopoiesis [87, 117]. Mice lacking *Flk-1* fail to develop definitive HSCs and die between E8.5 and E9.5 [5]. Consistent with this study, *Flk-1*^{-/-} ES cells do not contribute to primitive or definitive hematopoiesis in chimeric embryos [118].

VEGF is also essential for definitive hematopoiesis in the zebrafish embryo. This is supported by the expression of Flk-1 in *Runx 1* expressing blood cells [78]. In zebrafish,

VEGF is downstream to Hh signaling pathway, but can also be regulated by canonical BMP pathway as its promoter contains BMP activated Smad-binding elements [78, 119].

During mouse development, VEGF is produced by the visceral endoderm (as is *Ihh*). *Gata4*^{-/-} ES cells differentiated to embryoid bodies demonstrate a disruption in the formation of the visceral endoderm, VEGF and *Ihh* expression and definitive hematopoietic progenitor growth. However, exogenously added VEGF and *Ihh* can rescue definitive hematopoietic progenitor production in *Gata 4*^{-/-} embryoid bodies [120]. Also, VEGF can act in synergy with bFGF in human ES cell cultures to specify hemogenic endothelial and hematopoietic cells. In serum-free conditions a combination of BMP4 and VEGF induce hematopoiesis from murine ES cells and induced pluripotent stem cells [121-123].

VEGFA is also required to maintain adult hematopoietic stem cells. Although VEGF is present in the host environment, *VEGF* deficient HSCs do not engraft adult irradiated recipient mice suggesting that HSC are regulated through an autocrine mechanism [124]. However, adult HSCs express the VEGFR and other experiments suggest that HSC engraftment can also be dependent on the VEGF signaling pathway [125-126].

3.5 Notch

Notch signaling is required for the specification and generation of definitive HSCs from hemogenic endothelium during development [127-129]. Inhibition of different components of Notch signaling by mutation or targeted deletion results in severe vascular defects. Arterial vessels, the location of the first generated definitive HSCs, are lacking and there is a total absence of HSCs [52, 128, 130]. In contrast to the severe defects in definitive hematopoiesis in *Notch1*^{-/-} embryos, YS hematopoietic development is not affected. Thus, Notch1 is required only for definitive hematopoietic progenitor and HSC generation [127, 129]. In mouse embryo, Notch1 activates *Gata2* expression (a direct Notch target) in the aorta. *Notch1* is expressed in some endothelial cells lining the ventral wall of the aorta and transcriptional transactivation studies suggest regulation of AGM HSCs through activation of *Gata2* expression [129].

In Zebrafish *Notch* mutants, *Runx1* (used as a hematopoietic progenitor/stem cells marker) is not expressed. However, when heat shock inducible Notch1a intracellular domain is transiently activated during embryogenesis, the number of *Runx1* expressing cells as compared to control is expanded, suggesting that *Runx1* is a Notch target gene in hematopoietic progenitor/stem cells [131]. Downregulation of Notch 1 by the morpholino approach results in highly altered definitive myelopoiesis from the early stages of development [132]. Whereas some Notch ligands may be expressed in the aorta, the somitic expression of the Notch ligands is required for the establishment of definitive haematopoiesis, as recently shown in the zebrafish embryo [133].

The Notch signaling pathway cooperates with other pathways to control hematopoiesis. Studies in zebrafish and mice have shown that Notch specification of HSCs is regulated by VEGFA, downstream of Hh signaling pathway (reviewed by [128]). Moreover in *Drosophila*, Notch gain and loss of function phenotypes are similar to that of hypoxic genes controlling hematopoietic cells. Data recently showed that interactions between Notch and Hif-signaling pathways control hematopoietic development [134].

4. Future Perspectives

The challenges currently at the leading edge of research into HSC development, are to define *in vivo* the biochemical and cellular processes that affect the onset and implementation of the hematopoietic gene expression program in each of the *de novo* waves

of hematogenesis, and to identify the specific multistep program and network of interactions that lead to the genesis of adult HSCs in the 3rd wave. Increasingly complex molecular programs directed by pivotal transcription factors *Scl*, *Gata2* and *Runx1* culminate in the generation of HSCs and the adult hematopoietic system and are only the starting point in our understanding of how HSCs are made. Taking a multidisciplinary approach and using state-of-the-art molecular, cellular and microscopic (3-D whole embryo confocal and real-time vital confocal imaging) techniques, a wide variety of reagents and mouse models, it should be possible to track, isolate and characterize presumptive HSCs during all ontogenic stages as these cells metamorphose through the mesoderm, endothelium, hemogenic endothelium and emerge as HSCs. Important issues include whether the presumptive cells simultaneously express the pivotal transcription factors *Scl*, *Gata2* and *Runx1* or whether there is a sequential/orderly factor requirement; how levels of transcription factors affect the expression of downstream targets, how many signaling pathways impact on HSC development and what are the specific effects of each pathway and how the pathways synergise. The results of such studies will lead to insights allowing the development of novel strategies for the generation of HSCs from human vascular tissue or human iPS cells.

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Highlights

1. Current knowledge about hematopoietic stem cell ontogeny in vertebrate embryos
2. Intrinsic/transcription factors – SCL, Runx, Gata2 – that direct HSC generation
3. Developmental morphogens/factors that are extrinsic cues for HSC generation

Figure 1.

Hematopoietic stem cell development in the mouse embryo. A) Depiction of a mouse embryo at day 10.5 at the time when the first hematopoietic stem cells are generated in the aorta. Sites harboring (and/or generating) hematopoietic cells are shown: the extraembryonic yolk sac and placenta, the intraembryonic aorta and liver, and the umbilical and vitelline vessels that respectively connect the placenta and yolk sac to the aorta. The dotted line through the trunk of the embryo indicates the transverse section shown in panel B. B) Depiction of a transverse section through an E10.5 mouse embryo with the AGM (aorta-gonad-mesonephros/ aorta and urogenital ridges) region in the red rectangle. The AGM is flanked on the dorsal side by the neural tube and the somites, and on the ventral side by the

gut and peritoneum. A hematopoietic cluster is indicated on the ventral wall of the dorsal aorta. Hematopoietic stem cells are localized in the clusters. C) A close up of the ventral wall of the aorta showing cluster formation. A hemogenic endothelial cell is undergoing the transition from endothelial cell to a hematopoietic cell.

Figure 2.

De novo generation of hematopoietic cells begins shortly after mesoderm formation and continues through mouse midgestation. Extrinsic factors that include morphogens such as FGF, Hh and BMPs produced in surrounding microenvironment affect mesodermal cells in hematopoietic fate choice and differentiation. These signaling pathways, as well as the Notch and VEGF pathways, impact directly or indirectly on the expression of several hematopoietic transcription factors in the presumptive hematopoietic cells in different hematopoietic sites and stages of development. The transcription factors directing hematopoietic fate and blood cell production include SCL, Gata2 and Runx1, amongst others. The specific temporal and spatial sequence of extrinsic signals, the combination and/or the levels of extrinsic signals play a role in the differential transcription factor expression and production of the distinct waves of hematopoietic cells in the developing embryo.