Review

Hematopoietic stem cell development and regulatory signaling in zebrafish ☆

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ABSTRACT

Background: Hematopoietic stem cells (HSCs) are a population of multipotent cells that can self-renew and differentiate into all blood lineages. HSC development must be tightly controlled from cell fate determination to self-maintenance during adulthood. This involves a panel of important developmental signaling pathways and other factors which act synergistically within the HSC population and/or in the HSC niche. Genetically conserved processes of HSC development plus many other developmental advantages make the zebrafish an ideal model organism to elucidate the regulatory mechanisms underlying HSC programming.
Scope of review: This review summarizes recent progress on zebrafish HSCs with particular focus on how developmental signaling controls hemogenic endothelium-derived HSC development. We also describe the interaction of different signaling pathways during these processes.
Major conclusions: The hematopoietic stem cell system is a paradigm for stem cell studies. Use of the zebrafish model to study signaling regulation of HSCs in vivo has resulted in a great deal of information concerning HSC biology in vertebrates.
General significance: These new findings facilitate a better understanding of molecular mechanisms of HSC programming, and will provide possible new strategies for the treatment of HSC-related hematological diseases, such as leukemia. This article is part of a Special Issue entitled Biochemistry of Stem Cells.

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1. Introduction

Stem cells are characterized by their ability to self-renew and differentiate into multiple cell lineages. As with other stem cells, hematopoietic stem cells (HSCs) are capable of self-renewal and have the potential to generate all blood lineages, including erythrocytes, megakaryocytes, myelocytes (monocytes and granulocytes), and lymphocytes. HSCs, the cells at the top of the hierarchy of the 'blood lineage tree', have become the archetype for the study of basic mechanisms of stem cell biology and more importantly may contribute to the design of new therapeutic methods to treat stem cell related disease. For example, HSC transplantation (including bone marrow, peripheral blood and cord blood transplantation) has been successfully performed in the clinic to treat many blood diseases, such as leukemia. However, the limited sources of HSCs restrict its application worldwide. Therefore, new methods for the production of transplantable HSCs in vitro or ex vivo will be essential for future widely applied clinical use.

During the last two decades, the zebrafish (*Danio rerio*) has developed as a powerful model for HSC studies due to many appealing advantages. First, external fertilization and transparent embryos allow researchers to examine the whole process of embryogenesis and directly visualize the development, differentiation and migration of individual cells in vivo. Second, high fecundity and rapid growth facilitate large-scale forward genetic screens to identify hematopoiesis-defective mutants. Third, availability of many different transgenic lines makes the zebrafish embryo an unparalleled model for observing and tracing hematopoietic dynamics and processes over time in vivo. More importantly, key genes and signaling pathways in mammals are highly conserved in zebrafish, ensuring its significant research value and making it complementary to other models [1–5].

In vertebrates, the process of hematopoiesis can be divided into two consecutive waves: primitive hematopoiesis and definitive hematopoiesis. In mammals, the primitive wave occurs in the extraembryonic yolk sac blood islands, and mainly generates erythrocytes. Although there is still a debate about whether the yolk sac can contribute to definitive hematopoiesis, it is generally believed that primitive and definitive hematopoiesis have separate origins in mammals [6,7]. The primitive wave is transient, and is replaced by the definitive wave at about E10.5 in mice when HSC specification begins in the aorta–gonad–mesonephros (AGM) region. Once formed, the HSCs firstly migrate to the fetal liver for self-expansion. Eventually, induced by several signaling pathways and chemokines, these HSCs will lodge in the bone marrow, which serves as a lifelong hematopoietic organ [8].

In zebrafish, the primitive wave begins at two intraembryonic sites: the anterior lateral mesoderm (ALM) and the posterior lateral mesoderm (PLM). Primitive myelocytes are mainly generated in the ALM, while primitive erythrocytes are generated in the PLM, which

will form the intermediate cell mass (ICM). The existence of hemangioblasts, bi-potential progenitors of both blood and endothelial cells, has been suggested in both the ALM and the PLM [9–11]. Current data is consistent with primitive hematopoiesis not contributing to the definitive HSC pool in zebrafish [8,12]. By 24 hpf, the specification of HSCs in the zebrafish AGM region indicates the initiation of definitive hematopoiesis. Then at about 30 hpf, the earliest HSCs are generated from the ventral wall of the dorsal aorta (VDA) through a process known as endothelial hematopoietic transition (EHT) [13,14]. During EHT, accompanied by changes in cell morphology, the HSCs egress into the mesenchyme between the dorsal aorta and the posterior cardinal vein, and sequentially enter into the vein, then the caudal hematopoietic tissue (CHT) through blood circulation (Fig. 1). After a short stay in the CHT, the expanded HSCs continue their migration journey either to the thymus, where some HSCs differentiate into T lymphocytes, or to the kidney marrow to sustain life-long hematopoiesis [4]. Alternatively, the earliest HSCs, once egressed from the VDA, enter into the neighboring pronephric ducts underneath the dorsal aorta, then colonize the pronephros through a novel posterior-to-anterior migration route which is distinct from the AGM–CHT mode [15].

During these successive hematopoietic processes, many signaling pathways, such as Wnt, Hedgehog, Vegf, Notch, BMP and FGF, have been identified and demonstrated to be critical at different developmental stages of HSCs and their progenitors, from specification and proliferation to migration and maintenance in vertebrates. However, the detailed molecular mechanisms still remain a huge challenge and warrant further studies. Here, we review recent progress to summarize how these signaling pathways function and cooperate to regulate the different stages of hematopoiesis in zebrafish (Table 1; Fig. 1).

2. Hemangioblast and ventral mesoderm patterning

The existence of hemangioblasts, the common progenitors for both hematopoietic cells and endothelial cells, has been suggested by the co-expression of many hematopoietic and endothelial markers in zebrafish [10,16,17]. Single cell labeling and lineage tracing have evidenced their presence and their hematopoietic/endothelial potentials in zebrafish [9,11].

Hemangioblasts are firstly derived from the ventral mesoderm at an early stage of embryogenesis [18,19]. Therefore, it is critical to figure out how the ventral mesoderm is formed and then differentiates into different cell types. FGF signaling is required for mesoderm induction, and BMP signaling is needed to determine ventral mesoderm patterning, while Wnt signaling can influence the dorsal–anterior mesoderm formation [20]. After ventral mesoderm formation, BMP signaling induces the mesoderm to differentiate into hematopoietic cells, while FGF negatively regulates this process and promotes endothelial cell fate determination in vertebrates including *Xenopus*, chick and mice [21–23]. Recently, a novel function of Notch signaling was reported in the cell fate switch between the endothelial and hematopoietic lineages in the mesoderm, because inhibition of Notch signaling at an early stage could promote endothelial cell production at the expense of hematopoietic lineages in zebrafish [24].

3. Artery specification

Angioblasts, the precursors of endothelial cells, are derived from the hemangioblast and then these angioblasts migrate to the midline, where they form the dorsal aorta and cardinal vein. The determination of arterial versus venous fate has been demonstrated to rely on the Vegf–Notch pathway. In zebrafish, there are four Vegf receptors: Flt1, Kdr, Flt4 and Flk1/Kdr-like (Kdrl) [25,26]. Artery specification is mainly regulated by the Vegfa–Kdr/Kdrl pathway. Once bound to Kdr or Kdrl, the somite-expressed Vegfa will induce endothelial cell differentiation and primary sprouting of intersegmental arteries (ISA) via transmitting signals to PLCγ–ERK, which specifies arterial fate [27,28]. It is well known that the Hedgehog pathway acts upstream of Vegf to regulate the arterial program. Both *shh* and *gli2* mutants have defects in arterial differentiation, which are similar to the phenotype caused by the deletion of *vegfa*, while overexpression of *vegfa* mRNA can rescue the artery defect in Hedgehog signaling-defective mutants [29].

Vegf acts upstream of the Notch pathway during arterial specification, since the activation of the Notch signaling pathway can rescue the arterial defects caused by the absence of Vegf signals [30]. The Notch signaling cascade is triggered by the binding of the single-pass membrane receptor, Notch (Notch1 to 4, zebrafish Notch5 was previously known as Notch3), to the integral membrane ligands (Jagged1/2, delta like 1/4 and deltaC in zebrafish), which are expressed mainly in arterial endothelial cells [31]. In the *mindbomb* mutant, which is defective in Notch signaling, the expression of the artery marker *ephrinB2* is reduced; while the expression of the venous marker *flt4* expands into the artery [32]. Moreover, loss of *hey2* (*gridlock* in zebrafish), which was considered downstream of
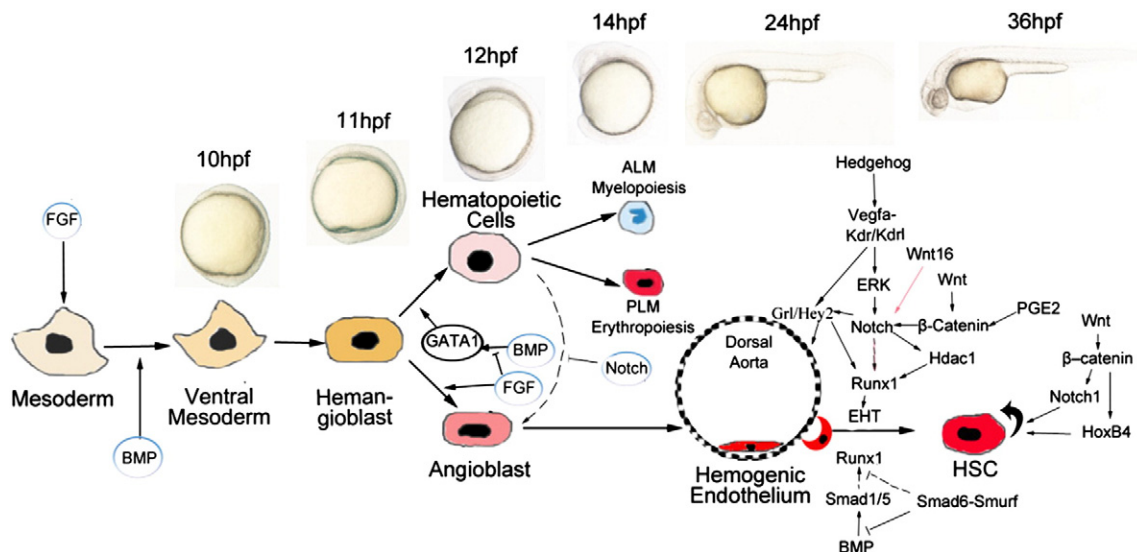

Fig. 1. The signaling inputs during zebrafish hematopoiesis. Different signaling pathways are involved during early embryogenesis and at various stages of hematopoiesis.

Table 1
Expression pattern and function of HSC signaling pathways in zebrafish.

Signaling pathways	Expression pattern	Function	References
Wnt	Wnt3/8/16: brain and somite etc. Fzd2: head, tailbud, mesoderm, axial vasculature and hypochord	Vascular specification, HSC formation and self-renewal	[36,43,46,54,66,67]
Hedgehog	Shh: notochord Ptc1: notochord, somite and dorsal wall of the dorsal aorta	Vascular specification, HSC formation	[30]
Vegf	Vegf-a: somite Vegfc: first in the hypochord and then in dorsal aorta Kdr/kdrl: blood vessel endothelial cell Flt4: ISA, dorsal aorta and vein	Artery/vein specification, hematopoietic cell fate determination	[28]
Notch	Delta C/dll4: arterial endothelial cells Notch5: artery	Artery specification, hematopoietic cell fate determination	[24,32,40,41]
BMP	BMP4: somite ventral region etc. Smad1/5: somite	Ventral mesoderm induction and HSC formation	[20,39,47]
FGF	FGF21: notochord	Myeloid–erythroid progenitor cell fate determination	[53]

the Notch pathway, resulted in a disrupted artery which might be caused by inhibiting angioblast recruitment from the PLM to the dorsal aorta [33]. Hedgehog and Vegf signaling can maintain *hey2* expression in the dorsa aorta, and overexpression of the Notch intracellular domain (NICD) can rescue the arterial phenotype caused by *hey2* deficiency, suggesting that hey2 also acts upstream of Notch signaling [34]. However, it is unclear how Vegf regulates the Notch pathway, though studies in mouse showed that Vegf-activated ERK signaling can induce the expression of *dll4* through *foxc1* [35]. Besides Hedgehog–Vegf signaling, Wnt/β-catenin has been shown to modulate vascular specification and remodeling in mouse. β-Catenin can upregulate Dll4–Notch signaling via direct binding to the *dll4* promoter [36].

4. Hemogenic endothelium and EHT

Recently, studies in zebrafish have shown that HSCs are derived directly from aortic endothelium, through a process called EHT. During EHT, single endothelial cells bend and then egress from the dorsal aorta ventral wall into the sub-aortic space [13,14]. This was the first visualization of HSC initiation and provided direct in vivo evidence for the presence of hemogenic endothelium, a specialized endothelial population that has the potential to generate HSCs [5,16]. However, which factors and signaling pathways can determine hemogenic endothelial cell fate? The hematopoietic factor, Runx1, is expressed in hemogenic endothelium in zebrafish and mice [37,38] and *runx1*-deficient zebrafish embryos initiate EHT very poorly [13]. In the few embryos that could initiate EHT, the hemogenic endothelial cells broke into pieces, thereby aborting the process [13]. The other factors required for hemogenic endothelial cell fate determination are currently unknown. Signaling pathways, such as BMP and Hedgehog, which function ventrally and dorsally respectively in the AGM region [39], appear to determine which group of endothelial cells is transformed into HSCs and which maintains vascular endothelial fate in zebrafish.

5. HSC initiation and maintenance

The initiation and maintenance of definitive hematopoiesis result from the cooperative regulation of many signaling pathways. Notch is well known for its role in HSC emergence. Studies using the Notch inhibitor DAPT, the *mindbomb* mutant and an hsp70:gal4;uas:NICD overexpression system showed that the expression of the HSC markers, *runx1* and *cmyb*, was absent in the mutant and DAPT treated embryos, while greatly expanded in the overexpression system in zebrafish [40,41]. Moreover, by treating the hsp70:gal4;uas:NICD embryos for

various periods of time to control the expansion of *ephrinB2*, it was found that the effect of Notch on artery and HSC development could be uncoupled [41]. But a question still remaining is how Notch regulates *runx1* transcription. Hdac1, a novel hematopoiesis regulator, has been shown to act downstream of Notch signaling to regulate HSC initiation, because the *hdac1* mutation in the hsp70:gal4;uas:NICD overexpression background still displayed loss of *runx1* and *cmyb* expression after artery specification in zebrafish [42]. Recent studies showed that Hey2 not only functions in artery specification, but also plays a role in HSC formation. Hey2-deficient embryos displayed reduced expression of HSC markers, such as *runx1* and *cmyb*. In contrast to previous studies [33], Hey2 was shown to act downstream of PLCγ and upstream of the Notch pathway to govern HSC formation [34].

The Wnt/β-catenin pathway has been studied in zebrafish for its role in HSC development. Goessling et al. found that Wnt was required for HSC formation [43]. Overexpression of Dkk, a membrane-level inhibitor of Wnt signaling and Axin, a member of the β-catenin destruction complex, led to diminished expression of *runx1*. They also revealed the interaction between PGE2 and the Wnt signaling pathway. PGE2 can phosphorylate β-catenin and GSK3β via cAMP/PKA signaling in vivo, and this interaction functions in HSC specification in vertebrates [43]. It was further revealed that the interaction of both Notch and Wnt with NO signaling could affect HSC formation and maintenance [44]. We have recently demonstrated that NO signaling is directly regulated by a blood flow-dependent transcription factor, Klf2a, in controlling the maintenance but not the initiation of HSC programming in zebrafish [45]. In addition, non-canonical Wnt also plays important roles in HSC specification. For example, Wnt16, though expressed in somites, controls the establishment of definitive HSCs via regulating the somitic expression of the Notch ligands, *deltaC* (*dlc*) and *deltaD* (*dld*), which are required for arteriovenous and HSC specification. However, loss of Wnt16 only resulted in an HSC defect rather than vascular abnormality. So the Wnt16–delta–Notch pathway seems to act separately from the traditional Hedgehog–Vegf–Notch cascade in controlling Artery–Vein specification and HSC emergence [46].

Using an inducible BMP dominant-negative zebrafish mutant, Wilkinson et al. found that BMP signaling was required for the initiation and maintenance of definitive HSCs, but not for vascular development at late somitogenesis stages [39]. Smad1 and Smad5 are key mediators of the BMP signaling pathway, and loss of either *smad1* or *smad5* expression leads to defects in the generation of definitive HSCs [47]. Nevertheless, the underlying molecular mechanism remains to be elucidated in zebrafish. In Cos-7 and 416 B cell lines, Smad1 binds to and activates the *Runx1* promoter, while Smad6 inhibits *Runx1* promoter activity with the help of Smurf1, indicating a direct link between Smads and Runx1 [48].

So far, the role of the FGF pathway in embryonic hematopoietic development and regulation of adult HSCs is still uncertain. An in vitro study showed that the FGF signaling pathway acts downstream of HOXB4, and negatively regulates HOXB4-mediated mouse HSC development or ES cell-derived hematopoietic progenitor expansion [49]. In zebrafish, Cdx–Hox signaling activated by BMP and Wnt is required for primitive blood specification, however, the role of Cdx–Hox signaling in HSC development is unknown [50–52]. Chemical inhibition of FGF signaling augmented the long-term repopulation activity of HOXB4-expressing HSCs/hematopoietic progenitor cells (HPCs) expanded in vitro, while suppressing the repopulation activity of control HSCs/HPCs [49]. FGF21 is a newly identified factor essential for the determination of myelo-erythroid progenitor cell fate in zebrafish, but *fgf21* knockdown embryos have normal blood vessels, lymphoid cells and HSCs, indicating that other FGF ligands might be involved in HSC regulation [53].

Despite extensive studies on the signaling pathways involved in HSC initiation and maintenance, whether and how these signaling pathways regulate the hemogenic-endothelium specification, EHT or subsequent processes remain to be explored.

6. HSC self-renewal, trafficking and differentiation

After HSC program initiation, signaling pathways are required to maintain HSCs, their self-renewal, trafficking and differentiation. Wnt regulates HSC self-renewal [54]. Specifically, Wnt/β-catenin signal is thought to be required for normal HSC proliferation and self-renewal via the upregulation of Notch1 and HOXB4 in mouse HSCs [54]. Both Notch receptors and target genes can be upregulated by the stimulation of Wnt signaling, and inhibition of Notch signaling can promote HSC differentiation and disturb the maintenance of HSCs [55]. However, the role of Notch in maintenance of HSCs has been disputed [56,57]. BMP signaling can maintain proliferation and pluripotency of mouse embryonic stem cells (ESCs) through regulating Wnt/β-catenin signaling and Akt phosphorylation [58]. Self-renewal of mouse ESCs regulated by BMP signaling also involves inhibition of the ERK/p38 pathways, which is required for differentiation [59,60]. However, whether there is a similar regulatory mechanism during HSC differentiation in vertebrates is unclear. Smad7, one of the inhibitory Smads downstream of BMP signaling, has been recently shown to play an important role in mouse HSC self-renewal. Overexpression of Smad7 in HSCs increased self-renewal in vivo by blocking all Smad signaling [61].

In zebrafish, once HSCs have formed in the VDA, these cells will migrate to a series of successive definitive hematopoietic organs, such as the CHT, thymus and pronephros. Recently, Wen and colleagues have elegantly demonstrated that HSC trafficking from the VDA is regulated by cMyb, an essential regulator of HSC development [62]. Definitive hematopoiesis was initiated normally in the $cmyb^{-/-}$ mutant; however, the HSPCs could not migrate from the AGM region due to the upregulation of Sdf1a which acts as a strong retention signal for HSCs in the BM of mice [62]. Surprisingly, the expression of the Sdf1a receptor, *cxcr4a/4b* was not affected and the addition of CXCR4 inhibitor could not rescue the HSPC mobilization defects in $cmyb^{-/-}$ mutant indicating that there might be other receptors downstream of Sdf1a.

7. Perspective

The hematopoietic stem cell system is a paradigm for stem cell studies. Using the zebrafish model to study signaling regulation of HSCs in vivo has been feasible. The extracellular signals are transmitted into the nucleus by a complex of signaling pathways, and most of them have multiple roles during embryonic development. But how can they regulate a specific system like HSCs synergistically? Hedgehog, Vegf and Wnt ligands are expressed in non-hematopoietic tissues, but

they can transmit the signals through vascular endothelial cells expressing receptors and then regulate the Notch pathway, which functions in the vessel, and HSC development. Besides the intercellular communications between ligands and receptors, BMP and Wnt signaling pathway components can also regulate specific cells by co-occupancy on regulatory elements of lineage specific master regulators [63,64]. For instance, Smad1 and TCF7L2, downstream transcription factors of BMP and Wnt signaling, respectively, can co-occupy the regulatory elements of two erythroid master regulators, GATA1 and GATA2, and then regulate erythroid specific gene expression [63]. This newly found regulatory model may explain cell-type-specific regulation by extracellular signals. Further studies are needed to find out whether there are some regulatory elements in HSC master regulators which are co-occupied by key factors downstream of the signaling pathways.

On the other hand, signals for HSC development may be maintained at a medium level, whereby either too high or too low levels may lead to a defect. The Wnt signaling pathway can serve as a good example. Absence of Wnt signaling can lead to abnormal HSC proliferation and survival, while augmented Wnt signaling can also induce disrupted reconstitution after HSC transplantation [65]. Therefore, the discovery of regulators which can maintain HSC related signals at a normal range may provide useful therapeutic strategies for some blood diseases. Moreover, although discovery of the EHT process helps explain HSC emergence from the ventral wall of dorsal aorta, increasing evidence has shown that some signaling pathways regulate artery and HSC development through separate mechanisms. For example, several reports have demonstrated the roles of the Vegf signaling pathway in artery specification, while little is known about the mechanism by which Vegf regulates HSC initiation. Therefore, it is critical to study the two processes separately by designing novel approaches.

Taken together, elucidation of regulatory mechanisms of HSC biology using zebrafish and other animal models has provided a great deal of information in the past. A better understanding of HSC development under normal physiological and pathological conditions will offer new therapeutic applications in treating many human blood diseases.

Acknowledgements

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