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On signaling pathways: hematopoietic stem cell specification from hemogenic endothelium

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Hematopoietic stem cells (HSCs) are specified and generated during the embryonic development and have remarkable potential to replenish the full set of blood cell lineages. Researchers have long been interested in clarifying the molecular events involved in HSC specification. Many studies have reported the development of methods for generating functional hematopoietic cells from pluripotent stem cells (PSCs-embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)) for decades. However, the generation of HSCs with robust long-term repopulation potential remains a swingeing challenge, of which a major factor contributing to this failure is the difficulty to define the intraembryonic signals related to the specification of HSCs. Since HSCs directly derive from hemogenic endothelium, in this review, we summarize both *in vivo* and *in vitro* studies on conserved signaling pathways that control the specification of HSCs from hemogenic endothelial cells.

HSCs, hemogenic endothelium, signaling pathways

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Hematopoietic stem cells (HSCs) emerge in the definitive hematopoiesis of early embryos. Definitive hematopoiesis initiates through the formation of transient erythromyeloid progenitors (EMPs), then the HSCs arise. EMPs and HSCs not only share many phenotypic traits, but also both have multilineage potential. Typically, they can be distinguished by their lymphoid potential or self-renewal capacity, as well as the Notch signal profile [1]. From the anatomy level, evidences provided by *in vitro* studies have demonstrated that HSCs specification can be detected in various embryonic sites, including umbilical and vitelline arteries [2], the placenta [3], the fetal head [4] and the yolk sac [5]. From cell level, HSCs fate has been determined from formation of the primitive steak as the beginning, then followed by initiation of hemangioblasts, hemogenic endothelium and HSCs in the end [6,7]. Along the passage of embryonic definitive hematopoiesis, the signaling environment plays a major role in governing HSCs fate.

1 The general route of developmental events of HSCs

In early embryos, HSCs develop from the ventro-posterior floor of the lateral plate mesoderm and the first definitive HSCs are detected in the aorta-gonad-mesonephros (AGM) region in mouse E10.5 embryos [8]. The earliest hematopoietic mesoderm cells, which display both hematopoietic and vascular potential, were first identified in the *de novo* embryoid body (EB) forming system [9]. Signaling pathways that control posterior mesoderm specification are distinctive and include bone morphogenetic protein (BMP) [10], fibroblast growth factor (FGF) [11], Nodal [12] and Wnt [13].

The second step of HSCs development is the formation of hemogenic endothelium, which control HSCs fate via an

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endothelial-hematopoietic transition process confirmed in mouse studies [14]. The concept of hemogenic endothelium was initially developed based on AGM studies, and later it was proved to exist in varied embryonic and extraembryonic sites, including umbilical arteries [15], the placenta [3], the head [4] and nascent yolk sac capillaries [16]. A mouse study found that hemogenic endothelial cells are retinoic acid (RA) responsive, and defined several critical signals downstream RA, including c-Kit, Notch and p27 that are required for hemogenic endothelium specification [17].

The final step is the specification of HSCs from hemogenic endothelium. It seems to exist transiently and is characterized by changes in gene expression and shape of ventral aortic endothelial cells [1]. In order to clarify the trigger signals of this process, the identification of direct precursors of HSCs is crucial. Recent studies on HSCs fate determination demonstrate that the transition of both primitive and definitive program are from $CD34^{+}CD43^{-}$ hemogenic endothelial cells, while the difference is that only KDR^{+} $CD235a^{-}$ mesodermal cells derived $CD34^{+}CD43^{-}$ population give rise to definitive HSCs compared to the $KDR^{+}CD235a^{+}$ mesodermal cells [18,19]. Thus, it is challenging to distinguish definitive hemogenic endothelium from the differentiated clusters only on the basis of cell surface markers, therefore more molecular details are required to unveil the emergence of direct precursors of HSCs. Classical signaling pathways related to the commitment of HSCs from hemogenic endothelium include Sonic hedgehog (Shh) [20], Notch [21],VEGF [22], and Wnt [23].

2 The accumulating data of different signaling patterns of HSCs fate determination from hemogenic endothelium

Studies have provided direct evidence that the formation of HSCs from hemogenic endothelium involved endothelial-hematopoietic transition (EHT) [24]. This process depends strictly on the balance of intrinsic and extrinsic molecular signals [25]. Here, we list current knowledge of intraembryonic signaling pathways that control HSCs fate determination from hemogenic endothelium.

2.1 Notch signaling

Notch signaling is highly conserved across the metazoan [26]. In mammals, there are at least 5 Notch ligands (Jagged1, Jagged3, Delta1, Delta3 and Delta4) interacting with transmembrane receptors (Notch1, Notch2, Notch3, and Notch4 in mice; Notch1a, Notch1b, Notch2, and Notch3 in zebrafish) on adjacent cells, leading to activation of Notch signaling by liberation of the Notch intracellular domain (NICD). Released NCID translocates into the nucleus and modulates target gene expression [27].

Notch signaling is required for vascular patterning, HSC specification and cell fate determination of blood cells. The endothelial-hematopoietic transition also displays a Notch-dependent manner. Both *in vivo* and *in vitro* studies have manifested that the requirement for Notch1 is vital and cell-autonomous in the establishment of HSCs, moreover disturb of notch signaling pathway *in vivo* directly led to loss or decrease of HSCs [28,29]. The $Notch1^{-/-}$ embryo displayed a severely impaired hematopoietic cell development but had no cell number decrease in hemogenic endothelial cells [30], and the $Notch1^{-/-}$ mouse embryonic stem cells kept the capacity to differentiate into flk1 mesodermal cells but failed in produce HSCs [31]. Inactivated Notch signaling in zebrafish had no effects on vascular function, meanwhile dose-dependent activation of Notch signal led to considerable expansion of HSCs without any change of some arterial markers, which manifested that Notch signaling acts through independent pathways to regulate induction of each cells fate [28]. Notch1a and Notch1b, two orthologues of Notch1, are both required autonomously in hemogenic endothelium for HSC generation, meanwhile, Notch3 is proved to function in a non-cell-autonomous manner [32]. Jagged1 knockout mice had decreased number of Gata2 and Runx1 expressing HSCs in the E10.5 embryo compared with the normal ones, but it remained normal arterial formation, suggesting unique Notch requirements between HSCs and arterial fate [33]. Notch1 activates downstream *Runx1* gene expression indirectly through Gata2, which is required in definitive hematopoiesis from endothelial cells [30]. Meanwhile Notch1 acts via Foxc2 in hemogenic endothelium to promote definitive hematopoiesis [21]. However, more Notch-regulated elements controlling HSC generation need to be discovered, such as the unique targets downstream of each required Notch receptors that dominates specification of HSCs.

2.2 Wnt/β-catenin signaling

The signaling contains Wnt proteins, which involves at least 19 highly conserved secreted glycoproteins function as ligands, 10 G protein-coupled Frizzled (Fzd) receptors and 2 low-density lipoprotein receptor-related proteins (LRP) co-receptors [34]. The canonical Wnt pathway involves two core components, which are β-catenin and members of the T cell factor (TCF)/lymphocyte enhancer binding factor (LEF) transcriptional factor family [35]. Wnt ligands bind to the receptor complex, activate the pathway by elevating cytoplasmic level of β-catenin, then migrate to the nucleus and bind to TCF/LEF transcription factor, thereby activating the transcription of target genes [36,37].

Wnt/β-catenin controls many biological processes, including cell fate determination, cell proliferation and self-renew. The role of certaining Wnt proteins in HSC specification has remained elusive. Wnt3α-knockout mice had decreased numbers of HSCs and an impaired reconstruction ability in the secondary transplantation assay [38]. Another mouse study shown that Wnt3α deficiency

did not significantly influence the expression of the other *Wnt* genes, and Wnt3α$^{-/-}$ LSKs displayed a complete abolishment of canonical Wnt signaling, based on which we can speculate that Wnt3α might be the only Wnt protein able to activate canonical Wnt signaling in the HSCs [39]. However, direct effects of Wnt3a on the specification of HSCs from hemogenic endothelium have not been reported. Activation of β-catenin was shown to be vital for HSC specification in the zebrafish AGM through a cyclic AMP (cAMP)/protein kinase A (PKA)-dependent mechanism downstream of prostaglandin E2 (PGE2) [40]. The same result were found in mice that Wnt/β-catenin activity was restricted to a very small number of endothelial cells in the E10.5 AGM, and was required in a dose- and time-dependent manner to produce functional HSCs [41]. All those data indicate that Wnt signaling maybe not be decisive in direct control of HSCs fate, but it is crucial for the initial specification of HSCs.

2.3 Bmp4 signaling

Bone morphogenetic proteins (BMPs) acts as multi-functional growth factors and belongs to the transforming growth factor beta (TGF) superfamily, which regulate many cellular processes including cell fate determination during early embryonic development. The activation of signaling pathways begins with the heterodimerization of Type I and II receptors upon binding to Bmp ligands, which are followed by cytoplasmic R-Smads phosphorylation and in turn regulates multiple genes expression [42]. Signal transduction studies have revealed that Smad1, 5 and 8 are the immediate downstream proteins of Bmp receptors, and they play central roles in the pathway [43].

Bmp4 is a key determinant for the Runx1-mediated emergence of HSCs from hemogenic endothelium, as confirmed by a conditional knockdown assay in zebrafish that resulted in a loss of HSCs in the ventral wall of the dorsal aorta, while the arterial program was unaffected [44]. Recently, a novel PKA-cAMP response element-binding protein (CREB)-Bmp signaling pathway downstream of shear stress was proved to function in HSC emergence in the AGM through the endothelial-hematopoietic transition [45]. Knowing the regulation of downstream signals of the pathway is relatively more crucial. For years, studies conducted on cytoplasmic like Smad1, Smad5 and Smad9 confirmed that Bmp4 signal only functioned in HSC formation instead of later lineage commitment [46–48]. Interaction between Smad1/5 and extracellular signal-regulated kinase (ERK) signaling is essential for endothelial-hematopoietic transition, shown by the defects in HSC formation induced by ERK activation via knockdown of Smad1/5 [47]. These findings manifest the different requirements in Bmp signaling during hematopoietic commitment from endothelium.

2.4 Other signals

Multiple additional signals also influence the specification of HSCs from hemogenic endothelium. Studies in zebrafish have proved that Notch regulated HSC specification was directly controlled by Shh-VEGF signaling (vascular endothelial growth factor acts downstream of sonic hedgehog) during arterial endothelial differentiation [20,22]. A zebrafish study menifests that the FGF signaling regulates HSC fate through repressing BMP activity and this negative regulation is independent from arterial specification during the convergence of vascular precursor cells to the midline [49]. Meanwhile, it has been proved that Wnt16 acts upstream of FGF signaling pathway through FGF receptor 4 (Fgfr4) to relay signals to Notch ligand deltaC (dlc) in fate HSCs during the endothelial-hematopoietic transition process [50]. Recent works have uncovered several previously unknown signals required for HSC specification including the PGE2-cAMP/PKA signaling axis [51–53] which was activated by biomechanical forces, and the inflammatory signaling toll-like receptor 4 (TLR4)/nuclear factor-kappa B (NF-κB) which regulates HSC determination via promoting Notch activity [54]. Advances in new signaling pathways that relates to the fate determination of HSCs may help improve the whole molecular patterns of certain precursor cells. Collectively, these findings indicate that the precursors of HSCs must emergence in time in the dorsal aorta to accept specified signals to fulfill the final specification step.

2.5 Interactions of different signaling pathways

The expression pattern of these required signals is dynamic in location and timing, which is closely related to their biological roles. As to HSC specification, direct cell-to-cell contact through Notch receptors and ligands in proper time is needed, so does those signals such as Bmp4 that function in somites. The interaction models of key signaling pathways that dominant HSC emergence from hemogenic endothelium both *in vivo* and *in vitro* can be concluded below (Figure 1). Canonical Wnt signaling also interact with other pathways, such as the PGE2, in a β-catenin-dependent manner [40]. Shh signaling induces vascular endothelial growth factor A (VegfA) expression in somites, which in turn activates the expression of Notch receptors in endothelial cells, thus promoting the possibility to activate Notch signaling in HSC specification [22]. FGF signaling function as an intermediate role in somites between Wnt16 and Notch signaling pathway in HSC specification from hemogenic endothelium. In conclusion, coordination between multiple signaling pathways leads to the inflexible time- and dose-dependent requirement of molecular signals during HSC specification.

3 Summary

Hematopoietic regulation is a complex dynamic network controlled by both intrinsic and extrinsic factors in a three

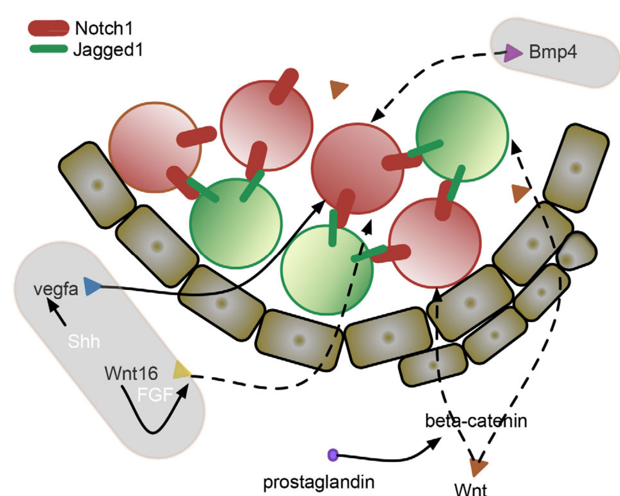

Figure 1 Major cell signaling pathways involved in HSC specification from hemogenic endothelium. In the dorsal aorta, hemogenic endothelium (Red) receives specified signals from adjacent cells (Green) and somites (Grey) to gain HSCs fate. Key molecular signals involved in this process include Notch; non-canonical/canonical Wnt; Shh; Bmp4. Solid lines indicate there are genetic evidence of interacting proteins, while dotted lines represent unproved but plausible interactions.

dimensional condition [55]. In recent years, efforts have been made in developing methods for producing specialized blood cells from hPSCs [56]. However, the clinical application of hPSCs derived blood cells such as the most promising red blood cells (RBCs) requires further development [57]. And when it comes to *in vitro* generation of HSCs, the most hopeful process is to define the direct precursors of HSCs in *de novo* systems. The fact that the embryonic vascular development is closely associated with HSC generation, which makes it vital to clarify the mechanical differences within these two processes. A recent study shown that genetic loss of Sox17 and Notch1 (arterial genes) during EHT result in increased number of hematopoietic cells suggested that EHT is actively repressed in a sub set of endothelial cells [58].

As to hematopoietic differentiation of pluripotent stem cells (PSCs), signals such as Bmp4, VEGF, and Wnt have been used in combination with other factors in well-defined conditions *in vitro* to induce function hematopoietic progenitors. However, these approaches have not yet led to the formation of functional HSCs. The accumulating data have proved that not all of the hemogenic endothelial cells of *in vitro* systems can turn on definitive program [59,60]. The erythrocytes derived from hESC firstly show the primitive properties, then turn to obtain definitive properties in a clonal tracing assay, implying that there may be a definitive switching mechanism within hESC-derived hematopoiesis [61]. And a recent study also showed that hPSCs derived hemogenic endothelial cells are distinctive to *in vivo* studied hemogenic endothelium [62]. The existence of heterogeneous cell groups within HE cells, which cannot be identified simply by known cell surface markers, implys that more molecular mechanisms like the regulation of transcriptional and epigenetic factors (like Gata2 [63], Gata3 [64] and AML1 [65]) need to be re-discovered, so do the conserved signaling pathways which need to be reviewed. In this review, we have discussed a subset of known signaling pathways related to HSC specification from hemogenic endothelium. Upon combining with an efficient induction system as well as certain reporter hPSCs cell lines, the signaling pathways confirmed in animal studies need to be further tested *in vitro*, thus prompting the generation of HSCs *in vitro*. In summary, operating molecular networks such as signaling pathways will be the most promising method to explore the normal HSC development and finally to find ways to recapitulate it *in vitro*.

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