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Review Article

Complex regulation of HSC emergence by the Notch signaling pathway

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ABSTRACT

Hematopoietic stem cells are formed during embryonic development, and serve as the foundation of the definitive blood program for life. Notch signaling has been well established as an essential direct contributor to HSC specification. However, several recent studies have indicated that the contribution of Notch signaling is complex. HSC specification requires multiple Notch signaling inputs, some received directly by hematopoietic precursors, and others that occur indirectly within neighboring somites. Of note, proinflammatory signals provided by primitive myeloid cells are needed for HSC specification via upregulation of the Notch pathway in hemogenic endothelium. In addition to multiple requirements for Notch activation, recent studies indicate that Notch signaling must subsequently be repressed to permit HSC emergence. Finally, Notch must then be reactivated to maintain HSC fate. In this review, we discuss the growing understanding of the dynamic contributions of Notch signaling to the establishment of hematopoiesis during development.

1. Introduction

Hematopoietic stem cells (HSCs) are the foundation of the vertebrate blood system. These stem cells self-renew to maintain a steady pool of HSCs that can differentiate as needed into cells of the lymphoid, myeloid and erythroid lineages, and thereby maintain blood cell homeostasis for life. Thus far it has not been possible to derive HSCs *in vitro* from pluripotent precursors, necessitating a deeper understanding of the mechanisms leading to their development *in vivo*. HSCs are born during embryonic development, though the molecular mechanisms governing their emergence are not completely understood. In recent years, it has become clear that the dynamic context of the developing vertebrate embryo provides numerous molecular and environmental cues critical for HSC formation, including the Notch signaling pathway (Clements and Traver, 2013).

The generation of HSCs during embryonic development is dependent upon Notch signaling (Kumano et al., 2003). Within wild-type/Notch1 −/− chimeric mice, Notch1-null cells fail to contribute to hematopoiesis, indicating that the Notch1 receptor is required cell-autonomously, for HSC potential (Hadland et al., 2004). In both mice and zebrafish, Notch signaling functions genetically upstream of the transcription factor *Runx1* (Burns et al., 2005; Nakagawa et al., 2006), which is essential prior to HSC emergence (Chen et al., 2009; Kissa and Herbomel, 2010). Although Notch signaling provides direct transcriptional regulation of several genes that are important for HSC formation, including *Hes1*, *Hes5*, *Hey2* and *Gata2* (Davis and Turner, 2001; Guiu et al., 2013; Tsai et al., 1994; Iso et al., 2003; Robert-Moreno et al., 2005), Notch does not appear to directly regulate *Runx1* expression (Robert-Moreno et al., 2005; Nottingham et al., 2007). Rather, a transcriptional complex including Gata2 drives *Runx1* expression within the hemogenic endothelium from which HSCs derive (Nottingham et al., 2007), providing a defined, cell-autonomous link between receipt of Notch activation and the emergence of HSCs. Interestingly, the hematopoietic defect that occurs in the absence of Notch signaling can be rescued by the artificial induction of *Runx1* but not *Gata2* (Nakagawa et al., 2006). This strongly indicates that Notch signaling regulates additional, unknown factors required for *Runx1* induction.

Although it is well established that Notch signaling is required cell-autonomously for HSC formation (Kumano et al., 2003; Hadland et al., 2004), recent evidence has revealed that the Notch signaling pathway exerts complex regulation of HSC specification, emergence and maintenance in the developing embryo. It is now apparent that multiple, distinct Notch signaling events act in both direct and indirect ways, and provide both positive and negative regulation over the establishment of the adult hematopoietic system.

2. Mechanisms of Notch signaling

Notch signaling allows for communication between cells in close contact, through the binding of ligands and receptors on

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adjacent cells. Notch family receptors consist of single-pass transmembrane proteins, including an extracellular domain made up of ligand-binding EGF repeats, a membrane-tethered transcriptionally active intracellular domain and multiple proteolytic cleavage sites allowing for separation of these components upon ligand binding. The Notch signal initiates when a ligand of the Delta/Serrate/Lag-2 (DSL) family on the signal-sending cell interacts directly with a Notch receptor on the signal-receiving cell. Within the signal-sending cell, ubiquitination of the ligand by the E3 ubiquitin ligases Mindbomb (Chen and Corliss, 2004; Itoh et al., 2003) and Neuralized (Deblandre et al., 2001; Yeh et al., 2001; Pavlopoulos et al., 2001; Lai et al., 2001) promote Notch activation by stimulating endocytosis of the receptor-bound ligand. The resulting tension exposes the S2 proteolytic cleavage site at the base of the Notch receptor extracellular domain, near the cell membrane, facilitating S2 cleavage by ADAM family metalloproteases (Gordon et al., 2007; Nichols et al., 2007; Parks et al., 2000). Subsequently, the remaining membrane-tethered receptor is cleaved by γ-secretase from the S3 to S4 cleavage sites, releasing the Notch intracellular domain (NICD) and allowing its translocation to the nucleus. In the most established model of Notch transcriptional regulation, the Notch transcriptional partner RBPjK recruits nuclear corepressor (NcoR) and histone deacetylases (HDACs) and holds Notch target genes in a transcriptionally repressed state in the absence of Notch signaling (Fig. 1A) (Kao et al., 1998). Upon Notch activation, nuclear NICD displaces these transcriptional corepressors and recruits coactivators such as Mastermind, initializing transcription of direct Notch targets. Although according to this traditionally accepted model, RBPjK actively represses activation of Notch targets in the absence of Notch signaling, recent work has indicated that Notch transcriptional regulation may be more complex than previously realized. Rather than constantly occupying Notch-responsive enhancer elements, in many cases RBPjK is recruited alongside NICD, suggesting that RBPjK does not repress all Notch targets prior to Notch activation (Fig. 1B) (Castel et al., 2013). It remains unclear whether RBPjK functions as a steady-state repressor for direct Notch targets in the context of HSC formation.

Induction of Notch signaling allows coordinated cell fate decisions amongst neighboring cells, through lateral inhibition or lateral induction mechanisms (Lewis, 1998; Artavanis-Tsakonas et al., 1999). Lateral inhibition occurs when stochastic differences between cells result in Notch activation in one neighbor, and this activation results in the transcriptional repression of Notch ligand in the signal receiving, Notch-active cell. The resulting negative feedback loop enforces directionality of Notch activation, and can drive adjacent cells of shared developmental origin to distinct fates. In contrast, lateral induction occurs when Notch activation results in increased expression of Notch ligand on the signal receiving, Notch-active cell. In lateral induction, Notch signaling induces positive feedback. As Notch-active cells become Notch-activators, elevated levels of Notch activity drive adjacent cells to the same fate. Lateral induction results in clusters of Notch-active cells, lateral inhibition creates a “salt and pepper” arrangement of Notch-active cells amongst Notch-inactive neighbors by restricting Notch activation (Artavanis-Tsakonas et al., 1999; Bigas et al., 2010).

3. Origins of the hematopoietic system

Vertebrate hematopoiesis begins during embryogenesis with several independent waves of blood formation preceding the eventual emergence of definitive HSCs, which will establish and maintain the adult blood program. This process initiates with the independent generation of primitive myeloid and primitive erythroid cells, which both arise transiently without a sustained pool of progenitors. Primitive erythroid cells and endothelial cells arise

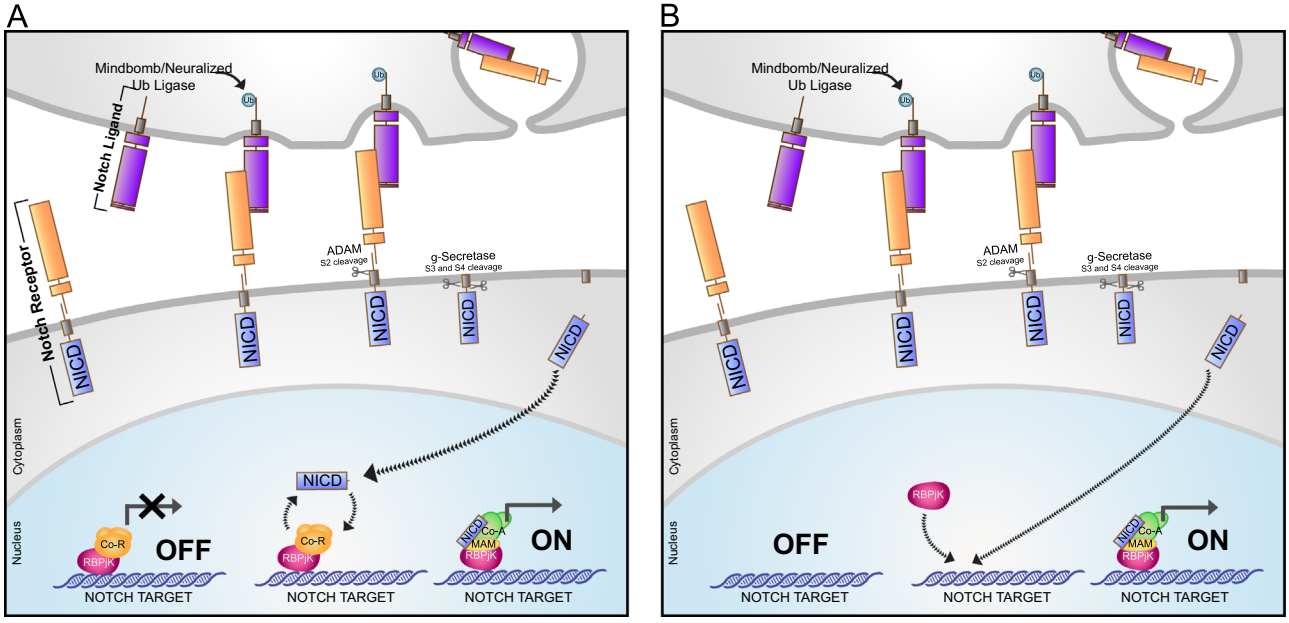


Fig. 1. Overview of Notch Signaling: (A) Notch receptors are activated by binding of the Notch extracellular domain (NECD) to Notch ligands of the Delta/Serrate/Lag-2 families on adjacent signal-sending cell. Ubiquitination of Notch ligands by the Mindbomb and Neuralized E3 ubiquitin ligases promotes endocytosis, facilitating cleavage by ADAM family metalloproteases through exposure of the S2 proteolytic cleavage site. S2 cleavage results in the separation of ligand-bound NECD, and the remaining transmembrane receptor. Subsequently, the ligand–NECD complex is taken up by the signal-sending cell. The remaining membrane-bound Notch receptor is cleaved at the S3 and S4 proteolytic sites by Gamma-Secretase, releasing the Notch intracellular domain (NICD) from the membrane tether and allowing NICD to translocate to the nucleus. In the absence of Notch receptor activation, Notch transcriptional targets are bound by RBPjK/CSL and transcriptional corepressors (Co-R), and held in a transcriptionally repressed state. Nuclear NICD binds RBPjK, displacing the corepressor complex and allowing for the recruitment of transcriptional partner Mastermind (MAM) and additional coactivators (Co-A), allowing for transcriptional activation of Notch target genes. (B) In some cases RBPjK occupies Notch transcriptional target sites in a more dynamic manner. In this case, RBPjK does not occupy the Notch target site in the absence of Notch activation, and is instead recruited upon activation alongside NICD and transcriptional co-activators.

concomitantly within the yolk sac of mammals and birds (Medvinsky et al., 2011). Primitive erythrocytes and endothelial cells derive from a shared "hemangioblast" mesodermal precursor, during gastrulation that differentiates into distinct hematopoietic and endothelial lineages prior to reaching the yolk sac (Huber et al., 2004). Embryo-derived primitive myeloid cells arise with restricted potential from the yolk sac shortly following primitive erythrocyte formation (Dzierzak and Speck, 2008; Bertrand et al., 2005). Multipotent erythromyeloid progenitor (EMP) cells, which give rise to both definitive erythroid and myeloid lineages, emerge shortly after primitive erythropoiesis and myelopoiesis, also from the yolk sac (Palis et al., 1999). Whereas primitive erythrocytes arise from a shared endothelial precursor, EMPs were recently suggested to derive from the endothelium itself in the murine yolk sac (Chen et al., 2011). Embryonic hematopoiesis culminates with the generation of HSCs, the only cells capable of long-term self-maintenance and differentiation into a full repertoire of hematopoietic lineages. In the mouse, HSCs have been reported to emerge from hemogenic endothelium primarily within the dorsal aorta, from the umbilical and vitelline arteries, and the placenta (Jaffredo et al., 1998, 2000; Rhodes et al., 2008; Gordon-Keylock et al., 2013; de Bruijn et al., 2000) HSCs have also been reported to emerge within the head vasculature and heart, although these findings are controversial (Li et al., 2012; Nakano et al., 2013).

Although lower vertebrates develop externally to the parent and lack an extraembryonic yolk sac, hematopoiesis likewise proceeds through four waves, via cellular and molecular mechanisms that appear to be largely conserved. In this regard, work in zebrafish and Xenopus has helped clarify the developmental origins of embryonic and adult blood cells. In mammals, lineage restricted primitive erythrocytes and primitive myeloid cells arise during a similar period within the yolk sac, however work in zebrafish has defined distinct mesodermal origins of these two populations. Primitive myeloid cells derive from the anterior lateral plate mesoderm (ALM), and differentiate within the rostral blood island, on the anterior region of the yolk ball (Herbomel et al., 1999). In contrast, primitive erythrocytes, EMPs and HSCs are thought to share a common origin within the posterior lateral plate mesoderm (PLM) of zebrafish, which is analogous to the dorsal lateral plate (DLP) of Xenopus. In both species, cells within this bilateral mesodermal population undergo commitment to primitive hematopoietic or endothelial fate, followed by medial migration, convergence at the midline, and the initiation of vasculogenesis (Medvinsky et al., 2011). The formation of HSCs from hemogenic endothelium of the dorsal aorta is conserved from humans to zebrafish (Medvinsky et al., 2011). Of the four embryonic waves of vertebrate hematopoiesis, Notch signaling is essential only for the establishment of HSCs (Kumano et al., 2003; Burns et al., 2005; Bertrand et al., 2010; Gering and Patient, 2005), although it also appears to influence the development of primitive erythrocytes (Robert-Moreno et al., 2007).

4. A role for Notch signaling in hematovascular fate determination

Signaling involved in mesodermal commitment to an endothelial fate precedes HSC development. In both mice and zebrafish, primitive erythrocytes and endothelium arise from a shared hemangioblast precursor (Huber et al., 2004; Vogeli et al., 2006). Multiple studies in zebrafish have postulated that Notch signaling influences the development of endothelial and primitive erythroid cells from the PLM (Chun et al., 2011; Lee et al., 2009). In one study, Notch activation in the early zebrafish embryo led to increased numbers of primitive erythrocytes and a corresponding decrease in endothelial cells, suggesting that Notch signaling influences the determination of endothelial versus primitive erythroid fate (Lee et al., 2009), consistent with its role in lateral inhibition. More recently, however, Notch signaling has been reported to promote the proliferation of endothelial precursors within the PLM (Chun et al., 2011). Additionally, the gain of Notch signaling that occurs with the loss of Notch inhibitors *numb* and *numb-like* results in primitive erythroblasts that fail to mature and instead undergo apoptosis (Bresciani et al., 2010), indicating that Notch signaling inhibition promotes primitive erythrocyte survival. Accordingly, murine *RBPjK* mutants experience an elevated number of primitive erythrocytes, due to reduced levels of apoptosis (Robert-Moreno et al., 2007), whereas the zebrafish Notch signaling mutant *mindbomb* shows no noticeable reduction in primitive erythrocytes (Burns et al., 2005). Presently, it appears that Notch signaling may independently promote endothelial lineage expansion within the PLM and the maintenance of primitive erythrocytes as they undergo differentiation. Further study is necessary to elucidate any contribution of Notch signaling to fate determination within lateral plate mesoderm.

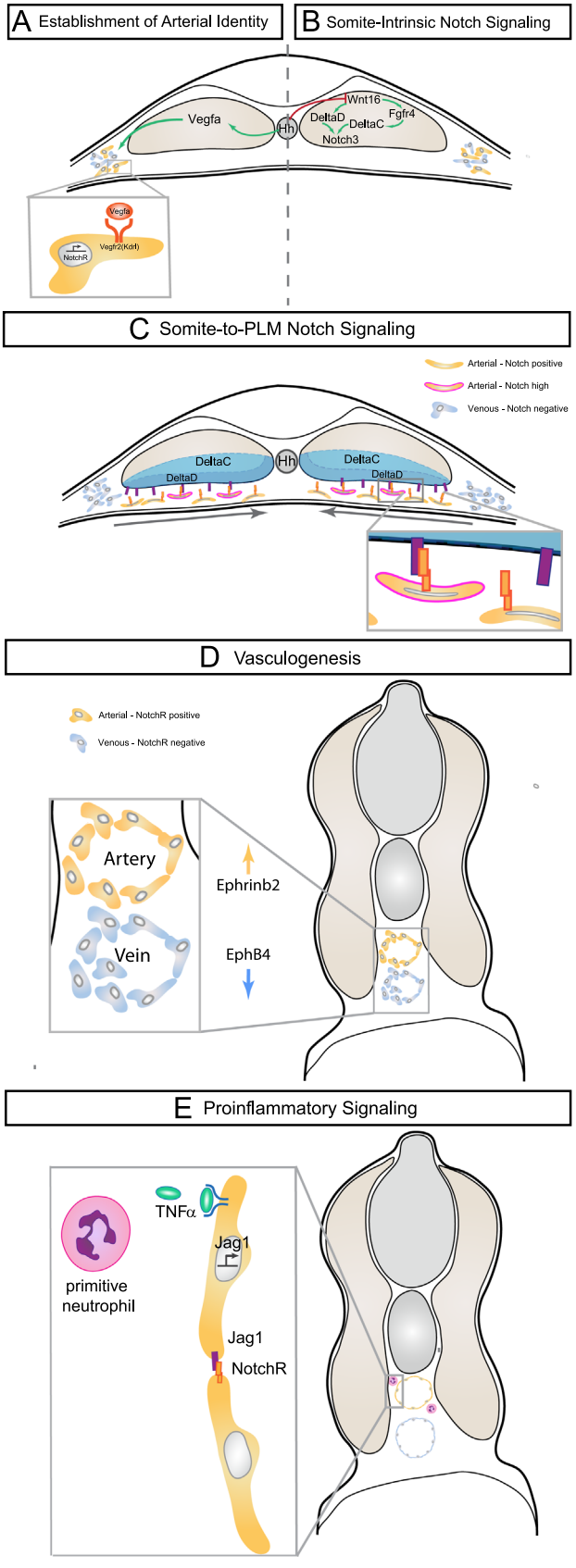
5. Notch signaling in arterial specification

Hematopoietic stem cells are produced from the major arterial vessels, most robustly from the ventral wall of the dorsal aorta, with additional contributions from the vitelline and umbilical arteries in the mouse (Jaffredo et al., 1998, 2000; Gordon-Keylock et al., 2013; de Bruijn et al., 2000). Early observations of the close association between the major arterial vessels and the earliest hematopoietic clusters (Jordan, 1917) and the determination that these regions are the first to generate HSCs, led to the long-standing hypothesis that arterial specification is a prerequisite to HSC formation (de Bruijn et al., 2000; Garcia-Porrero et al., 1995). Endothelial cells and HSCs share a common mesodermal origin across vertebrate species (Huber et al., 2004; Ciau-Uitz et al., 2010; Nishikawa et al., 1998; Pola et al., 2001). Within the endothelial lineage, the major arterial vessels in particular give rise to HSCs, suggesting arterial specification may be an intermediate step necessary for HSC formation. This idea has been bolstered by the lack of venous contribution to HSCs, and by shared requirements for Hedgehog, VEGF, and Notch signaling in both arterial specification and HSC emergence.

Mechanistically, work in zebrafish has shown that Hedgehog secreted from the notochord and floor plate stimulates the production of vascular endothelial growth factor a (Vegfa) from somitic tissues (Lawson et al., 2002). Both Hedgehog and VEGF signaling pathways are required for the migration of endothelial progenitors, lumenization during primary vasculogenesis, and arterial specification (Lawson et al., 2002; Williams et al., 2010; Vokes et al., 2004; Brown et al., 2000; Wilkinson et al., 2012; Cleaver and Krieg, 1998). Hedgehog and Vegfa both function upstream of Notch signaling in the specification of arterial identity (Lawson et al., 2001, 2002). Notch promotes arterial specification at least in part through the direct regulation of the arterial ligand *EphrinB2* (Grego-Bessa et al., 2007), which together with its venous receptor, EphB4, mediates the segregation of venous and arterial cells into distinct vessels by forward and reverse Eph/Ephrin transmembrane signaling (Adams et al., 1999, 2001; Foo et al., 2006; Wang et al., 1998; Herbert et al., 2009). Downstream of Hedgehog and Vegfa, Notch activation bestows both arterial identity (Gering and Patient, 2005; Herbert et al., 2009; Lawson et al., 2003) and HSC fate from hemogenic endothelium (Gering and Patient, 2005; Kim et al., 2013; Burns et al., 2009), placing Notch signaling as an essential downstream effector of this signaling axis (Fig. 2A).

Establishment of arterial fate begins far in advance of

vasculogenesis, initiating as early as the 5-somite stage in the zebrafish embryo (Quillien et al., 2014). In developing zebrafish embryos, the Hes-related transcription factor *hey2* promotes arterial fate determination within the early PLM, and loss of Hey2 results in severe defects in vasculogenesis and lack of arterial expression (Zhong et al., 2001; Weinstein et al., 1995; Zhong et al., 2000; Rowlinson and Gering, 2010). Although murine *Hey2* mutants have only mild vascular defects (Iso et al., 2003; Gessler et al., 2002), the combined loss of *Hey1* and *Hey2* results in severe defects in arterial specification and vascular morphogenesis, similarly to zebrafish *hey2* mutants and reminiscent of murine *Notch1*-null embryos (Fischer et al., 2004; Kokubo et al., 2005), suggesting a conserved role of Hey transcription factors in the establishment of functional vasculature.



In zebrafish, Hey2 functions downstream of Hedgehog and Vegfa, but its expression is maintained in *mindbomb* Notch signaling mutants (Lawson et al., 2001; Rowlinson and Gering, 2010). Hey2 is required for arterial expression of *notch1b* and *ephrinB2a*, and hemogenic endothelial expression of *runx1* and *cmyb* (Weinstein et al., 1995; Zhong et al., 2000; Rowlinson and Gering, 2010). Both arterial and hematopoietic defects in *hey2* morphants are rescued by restoration of Notch signaling, placing Hey2 as a key initiator of arterial specification in zebrafish (Rowlinson and Gering, 2010). Although Hey2 has not been reported to function upstream of Notch signaling in mice, the Hey2 transcriptional co-regulators Foxc1 and Foxc2 are required for expression of *Notch1*, *Notch4, Dll4, Jag1*, as well as *EphrinB2* (Seo et al., 2006; Hayashi and Kume, 2008), allowing for the possibility that Hey2 may act upstream of Notch signaling during the initiation of mammalian arterial specification.

Several ligands and receptors of Notch signaling are expressed in the dorsal aorta during the period of arterial and hematopoietic specification. Within the E9.5-10.5 aorta, at the time HSPC clusters are formed, the *Dll4*, *Jag1* and *Jag2* ligands and *Notch1* and *Notch4* receptors are arterially expressed (Robert-Moreno et al., 2008). Notch1 is essential for arterial specification, and is required cell-autonomously for HSC formation (Kumano et al., 2003; Hadland et al., 2004; Krebs et al., 2000, 2004). Although Notch4 is dispensable for normal arteriogenesis, it may provide some contribution to vascular development as *Notch1*/*Notch4* double mutants fail to form lumenized vessels and have a more severe phenotype than *Notch1* mutants alone (Krebs et al., 2000). *Dll4* expression is initiated within arterial cells downstream of Vegfa (Hayashi and Kume, 2008). Both homozygous and heterozygous *Dll4* mutants are embryonic lethal, with broad vascular defects prior to HSC emergence (Krebs et al., 2004; Duarte et al., 2004). In contrast, *Dll4* overexpression results in the ectopic expansion of arterial markers to the venous endothelium, including *Hey1*, *Notch1*, and *EphrinB2*, suggesting that Dll4 is sufficient to initiate the arterial Notch program within endothelial cells (Trindade et al., 2008).

Notch signaling itself is required for the expression of a number

Fig. 2. Multiple contributions of Notch signaling surrounding HSC specification in zebrafish embryos. (A) Establishment of Arterial Identity: Hedgehog secreted by the notochord stimulates the production of Vegfa from the somites, initiating the arterial program including Notch ligand and receptor expression. (B) Somite-Intrinsic Notch Signaling: Non-canonical Wnt ligand Wnt16 controls the pro-hematopoietic somitic expression of Notch ligands *deltaC* and *deltaD*. Fgf signaling downstream of Wnt16 is required for somitic *deltaC*, but not *deltaD* expression. Activation of the somitic Notch3 receptor, possibly via the DeltaC/D ligands, promotes HSPC specification, possibly by regulation of the organization or function of the sclerotome compartment. (C) Somite-to-PLM Notch signaling: During medial convergence of the posterior lateral plate mesoderm, direct cell contact allows for Notch signaling between DeltaC and DeltaD ligands on the ventral face of the somite and Notch receptors on migrating arterial cells. (D) Vasculogenesis: Direct arterial Notch target EphrinB2, together with venous EphB4, promotes arteriovenous segregation and the vasculogenesis. Formation of intact vasculature is required for the establishment of circulation and for circulation-dependent hemogenic endothelial maintenance. F. Proinflammatory signaling. Production of TNFα by primitive neutrophils signals through Tnfr2 to promote arterial expression of *jag1a*. Jag1a is required for HSPC formation, possibly through endothelial-intrinsic signaling with the Notch1a receptor.

of Notch ligands and receptors within the dorsal aorta. In mice, Notch signaling is required for arterial expression of the ligands *Jag1* and *Jag2*, and for expression of the *Notch1* receptor (Robert-Moreno et al., 2005), suggesting these factors might be regulated by a primary Notch signaling event within the endothelium. Similarly, Notch signaling is required for the expression of *deltaC* and *notch3* within the dorsal aorta of embryonic zebrafish (Lawson et al., 2001). The conserved regulation of Notch ligands and receptors by Notch signaling suggests that multiple, sequential Notch signaling events occur within the dorsal aorta.

Jag1 mutant embryos undergo normal arterial development, but within the dorsal aorta, both hematopoietic gene expression within the hemogenic endothelium and hematopoietic cluster formation are reduced (Robert-Moreno et al., 2008). *Notch1* and *Jag1* are expressed in a salt-and-pepper fashion within the arterial endothelium (Robert-Moreno et al., 2005). This finding, paired with the close proximity of *Jag1*- and *Notch1*-expressing cells, is suggestive of inductive signaling between adjacent endothelial cells. The stochastic development of Notch-sending and Notch-receiving cells within the hemogenic endothelium may be regulated by Sox17, which directly regulates *Notch1* through conserved binding sites in the *Notch1* promoter and has opposing effects on *Notch1* and *Jag1* expression (Clarke et al., 2013). Notch signaling through Jag1-Notch1 interaction results in direct transcriptional activation of *Gata2* within the hemogenic endothelium. However, the hematopoietic defect of *Notch1* mutants is more severe than the defect of *Jag1* mutants, suggesting involvement of additional Notch ligands (Robert-Moreno et al., 2005, 2008). Dorsal aorta formation and hematopoietic cluster formation appear normal in homozygous *Jag2* mutants (Robert-Moreno et al., 2008).

Finally, Dll1 maintains arterial identity through regulation of Neuropilin-1 (Nrp1) and the downstream responsiveness to VEGFA, but its expression in the endothelium initiates around E13.5, far after hematopoietic specification (Sorensen et al., 2009). While *Dll1* is not expressed within arterial endothelium at E10.5, it is expressed in cells dispersed throughout the subaortic region (Yoon et al., 2008). Coculture with Dll1-expressing OP9 cells is sufficient to rescue hematopoietic colony forming capacity of ligand-signaling deficient *Mib*−/− para-splachnopleuric explants (Yoon et al., 2008). Although these findings suggest non-endothelial cells near the aorta may contribute to pro-hematopoietic Notch signaling within the endothelium, it remains unclear whether Dll1 is important for HSC formation, or whether exogenous Dll1 is simply sufficient to rescue the function of a different ligand.

Similar to Notch, Vegfa is also required for both arterial and hematopoietic specification, and recent studies have demonstrated distinct contributions of Vegfa to these processes. Of three splice isoforms of Vegfa expressed during embryogenesis, the Vegfa short isoform, *Vegfa121*, is sufficient to rescue both *Hey2* and *runx1* expression following Hedgehog inhibition (Rowlinson and Gering, 2010). In Xenopus, somitic Eto2 is required for expression of the medium and long isoforms of *Vegfa*, that, while dispensible for vasculogenesis and arterial specification, are required for expression of the hemogenic endothelial markers *Runx1*, *Scl*, *Gata2*, and *Gfi1* (Leung et al., 2013). Similarly, knockdown of *eto2* in zebrafish inhibits formation of hemogenic endothelium without alteration to arterial expression of *deltaC*, *dll4*, *notch1b* and *notch3* (Leung et al., 2013). Therefore, rather than filling a single requirement, Vegfa makes both shared and distinct contributions to arterial specification and hemogenic endothelial patterning.

6. Is Notch key to the hemogenic potential of arterial endothelium

In a number of cases, the extension of arterial gene expression to venous endothelium confers expression of hematopoietic genes, and even hematopoietic cell emergence from venous vessels, providing some insight into the molecular mechanisms that confer hemogenic potential. In one example, Nrp1 acts as a co-receptor to VEGFR-2, enhancing the affinity and level of response to Vegfa within arterial endothelium (Becker et al., 2005; Soker et al., 1998). Expression of *Nrp1* is inhibited in the venous endothelium by COUP-TFII, which prevents a response to VEGF signaling in these cells (Gridley, 2007). Endothelial-specific deletion of COUP-TFII results in venous expression of arterial markers, including *Nrp1*, *Hey1*, *Jag1* and *Notch1*, as well the formation of ectopic *c-Kit*+ *CD45*+ hematopoietic clusters (You et al., 2005). Similarly, venous hematopoietic cluster formation accompanies the ectopic expression of arterial genes in *Alk1* and *Endoglin* mutants (Sorensen et al., 2003; Urness et al., 2000). In a third instance, constitutively activated Hedgehog signaling in zebrafish *ptc1;ptc2* mutants results in the expansion of arterial expression to the venous endothelium, and this is accompanied by ectopic *runx1* expression in the posterior cardinal vein (Wilkinson et al., 2012). Finally, whole-embryo activation of Notch signaling by heat-shock induction of NICD1 results in an expansion of both arterial and hemogenic endothelial expression in the posterior cardinal vein (Burns et al., 2005). Therefore, it is possible that the molecular specification of arterial endothelium creates a permissive environment for hematopoietic development by initiating the expression of Notch ligands and receptors essential to HSC formation.

Within the context of hematopoiesis, Notch signaling seems to be a unique element driving HSC potential. To this end, EMPs, which arise from endothelial cells similarly to HSCs, emerge independent of Notch signaling (Kumano et al., 2003; Hadland et al., 2004; Bertrand et al., 2010). Recently, HSCs and EMPs were suggested to arise from distinct endothelial populations, with expression of *Ly6A* differentiating aortic endothelial cells with HSC potential (Chen et al., 2011). *Jag1* mutants, which have normal arterial specification, but are deficient in HSC formation, fail to generate *Ly6a*-positive cells within the dorsal aorta (Robert-Moreno et al., 2008). Similarly, *in vitro* programmed hemogenic endothelium requires *Notch1* expression to maintain lymphoid potential, with Sox17-Notch-deficient cells generating only definitive erythroid and myeloid lineages (Clarke et al., 2013). Taken together, these reports indicate a key role for Notch signaling in establishing the hemogenic capacity of the dorsal aorta.

7. Somitic signaling contributes to hematopoiesis

Recently, the somites have become implicated as essential contributors to the hematopoietic specification process. The β-catenin-independent Wnt16 ligand is expressed within the somites and is required for HSC specification independently of arterial specification (Clements et al., 2011). Somitic expression of the Notch ligands *deltaC* and *deltaD* are downregulated following *wnt16* knockdown. Combined rescue of both ligands is sufficient to restore expression of hemogenic endothelial markers *runx1* and *cmyb* within the floor of the dorsal aorta, while neither alone is sufficient for rescue (Clements et al., 2011). Thus far it is not clear why both Notch ligands are needed within the somite. The DeltaC and DeltaD ligands interact homo- and heterophilically during somite segmentation (Wright et al., 2011). Although both ligands are required in this process, DeltaC is sufficient to permit Notch activation while DeltaD is not, suggesting that signaling occurs through DeltaC–DeltaC and DeltaC–DeltaD dimers (Wright et al., 2011). In HSC specification, it is unclear whether DeltaC and DeltaD function redundantly, as heterodimers, or independently with distinct functions.

The constitutive Hedgehog signaling that occurs in *ptc1;ptc2*

double mutants results in an increase in arterial endothelium and a concomitant 89% decrease in *runx1* expressing endothelial cells, due to a simultaneous increase of *Vegfa* expression and decrease of *wnt16*, *deltaC* and *deltaD* expression within the somites (Wilkinson et al., 2012).

Recent studies in the zebrafish embryo have demonstrated dynamic regulation of HSC specification by Fgf signaling, which first promotes hematopoietic specification through somitic signaling at 14–17 h post fertilization, and then from creating a restrictive environment for HSC emergence from 20.5 h, after the formation of the vascular cord (Lee et al., 2014; Pouget et al., 2014). Within the early, positive window, Fgf signaling regulates somitic expression of *deltaC*, but not *deltaD*, through the Wnt16-dependent Fgfr4 receptor (Lee et al., 2014), indicating distinct regulation of *deltaC* and *deltaD* downstream of somitic Wnt16.

Two somitic Notch signaling events are required for HSC formation within the zebrafish embryo: somite-intrinsic Notch signaling and somite-to-PLM Notch signaling (Fig. 2). Interaction between the migrating PLM cells and the ventral surface of the somite is mediated by the junctional adhesion molecule Jam1a, on PLM cells, and its binding partner Jam2a on somitic cells (Kobayashi et al., 2014). Direct contact between the somite and PLM is required for efficient Notch signal transduction to the PLM. When the function of either *jam1a* or *jam2a* is lost, reduced Notch signal transmission from the somite to the PLM disrupts hemogenic endothelial programming, whereas arterial differentiation occurs normally. The hematopoietic defect resulting from insufficient somite–PLM contact can be partially rescued by the provision of exogenous DeltaC, however it is fully rescuable by DeltaD (Kobayashi et al., 2014). Given the inability of DeltaD to individually activate Notch signaling during somite segmentation, it is possible that DeltaD may signal more effectively to the adjacent PLM.

Gata2 is a direct transcriptional target of Notch signaling and is required cell-autonomously for HSC formation prior to the endothelial–hematopoietic transition (Kumano et al., 2003; Hadland et al., 2004; Robert-Moreno et al., 2005; de Pater et al., 2013). In zebrafish, the Gata2 homolog Gata2b is specifically required for HSC formation (Butko et al., 2015). Expression of *gata2b* initiates in the vascular cord during PLM convergence, and is dependent upon both Wnt16 and endothelial Notch receptors, suggesting that somite-to-PLM Notch signaling directly specifies hemogenic endothelium through Gata2b function.

An in-depth analysis of the tissue-specific requirements for the various Notch receptors recently revealed that Notch signaling must be activated both within the endothelium and the somites in order for HSC specification to proceed normally (Kim et al., 2014). Of the four Notch receptors in the zebrafish embryo, Notch1a, Notch1b, and Notch3 are independently required for HSC formation, whereas Notch2 is not required. Whereas the Notch1 homologs Notch1a and Notch1b function directly within the endothelium, Notch3 is required within the somite and regulates HSC formation in a non cell-autonomous manner. Notch3 functions epistatically downstream of Wnt16, and combinatorial low-level knockdowns indicate *notch3* is required synergistically with *deltaC* and *deltaD*, suggesting that Notch3 may be activated within the somite by one or both of these ligands (Fig. 2B). While it is still unclear how somite-intrinsic Notch activation affects HSC emergence, both Wnt16 and Notch3 are required for the proper formation of the sclerotomal compartment of the somite. The transcription factor Pax9, which marks the sclerotome (Nornes et al., 1996), was recently shown to be important for HSPC emergence. Knockdown of *pax9* results in the decreased generation of *cmyb*-positive cells within the dorsal aorta (Charbord et al., 2014), further supporting a contribution to HSC generation by the sclerotome. Although several lines of evidence have indicated the importance of sclerotome for HSPC specification, the exact contribution this tissue makes is not known. One likely explanation is that correct somite morphology or organization is necessary for effective transmission of Notch signaling from the somite to the PLM. It has also been proposed that Notch3-activated somitic cells may interact directly with the hemogenic endothelium following dorsal aorta formation, either by incorporating into the aortic wall or giving rise to the smooth muscle precursors that envelop the dorsal aorta. In chicken embryos, somite derived cells incorporate into the aortic endothelium in a Notch-dependent manner (Pouget et al., 2006; Ohata et al., 2009; Sato et al., 2008). This process occurs in the period leading up to HSC emergence, and it is conceivable that these cells may relay an unknown hematopoietic inductive signal. In both mice and chicken embryos, the sclerotome gives rise to vascular smooth muscle cells that directly surround the aorta (Pouget et al., 2008; Wasteson et al., 2008). However, sclerotome-derived smooth muscle cells are not required for aortic hematopoiesis (Richard et al., 2013), making it unlikely that the sclerotomal contribution occurs through this population.

8. Proinflammatory signaling and HSC emergence

Although embryonic development occurs in a protected environment under relatively sterile conditions, HSC formation is preceded by a transient wave of functional primitive myeloid cells. Recently, inflammatory signals produced by these primitive myeloid cells were found to be critical for the establishment and expansion of HSPCs, in part through the regulation of the Notch pathway.

Proinflammatory signaling pathways are heavily activated within HSPC clusters associated with the mouse dorsal aorta (Li et al., 2014). The innate inflammatory cytokine *Interleukin-1* is expressed in the dorsal aorta and budding hematopoietic cells, and promotes the activity of nascent HSPCs (Orelio et al., 2008). Types I and II Interferon produced by primitive myeloid cells activate innate inflammatory signaling within the hemogenic endothelium (Li et al., 2014). Interferon signaling is required for the formation and expansion of HSPCs, whereas EMP formation occurs independently of this signal in both mice and zebrafish (Li et al., 2014). Combined knockdown of *tnfa* and *ifng* results in more severe reduction of *runx1* expression than loss of either cytokine individually, suggesting cooperative action by multiple proinflammatory cytokines in HSC formation (Li et al., 2014). Tnf, in particular, is provided by primitive neutrophils, and signals through Tnfr2 to activate expression of the Notch ligand *jag1a* in the aortic endothelium (Espin-Palazon et al., 2014). Similar to the requirement for Jag1 in mice, zebrafish Jag1a is required for HSC formation but not arterial specification (Robert-Moreno et al., 2008; Espin-Palazon et al., 2014). Of the two Notch1 homologs, low level knockdowns of *jag1a* with *notch1a* results in a more severe downregulation of hematopoietic expression, while combined knockdown of *jag1a* with *notch1b* does not. This suggests that Jag1a may signal through the Notch1a receptor to promote the formation and expansion of HSCs (Fig. 2D). Interestingly, aortic expression of *Jag1* is dependent on Notch signaling in mice (Robert-Moreno et al., 2005). If *Jag1* regulation is conserved, it is possible that it may represent an intersection of successive hematopoietic Notch requirements.

9. Downregulation of Notch signaling in emerging HSCs

As discussed previously, multiple Notch signaling events positively regulate HSC specification. Notch transcriptional targets, including Gata2, Hes1, Hrt1 and Hrt2 are expressed in the

endothelium and budding clusters within the dorsal aorta (Guiu et al., 2013; Robert-Moreno et al., 2005; Fischer et al., 2004). Recent evidence from mice suggests that Notch signaling negatively regulates HSC emergence following the specification of hemogenic endothelial cells and must be down regulated for HSC emergence to progress. VE-Cadherin:Cre mediated permanent induction of *NICD1* in the endothelium and endothelial-derived cells results in a surprising lack of hematopoietic clusters within the dorsal aorta in E10.5 embryos, and a deficiency in fetal liver colonization by Notch-active cells (Tang et al., 2013). Although total loss of Notch signaling inhibits HSC specification, deletion of either Notch1 or Notch1-regulator Sox17 from E9.5 mouse AGM explants increases the hematopoietic output of the dorsal aorta (Lizama et al., 2015). Concordantly, late chemical inhibition of Notch signaling inhibition results in increased production of CD45+ cells in both mouse para-splanchnopleura explants and in avian embryos (Richard et al., 2013). Chemical inhibition of Notch signaling also accelerates hematopoietic cluster formation, suggesting that Notch actively delays the onset of the budding process. However, this Notch inhibition-mediated hematopoietic expansion is transient, with newly formed cells undergoing apoptosis under continued Notch inhibition (Richard et al., 2013).

During normal hematopoietic cluster formation in both mouse and chicken embryos, Notch activity is downregulated specifically in hematopoietic clusters, but maintained in the surrounding aortic endothelium (Richard et al., 2013; Del Monte et al., 2007). This drop in Notch activity may result from loss of Sox17, which promotes Notch1 expression in arterial endothelium while actively repressing expression of hematopoietic transcription factors Runx1 and Gata2 (Lizama et al., 2015). The expression of VE-Cadherin and Dll4 are also downregulated in hematopoietic clusters relative to the aortic endothelium (Richard et al., 2013), suggesting that hematopoietic clusters experience a general loss of arterial and endothelial identity during EHT. Although both Notch1 and Notch4 are expressed in the murine dorsal aorta, only Notch1 must be downregulated, whereas enforced activation of Notch4 signaling by ectopic NICD4 impairs vascular remodeling but does not inhibit hematopoiesis (Tang et al., 2013). Consistent with this, the Notch1 target Hes1 is progressively downregulated in emergent murine HSPCs as they mature (Richard et al., 2013), indicating a requirement for down-regulation of the Notch signal for HSC emergence.

The downregulation of Notch activity in chicken embryos is directly observable by the reduced fluorescence of the Tp1:Venus Notch reporter in budding hematopoietic clusters (Richard et al., 2013). In zebrafish embryos, Tp1:GFP is expressed in arterial endothelium in advance of HSC formation, and is not downregulated during HSPC budding (Fig. 3). However, epistatic studies in zebrafish have recently suggested that the requirement for Notch downregulation may be conserved in this system. In zebrafish embryos, Cfos acts genetically upstream of the Notch-mediated maintenance of *dll4* and *ephrinb2a* following arterial specification (Wei et al., 2014). Deacetylation of *cfos* by the transcriptional repressors Ncor2 and Hdac3 is required for HSC emergence, suggesting that the formation of HSCs may be tied to the down-regulation of arterial identity in zebrafish as well (Wei et al., 2014). In zebrafish, it is likely that HSPC emergence proceeds too quickly to yield a noticeable decrease in Tp1:GFP fluorescence. *In vivo* visualization of this process may instead require new tools allowing for the detection of Notch receptor activation in real time.

Tight regulation of the response to Notch1 receptor-mediated signaling is provided by a type-1 incoherent feed forward loop (Guiu et al., 2013). To this effect, Notch1 simultaneously controls expression of transcriptional activators, such as *Gata2*, and transcriptional repressors, such as *Hes1*. Following simultaneous activation of both *Gata2* and *Hes1*, Hes1 provides direct inhibition of *Gata2* expression, resulting in a restricted pulse of the positive hematopoietic regulator. Loss of *Hes1*, combined with a loss of *Hes5*, results in the formation of large hematopoietic clusters that lack functionality (Guiu et al., 2013). This phenotype is reminiscent of the robust, accelerated formation of non-functional hematopoietic clusters formed under Notch inhibition, suggesting that Notch-dependent repression of cluster formation occurs via Hes1 and Hes5. Repression of premature cluster formation is likely a hematopoietic-specific process, as Hes1 and Hes5 are dispensable for arterial differentiation (Guiu et al., 2013) and late

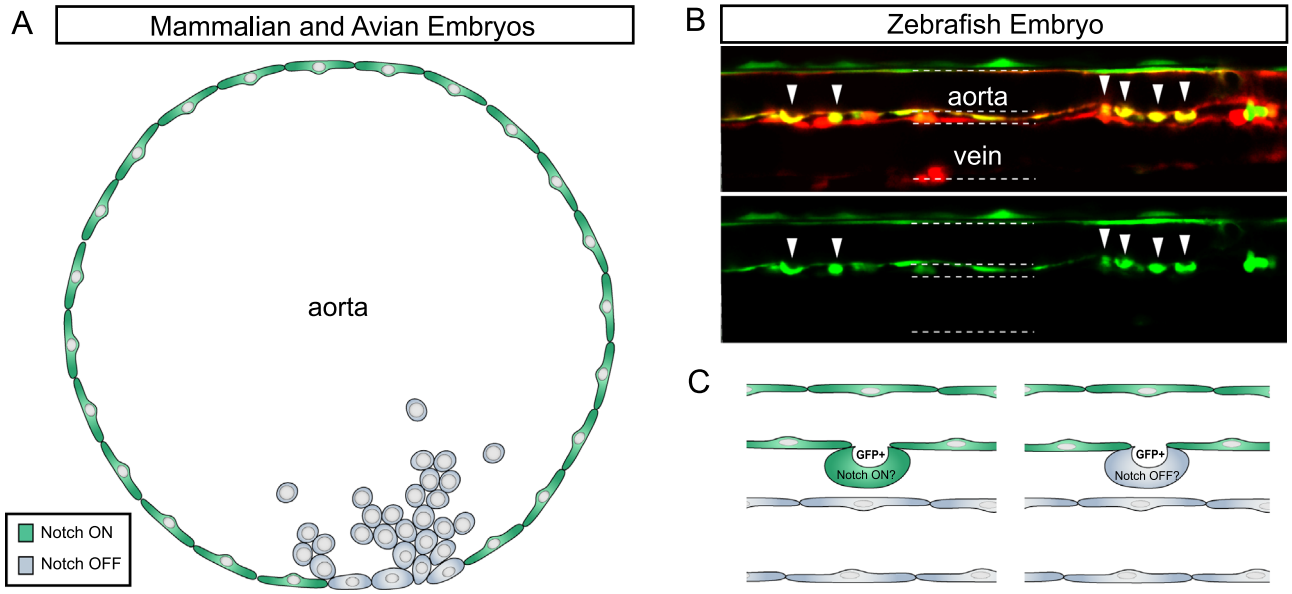


Fig. 3. Notch activity in budding hematopoietic cells. (A) Notch signaling is inactivated in the budding hematopoietic clusters of higher vertebrates. Green coloring indicates cells in a "Notch-on" active Notch signaling state. Gray coloring indicates cells in a "Notch-off" inactive Notch signaling state. (B) Confocal microscopy of HSPC budding in transgenic zebrafish. *Kdrl:Cre; bactin:DsRed* labels vascular and vascular-derived cells, and *Tp1:GFP* labels cells responsive to Notch activity. White arrows indicate cells undergoing EHT. Notch signaling is active throughout the dorsal aorta, and budding HSPCs have high levels of Notch reporter GFP at 48hpf. (C) Due to the rapid development of the zebrafish embryo, it remains unclear whether Tp1:GFP+ budding HSPCs are truly Notch-active, or whether these cells have inactivated Notch signaling while the GFP protein persists.

Notch inhibition does not induce hematopoietic clusters in the vein (Richard et al., 2013).

10. Post-emergence hematopoietic contributions of Notch signaling

Although Notch activity must be downregulated in order to form hematopoietic clusters that contain functional HSPCs, Notch signaling is required in multiple capacities post-emergence. Notch signaling has a well-characterized role in lymphocyte development (Radtke et al., 2013). Additionally, a novel role for Notch signaling was recently identified in HSPCs following emergence from the dorsal aorta region. Notch1 hypomorphs successfully generate functional HSCs that seed the fetal liver, however, these cells have impaired reconstitutive capacity. Similarly, conditional deletion of RBPjK following the endothelial to hematopoietic transition results in similarly impaired competitive reconstitution. Together, these results suggest a critical, cell-autonomous requirement for Notch signaling that occurs post-emergence, although the underlying molecular mechanism(s) has not been defined. Within the bone marrow niche, Notch signaling induced by endothelial Jag1 balances the quiescence and self-renewal of long-term (LT) HSCs, thereby preventing exhaustion of the LT-HSC population (Poulos et al., 2013). Similarly, Notch activation restricts differentiation of primary cord-derived hematopoietic cells while maintaining normal levels of proliferation (Carlesso et al., 1999). It is conceivable that Notch signaling may have a similar role in maintaining a steady pool of the nascent HSC population.

11. Conclusion

HSCs are formed through a complex process that has yet to be fully recapitulated *in vitro*, suggesting that we still do not understand all requisite aspects of their development. Although Notch signaling has been known to be an essential component for HSC generation, recent studies suggest that it makes a far more complex contribution than was previously appreciated. Leading up to HSC specification, Notch signaling promotes the proliferation of early endothelial cells. In establishing the arterial program, Notch signaling makes two important contributions to the establishment of HSCs. First, it allows proper morphological development of the vasculature, permitting the establishment of circulation, which is necessary for HSC development, and second, it promotes the expression of Notch receptors and ligands that are subsequently required for HSC development. Somite-intrinsic Notch signaling establishes the sclerotomal compartment of the somite, which makes an essential, but poorly understood contribution to HSC specification (Kim et al., 2014). Our current understanding suggests the sclerotome presents Notch ligand(s) to the shared vascular precursors of HSCs as they migrate across the ventral face of the somites to form the vascular cord at the embryonic midline. In this case, somite-intrinsic and arterial Notch signaling act hierarchically upstream of the somite-to-PLM signal, on the signal-sending and signal-receiving cells, respectively. An additional Notch ligand, Jag1, is induced by proinflammatory signaling and activates Notch1 in an endothelial-intrinsic manner (Robert-Moreno et al., 2008; Espin-Palazon et al., 2014). The number of Notch signaling events at present represents a puzzle, as we do not yet understand how the multiple intrinsic Notch signals (arterial, somite-to-PLM, and interendothelial) differ. Do these distinct Notch signaling events activate different downstream targets, and how is this achieved? Do they instead raise the overall level of Notch signaling, allowing for greater target activation? In zebrafish, where the two involvements of the somite have been described, three of the four Notch receptors are required for hematopoiesis (Kim et al., 2014), and there is some separation of ligands and receptors involved in arterial specification, somitic signaling, and interendothelial Notch signaling. However, thus far in mice, only Notch1 appears essential to the establishment of HSCs. It is not yet clear whether somite-intrinsic and somite-to-PLM Notch signaling events are conserved in mammalian species, and it is also unclear whether Notch1 mediates each signaling event or whether other receptors may be involved. Further studies focused on ligand–receptor specificity, downstream targets, and effects of Notch signaling level on hematopoiesis are necessary. Furthermore, the need for Notch downregulation within budding hematopoietic clusters is just beginning to be appreciated. Defining the molecular mechanisms of Notch shutdown, and the reason it is required for HSC establishment, may inform future in vitro HSC derivation strategies.

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