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Discrete Notch signaling requirements in the specification of hematopoietic stem cells

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by

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ABSTRACT OF THE DISSERTATION

Discrete Notch signaling requirements in the specification of hematopoietic stem cells

by

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Hematopoietic stem cells (HSCs) are generated during embryonic development and possess the ability to reconstitute all adult blood lineages and self-renew for the life of an organism. Despite efforts to generate HSCs *in vitro* in a manner that recapitulates embryonic development, all attempts to date have failed indicating that our knowledge of this process is incomplete. Cell signaling pathways are crucial for the formation of HSCs during embryonic development. Understanding the requirements for cell signaling are complicated by the fact that there is crosstalk

between pathways during development, necessitating a clear understanding of these relationships. Notch signaling is essential for HSC formation, as evidenced by the fact that many Notch pathway proteins are required for this process including the specific Notch1 receptor that functions cell-autonomously in mouse. Additionally, recent data from the zebrafish indicate that Notch signaling downstream of the non-canonical Wnt protein Wnt16 is also required in the somitic environment for HSC and sclerotome specification, suggesting that these two developmental events are related. We asked if the remaining Notch receptors perform any role in HSC emergence, here we present evidence that Notch3 is required in zebrafish. Tissue and temporal-specific activation of Notch intracellular domain (NICD) rescue experiments in Notch3 knockdown embryos indicate that Notch3 is required in somitic tissues for the generation of HSCs and sclerotome well before establishment of the HSC program, conversely Notch1 homologues Notch1a and Notch1b are required in the endothelium for dorsal aorta (DA) and HSC specification just before HSCs are generated. We identified that the function of Notch3 lies downstream of Wnt16, Dlc, and Dld in a linear genetic pathway. In contrast, endothelial expression of Notch1b requires the activity of Pdgfra receptor in a distinct signaling cascade from Wnt16-Notch3. Collectively, these findings demonstrate that multiple inputs from the Notch pathway are required at different times and places during development, with distinct inputs regulated by specific signaling pathway.

Introduction

Cell signaling pathways involved in hematopoietic stem cell specification

Generation of HSCs for regenerative medicine

Hematopoietic stem cells (HSCs) are self-renewing, tissue-specific stem cells that give rise to all mature blood cell types. The capacity of HSCs to reconstitute the entire adult hematopoietic system after transplantation makes them invaluable for the treatment of various blood disorders. A significant limitation of this treatment is the need for immune compatibility between donor and host, thus there has always been an acute need for reliable cultivation or generation of HSCs. The recently demonstrated ability to generate induced pluripotent stem (iPS) cells that resemble embryonic stem cells (ESCs) now make generation of HSCs from stem cells a realistic goal. To date, pluripotent stem cells have been instructed by a variety of experimental approaches to recapitulate waves of hematopoiesis such as primitive and transient definitive cells (Sturgeon et al, 2014), myelomonocytic cells (Choi et al, 2009), and multilineage progenitors with lymphoid potential (Kennedy et al, 2012) (Figure 1A). Surprisingly, concerted efforts to generate functional HSCs *in vitro* from pluripotent stem cells have thus far proven unsuccessful, indicating that our understanding of de novo generation of HSCs is insufficient (Murry & Keller, 2008) (Figure 1B). Therefore, it is crucial to precisely characterize the mechanisms of cell signaling events that occur *in vivo* to form functional HSCs. Importantly, recent studies mapping the process of HSC generation in vertebrate embryos demonstrated that HSCs emerge from hemogenic

endothelium present in the floor of the dorsal aorta (DA) (Bertrand et al, 2010a; Boisset et al, 2010; de Bruijn et al, 2000; Kissa & Herbomel, 2010; Zovein et al, 2008). For this reason, the generation of hemogenic endothelium likely represents a critical prerequisite for successfully generating HSCs *in vitro*. While many major cell-signaling pathways conserved throughout the animal kingdom have been demonstrated as requirements for DA and/or HSC formation, the molecular mechanisms that each required effector molecule exerts in this context is unclear. In this chapter we summarize the roles of select cell-signaling pathways in HSC generation in the embryo and provide perspective on the in vitro instruction of HSCs fate for use in regenerative medicine.

Hematopoietic stem cell emergence in the vertebrate embryo

The HSCs that maintain homeostasis of the adult hematopoietic system are generated during embryogenesis, but are not the first blood cells to be formed in the embryo. HSC emergence is preceded by primitive and definitive waves that are defined by limited differentiation potentials. Primitive myeloid and erythroid cells are the first hematopoietic cells to emerge, but unlike adult blood progenitors, do not possess multilineage potential or the capacity to self-renew (Palis et al, 1999; Tober et al, 2007) (Figure 1C). Following these primitive waves, the first transient definitive progenitors arise that possess multipotent erythromyeloid potential (EMPs) (Bertrand et al, 2007; Chen et al, 2011; Palis et al, 1999). EMPs are similar to HSCs in that they have multilineage potential, but are separated by the fact that they do not possess

lymphoid potential or the capacity to appreciably self-renew (Figure 1D). The anatomical sites of emergence from which these waves arise vary according to species as shown by transplantation, imaging, and lineage tracing studies. The first three hematopoietic waves are found in the yolk sac in mammals and birds, in anterior/posterior ventral blood islands in frogs, and anterior/posterior lateral mesoderm in fish, the details of which are reviewed elsewhere (Chen & Zon, 2009; Ciau-Uitz et al, 2014). In contrast, HSCs emerge from hemogenic endothelium within the floor of the dorsal aorta in a process termed endothelial to hematopoietic transition (EHT) in all vertebrate species analyzed (Bertrand et al, 2010b; Boisset et al, 2010; Kissa & Herbomel, 2010; Zovein et al, 2008) (Figure 1E). Nascent HSCs have been defined by their capacity in mammals to long-term reconstitute immune-deficient adult recipients and colonize adult hematopoietic organs (Medvinsky & Dzierzak, 1996; Muller et al, 1994). Developmental hematopoiesis thus progresses through four ordered waves, the last of which generates HSCs.

A major question regarding the ontogeny of the hematopoetic system is how different regions of mesodermal derivatives are specified into each of these related but distinct fates. Transplantation experiments performed in frogs demonstrate that mesodermal precursors to blood cells experience bipotency for either primitive or definitive fate potential until the neurula stage (Turpen et al, 1997), indicating that environmental signals are important during key stages of programming to different fate outcomes. Many of the signaling molecules involved in HSC specification are dispensable for other hematopoietic waves, indicating that the combined inputs that

bestow HSC potential may be specific. Here we highlight the major signaling pathways involved in HSC emergence that are conserved across vertebrates.

Bmp Signaling

Bmp signaling is part of the transforming growth factor-β (TGFβ)(Choi et al, 2009) superfamily that regulates many cellular processes and fate decisions during early embryonic development. Bmps signal through Type I and II receptors that heterodimerize in response to ligand binding. As activated complexes, these proteins phosphorylate Smad proteins that regulate the expression of a multitude of genes (Schmierer & Hill, 2007). Bmp is required for embryogenesis during gastrulation and for the specification of mesoderm (Mishina et al, 1995; Winnier et al, 1995), but has also been implicated in a later role in HSC emergence. Bmp4 is expressed in the mesenchyme surrounding the developing dorsal aorta, and antisense knockdown leads to a loss of HSCs in zebrafish (Wilkinson et al, 2009), suggesting it is a key determinant of HSC fate. In mammals, chemical inhibition of Bmp signaling reduced HSC numbers contained within the aorta-gonads-mesonephros (AGM) region, indicating that this requirement for Bmp4 is conserved in vertebrates (Durand et al, 2007). In addition, mouse AGM explants that contain nascent HSCs were enhanced for repopulating potential following the addition of Bmp4 (Durand et al, 2007). These results suggest that Bmp promotes the generation, homing efficiency, and/or survival of HSCs. Downstream effectors of Bmp signaling, Smad1 and Smad5, are expressed in tissues around the sites of HSC emergence (Blank et al, 2008). Endothelium-

specific inactivation of Smad1/5 results in embryonic lethality in mice before HSC specification (Moya et al, 2012), but recent studies in zebrafish demonstrate that low-dose knockdown of Smad1/5 that bypass early embryonic requirements have specific defects in HSC formation (Zhang et al, 2014). Specific genetic excision of Smad1 and Smad5 in specified blood had no effect on hematopoiesis (Singbrant et al, 2010), suggesting that Bmp signaling is dispensable after HSC commitment. These findings position the requirement for Bmp signaling during two distinct time windows during differentiation; first during mesoderm commitment, and later just prior to HSC specification in the local HSC microenvironment (Figure 2A).

Hedgehog Signaling

Hedgehog (Hh) signaling is involved in a wide range of activities during development that are conserved throughout the animal kingdom, including axis and segment patterning of the vertebrate body plan. Hh binds to the Patched transmembrane receptor (Ingham et al, 1991), that in the absence of ligand-binding, inhibits a required transmembrane signal transducer Smoothened (Ingham et al, 1991). Uninhibited Smoothened is then free to activate the zinc-finger transcription factor Cubitus interruptis (Ci) that becomes phosphorylated and available to interact with and activate a wide range of kinase pathways, ultimately translocating to the nucleus to activate expression of Hh target genes (Dominguez et al, 1996). In mammals, the loss of Sonic Hedgehog (Shh) or Smoothened results in embryonic lethality. However, murine AGM explants cultured with exogenous Hh generated HSCs with increased

transplantation efficiency (Peeters et al, 2009). In zebrafish, the notochord and floor plate are the main sources of Hh ligand during the time window of HSC specification, suggesting that these tissues play important signaling roles during HSC formation. Importantly, genetic loss of function or chemical inhibition of Hh signaling by cyclopamine results in a specific loss of HSC specification in the DA (Gering & Patient, 2005; Wilkinson et al, 2009). As demonstrated by epistatsis experiments, Hh signaling is genetically upstream of vascular endothelial growth factor (Vegf) signaling, which controls Notch activation in the endothelium (Lawson et al, 2002) (Figure 2B). Collectively, these studies indicate that Hh signaling is a key regulator of an essential signaling cascade responsible for vascular patterning and the subsequent generation of hemogenic endothelium.

Vegf Signaling

Unlike the previous pathways mentioned, Vegf signaling is required after gastrulation and axis formation and exclusively by endothelial cells. There are four Vegf ligands (VegfA, B, C, and D), and multiple known isoforms of VegfA are produced by alternative splicing, which play distinct roles in regulating proliferation, migration, survival, and/or permeability (Houck et al, 1991; Tischer et al, 1991). Vegf ligands bind with variable affinity to the Vegf receptor tyrosine kinases, Vegfr-1, 2, or 3, leading to the hetero- or homo-dimerization of receptors and subsequent activation by autophosphorylation (Cebe-Suarez et al, 2006). In mice, the loss of a single Vegf allele results in severe vascular defects, resulting in embryonic lethality before or

during HSC specification (Carmeliet et al, 1996; Shalaby et al, 1995). In zebrafish, the Vegf receptor Kdrl is expressed throughout the vasculature and genetic or pharmacological inhibition results in the combined loss of DA and HSC specification (Gering & Patient, 2005; Lawson et al, 2002). Studies in Xenopus elucidated that VegfA is required for HSC formation through multiple inputs; longer $VegfA_{170}$ and $VegfA_{190}$ isoforms are required for HSC specification but dispensable for DA specification, whereas the shorter diffusible $VegfA_{122}$ isoforms lacking extracellular matrix-binding domains are required for both processes, as shown by analysis of isoform-specific mutants (Leung et al, 2013). In addition, VegfA production in lateral plate mesoderm and somitic tissues is dependent upon the transcriptional activity of Tel1. Dorsal lateral plate (DLP) mesoderm that normally give rise to HSCs *in vivo* are capable of hematopoiesis *in vitro* when co-cultured with wild-type somites, but Tel1-deficient somitic cells that do not secrete VegfA are deficient in promoting hematopoiesis from wild-type DLP (Ciau-Uitz et al, 2010). This data is in agreement with the observation that VegfA is produced in the somites of zebrafish (Lawson et al, 2002). Thus, Vegf signaling is important for the formation of the DA and HSCs from endothelial precursors (Figure 2C).

Notch Signaling

Notch signaling is a cell-to-cell signaling pathway involved in a wide range of cellular fate decisions including lineage commitment, lateral inhibition between neighboring cells, and maintenance of homeostasis (Lai, 2004). Key proteins involved

in Notch signaling include Notch receptors (Notch1, Notch2, Notch3, and Notch4 in mammals), their cognate Jagged/Delta ligands that vary in number across species, enzymes that modify Notch ligands during activation (Mindbomb), proteases that cleave activated receptors (gamma secretase/ADAM TACE) to release a transcriptionally active Notch intracellular domain (NICD), as well as an array of intracellular proteins that facilitate transcriptional repressive (RBPj/CSL) and/or activating complexes (Mastermind and Mastermind-like) [reviewed in depth in (Kopan & Ilagan, 2009; Lai, 2004). Many Notch signaling pathway proteins are required for HSC specification. Loss of Mindbomb and RBPj, both of which are essential for Notch signaling, leads to loss of HSCs in developing embryos (Burns et al, 2005; Robert-Moreno et al, 2005; Yoon et al, 2008). Additionally, the Notch1 receptor is required in a cell-autonomous manner to specify HSCs as shown by blastula chimera experiments in (Hadland et al, 2004; Kumano et al, 2003); mouse mutants also display vascular and aortic defects (Krebs et al, 2000). The necessity for Notch1 in both of these processes may reflect a dual requirement for Notch, since many studies have implicated, but not directly shown, that DA specification is a functional prerequisite for HSC specification. Unlike Notch1 mutants, mutants for the Notch ligand Jagged1 are not defective in DA formation but similarly fail to specify HSCs, suggesting that there are likely multiple requirements for Notch signaling in HSC specification (Robert-Moreno et al, 2008) (Figure 2D). Recently our laboratory has uncovered, through loss of function and spatiotemporally-controlled NICD rescue experiments, that Notch3 is required in the somites to specify HSCs (Kim et al, 2014b). This non-cell-autonomous requirement is genetically downstream of a

previously indentified Wnt16 regulated somitic signaling cascade (Kim et al, 2014b) (Figure 2E). Collectively these findings indicate that Notch signaling orchestrates intrinsic as well as environmental programs to instruct HSC fate.

Wnt Signaling

Canonical Wnt signaling is involved in the specification and homeostasis of many tissues. In mammals, the Wnt pathway is comprised of 19 secreted ligands that directly associate with Frizzled receptors and co-receptors expressed on the surface of many diverse cell types (Bhanot et al, 1996; Yang-Snyder et al, 1996). In the absence of ligand binding, b-catenin is normally targeted for degradation by a 'destruction complex' of proteins (Aberle et al, 1997; Gao et al, 2002). However, upon the ligand-induced activation of Wnt receptors, this protein complex is inactivated and b-catenin translocates to the nucleus to bind the TCF/LEF transcription factors that activate target gene transcription (Angers & Moon, 2009). In mice, genetic deletion of b-Catenin in VE-Cadherin^{+} endothelium results in hematopoietic defects, but has no effect when genetically deleted in Vav1^{+} committed blood cell precursors, suggesting that this requirement for Wnt signaling is in cells during or just before they become hemogenic endothelium (Ruiz-Herguido et al, 2012). Interestingly, in this context arterial specification is unaffected in the endothelium of mutant embryos, suggesting that Wnt signaling is dispensable for the aortic program. In contrast, gain-of-function studies demonstrate that over-activation of b-catenin results in an upregulation of

arterial markers. These data indicate that Wnt signaling plays discrete roles in HSC and arterial fates (Figure 2F).

Interaction between Signaling Pathways

The fact that specific molecules from Bmp, Hedgehog, Vegf, Notch and Wnt signaling pathways are required for HSC formation during development raises important questions about when and where each is required, and regarding interactions between pathways. The expression pattern of most of these required molecules is dynamic, suggesting that location and timing of these signals is tightly regulated and related to their functional roles. The fact that diffusible Hh and Vegf are secreted from tissues physically separated from the DA, while direct cell-to-cell contact through Notch and Jagged1 occurs proximal to the DA, suggest that these diverse signals must be initiated and received at specific spatial locations during ontogeny (Compare Figure 2C to 2D). Additionally, timing is an important consideration, as evidenced by the requirement for non-canonical Wnt16 function in the regulation of somitic expression of DeltaC/DeltaD in zebrafish (Clements et al, 2011). The failure to specify HSCs in Wnt16-deficient embryos can be rescued by ectopic activation of Notch signaling during mid-somitogenesis, despite the fact that HSCs are not specified for many hours afterwards (Figure 2E). This surprising inflexibility in the timing of this molecular requirement may be attributed to coordination between multiple signaling pathways. Canonical Wnt signaling also interacts with other signaling pathways to specify HSCs, as demonstrated by the required for prostaglandin via a b-catenin-dependent

mechanism (Goessling et al, 2009) (Figure 2F). It is likely that other signaling pathways are utilized iteratively for HSC specification at multiple sites and/or times during ontogeny that trigger different genetic, cellular, and morphogenetic outcomes.

Conclusion

While only a subset of the known signaling requirements in HSC specification have been discussed in this review, many have been utilized in attempts to recapitulate hemogenesis *in vitro*. Wnt, Bmp, Vegf, and Notch have been utilized in combination with other factors in well-defined supportive conditions *in vitro* to generate multilineage blood cell precursors from pluripotent stem cells. These approaches have not yet, however, led to generation of engraftable HSCs (Kennedy et al, 2012; Rafii et al, 2013; Sturgeon et al, 2014). One possible explanation for these difficulties is that the growth conditions utilized are missing key signals and/or that some of these signals are provided in an inappropriate context for HSC emergence. Investigation of the mechanisms involved in HSC specification in the embryo is a rapidly advancing field (Figure 3A), therefore strategies for the generation of HSCs must continue to evolve accordingly.

Alternative strategies involving reprogramming also hold great promise for generating HSCs. Recently, a study demonstrated that terminally differentiated myeloid and lymphoid cells could be dedifferentiated back to HSCs by brief induction of transcription factors (Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37) and subsequent transplantation (Riddell et al, 2014), indicating that HSC identity can be reacquired in

blood cells by molecular reprogramming and may thus represent a source of HSCs in the foreseeable future (Figure 3B). Surprisingly, even non-hematopoietic differentiated fibroblasts have been transformed to hematopoietic progenitor fates (Figure 3C), as shown by ectopic expression of transcription factors Oct4 or Gata2, Gfi1b, cFos, and Etv6 in fibroblasts (Pereira et al, 2013; Szabo et al, 2010). Combining reprogramming along with supportive microenvironmental cells is also a promising strategy; recently, human umbilical vein cells induced with transcription factors (Fosb, Gfi1, Runx1, and Spi1) plated on supportive vascular monolayers designed to mimic the hemogenic endothelial niche acquired hematopoietic fate and engrafted into immune-deficient mice (Sandler et al, 2014). In summary, the major goals now in the fields of regenerative medicine and HSC biology are to understand how certain tissues can be reprogrammed to an HSC-like fate, how reprogramming and normal embryonic programming of HSCs compare to one another, and how normal HSC development can be recapitulated in vitro without the use of potentially oncogenic gene transduction approaches (Figure 3). Key advancements in investigating and replicating HSC induction will involve the generation of reagents and protocols that allow unprecedented precision in the observation, induction, and manipulation of essential, cooperative molecular inputs.

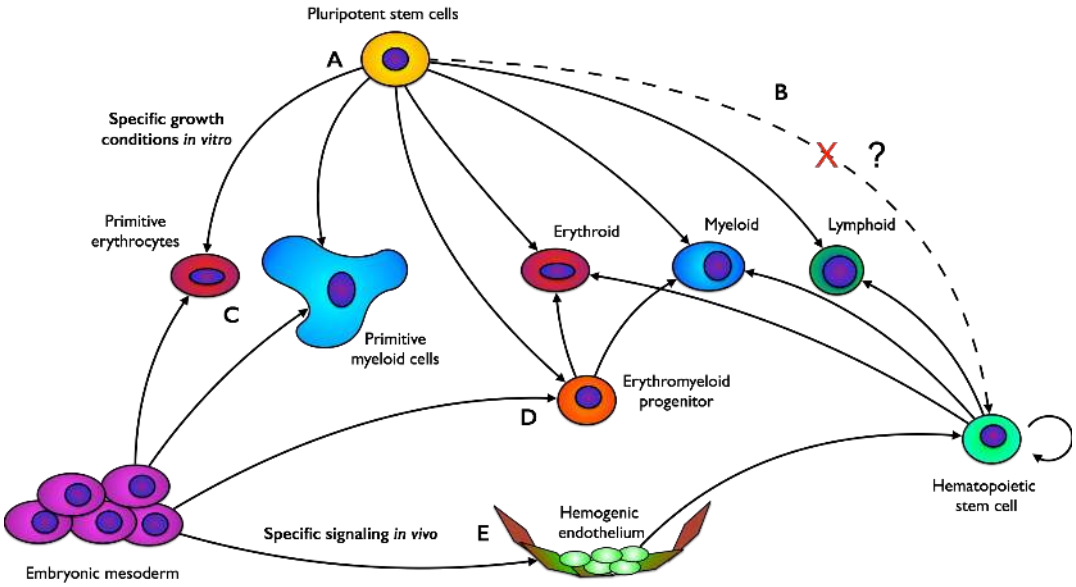

Figure 0-1 Pathways to hematopoietic differentiation *in vitro* and *in vivo*.

(A) Pluripotent cells from embryonic or induced pluripotent sources have not been successfully instructed to hematopoetic stem cell fate (B), but have been successful in generating primitive (C) and transient definitive blood (D) cell fates. Embryonic hematopoiesis proceeds in four ordered waves with primitive erythroid and myeloid waves preceding a definitive EMP wave, and culminates with the establishment of adult definitive hematopoiesis through specification of hematopoietic stem cells via ventral aortic endothelium (E).

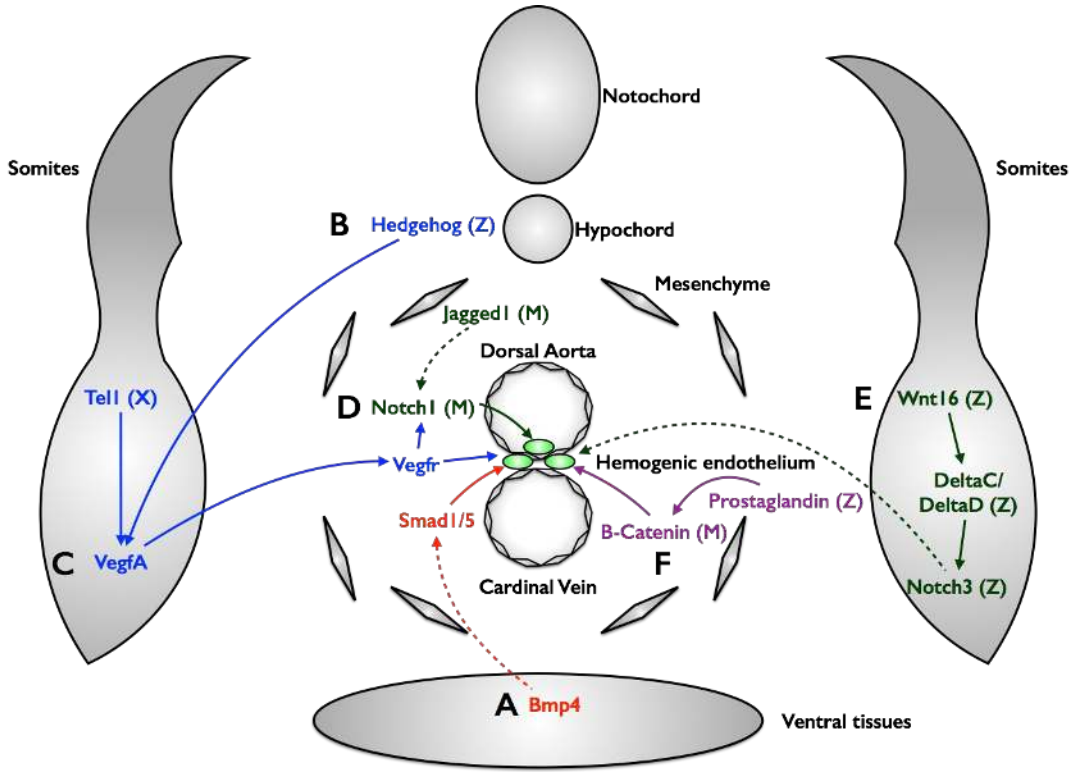

Figure 0-2 Cell signaling pathways involved in HSC specification, at a glance.

A virtual cross section through the vertebrate embryo (zebrafish) with Bmp (A), Hedgehog (B), Vegf (C), Notch (D), non-canonical Wnt/Notch (E), and canonical Wnt signaling requirements depicted (F). Genes required for HSC emergence are annotated, including their anatomical site of expression and known epistasis within each pathway. Species is indicated in instances where gene function has only been identified in mouse (M), Xenopus (X), or zebrafish (Z). Solid lines indicate that there is evidence for a genetic relationship between proteins, while dotted lines indicate uninvestigated but plausible relationships.

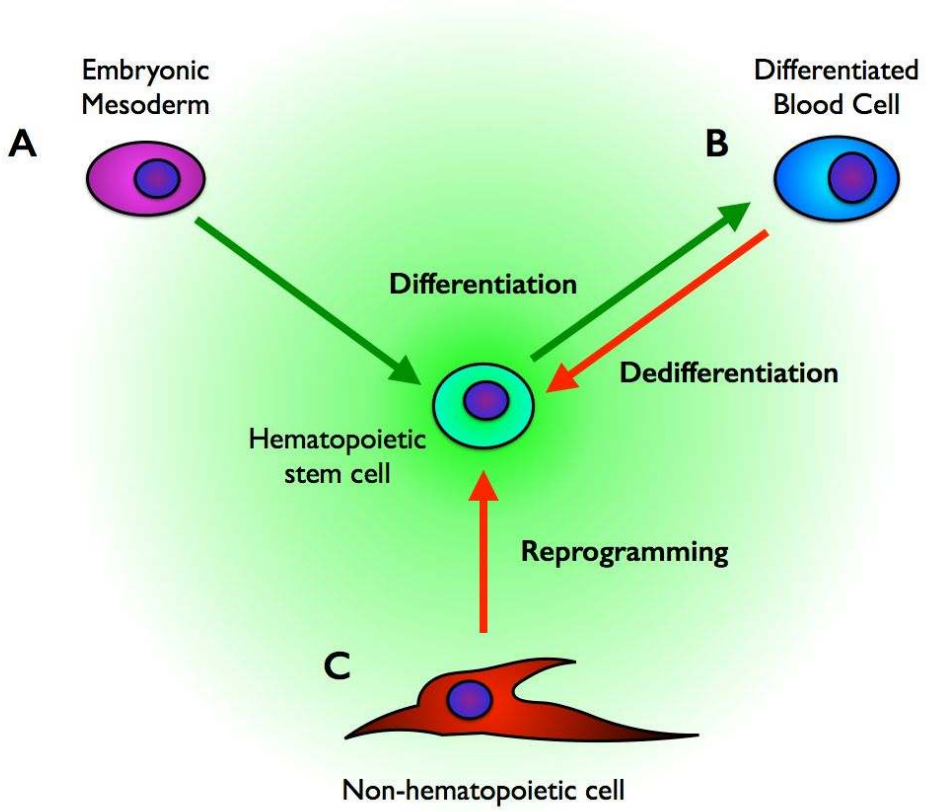

Figure 0-3 Multiple sources for HSC generation.

Normal embryonic developmental processes specify HSCs from mesoderm, and differentiated blood from committed progenitors with instrinsic and extrinsic signals denoted by green arrows (A). Recent studies have established conditions using ectopic induction of transcription factors and supportive signaling conditions denoted by red arrows to dedifferentiate hematopoietic cells (B) or reprogram non-hematopoietic lineages to HSC-like fates (C).

CHAPTER 1

Discrete Notch signaling requirements in the specification of hematopoietic stem cells

Abstract

Hematopoietic stem cells (HSCs) require multiple molecular inputs for proper specification, including activity of the Notch signaling pathway. A requirement for the Notch1 and dispensability of the Notch2 receptor has been demonstrated in mice, but the role of the remaining Notch receptors has not been investigated. Here, we demonstrate that three of the four Notch receptors are independently required for the specification of HSCs in the zebrafish. The orthologues of the murine Notch1 receptor, Notch1a and Notch1b, are each required intrinsically to fate HSCs, just prior to their emergence from aortic hemogenic endothelium. By contrast, the Notch3 receptor is required earlier within the developing somite to regulate HSC emergence in a non-cell-autonomous manner. Epistatic analyses demonstrate that Notch3 functions downstream of Wnt16, which is required for HSC specification through its regulation of two Notch ligands, *deltaC* and *deltaD*. Collectively, these findings demonstrate for the first time that multiple Notch signaling inputs are required to specify HSCs, and that Notch3 performs a novel role within the somite to regulate the neighboring precursors of hemogenic endothelium.

Introduction

The developmental ontogeny of the hematopoietic system is complex and proceeds through four ordered, temporal waves during vertebrate development. The first blood cells specified during embryogenesis are primitive erythroid and myeloid cells, which arise through the direct specification of mesoderm to rapidly generate cells capable of transporting oxygen throughout the developing embryo and providing immunity, respectively (Davidson & Zon, 2004; Le Guyader et al, 2008; Orkin & Zon, 2008; Tober et al, 2007). These primitive waves are followed by specification of definitive waves, the first of which are erythromyeloid progenitors (EMPs), transient precursors that give rise to cells of the erythroid and myeloid pathways (Bertrand et al, 2007; Palis et al, 1999). Finally, hematopoietic stem cells (HSCs), which have both the ability to self-renew and differentiate into the complete repertoire of mature blood cells for an organism's lifespan, are generated. HSC specification is spatially conserved across vertebrate species, and involves the transdifferentiation of hemogenic endothelium in the ventral wall of the dorsal aorta (DA) (Bertrand et al, 2010a; Boisset et al, 2010; de Bruijn et al, 2000; Kissa & Herbomel, 2010; Zovein et al, 2008). Numerous key studies have demonstrated that HSC specification requires specific molecular inputs from a number of signaling pathways, including Notch.

Notch signaling is a conserved cell-to-cell signaling pathway responsible for a multitude of critical cell-fate decisions during the lifespan of metazoan organisms (Kopan & Ilagan, 2009; Lai, 2004). In mammals and zebrafish, Notch signaling occurs through the interaction of many proteins. First, one of four transmembrane Notch

receptors (Notch1, Notch2, Notch3, and Notch4 in mice; Notch1a, Notch1b, Notch2 and Notch3 in zebrafish) on a signal-receiving cell binds to a Notch ligand, termed Jagged and Delta, on a signal-emitting cell (Rebay et al, 1991). Ligand-dependent activation of Notch signaling requires cleavage of the Notch receptor, first by members of ADAM TACE metalloproteases at the S2 site (Bozkulak & Weinmaster, 2009; Brou et al, 2000), then by g-secretase at the S3 site to release a Notch intracellular domain (NICD), which translocates to the nucleus (Mumm et al, 2000) to modulate transcription of Notch target genes (Kopan & Ilagan, 2009). The specification, lineage commitment, and maintenance of many tissues require the precise regulation of Notch signaling.

Notch signaling is especially important for the formation of the hematopoietic system during embryogenesis. While Notch signaling is dispensable for the generation of transient, embryonic blood cells (Bertrand et al, 2010b), it is absolutely required for the generation of HSCs across vertebrate phyla (Burns et al, 2005; Hadland et al, 2004; Krebs et al, 2000; Kumano et al, 2003; Robert-Moreno et al, 2005). Several Notch pathway mutants that fail to specify the DA have defects in HSCs (Duarte et al, 2004; Krebs et al, 2004; Krebs et al, 2000; Lawson et al, 2001), suggesting that the DA is a morphogenetic prerequisite to HSCs. However, several studies have demonstrated that HSC specification can be rescued even in the context of impaired DA formation (Burns et al, 2005; Ren et al, 2010), confounding a clear necessity for a properly formed DA in subsequent HSC formation. Furthermore, mutants in the Notch ligand Jagged1 are deficient in HSCs but have normal arterial formation, suggesting that HSC formation has unique Notch requirements distinct from those required for

arterial fate (Robert-Moreno et al, 2008). Previous work from our laboratory demonstrated that Wnt16 regulates the somitic expression of two Notch ligands, *dlc* and *dld.* While the somitic expression of *dlc* and *dld* was dispensable for DA specification, it was required for the formation of the sclerotome compartment of the somite and subsequent HSC specification (Clements et al, 2011). We reasoned that if Notch signaling performs differential functions in the somites versus the endothelium, then this specificity might be achieved through the discrete use of specific Notch receptors during these different processes.

In this report we investigated which of the four Notch receptors are required for HSC specification, and when and where each of these requirements is needed. We have determined that Notch1a and Notch1b are autonomously required in the precursors of hemogenic endothelium, whereas Notch3 is dispensable in the endothelium and instead required in the somites to indirectly specify the HSC program. Furthermore, we demonstrate that this novel Notch requirement functions within the Wnt16/Notch signaling pathway that we previously showed is necessary to specify the sclerotome and HSCs.

Results

Notch3 is required for HSC specification

Previous studies have investigated the role of two of the four murine Notch receptors in HSC specification. Notch1 is required cell-autonomously for HSC specification, while Notch2 is dispensable (Hadland et al, 2004; Kumano et al, 2003). However, the role of Notch3 or Notch4 had not previously been explored. First, we characterized the expression pattern of *notch3* in zebrafish embryos at 13, 19, and 24 hours post fertilization (hpf) and identified expression in HSC-related tissues. *Notch3* was expressed widely throughout posterior lateral mesoderm (PLM) and somites at 13 hpf (Figure 1A). At 19 hpf, *notch3* expression was reduced in mature somites but maintained in the 3-5 youngest somites and nascent endothelium. At 24 hpf, *notch3* was largely restricted to the endothelium. To examine if *notch3* was required for HSC specification, we knocked down its expression with a *notch3* splice-blocking morpholino (Ma & Jiang, 2007). Whole mount *in situ* hybridization (WISH) analysis of *runx1* and *cmyb,* two markers of HSC specification in the DA, were greatly reduced in *notch3* morphants compared to uninjected control embryos (Figures 1B and 1C). Consistent with these WISH results, confocal imaging of *cmyb:GFP; kdrl:RFP* embryos indicated that $cmyb^{+}kdrl^{+}$ double-positive HSCs (Bertrand et al, 2010a) that normally emerge from hemogenic endothelium were absent at 48 hpf in *notch3* morphants (Figure 1D). Furthermore, the generation of *rag2:GFP*$^{+}$ T lymphocytes, which are dependent upon upstream HSC precursors, were completely absent at 4 dpf

in *notch3* morphants (Figure 1E). Quantitation of the number of cmyb^{+}kdrl^{+} double-positive HSCs showed that the difference between uninjected and *notch3* morphants is statistically significant (Figure 1F). Together, these results indicate that *notch3* is required for the specification of HSCs.

To investigate whether the reduction in HSCs in *notch3* morphants was caused by defects in vasculature formation, we examined several markers of endothelium and DA specification by WISH (Figure 2). *Kdrl* expression in intersomitic vessels was reduced but expression in trunk endothelium was moderately upregulated, whereas aortic expression of *efnb2a,* and *dlc* were unaffected in *notch3* morphants, indicating that DA formation occurs normally and is not likely an explanation for reduced HSC number (Figure 2C to 2E). As our previous work indicated that somite and sclerotome formation might be linked to HSC formation (Clements et al, 2011), we investigated the expression of the somite marker *myod* and the sclerotome-specific markers *foxc1b* and *twist1b* in *notch3* morphants. The somites in *notch3* morphants were specified but exhibited moderate upregulation of *myod* expression in ventral domains (Figure 2F). In contrast, sclerotomal expression of *foxc1b*$^{+}$ and *twist1b*$^{+}$ were greatly reduced in *notch3* morphants compared to uninjected embryos (Figure 2G and 2H). We confirmed that a loss of HSCs and sclerotome observed in *notch3* morphants is due a specific loss of function of the *notch3* gene and not due to off-target effects from morpholino injection as evidenced by similar defects in *runx1* and *foxc1b* in *notch3*^{fh332} mutants (Figure S1). Earlier sclerotomal defects were observed in *notch3* morphants and *notch3*^{fh332}mutants at 17 hpf by reduction of *foxc1b*, *twist1a*, and *twist1b* expression indicating that sclerotome specification is impaired (Figure S2).

These results indicate that *notch3* is essential for sclerotome and HSC specification, but is largely dispensable for DA formation.

We next wished to determine possible roles for the remaining Notch receptors *notch1a*, *notch1b*, and *notch2* in HSC specification. The expression pattern of *notch1a* and *notch1b* were similar to that of *notch3*, whereas *notch2* was exclusively observed in the somites at these developmental stages (Figure S3A to S3C). We utilized a splice-blocking morpholino for *notch1a* (Ma & Jiang, 2007) and designed splice-blocking morpholinos for *notch1b* and *notch2* (Figure S3D and S3E). Loss of function of *notch1a* and *notch1b,* but not *notch2*, resulted in loss of *runx1* expression in the DA (Figure S4A), consistent with the requirement for Notch1 but not Notch2 in murine HSC specification (Kumano et al, 2003). Notch1 mutant mice have vascular defects including a failure to specify DA (Krebs et al, 2000). In agreement with these findings, we observed variable loss of intersomitic vessels and defective aortic *efnb2a* and *dlc* expression in *notch1a* morphants, whereas *notch1b* morphants had only mild defects in *dlc* (Figure S4B to S4D). *Notch2* morphants displayed loss of intersomitic *kdrl* and *dlc* expression but maintained trunk endothelium and aortic markers. *Notch1a, notch1b,* and *notch2* morphants showed normal $myod^{+}$ somites and $foxc1b^{+}/twist1b^{+}$ sclerotome (despite affected somite boundaries in *notch1a* morphants), suggesting that formation of somites does not require these Notch receptors (Figure S4E to S4G). We confirmed that the tissue-specific defects we observed in *notch1a* and *notch2* morphants are not due to off-target morpholino effects as evidenced by similar aortic defects on *runx1* and *efnb2a* expression in $notch1a^{b420}$ mutants and loss of intersomitic vessel expression of *dlc* but maintained

aortic expression of *runx1* in *notch2*^{el517} mutants (Figure S5A to S5D). Although singular loss of *notch1b* or *notch3* does not result in a loss of aortic *efnb2a*, a recent study demonstrated that both receptors are required synergistically for aorta specification (Quillien et al, 2014). Injection of *notch1b* morpholino #2 from Quillien et al., resulted in a reduction of HSCs but did not affect *efnb2a* validating our observed *notch1b* morphant phenotype. Furthermore coinjection of *notch1b* morpholino #2 and *notch3* morpholino resulted in a loss of aortic *efnb2a*, supporting the previous claim that loss of *notch3* alone is tolerated by the aortic program in the presence of functional *notch1b* (Figure S5E and S5F). Consistent with the endothelial-specific effects observed in *notch1a* and *notch1b* morphants, we found coexpression of *notch1a/notch1b* within and around *efnb2a* and *runx1* expression domains in wild-type embryos (Figure S6A to S6D). These data suggest that the role of the paralogous *notch1a* and *notch1b* genes in zebrafish is functionally conserved to that described for the murine *Notch1* gene.

Notch3 is required non-cell-autonomously for HSC specification

By temporal induction of an NICD transgene, we previously showed that a Notch signal downstream of *wnt16* in the somite was required for HSC specification during a brief permissive window beginning at 14 hpf. This finding helped us determine that this requirement was non-cell-autonomous, since the earliest Notch signaling events in HSC precursors were not detectable until 20-22 hpf (Clements 2011). We therefore investigated if similar induction of NICD could rescue HSCs in

notch3 morphants. To perform these experiments, we utilized *hsp70:gal4; UAS:NICD-myc* double transgenic animals to temporally control Notch signaling. Expression of NICD-Myc protein was detected by whole-mount immunofluorescence within an hour and up to 24 hours after induction as previously reported (Clements et al, 2011). Induction of NICD in uninjected embryos did not affect the number of *runx1*$^{+}$ HSCs (Unpublished observations). Early induction of NICD at 14 hpf rescued *runx1*$^{+}$ HSCs in *notch3* morphants, while late induction at 20 hpf did not (Figure 3A to 3C), suggesting that Notch3 may mediate the early, non-cell-autonomous HSC specification requirement. In contrast, *notch1a* and *notch1b* morphants were robustly rescued by 14 hpf induction, and more importantly, by 20 hpf induction (Figure S7A to S7D).

The observation that *notch3* morphants had different temporal requirements for NICD-mediated HSC rescue when compared to *notch1a* and *notch1b* morphants suggests that there are at least two temporal windows in which Notch signaling was important for HSC specification. We predicted that global Notch inhibition that spanned 14 hpf would phenocopy loss of *notch3*, while inhibition at 20 hpf would phenocopy loss of either *notch1a* or *notch1b*. To pharmacologically inhibit Notch signaling, we utilized the g-secretase inhibitor DBZ, treating embryos during 6-15 hpf or 15-26 hpf developmental time windows and subsequently assaying for tissue-specific effects. We observed a reduction in *runx1* intensity in the DA of embryos treated during either drug treatment window, indicating that Notch signaling was required for HSC specification during both windows (Figure 4A). We observed sclerotome malformation when embryos were treated between 6-15 hpf that

phenocopied *notch3* morphants, but no affect when embryos were treated between 15-26 hpf (compare Figure 4B and Figure 2E). In contrast, effects on the DA transcripts *efnb2a* and *dlc* were severely reduced during the 15-26 hpf window resembling *notch1a* and *notch1b* morphants (compare Figure 4C and 4D to Figure S4C and S4D), but unaffected by drug treatment during 6-15 hpf. Interestingly, the loss of *efnb2a* and *dlc* caused by DBZ treatment from 15-26 hpf was more dramatic than that observed in *notch1a* or *notch1b* morphants, suggesting that each may have non-redundant requirements or that remaining Notch receptors, likely Notch3 (Figure S5F), may partially compensate for the loss of either Notch1a or Notch1b during DA specification. These results indicate that Notch signaling performs transient and non-redundant roles during somitogenesis compared to DA formation that are both essential for HSC production.

To determine if spatially restricted expression of NICD was sufficient to rescue HSCs in *notch3* morphants, we utilized tissue-specific drivers of Gal4. To drive NICD within the HSC lineage, we utilized a *kdrl:gal4* transgenic line whereby expression is targeted to the vasculature, including hemogenic endothelial cells (Bertrand et al, 2010a), (Figure S8A). Since *notch3* is expressed in the sclerotome (Figure S8B), and since *notch3* morphants displayed defects in both sclerotome and HSCs, we asked if enforced somitic expression of NICD could rescue HSCs in *notch3* morphants. To perform these experiments, we utilized the *phldb1:gal4-mCherry* transgenic line (Distel et al, 2009), which drives robust expression specifically in the somite (Figure S8C to S8G). Double transgenic *α-actin:GFP; phldb1:gal4-mCherry* embryos showed high level of mCherry in all GFP+ cells by fluorescence activated cell sorting (FACS),

indicating *phldb1:gal4-mCherry* is expressed widely in somitic tissues (Figures S8G). Enforced expression of NICD in the somite rescued *runx1*$^{+}$ HSCs in *notch3* morphants with significantly greater frequency than endothelial-driven NICD did in our analyses (Figure 5A to 5C). In contrast, HSCs in *notch1a* and *notch1b* morphants were rescued with vascular-specific *kdrl:gal4* driven NICD but not with somite-specific *phldb1:gal4-mCherry* driver in our analyses (Figure S9A to S9D). These findings indicate that *notch1a* and *notch1b* are required for activation of Notch signaling within the endothelium, but not the somites, to specify HSCs. We next asked if the induction of NICD is sufficient to rescue HSCs in *notch3* or *notch1a* morphants could also rescue the defects observed in the sclerotome and DA, respectively. NICD induction globally at 14 hpf or somitically using the *phldb1:gal4-mCherry* driver in *notch3* morphants also restored expression of *twist1b* in the sclerotome *(*Figure S10A and S10B). Similarly, global NICD induction at 20 hpf or in vascular cells using the *kdrl:gal4* driver restored expression of *efnb2a* in the DA (Figure S10C and S10D). Together, these results demonstrate that Notch3 is required in the somite at 14 hpf to specify sclerotome and HSCs, and that Notch signaling is then needed again for HSC fate via subsequent function of Notch1a and Notch1b in the vasculature at 20 hpf .

Notch3 is required downstream of somitic *dlc*, *dld*, and *wnt16* for HSC specification

Because *wnt16*, *dlc*, *dld* (Clements et al, 2011), and *notch3* are each required for sclerotome formation, and because loss of *wnt16* or *notch3* can be rescued only by

early NICD induction, we investigated the functional relationship between *dlc*, *dld*, and *notch3* in HSC specification. We tested if there was synergy between *dlc* or *dld* and *notch3* by combinatorial low-dose knockdown experiments and assessed the severity of affected phenotypes. Heterozygotes from the *dlc* mutant, *beamter* (Julich et al, 2005), low dose (5ng) *dld* morphants, or low dose (5ng) *notch3* morphants each showed a partial loss of *runx1, twist1b,* and *foxc1b* expression compared to controls (Figure 6A to 6D). Combinatorial knockdown of *dlc*/*notch3* or *dld*/*notch3*, however, had more severe effects on HSCs and sclerotome than any of the single knockdown controls, suggesting that *dlc* and *dld* interact with *notch3* (Figure 6E and 6F). To test if *notch3* was genetically upstream of *dlc* and *dld* expression in somites, we examined if *notch3* morphants had defects in somitic *dlc* or *dld* expression. We observed no significant reduction in *dlc* or *dld* in *notch3* morphants compared to uninjected embryos, suggesting that *notch3* is not required to induce *dlc* or *dld* expression (Figure S11A and S11B). In addition, HSC formation in *notch3* morphants was not rescued by combined *dlc/dld* mRNA injection, a strategy that was able to rescue HSCs following loss of *wnt16* (Figure 7A to 7D) (Clements et al, 2011). Collectively, these findings suggest that Notch3 function lies downstream of DeltaC/DeltaD function. This hypothesis is supported by the finding that loss of *notch3* inhibited the rescue of HSCs by *dlc/dld* mRNA in *wnt16* morphants (Figure 7E). These results suggest that Notch3 is necessary to receive signals from DeltaC and/or DeltaD in the sclerotome to relay further signals to the precursors of HSCs that are required for their proper specification (Figure S12).

Discussion

Previous studies have demonstrated that Notch signaling is required for HSC specification (Bertrand et al, 2010b; Burns et al, 2005; Hadland et al, 2004; Kumano et al, 2003; Robert-Moreno et al, 2005; Robert-Moreno et al, 2008; Yoon et al, 2008). Of the four murine Notch receptors, Notch1 is required cell-autonomously while Notch2 is dispensable (Hadland et al, 2004; Kumano et al, 2003); the roles of the remaining receptors have not been addressed. Here, we demonstrate that Notch3 is required to activate Notch signaling in the somite by 14 hpf, and that this activation is required for HSC specification.

Despite the fact that Notch receptors are widely conserved across vertebrate species, there are evolutionary differences in the Notch receptor genes of mammals and zebrafish. One of the most notable is the presence of two Notch1-related homologues, *notch1a* and *notch1b* in zebrafish. Notch1 has high amino acid identity to both Notch1a and Notch1b, and phylogenetic reconstruction analyses have suggested that *notch1a* and *notch1b* arose from a gene duplication event that occurred early during teleost evolution (Kortschak et al, 2001; Westin & Lardelli, 1997). Despite Notch1 and Notch2 genes sharing high amino acid similarity, Notch2 is dispensable for HSC specification in the mouse (Kumano et al, 2003). We show that Notch2 is also dispensable in zebrafish for HSC generation, indicating that the individual roles of Notch receptors may be conserved across vertebrates. Supporting this hypothesis, our experiments show that the combinatorial actions of Notch1a and Notch1b in zebrafish functionally phenocopy the activity of Notch1 in other vertebrates.

Several lines of evidence indicate that Notch1a and Notch1b have distinct but overlapping roles in hemogenic endothelium. We show that *notch1a* and *notch1b* are both expressed in endothelium by FISH, but in other tissues each receptor's expression pattern is more distinct but additively resemble the wider expression pattern of murine Notch1 (Westin & Lardelli, 1997). We show that *notch1a* morphants have reduced HSCs and reduced aortic *efnb2a* and *dlc* expression, while *notch1b* morphants had reduced numbers of HSCs and reduced aortic *dlc* levels while displaying normal *efnb2a* expression that is contingent on the presence of functional Notch3. Additively, these phenotypes resemble Notch1 mutant mice (Krebs et al, 2004), and suggest that the role for Notch1b is more HSC-specific. We also demonstrate that Notch1a and Notch1b have a functional role in the endothelial cells; when NICD was specifically expressed in the *kdrl*$^{+}$ endothelium we were able to rescue *runx1*$^{+}$ HSC formation in *notch1a* or *notch1b* morphants, as well as rescue aortic *efnb2a* expression in *notch1a* morphants. Additionally, Notch1a and Notch1b are required during the temporal window essential for HSC formation, shown by our rescue of HSCs with NICD induction at 20 hpf, when HSC precursors first experience Notch signaling (Clements et al, 2011). This timing is consistent with our findings that global pharmacological inhibition of Notch between 15-26 hpf specifically blocked DA and HSC specification. Our data demonstrate that like Notch1, Notch1a and Notch1b both perform a cell-autonomous role in HSC specification.

In contrast to Notch1, the role of Notch3 in HSC specification is poorly understood. We investigated if Notch3 acts cell-autonomously or non-cell-autonomously to specify HSCs by specific temporal and spatial induction of NICD in

notch3 morphants. In contrast to the rescue of HSCs by induction of NICD at 20 hpf in *notch1a* and *notch1b* morphants, *notch3* morphants could only be rescued by earlier NICD induction at 14 hpf. This suggests that Notch3 is required during a brief permissive window before HSC precursors experience Notch signaling directly. Confirming the requirement for Notch signaling during this permissive window, pharmacological inhibition of Notch signaling during 6-15 hpf showed a strong reduction in HSC numbers. Our results using tissue-specific drivers of NICD in *notch3* morphants showed that there were also precise spatial requirements for HSC rescue. Enforced expression of NICD in the somites was sufficient to rescue HSCs in *notch3* morphants, whereas endothelial-specific expression was not, despite the fact that *notch3* is expressed in the DA (Lawson et al, 2002). These results indicate that Notch3 activation is required in the somites, but not in endothelium, to specify HSCs. The temporal and tissue-specific rescue of HSCs by NICD in *notch3* morphants was also accompanied by the rescue of sclerotome-specific transcripts. These data suggest that the molecular requirements for sclerotome formation are closely linked to HSC specification. Collectively, our data demonstrate that Notch3 is required non-cell-autonomously in the somites to specify HSCs.

We established that there is a genetic relationship between *dlc*/*dld* and *notch3* during HSC specification by combinatorial low-dose knockdown of these genes. Furthermore, the partial knockdown of *dlc* or *dld* was synergistic with a low-dose knockdown of *notch3,* indicating that each of these genes are involved in a linear genetic pathway. In this pathway, *wnt16* is genetically upstream of somitic *dlc*/*dld* but is dispensible for somitic *notch3* (Clements et al, 2011). However unlike *wnt16*,

notch3 is not genetically upstream of *dlc/dld* as evidenced by maintenance of *dlc/dld* in the somites of *notch3* morphants. This is bolstered by the finding that reduced numbers of HSCs in *notch3* morphants could not be rescued with coinjection of *dlc/dld* mRNA. Additionally, HSC rescue in *wnt16* morphants coinjected with *dlc*/*dld* mRNA was inhibited by the coinjection of the *notch3* morpholino. The simplest explanation is that Dlc and/or Dld directly activates Notch3. Why both ligands might be required to activate Notch3 is unclear. The roles of mammalian homologues Dll3 and Dll1 appear to be conserved with DeltaC and DeltaD respectively as evidenced by their conserved expression pattern in somitic tissues and loss of sclerotome and/or vertebral malformations in Dll3 and Dll1 loss of function animals (Chapman et al, 2010; Hrabe de Angelis et al, 1997; Takahashi et al, 2003), though a role in HSC specification has not been described. No studies to our knowledge have demonstrated that Notch3 is an obligate receptor for Dll3 or Dll1, on the contrary mammalian cell culture have demonstrated that Notch1 and Notch3 can bind a range of Delta and Jagged ligands, suggesting that binding between receptors and ligands is promiscuous (Shimizu et al, 2000). Direct binding has been reported between Dll3 and Notch1, however this interaction is inhibitory and occurs in *cis*, suggesting that one of the functions of Dll3 is to suppress Notch1 signaling cell-autonomously (Chapman et al, 2010; Ladi et al, 2005). Intriguingly Dll3 and Dll1 display non-redundant and even counteracting functions in somitogenesis (Geffers et al, 2007; Ladi et al, 2005; Takahashi et al, 2003). Studies in zebrafish may offer an explanation, since both DeltaC and DeltaD within the presomitic mesoderm (PSM) interact heterophilically and are endocytosed together from the plasma membrane, suggesting that both ligands

may be required to effectively activate Notch signaling (Wright et al, 2011). This hypothesis could explain our previous results demonstrating that both *dlc* and *dld* are required and sufficient for HSC formation in *wnt16* morphants (Clements et al, 2011). While the lack of a specific Notch3 antibody in zebrafish precludes testing whether or not Notch3 directly interacts with Dlc/Dld by biochemical or histological approaches, our results demonstrate that *wnt16*-induced *dlc/dld* requires the presence of *notch3* to promote HSC emergence.

Notch3 may be required for a specific morphogenetic process and/or activation of another signaling cascade required by HSCs. We have shown that *wnt16*, *dlc*, *dld*, and *notch3* are all required for sclerotome and HSC formation, but dispensable for DA specification. Notch3 function may be required to specify the sclerotome, which in turn is required to specify HSCs through provision of a relay signal to neighboring PLM cells. Another potential mechanism to explain the link between sclerotome and HSC specification is that sclerotome may give rise to vascular smooth muscle precursors that support the endothelium, as it does in chick and mouse (Pouget et al, 2008; Wasteson et al, 2008). This is an attractive hypothesis, as Notch signaling is necessary and sufficient for inducing somitic emigration to the dorsal aorta in chick (Sato et al, 2008). Recent studies performed in embryonic stem cells have confirmed that VSMCs do not directly give rise to hemogenic endothelium, indicating that if VSMCs have a role in HSC specification it is indirect (Stefanska et al, 2014). It is currently unknown if the sclerotome is specifically required for HSC specification, but previous studies have established that somites are, through their production of VEGF, required for HSC formation (Ciau-Uitz et al, 2010; Leung et al, 2013). Our data

elucidate an additional molecular pathway in which the somites are essential for the establishment of HSC fate.

Our study elucidates a previously unappreciated role for *notch3* in the somites that is required for HSC specification, and that the Notch1 homologues *notch1a* and *notch1b* are both required cell-autonomously in the hemogenic endothelium for this process. These data should prove essential for future studies focused on the identification of unique targets downstream of each required Notch receptor essential for HSC specification.

Materials and methods

Zebrafish husbandry

Zebrafish strains AB*, *Tg(UAS:myc-Notch1a-intra)*^{kca3} (Scheer & Campos-Ortega, 1999), *Tg(hsp70l:gal4)*$^{1.5kca4}$ (Scheer & Campos-Ortega, 1999), *Tg(actc1b:GFP)*^{zf13} (Higashijima et al, 1997), *Tg(-80.0myf5:EGFP)*^{zf37} (Chen et al, 2007), *Tg(rag2:EGFP)*^{zdf8} (Langenau et al, 2003), *Tg(phldb1:gal4-mCherry)* (Distel et al, 2009), *Tg(kdrl:EGFP)*^{la116} (Choi et al, 2007), *Tg(cmyb:EGFP)*^{zf169} (North et al, 2007), and *dlc*$^{tit446/tit446}$ (from Tübingen 2000 screen), *notch3*^{fh332} (Quillien et al, 2014), *notch1a*^{b420} (Gray et al, 2001), *notch2*^{el517} were maintained, injected, and staged as described (Westerfield, 2004) and in accordance with IACUC guidelines. *Tg(kdrl:miniGAL4)* was generated by cloning a 6 kb genomic fragment immediately upstream of the transcription start site from a plasmid carrying *kdrl*:*R-CFP* (Cross et al, 2003) and inserted into pCR8 (Invitrogen). The resulting plasmid was recombined into a Tol2 transgenesis vector *pColdHeart-Gtwy-miniGAL4* (Campbell et al, 2007) and coinjected with Tol2 mRNA into 1-cell stage embryos. A stable transgenic line with a single insertion was established. Heat shocks were performed at the times indicated for 45 min at 37ºC as previously described (Burns et al, 2005).

Microinjection of morpholinos, RT-PCR, and mRNA

The following morpholino antisense oligonucleotides were synthesized by Gene

Tools, LLC and suspended as 25mg/ml stocks in DEPC ddH_2O and diluted to injection strengths: 5ng *wnt16*-MO, 5ng *dld*-MO2 (Clements et al., 2011), 10ng *notch1a*-sp MO1, 10ng *notch3*-sp MO (Ma & Jiang, 2007), 10ng *notch1b*MO GTCGAGAATCTTATCACTTACTTGC, 10ng *notch2*MO TTCGAATGTGAAAGTCTTACCTGCA, 2.5ng *notch1b*MO2 (Quillien et al, 2014). For RT-PCR, RNA was isolated from groups of 30 uninjected or morpholino injected embryos at 26 hpf, and cDNA was prepared as previously described (Clements et al, 2009). PCR on cDNA was amplified with *notch1b*-sp-F TGCATCTTTTCTTCGTGAAAC, *notch1b*-sp-R GGATTGGAAGCAAGGGTTG, *notch2*-sp-F CAAAATATGGGCCAATTACCC, notch2-sp-R GACAGACATGCGTCCTCTTGC, b-actin-sp-F AAGATCAAGATCATTGCC, and b-actin-sp-R TTGTCGTTTGAAGTTTCTC with Taq polymerase (Invitrogen, Philadelphia, PA) as previously described (Clements et al, 2009). Full length dlc and dld mRNA was synthesized as described (Clements et al., 2011). Injections were performed as described previously (Clements et al., 2009). Genotyping of *notch3*^{fh332} after phenotypic analysis was performed as described previously (Quillien et al, 2014). Genotyping by PCR of *notch2el517* animals was performed with *notch2*-F GAGCAAGAGGACGCATGTCT-3', and *notch2*-R GCTGCGGTAAAATCCCATTA.

WISH, immunofluorescence, and microscopy

Single enzymatic and double fluorescence whole mount *in situs* were performed as previously described (Clements et al, 2011). Antisense RNA probes for the following genes were prepared using probes containing digoxigenin or fluorescein labeled UTP: *runx1, kdrl, efnb2a, dlc, myod, foxc1b, twist1a, twist1b, notch1a, notch1b,* and *notch3* as previously described (Clements et al, 2011). Whole-mount immunofluorescence was performed using anti-Myc monoclonal 9E10 antibodies at 1:200 (Covance) and Dylight488 AffiniPure donkey anti-mouse IgG secondary antibodies (Jackson Immunoresearch Laboratories) at 1:100 as described previously (Clements et al, 2011). Fluorescence images of transgenic embryos and embryo samples were imaged using confocal microscopy (Leica, SP5) and processed using Volocity software (Perkin-Elmer) as previously described (Bertrand et al, 2010a).

Pharmacological inhibition of Notch signaling

Dibenzazepine (DBZ) γ-secretase inhibitor (Calbiochem) was dissolved in DMSO at a concentration of 2mM. Zebrafish embryos were incubated in 3 ml of 4 μM DBZ solution in the dark from 6-15 or 15-26 hpf followed by fixation with 4% PFA.

Fluorescence-activated cell sorting (FACS)

Kdrl:GFP; *phldb1:gal4-mCherry,* and α*-actin:GFP*; *phldb1:gal4-mCherry* embryos were collected at 17 hpf and processed for FACS as previously described (Bertrand et al, 2007).

Figures

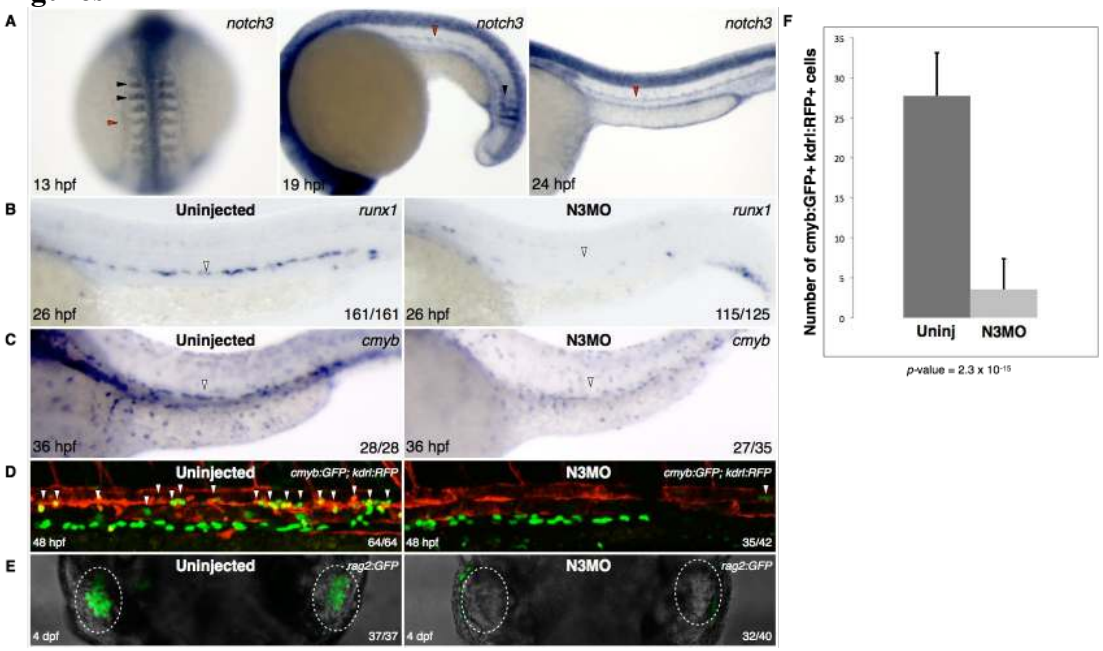

Figure 1-1 Notch3 is required for HSC specification

(A) WISH of *notch3* viewed dorsally at 13 hpf (left) and laterally in the trunk at 19 (middle) and 24 hpf (right). Black arrowheads denote somitic expression, red arrowheads denote PLM expression at 13 hpf and endothelial expression at 19 and 24 hpf. WISH of the HSC marker *runx1* at 26 hpf (B) and *cmyb* at 36 hpf (C) on uninjected and Notch3 morphants. Arrowheads indicate HSCs in the DA. Confocal fluorescence microscopy images of transgene reporter expression in *cmyb:GFP; kdrl:RFP* trunk region at 48 hpf (D) and *rag2:*GFP at 4 dpf (E) transgenics uninjected or with *notch3* morpholino injected. Arrowheads in *cmyb:GFP; kdrl:RFP* embryos indicate double positive HSCs, and dotted lines in *rag2:GFP* embryos outline the thymic lobes were GFP^+ lymphoid cells should reside. (F) Enumeration of *cmyb:GFP+; kdrl:RFP+* cells in the floor of the DA at 48 hpf. Bars represent mean ± S.E.M. of double positive cells for uninjected (n=12) and Notch3 morphants (n=20). $p=2.3 \times 10^{-15}$.

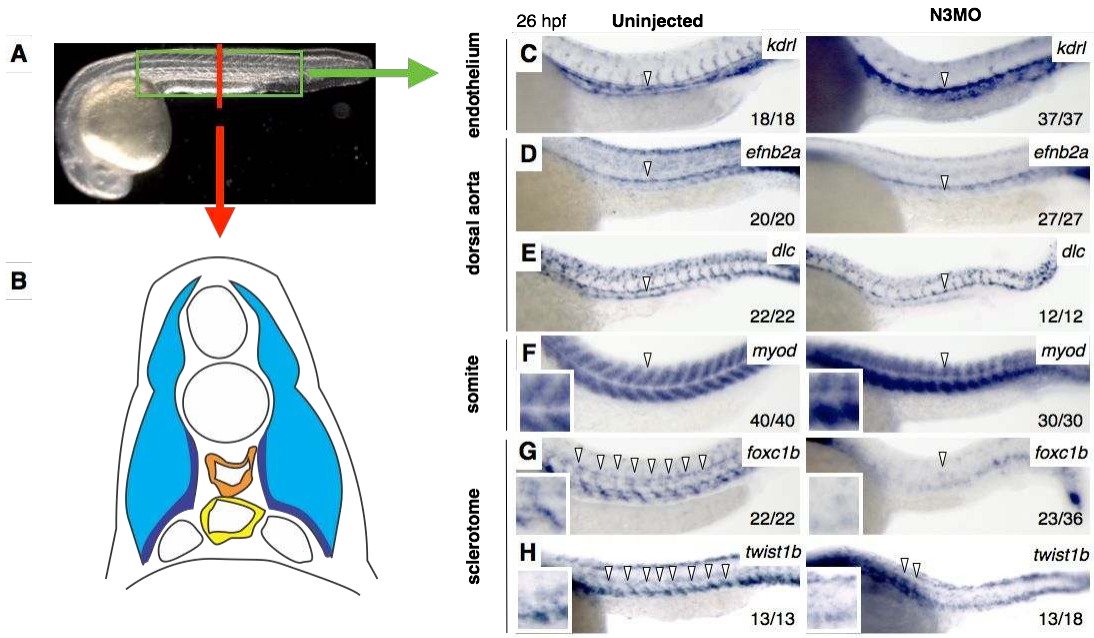

Figure 1-2 Notch3 is dispensable for DA, but required for sclerotome

(A). Brightfield image of a 26 hpf zebrafish. (B) Cartoonized cross-section of the embryonic trunk marking somites in light blue, sclerotome in purple, venous endothelium in yellow and aortic endothelium in orange. WISH of uninjected and *notch3* morphants at 26 hpf for the endothelial marker *kdrl* (C), dorsal aorta markers *efnb2a* (D) and *dlc* (E), the somite marker *myod* (F), and sclerotome markers *foxc1b* (G), and *twist1b* (H). Magnified panels are shown for somitic and sclerotomal markers in lower left corner. Arrowheads indicate tissue-specific gene expression.

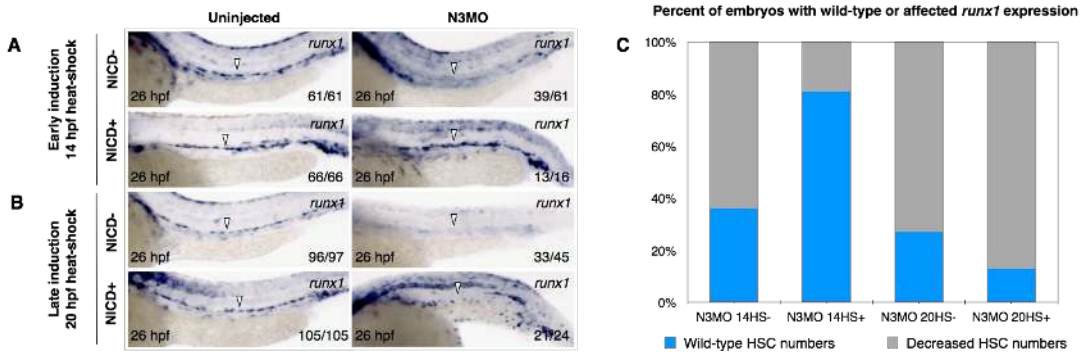

Figure 1-3 Specific temporal activation of Notch signaling is sufficient to rescue HSCs in *notch3* morphants

WISH for *runx1* in 26 hpf *hsp70:gal4; UAS:NICD-myc* uninjected or notch3 morphant transgenic embryos with heat-shock induction at 14 hpf (A) or 20 hpf (B), with or without enforced NICD expression. Arrowheads indicate presence or absence of HSCs at the midline. Quantitation of results recording percentages of embryos displaying normal or decreased numbers of *runx1*$^{+}$ HSCs at 26 hpf in *notch3* morphants with heat-shock induction conditions (C).

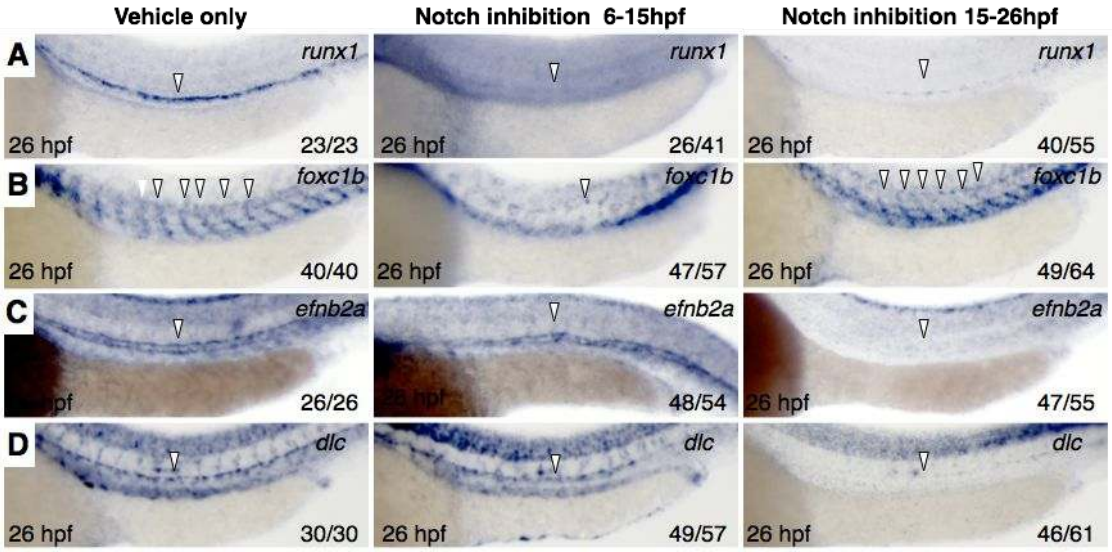

Figure 1-4 Notch signaling is required during two distinct time windows for specification of sclerotome and dorsal aorta

WISH for *runx1* (A), *foxc1b* (B), *efnb2a* (C), and *dlc* (D) in 26 hpf embryos treated with DMSO vehicle (left), 4uM g-secretase Notch inhibitor DBZ at 6-15 hpf (middle), or 15-26 hpf (right). Arrowheads indicate tissue-specific expression.

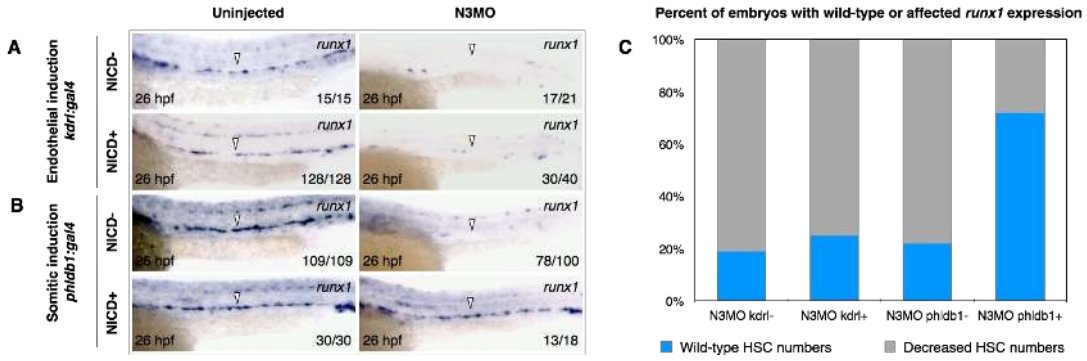

Figure 1-5 Specific spatial activation of Notch signaling is sufficient to rescue HSCs in *notch3* morphants

WISH for *runx1* in 26 hpf *kdrl:gal4* (A) or *phldb1:gal4-mcherry* (B) crossed to *UAS:NICD-myc* transgenic embryos either uninjected or injected with *notch3* morpholino, with or without enforced NICD expression. Arrowheads indicate presence or absence of HSCs at the midline. Quantitation of results recording percentages of embryos displaying normal or decreased numbers of *runx1*$^{+}$ HSCs at 26 hpf in *notch3* morphants with tissue-specific induction conditions (C).

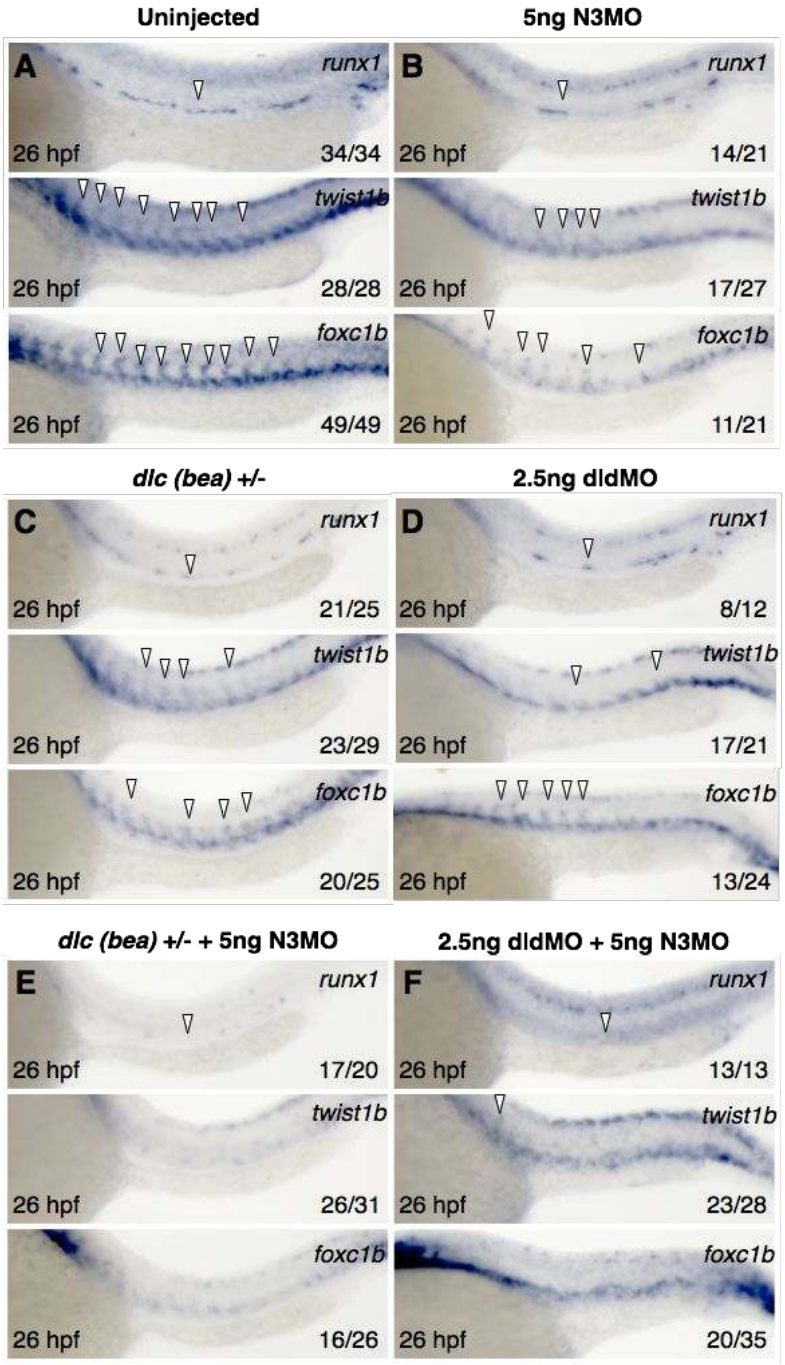

Figure 1-6 Notch3 cooperates synergistically with *dlc* and *dld* to specify HSCs

WISH of *runx1, twist1b,* and *foxc1b* at 26 hpf in uninjected (A), low-dose knockdown of *notch3* (B), heterozygotes for *dlc* mutant *bea* (C), low-dose knockdown of *dld* (D), *bea* heterozygotes with low-dose knockdown of *notch3* (E), and combinatorial low-dose knockdown of *dld* and *notch3* (F). Arrowheads indicate tissue-specific expression.

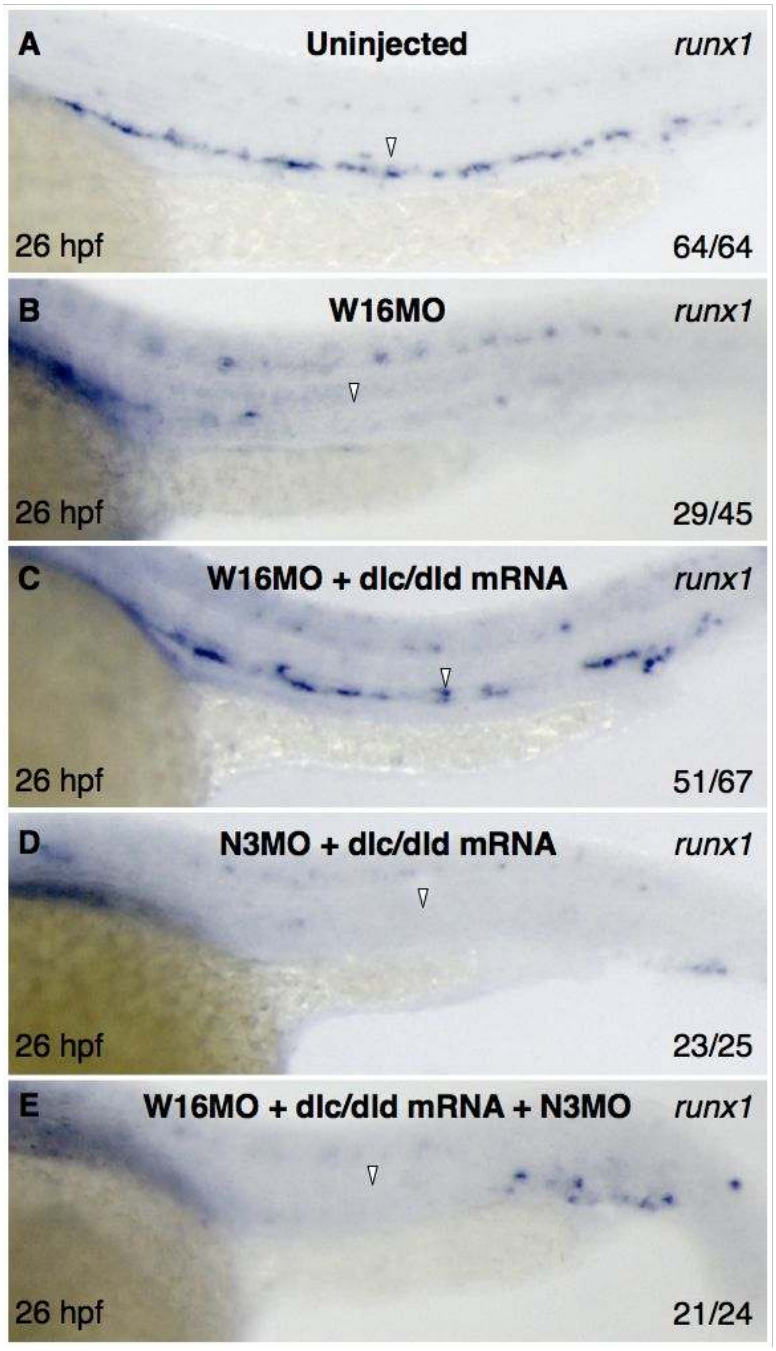

Figure 7. *Wnt16, dlc/dld*, and *notch3* function in a linear pathway to specify HSCs

Expression of *runx1* at 26hpf in uninjected (A) or injected with *wnt16*MO (B), *wnt16*MO with 50 pg *dlc*/*dld* mRNA (C), *notch3*MO with 50 pg *dlc*/*dld* mRNA (D), and *wnt16*MO/*notch3*MO with 50 pg *dlc*/*dld* mRNA (E). Arrowheads indicate presence or absence of HSCs.

Supplemental Figures

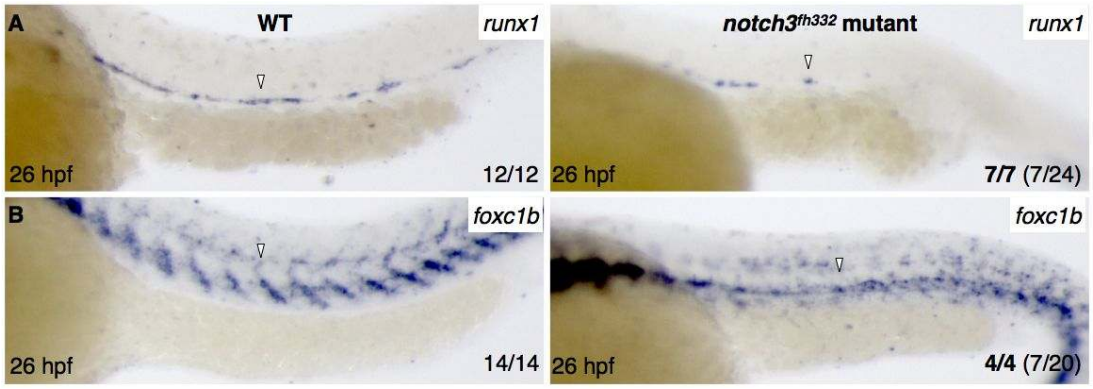

Figure 1-S1 Notch3 mutants are defective in HSCs and sclerotome

WISH of *runx1* (A) and *foxc1b* (B) in wild-type and *notch3*^{fh332} mutant embryos at 26 hpf. Counts in bold indicate number of genotyped *notch3*^{fh332} mutants with depicted phenotype out of total genotyped embryos, counts in parenthesis indicate number of progeny from adult *notch3*^{fh332} heterozygote matings that show depicted phenotype out of total sample size.

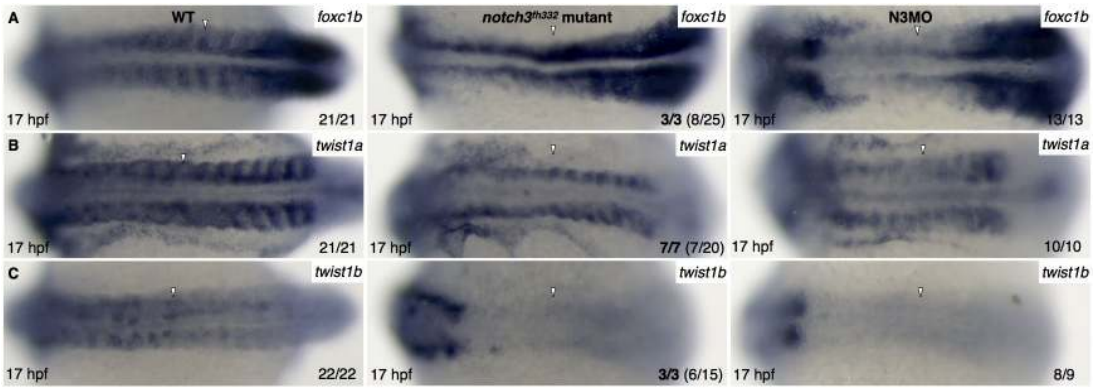

Figure 1-S2 Notch3 mutant and morphant embryos show early defects in sclerotome specification

WISH of *foxc1b* (A), *twist1a* (B), and *twist1b* (C) in wild-type, *notch3*^{fh332} mutant, and Notch3 morphant embryos at 17 hpf. *Notch3*^{fh332} mutants counts in bold indicate number of genotyped *notch3*^{fh332} mutants with depicted phenotype out of total genotyped embryos, counts in parenthesis indicate number of progeny from adult *notch3*^{fh332} heterozygote matings that show depicted phenotype out of total sample size.

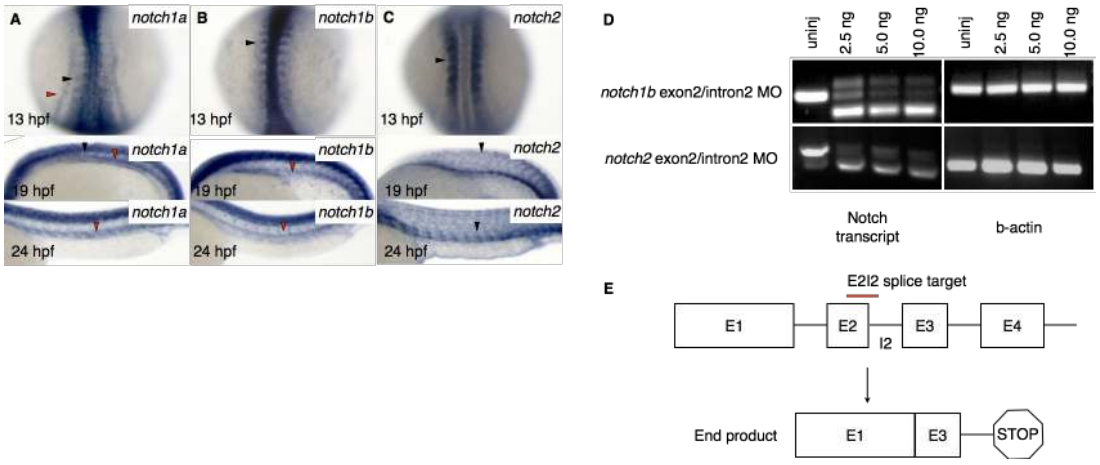

Figure 1-S3 *Notch1a, notch1b, and notch2* are expressed dynamically during development. Design and validation of specific morpholinos to *notch1b* and *notch2*

WISH of *notch1a* (A), *notch1b* (B), and *notch2* (C) viewed dorsally at 13 hpf and laterally in the trunk at 19 and 24 hpf. Black arrowheads denote somitic expression, red arrowheads denote PLM expression at 13 hpf and endothelial expression at 19 and 24 hpf. (D) cDNA from uninjected, *notch1b* morphant, or *notch2* morphant embryos with varying dosages of morpholino (2.5-10ng per embryo) subjected to RT-PCR analysis using specific primers which amplify wild-type or exon2 skipped amplicons that result in frame shift and premature stop codon (E). RT-PCR for *b-actin* was used as a loading control. Wild type and axon skipped products and were validated by sequencing. 10ng per embryo dose was the minimal dosage that consistently was used for both morpholinos for all experiments unless otherwise specified.

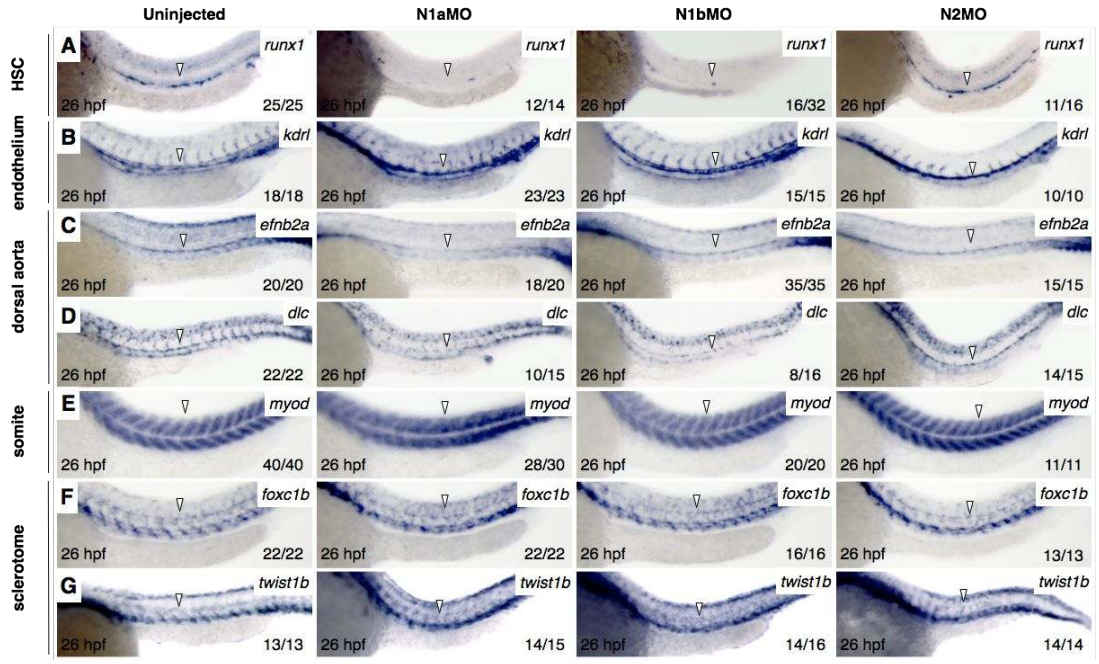

Figure 1-S4 *Notch1a* and *notch1b* are required for HSC specification but *notch2* is dispensable

WISH of uninjected, *notch1a* morphants, and *notch1b* morphants at 26 hpf for *runx1* (A), endothelial marker *kdrl* (B), dorsal aorta markers *efnb2a* (C) and *dlc* (D), the somite marker *myod* (E), and sclerotome markers *foxc1b* (F) and *twist1b* (G). Arrowheads denote tissue-specific expression.

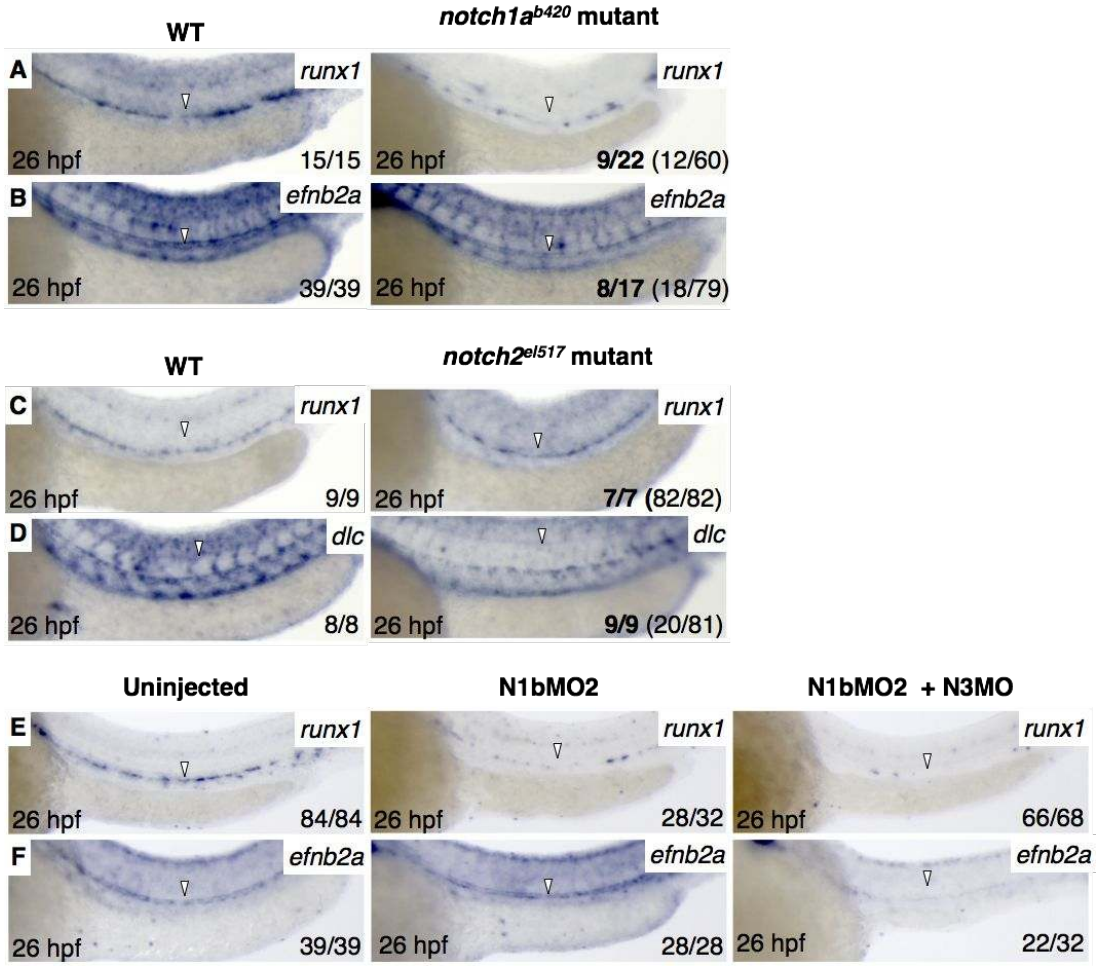

Figure 1-S5 Mutants for Notch1a and Notch2 resemble morphant phenotypes. Notch1b and Notch3 are synergistic for dorsal aorta specification.

WISH of *runx1* (A) and *efnb2a* (B) in wild-type and *notch1a*^{b420} mutant embryos at 26 hpf. Counts in bold indicate number of phenotypically screened mutants with depicted phenotype out of total screened mutants, counts in parenthesis indicate number of progeny from adult *notch1a*^{b420} heterozygote matings that show depicted phenotype out of total sample size. WISH of *runx1* (C) and *dlc* (D) in wild-type and *notch2*^{el517} mutant embryos at 26 hpf. Counts in bold indicate number of genotyped mutants with depicted phenotype out of total genotyped mutants, counts in parenthesis indicate number of progeny from adult *notch2*^{el517} heterozygote matings that show depicted phenotype out of total sample size. WISH of *runx1* (E) and *efnb2a* (F) in uninjected, *notch1b* morpholino #2 injected, and *notch1b* morpholino #2 and *notch3* morpholino coinjected embryos.

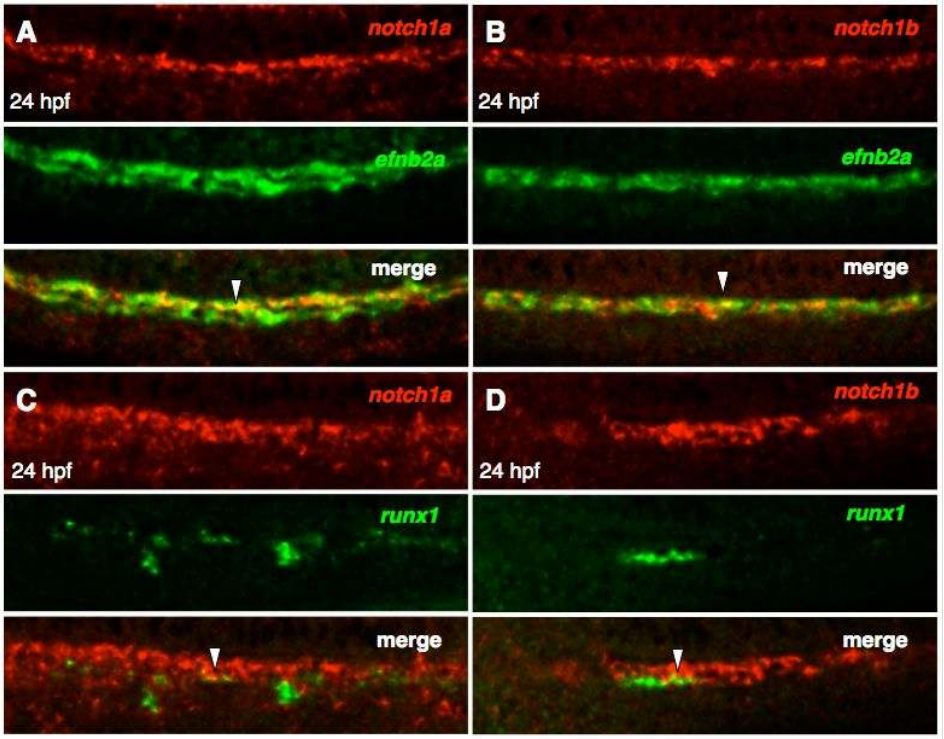

Figure 1-S6 *Notch1a* and *notch1b* expressed in dorsal aorta

Single plane confocal images with optical section thickness of 1.47 μm of whole mount two-color FISH of *efnb2a* with *notch1a* (A) or *notch1b* (B) viewed laterally in the endothelium at 24 hpf. Coexpression of *runx1* and *notch1a* (C) or *notch1b* (D) viewed laterally in the endothelium at 24 hpf. Bottom panels show merged fluorescence from both channels, arrowheads denote domains of coexpression.

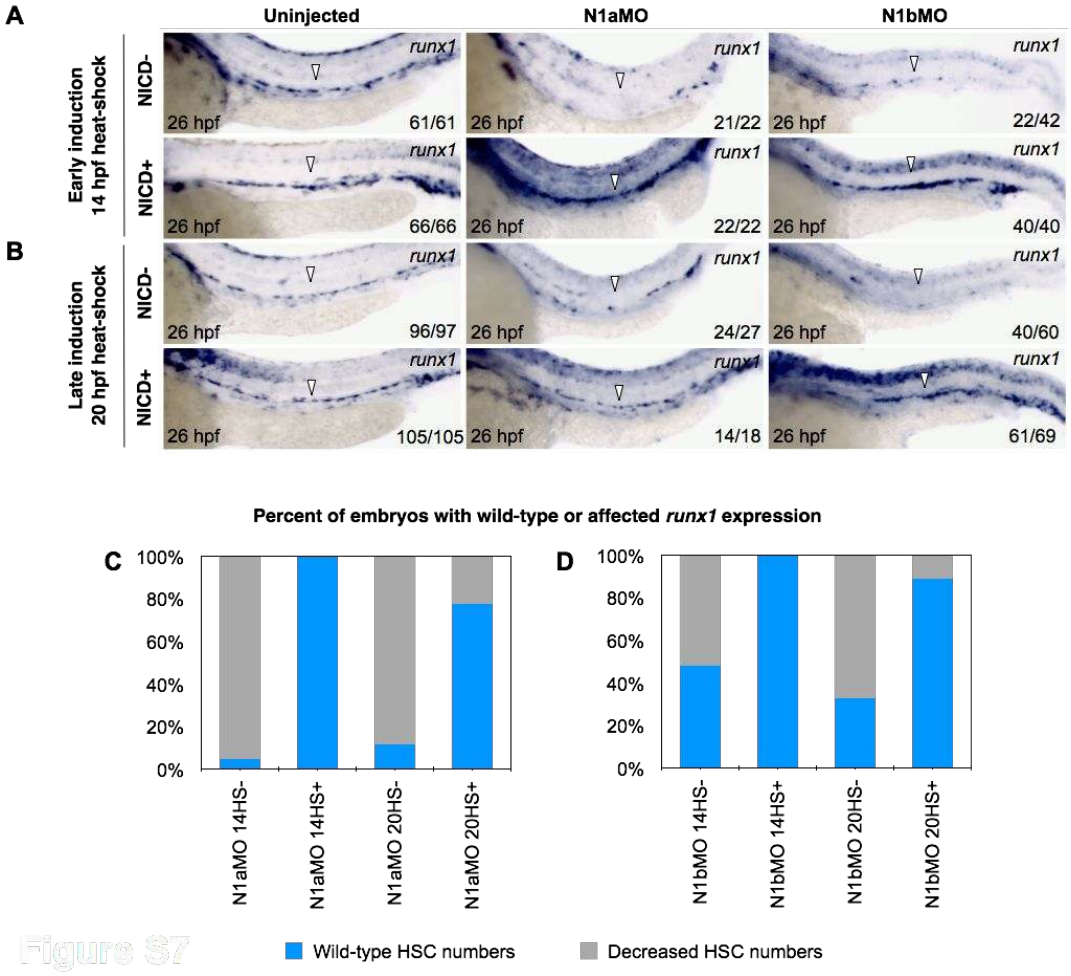

Figure 1-S7 Specific temporal activation of Notch signaling is sufficient to rescue HSCs in *notch1a* and *notch1b* morphants

WISH for *runx1* in *hsp70:gal4; UAS:NICD-myc* uninjected embryos or *notch1a* or *notch1b* morphants and heat-induced at 14 hpf (A) or 20 hpf (B) with or without enforced NICD expression at 26 hpf. Arrowheads denote presence or absence of *runx1*$^{+}$ HSCs in DA. Quantitation of results showing percentages of embryos displaying normal or decreased numbers of *runx1*$^{+}$ HSCs at 26 hpf by heat-shock induction in *notch1a* morphants (C) or *notch1b* morphants (D).

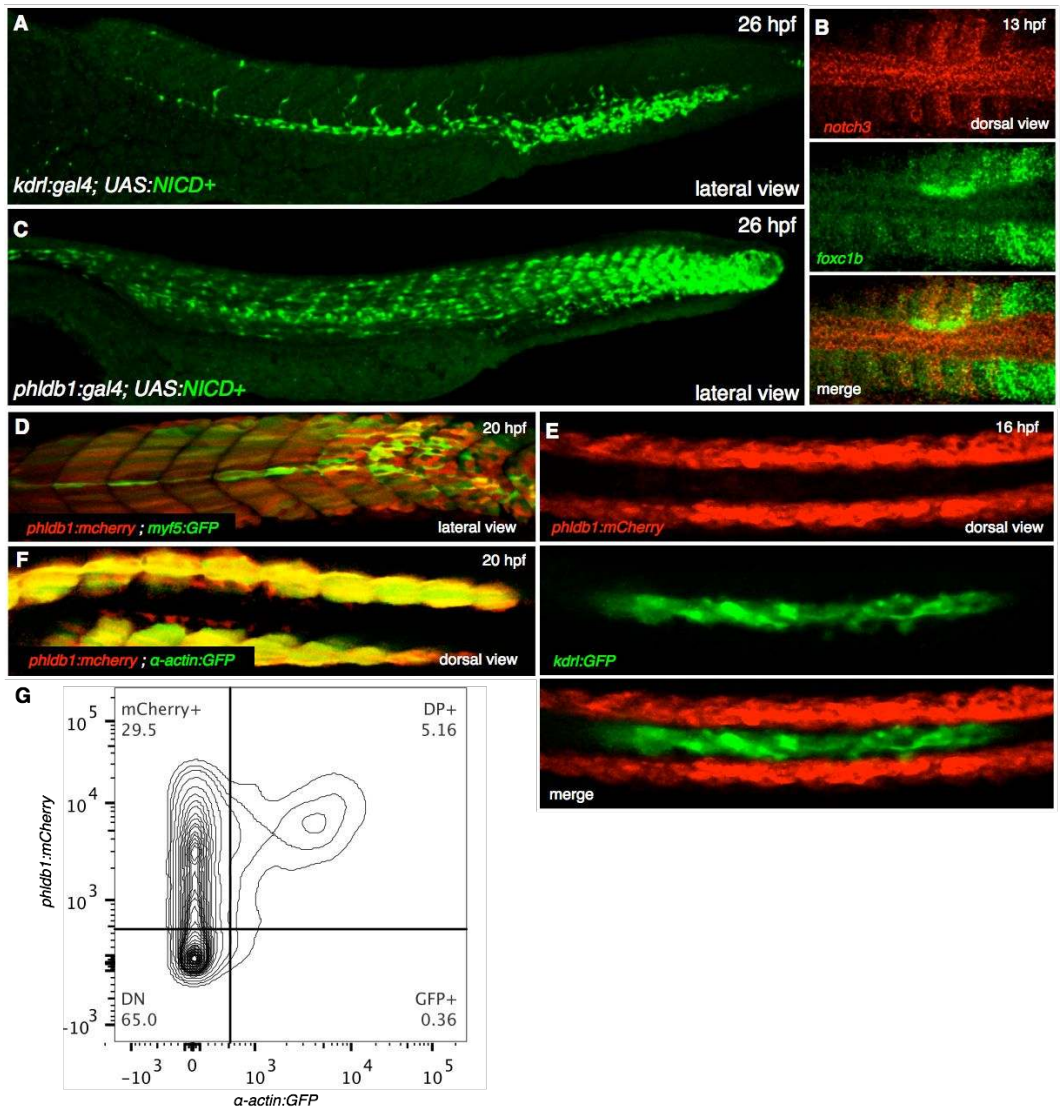

Figure 1-S8 Tissue-specific expression of *kdrl:gal4* and *phldb1:gal4* lines. Notch3 is expressed in the sclerotome.

(A) Whole mount immunofluorescence for MYC in *kdrl:gal4*; *UAS:NICD-myc* double transgenic embryos showing endothelial expression at 26 hpf. (B) Single plane confocal images with optical section thickness of 1.47 µm of whole mount two-color FISH of coexpression of *foxc1b* and *notch3* viewed dorsally in the somites at 13 hpf. (C) Whole mount immunofluorescence for MYC in *phldb1:gal4; UAS:NICD-myc* double transgenic embryos showing somitic expression at 26 hpf. Max projection confocal imaging of double transgenic *phldb1:gal4-mCherry* crossed to *myf5:GFP* (D), *kdrl:GFP* with single and merged channels (E), or *α-actin:GFP* (F) at stages and orientation indicated. (G) Flow cytometry of *phldb1:gal4-mcherry; α-actin:GFP* embryos at 17 hpf show GFP^{+} somitic cells are double positive for $mCherry^{+}$.

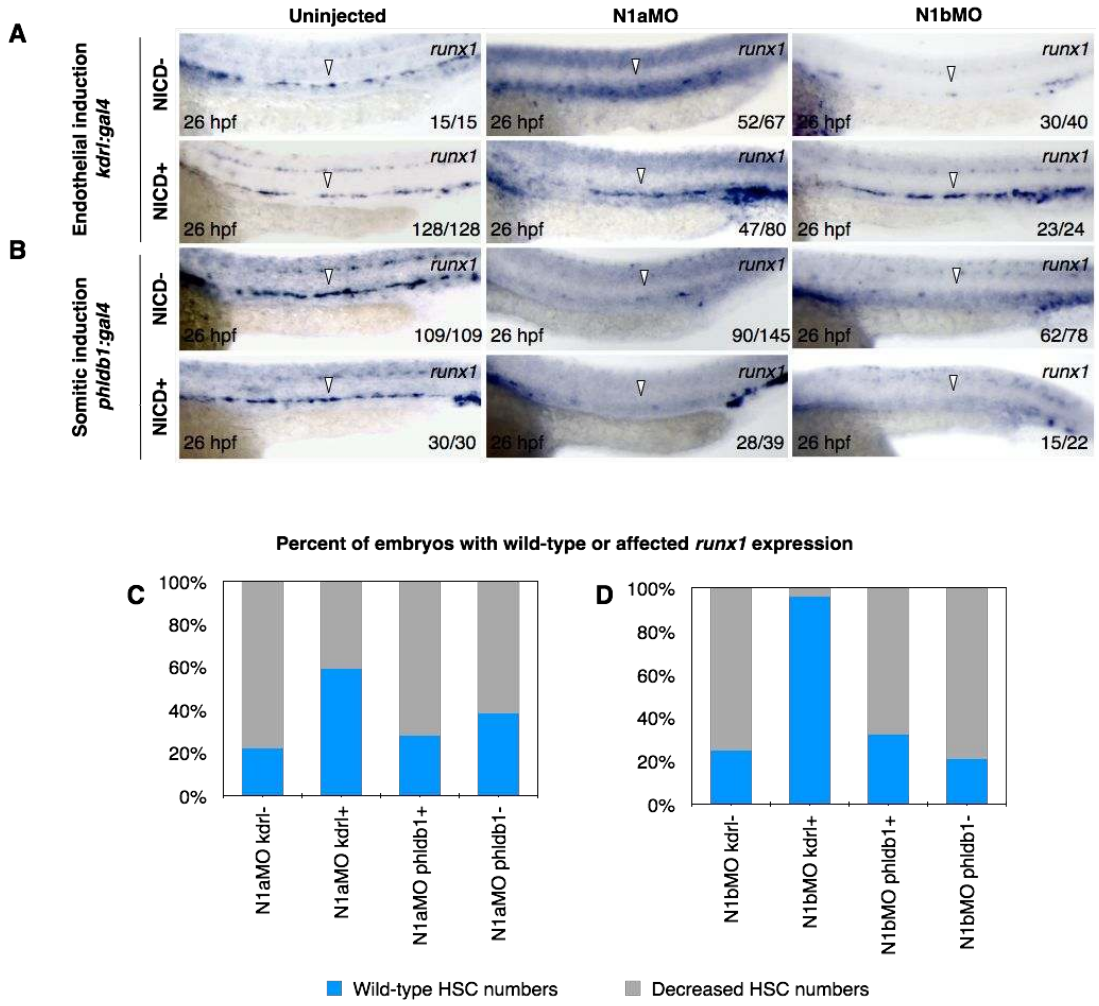

Figure 1-S9 Specific spatial activation of Notch signaling is sufficient to rescue HSCs in *notch1a* and *notch1b* morphants

Expression of *runx1* in *kdrl:gal4* (A) or *phldb1:gal4-mCherry* (B)*; UAS:NICD-myc* uninjected embryos, *notch1a* morphants, or *notch1b* morphants with or without enforced NICD expression at 26 hpf. Quantitation of results recording percentages of embryos displaying normal or decreased numbers of *runx1*$^{+}$ HSCs at 26 hpf in *notch1a* morphants (C) or *notch1b* (D) morphants with tissue-specific induction conditions.

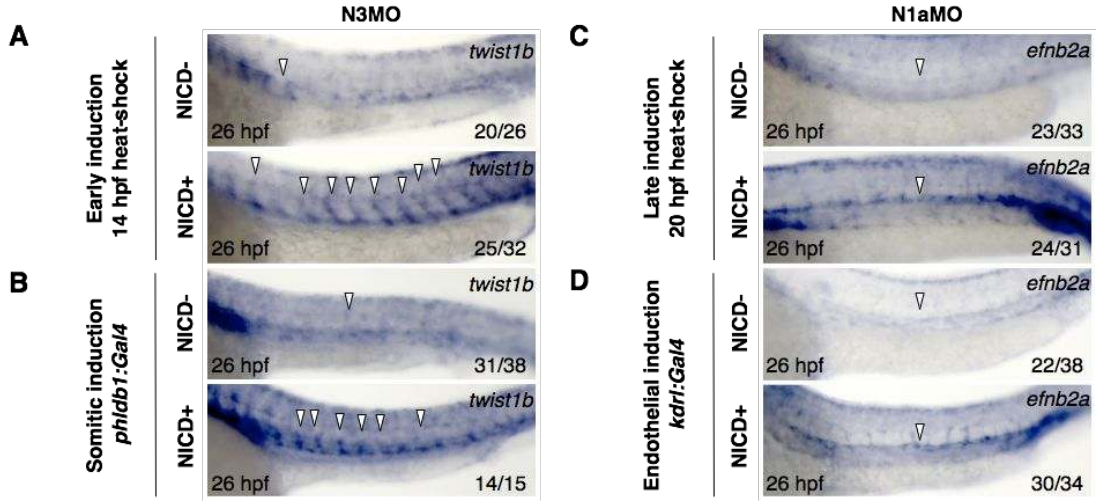

Figure 1-S10 Spatiotemporal activation of Notch is sufficient to rescue dorsal aorta and sclerotome with HSC specification in Notch receptor morphants

WISH for *twist1b* in *notch3* morphants at 26 hpf with 14 hpf induction induction of *hsp70:gal4; UAS:NICD-myc* (A) or *phldb1:gal4; UAS:NICD-myc* (B) with or without NICD. WISH of *efnb2a* in *notch1a* morphants at 26 hpf with 20 hpf induction of *hsp70:gal4; UAS:NICD-myc* (C) or *kdrl:gal4; UAS:NICD-myc* (D) with or without NICD. Arrowheads denote tissue-specific expression.

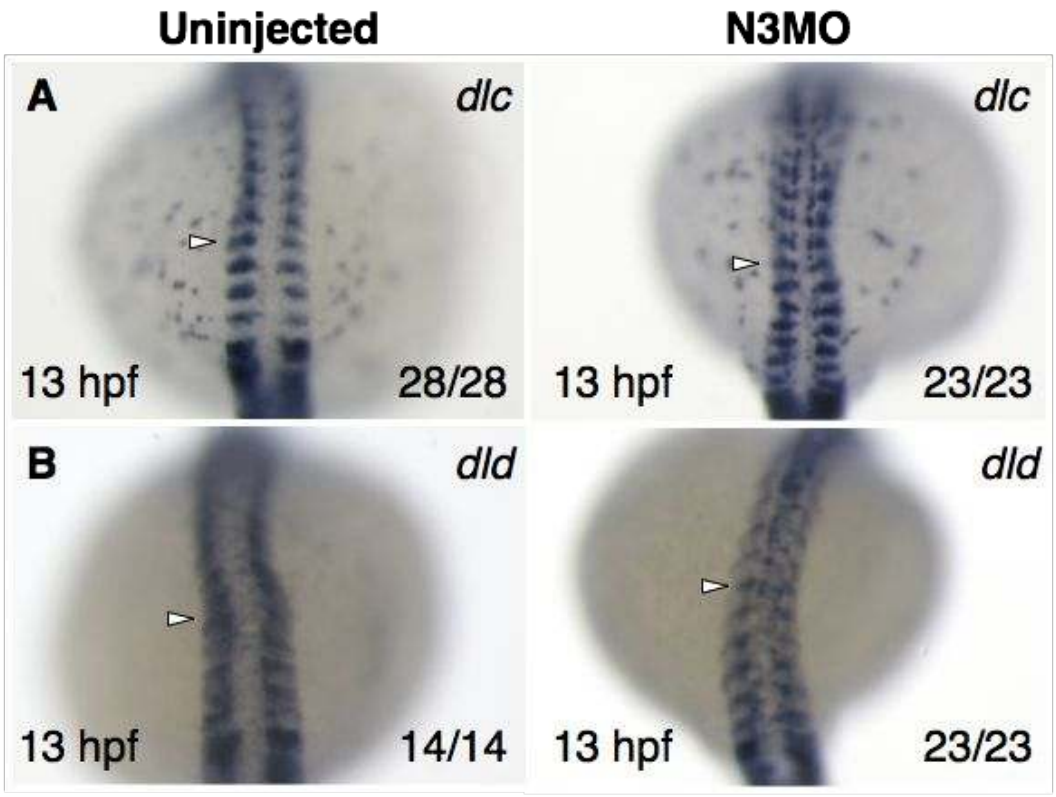

Figure 1-S11 *Notch3* is not required for somitic *dlc* or *dld* expression

WISH of *dlc* (A) or *dld* (B) at 13 hpf in uninjected or *notch3* morphants. Arrowheads denote somitic expression.

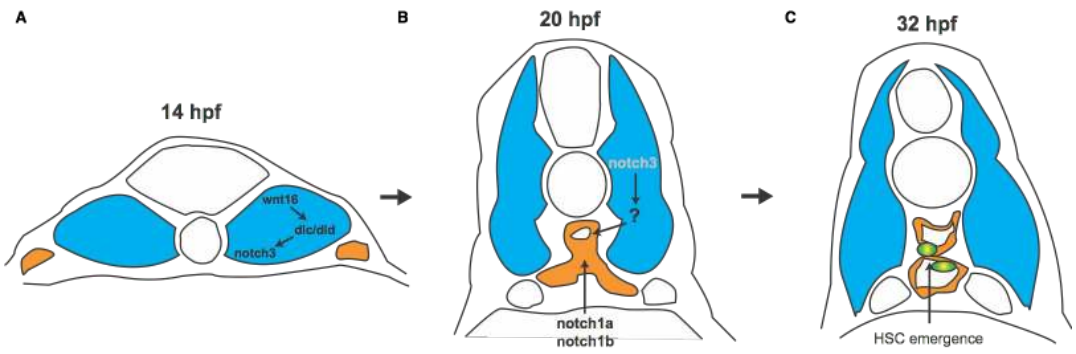

Figure 1-S12 Notch receptors act iteratively to specify HSCs

A schematic model for Notch requirement in HSC specification. (A) *Wnt16* regulates expression of somitic *dlc* and *dld*, these signals activate somitic *notch3* receptor during a permissive time window up until 14 hpf and is required for HSC specification. (B) In *kdrl*$^{+}$ endothelium *notch1a* is required for dorsal aorta specification while *notch1a* and *notch1b* are both required independently for HSC specification as late as 20 hpf. (C) Inputs from somitic *notch3* and endothelial *notch1a*/*notch1b* activation are parallel requirements for HSC specification and subsequent budding at 32 hpf.

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Author Contribution

A.D.K., W.K.C., and D.T. designed all experiments; Whole-mount in situs, whole-mount immunofluorescence, and double fluorescence in situs were performed by A.D.K. and C.H.M; Cell sorting experiments were performed by D.L.S. and A.D.K.; all other experiments were performed by A.D.K.; D.P. and C.M. provided the *Tg(kdrl:miniGAL4)* reporter line*;* M.D. provided the *Tg(phldb1:gal4-mCherry)* reporter line; The manuscript was written by A.K. and edited by D.L.S., W.K., and D.T. with critical input as described in the Acknowledgements.

Conflict of Interest

The authors declare that they have no conflicts of interest.

CHAPTER 2

Pdgfra is required for the specification of hematopoietic stem cells

Abstract

Our previous studies identified that multiple Notch receptors are involved in the specification of hematopoietic stem cells (HSCs) at distinct times and places; Notch3 is required first in the somites while Notch1a and Notch1b are required later in the endothelium. We asked if required Notch receptors are regulated in distinct ways from each other. We identified that Pdgf signaling mediated by Pdgfra is required for HSC specification and lies genetically upstream of endothelial *notch1b* expression. Enforced expression of Notch intracellular domain (NICD) within the endothelium of Pdgfra deficient embryos that lacked endothelial *notch1b* expression was sufficient to rescue *runx1*, supporting the claim that Pdgfra is required for HSC specification through activity of Notch1b. We identified that Pdgf signaling is required for *notch1b* expression and HSC specification during a narrow time window spanning 6-14 hpf by pharmacological inhibition experiments. During this developmental stage Pdgfra is expressed in somitic tissues and lateral plate mesoderm (LPM) by whole mount *in situ* (WISH). Expression of Pdgfra in LPM and requirement for endothelial expression of *notch1b* is consistent with a cell-autonomous requirement for Pdgra in HSC formation. Additionally, Pdgfra-deficient embryos also have specific defects in sclerotome that have been implicated in HSC formation. We asked if sclerotome defects observed in Pdgfra mutants could be explained loss of somitic expression *notch3, dlc,* or *dld*, however no defects were observed indicating that Pdgfra is a parallel requirement for

sclerotome along with somitic Notch3 signaling. In contrast to this finding, enforced expression of NICD within the somite compartment of Pdgfra partially rescued *runx1* expression in the dorsal aorta (DA), suggesting that overactivation of Notch signaling in somites can partially compensate for the loss of Pdgfra in HSC specification. These data indicate that Pdgfra signaling is required in two distinct roles for HSC formation; in parallel with Notch signaling in somites as to specify sclerotome, and required for endothelial expression of *notch1b*. These studies demonstrate the first functional requirement of Pdgf signaling in HSC formation during embryogenesis.

Introduction

The platelet-derived growth factor (PDGF) signaling pathway is conserved across the animal kingdom and shares evolutionary origins with the VEGF signaling pathway in invertebrates that possess a single PDGF/VEGF-like signaling pathway (Tarsitano et al, 2006). In conservation with VEGF signaling, PDGF signaling is mediated by signaling between diffusible disulfide-linked ligand dimers that are predominantly homodimers of PDGF-A, -B, -C, or –D, although heterodimers of PDGF-AB have been identified as described elsewhere (Fredriksson et al, 2004; Tallquist & Kazlauskas, 2004). These ligand dimers act via two receptor tyrosine kinases (RTKs) PDGFRα and PDGFRβ that share an extracellular immunoglobulin loop and intracellular receptor tyrosine kinase domain that is a conserved structural feature with VEGF receptors. PDGF ligand dimer binding induces PDGF receptor dimerization and subsequent autophosphorylation and activation of RTK domains (Kelly et al, 1991). Specific PDGF dimers bind to and activate distinct PDGF receptor dimers; PDGF-AA or PDGF-AB recruits PDGFR αα homodimers, PDGF-AB recruits PDGFR αβ hetermodimers, and PDGF-BB recruits PDGFR ββ dimers (Hammacher et al, 1989; Kanakaraj et al, 1991). Activation of PDGFR RTKs effect downstream signaling pathways by providing docking sites for molecules containing Src homology 2 (Src) domains and can activate Ras-MAP Kinase, PI 3-Kinase, Phospholipase C-γ signaling pathways activating a wide array of cell signaling responses. For comprehensive a review of PDGF signaling see Andrae, and Heldin and Westermark (Andrae et al, 2008; Heldin & Westermark, 1999).

PDGFRα and PDGFRβ perform many essential functions during embryogenesis and in the adult body including roles in nearly every major organ system including the vasculature, dermis, kidney, lung, testis, intestine, central nervous system, and is involved in wound healing, and tissue homeostasis as reviewed elsewhere (Betsholtz, 2004; Hoch & Soriano, 2003). The role of PDGFRβ and main associated ligand PDGF-B are best known for their roles in the vascular system and recruitment of vascular smooth muscle cells during early development and also in tumor pathogenesis (Abramsson et al, 2003; Hellstrom et al, 1999). In contrast PDGFRα is more widely involved in embryonic developmental processes, presenting difficulty in analyzing mutant phenotypes in mouse due to early lethality and plieotrophic effects.

A major organ system that PDGF signaling has not been well investigated for a role in is the hematopoietic system. The hematopoietic system is established during embryonic development through a series of waves generating primitive and definitive waves, ultimately culminating with the establishment of hematopoietic stem cells (HSCs) (Clements & Traver, 2013; Orkin & Zon, 2008). Although many signaling pathways are involved in specification of HSCs, no studies to date have identified a role of PDGF signaling in establishment of HSCs, although a role in definitive erythropoiesis downstream of HSC formation has been described (Li et al, 2006).

In this report we investigated in the zebrafish embryo if Pdgfra is required for HSC specification. We have determined by temporally-controlled pharmacological inhibition experiments that Pdgfra is required during mid-somitogenesis for HSC specification and is dispensable afterwards. We demonstrate that Pdgfra is involved in

HSC specification by two distinct mechanisms; first by establishment of the sclerotome that is involved for HSC emergence, and second by induction of Notch1b in HSC precursors in the endothelium.

Results

Pdgfra is required for HSC specification

Previous studies in mouse have demonstrated that PDGFRα is expressed in mesodermal tissues that give rise to endothelial, hematopoietic, and mesenchymal lineages (Ding et al, 2013; Yoshida et al, 1998). In zebrafish the homologue for PDGFRα gene Pdgfra is conserved, and is similarly expressed mesodermal tissues (Liu et al, 2002). We confirmed by whole mount *in situ* hybridization (WISH) that Pdgfra is expressed in the somites and lateral plate mesoderm (LPM) during mid-somitogenesis at 13 hpf, and found continued expression throughout 26 hpf (Figure 1A). By two-color fluorescent *in situ* hybridization (FISH) we found that *pdgfra* is coexpressed with early LPM expression of *fli1* at 13 hpf, but is downregulated in the endothelium later at 26 hpf and is mainly expressed adjacent to the endothelium at 26 hpf, consistent with its known expression pattern in mouse (Figure 1B). These expression patterns of Pdgfra in mammals and zebrafish are suggestive for a role in HSC specification, however no studies to date have investigated a functional role for Pdgfra in HSC specification.

To determine if Pdgfra signaling is required for HSC specification we analyzed Pdgfra mutant and morphant phenotypes for defects in HSC marker expression. WISH for *runx1* expression in the DA of Pdgfra mutants and morphants showed greatly reduced expression compared to wildtype embryos (Figure 2A and 2B), indicating that HSC specification requires Pdgfra. The fact that *runx1*+ cells are reduced in *pdgfra*

morphants to a similar level observed in Pdgfra mutants indicates that reduction of HSCs is not caused by off-target effects from the *pdgfra* morpholino. Consistent with these WISH results, confocal imaging of *cmyb:GFP; kdrl:RFP* embryos indicated that cmyb^{+}kdrl^{+} double-positive HSCs were reduced at 48 hpf in *pdgfra* morphants (Figure 2C). Furthermore, the generation of *rag2:GFP*$^{+}$ T lymphocytes, which are downstream progeny of HSCs, were reduced at 4 dpf in *pdgfra* morphants (Figure 2D). Quantitation of the number of cmyb^{+}kdrl^{+} double-positive HSCs showed that the difference between uninjected and *pdgfra* morphants is statistically significant (Figure 2E). The fact that all HSC markers analyzed were greatly reduced in Pdgfra-deficient embryos indicates that Pdgfra is required for HSC production.

Pdgfra signaling is required for Notch1b signaling in HSC precursors

We asked if the defects in HSC specification observed in Pdgfra-deficient embryos were caused by defects in Notch signaling. We previously demonstrated that precursors to HSCs experience elevated Notch signaling prior to and during HSC committment utilizing transgenic reporters driven by a Notch responsive promoter element TP1 (Clements et al, 2011; Kobayashi et al, 2014). Uninjected double transgenic *tp1:GFP; kdrl:RFP* embryos at 26 hpf show numerous double positive cells in the DA, however *pdgfra* morphants showed reduced numbers of double positive cells, indicating that Notch signaling in the endothelium is lowered in absence of Pdgfra (Figure 3A). We asked if DA specification, a Notch-dependent process, was impaired in *pdgfra* morphants by WISH for *efnb2a* at 26 hpf, however no significant changes in expression were detected (Figure 3B). We reasoned that if Notch signaling

in the DA was impaired in *pdgfra* morphants but arterial specification was intact, Pdgfra might be involved with specific signaling through Notch1b that is similarly required for HSC specification but is independently dispensable for arterial specification (Kim et al, 2014b). Consistent with this hypothesis expression of *notch1b* was specifically reduced in the DA, but not in neural tissues, of Pdgfra mutant embryos (Figure 3C). Furthermore activation of Notch signaling in endothelium that was previously shown to be sufficient to rescue *runx1* in the DA of *notch1b* morphants was also sufficient to rescue *runx1* in *pdgfra* morphants (Figure 4). These results indicate that Pdgfra specifically regulates Notch1b-mediated signaling in the DA to specify HSCs.

We previously demonstrated Notch1b is cell-autonomously required for HSC specification and is expressed in HSCs during the time HSCs are specified at 24-26 hpf (Kim et al, 2014b). Because we observed *pdgfra* expression in and around *fli1*+ endothelium 26 hpf (Figure 1B and 1C) we asked if at this developmental time if *pdgfra* and *notch1b* are expressed in the same cells in the DA. We performed two-color FISH for *pdgfra* and *notch1b* at 26 hpf and found that both genes are expressed in close proximity to each other in the DA (Figure 5A), similar to the expression pattern we observed with *pdgfra/fli.* To assess coexpression by an additional method, we analyzed transcript levels from sorted cell populations obtained from double transgenic *tp1:GFP; kdrl:RFP* embryos that enriched for Notch-responsive endothelium. This enrichment strategy was informed by the fact that this Notch-responsive double positive endothelial population was reduced in the absence of Pdgfra-deficient embryos (Figure 3A). Notch responsive endothelium contained high

levels of *efnb2a* and *runx1* transcripts, indicating that this fraction was enriched for DA and HSCs (Figure 5B and 5C). This double+ fraction contained high levels of *notch1b* transcript, but relatively lower levels of *pdgfra* compared to other sorted cell fractions (Figure 5D and 5E). Collectively these results indicate that Pdgfra is not strongly coexpressed in the same cells as Notch1b, but is highly expressed in immediately adjacent cells. Since Pdgfra is required for endothelial Notch1b expression, this close interaction between *pdgfra*+ and *notch1b*+ cells suggests that close cellular association could potentially be an important factor in this signaling relay.

We asked what intermediate mediators could be transducing signals between Pdgfra and Notch1b. Previous studies have demonstrated that Hey2, a hairy/enhancer-of-split-related basic-helix-loop helix transcription factor signaling considered to be a target of Notch signaling, acts genetically upstream of Notch1b expression in the endothelium (Rowlinson & Gering, 2010), bearing striking similarity to the phenotype we observed in Pdgfra-deficient embryos. We asked if Hey2 might be acting genetically downstream of Pdgfra and performed WISH in Pdgfra mutant embryos. Compared to wildtype embryos Pdgfra mutant clutches showed no alteration in *hey2* expression pattern, indicating that Hey2 does not serve as a mediator between Pdgfra signaling and Notch1b expression (Figure S1). These data indicate that Notch1b expression is dependent on at least two distinct molecular requirements for Pdgfra and Hey2.

Pdgfra is required during mid-somitogenesis for sclerotome specification

Our previous studies identified that the time window that Notch1b must be activated in order to specify HSCs is between 20-26 hpf by temporally controlled Notch intracellular domain (NICD) rescue experiments (Kim et al, 2014b). We therefore asked what temporal period Pdgfra signaling is required for induction of *notch1b* expression and HSC specification. To address this question we utilized a chemical inhibitor specific for Pdgfr signaling (Calbiochem) and treated embryos at time windows ranging from 6 hpf to 26 hpf and looked for defects in endothelial *notch1b* and *runx1*. Inhibition throughout 6-26 hpf led to a complete downregulation of both *notch1b* and *runx1* compared to DMSO vehicle treated controls (Figure 6A and 6B). Subdividing this range into 6-14 hpf and 14-26 hpf led to distinct outcomes, as early inhibition led to loss of *notch1b* and *runx1* equivalent to continuous drug treatment, however 14-26 hpf treatment had little to no effect on either marker (Figure 6C and 6D). These results indicate that Pdgf signaling is required during 6-14 hpf during mid-somitogenesis for expression of endothelial *notch1b* that is required between 20-26 hpf.

We asked if the loss of HSCs in Pdgfra-deficient embryos could be explained by defects in tissues related to HSC specification that are formed during 6-14 hpf. Because Pdgfra is expressed at 13 hpf in lateral plate mesoderm we analyzed if early LPM was affected in Pdgfra-deficient embryos. *Etsrp* is part of the ETS family of transcription factors that is expressed by and required for emergence of HSC precursors from the LPM and endothelium (Ren et al, 2010). Pdgfra mutants and embryos inhibited for Pdgfr activity during the 6-14 hpf critical window had no visible

defects in *etsrp* expression in the LPM (Figure S2). This result is consistent with our previous finding that Pdgfra is dispensable for arterial specification as evidenced by unaffected expression of *efnb2a* at 26 hpf in Pdgfra mutants compared to wildtype control embryos (Figure 3B). Therefore we concluded that overall vascular and arterial patterning was unimpaired and not the cause of loss of HSCs in Pdgfra-deficient embryos.

Previous studies have shown that Pdgfra is expressed in the sclerotome during the mid-somitogenesis in zebrafish (Liu et al, 2002) and is required for normal patterning of somites in mouse (Soriano, 1997), therefore we asked if Pdgfra might additionally be involved in HSC specification by establishment of the sclerotome. By two-color FISH we identified that *pdgfra* is coexpressed with sclerotomal *foxc1b* at 26 hpf (Figure 7A), indicating Pdgfra is coexpressed in the sclerotome until the developmental stage HSCs are specified. We then assessed if Pdgfra is required for sclerotome. Pdgfra-deficient embryos showed almost complete loss of *foxc1b*+ sclerotomal projections compared to control embryos (Figure 7B and 7C), similar to the phenotypes observed in Wnt16, Dlc, Dld, and Notch3-deficient embryos (Clements et al, 2011; Kim et al, 2014b). Because Pdgfra-deficient embryos had defects in sclerotome similar to embryos deficient in the Wnt16-Notch3 signaling cascade, we asked if Pdgfra is required for expression of members from this somitic Notch pathway. Somitic expression of *notch3, dlc* and *dld* were unaltered in Pdgfra-deficient embryos, indicating that the requirement for Pdgfra in sclerotome specification is not upstream of this somitic Notch signaling cascade (Figure S3). We asked if Notch signaling might regulate Pdgfra expression in a reversed epistatic

relationship, and analyzed Pdgfra expression patterns in Notch1a, Notch1b, and Notch3 morphant embryos that lack HSCs. Pdgfra expression in the sclerotome and LPM was largely unaffected in Notch-deficient embryos at 13 and 26 hpf, indicating that Pdgfra expression is not dependent on Notch signaling (Figure S4). Therefore the requirement for Pdgfra in sclerotome specification is independent of Notch signaling and is likely an additional independant role in HSC formation.

We previously demonstrated that sclerotome and HSC specification could be rescued in the absence of somitic-required Notch3 by ectopic activation of NICD in the somites (Kim et al, 2014b). Despite the fact that members of the Wnt16-Notch3 cascade were unaffected in Pdgfra-deficient embryos (Figure S3), we asked if ectopic activation of Notch signaling in the somites could enhance the lowered numbers of HSCs in Pdgfra-deficient embryos, effectively bypassing the requirement for Pdgfra. We found that activation of NICD in the somites of Pdgfra-deficient embryos was sufficient to increase the level of *runx1* compared to embryos that did not receive enhanced Notch signaling (Figure 8). These data suggest that Pdgfra and the Wnt16-Notch3 cascade are parallel requirements for sclerotome and HSC specification, and overactivation of Notch in a deficiency of Pdgfra is able to partially compensate for the reduction of HSCs. Furthermore, this role for Pdgfra in sclerotome specification is distinct from the function of activating cell-autonomously required Notch1b in the endothelium, however the two events may be linked by an unknown mechanism (Figure 9).

Discussion

Previous studies have demonstrated that Pdgf signaling is widely required for developmental processes during embryonic development, but has few known roles in the hematopoietic system. Pdgfra is expressed in the hematopoietic system (Ding et al, 2013) and is required for support of definitive erythropoiesis (Li et al, 2006), but a role for establishment of HSCs has not been described. Here we demonstrate that Pdgfra is required to HSC specification through multiple inputs; by activation of expression of Notch1b that is required cell-autonomously (Kim et al, 2014b), and by specification of sclerotome that is important in the microenvironment of HSCs (Clements et al, 2011).

We demonstrated that Pdgfra is involved in HSC specification by activation of cell-autonomously required Notch1b. Loss of function of Pdgra by mutation or chemical inhibition led to specific loss of Notch1b expression in the endothelium, but not in neural tissues. These data indicate that the regulation of Notch1b by Pdgfra is tissue-specific with respect to HSC emergence. The fact that chemical inhibition of Pdgf signaling during a narrow 6-14 hpf time window led to loss of both *notch1b* and *runx1* later at 24-26 hpf, while later inhibition left both markers intact suggest that the mechanism by which Pdgfra is involved in HSC formation is during early developmental processes well before the vasculature and HSCs are established. We observed no changes to early LPM, vascular, or arterial markers in Pdgfra-deficient embryos, indicating that vascular patterning was not affected. How Pdgfra signaling activates Notch1b expression in endothelial HSC precursors is unclear. We investigated if Pdgfra activates Notch1b expression via Hey2 that is required for

arterial specification, HSC specification, and Notch1b expression (Rowlinson & Gering, 2010), however we detected no alterations to *hey2* expression in Pdgfra mutant embryos. Downstream targets of Hey2 that are responsible for activation of Notch1b expression could potentially be targets of Pdgfa signaling, necessitating further investigation to identify intermediate mediators in this signaling pathway. The relationship between Pdgf and Notch signaling is better understood in pancreatic cancer cells. Inhibition of PDGF-D in cancer cells was reported to result in a loss of Notch1 and reduced cell migration and invasion (Wang et al, 2007), and Notch1 cleavage was shown to be dependent on the activity of PDGF signaling via c-Src (Ma et al, 2012). Pdgfra could regulate Notch1b in HSC specification in a similar fashion via Src as an intermediate, or by activating Notch1b via cleavage and inducing a feed-forward signaling loop.

We identified that Pdgfra signaling is required for specification of sclerotome, suggesting that Pdgfra signaling may be required for HSC specification in an additional way distinct from regulation of Notch1b. We recently demonstrated that specific receptors and ligands of the Notch pathway including Dlc, Dld, and Notch3 are required for specification of HSCs and sclerotome via their activation of Notch signaling within the somites (Clements et al, 2011; Kim et al, 2014b), we therefore asked if Pdgfra is involved in this signaling cascade. We found that loss of Pdgfra does not alter somitic expression of *dlc, dld*, and *notch3*, indicating that Pdgfra does not regulate the Dlc/Dld/Notch3 signaling cascade to specify sclerotome. In contrast with this finding, activation of Notch signaling in the somites that was previously shown sufficient to rescue sclerotome and HSCs in Notch3 morphants was able to

increase the levels of *runx1* in Pdgfra morphants. This result is surprising since we did not observe defects in somitic Notch receptor or ligand expression. A plausible interpretation of these findings is that sclerotome requires Pdgfra and Notch3-mediated signaling in parallel, and in the deficiency of one input overactivation of another is capable of restoring sclerotome specification. A recent study in mouse demonstrated that Pdgf ligand Pdgf-BB and Notch ligands Dll4 are required for conversion of skeletal myoblasts in the somites to perivascular cells, furthermore activation of NICD in the somites was sufficient to induce migration to the endothelium (Cappellari et al, 2013), similar to what has been observed in the avian embryo (Ohata et al, 2009). These studies suggest that somitic cells may require Notch and Pdgf signaling to become perivascular support cells for the embryonic vasculature, including hemogenic endothelium. Consistent with these findings hypothesis, a recent study described that a medial somitic compartment referred to as the endotome, directly contributes to aortic endothelium and induces HSC formation by expression of *cxcl12b* (Nguyen et al, 2014). Whether the requirement for Pdgfra in HSC formation is through regulation of endotome and/or cxcl12b signaling will require further investigation.

Our study demonstrates that HSC specification requires Pdgf signaling through the activation of Pdgfra during mid-somitogenesis. Pdgfra may be required cell-autonomously for HSC formation by activity within the LPM at 6-14 hpf that is required later for Notch1b expression, but could potentially be acting indirectly via a secondary signaling event that induces Notch1b. In addition, Pdgfra is required for specification of sclerotome that has been implicated in HSC formation. In theory one

of the functions of sclerotome is to serve as the microenvironment of emerging HSCs via an unknown a secondary signal. A comparison of molecular targets shared between somitic Wnt16, Notch3, and Pdgfra may be informative for identifying secondary messenges emanating from the sclerotome. In conclusion the role for Pdgfra in HSC specification is through multiple inputs that provide supportive signals as well as direct signals in HSC precursors.

Materials and methods

Zebrafish husbandry

Zebrafish strains AB*, *Tg(UAS:myc-Notch1a-intra)*^{kca3} (Scheer & Campos-Ortega, 1999), *Tg(rag2:EGFP)*^{zdf8} (Langenau et al, 2003), *Tg(phldb1:gal4-mCherry)* (Distel et al, 2009), *Tg(kdrl:RFP)*^{la4}, *Tg(TP1:GFP)*^{um14} (Parsons et al, 2009), *Tg(cmyb:EGFP)*^{zf169} (North et al, 2007), *Tg(kdrl:miniGAL4)* were maintained, injected, and staged as described (Westerfield, 2004) and in accordance with IACUC guidelines. The *pdgfra*^{ref} mutant line was generated from a forward genetic screen and mapped to the *pdgfra* locus. The *ref* mutation results in a missplicing event at exon14 that results in a truncated protein without a tyrosine kinase domain (Bloomekatz and Yelon et al., unpublished).

Microinjection of morpholinos, RT-PCR, and mRNA

The following morpholino antisense oligonucleotides were synthesized by Gene Tools, LLC and suspended as 25mg/ml stocks in DEPC ddH_2O and diluted to injection strengths: 8ng *pdgfra*-MO CACTCGCAAATCAGACCCTCCTGAT. Genotyping of *pdgfra*^{ref} mutants after phenotypic analysis was performed with Pd_Kpn1_F: GTAGGTAAAAGTAAAGCTGGTA Pdex14_R3: CAAGGGTGTGTTGAACCTGA primers.

WISH, immunofluorescence, and microscopy

Single enzymatic and double fluorescence whole mount *in situs* were performed as previously described (Clements et al, 2011). Antisense RNA probes for the following genes were prepared using probes containing digoxigenin or fluorescein labeled UTP: *pdgfra* (Eberhart et al, 2008)*, runx1, fli1, efnb2a, notch1b, foxc1b*, *hey2, etsrp, notch3, dlc,* and *dld* as previously described (Clements et al, 2011). Whole-mount immunofluorescence was performed using anti-Myc monoclonal 9E10 antibodies at 1:200 (Covance) and Dylight488 AffiniPure donkey anti-mouse IgG secondary antibodies (Jackson Immunoresearch Laboratories) at 1:100 as described previously (Clements et al, 2011). Fluorescence images of transgenic embryos and embryo samples were imaged using confocal microscopy (Leica, SP5) and processed using Volocity software (Perkin-Elmer) as previously described (Bertrand et al, 2010a).

Pharmacological inhibition of Notch signaling

Pdgfr Inhibitor V (Calbiochem) was dissolved in DMSO at a concentration of 2mM. Zebrafish embryos were incubated in 3 ml of 4 μM Pdgfr Inhibitor V solution in the dark from 6-26, 6-14 or 14-26 hpf followed by fixation with 4% PFA.

Fluorescence-activated cell sorting (FACS)

Kdrl:RFP; *TP1:GFP* embryos were collected at 26 hpf and processed for FACS as previously described (Bertrand et al, 2007).

Results

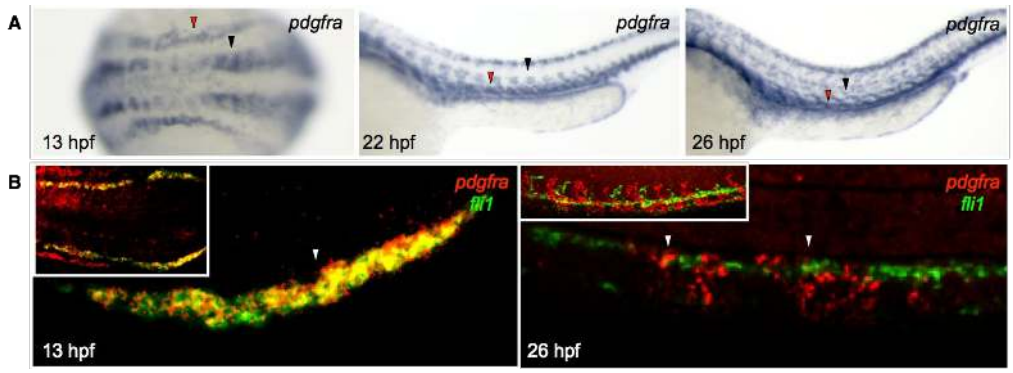

Figure 2-1 Pdgfra is expressed in somites and lateral plate mesoderm

(A) WISH of *pdgfra* viewed dorsally at 13 hpf (left) and laterally in the trunk at 22 (middle) and 26 hpf (right). Black arrowheads denote somitic expression, red arrowheads denote lateral plate mesoderm expression at 13 hpf and endothelial expression at 22 and 26 hpf. (B) Max projection (in cutout) and single plane confocal images with optical section thickness of 1.47 µm of whole mount two-color FISH of *fli1* in green with *pdgfra* in red viewed dorsally at 13 hpf and laterally in the endothelium at 26 hpf. Arrowheads denote domains of coexpression.

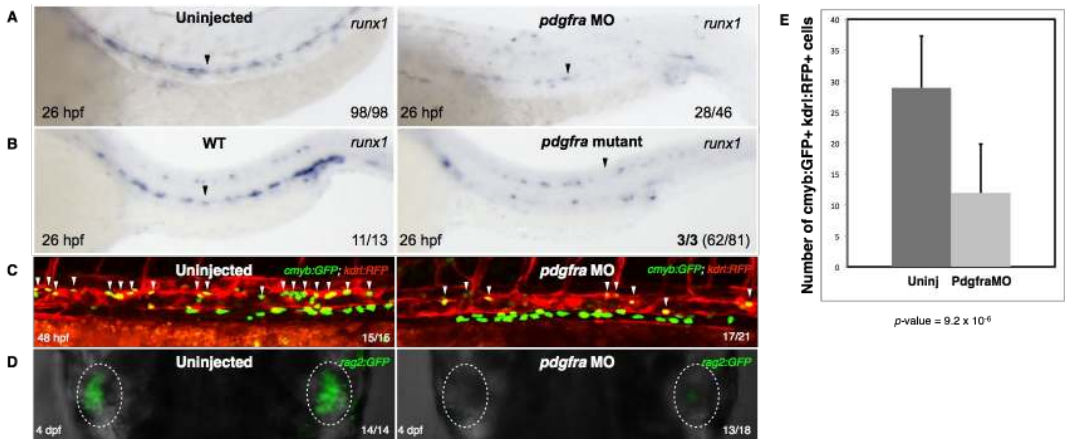

Figure 2-2 Pdgfra is required for HSC specification

WISH of the HSC marker *runx1* at 26 on uninjected and Pdgfra morphants (A) or mutants (B). Counts in bold indicate number of genotyped Pdgfra mutants with depicted phenotype out of total genotyped embryos, counts in parenthesis indicate number of progeny from adult Pdgfra heterozygote matings that show depicted phenotype out of total sample size. Arrowheads indicate HSCs in the DA. Confocal fluorescence microscopy images of transgene reporter expression in *cmyb:GFP; kdrl:RFP* trunk region at 48 hpf (C) and *rag2:*GFP at 4 dpf (D) transgenics uninjected or with *pdgfra* morpholino injected. Arrowheads in *cmyb:GFP; kdrl:RFP* embryos indicate double positive HSCs, and dotted lines in *rag2:GFP* embryos outline the thymic lobes were GFP^+ lymphoid cells should reside. (E) Enumeration of *cmyb:GFP+; kdrl:RFP+* cells in the floor of the DA at 48 hpf. Bars represent mean ± S.E.M. of double positive cells for uninjected (n=14) and pdgfra morphants (n=18). $p=9. \times 10^{-6}$.

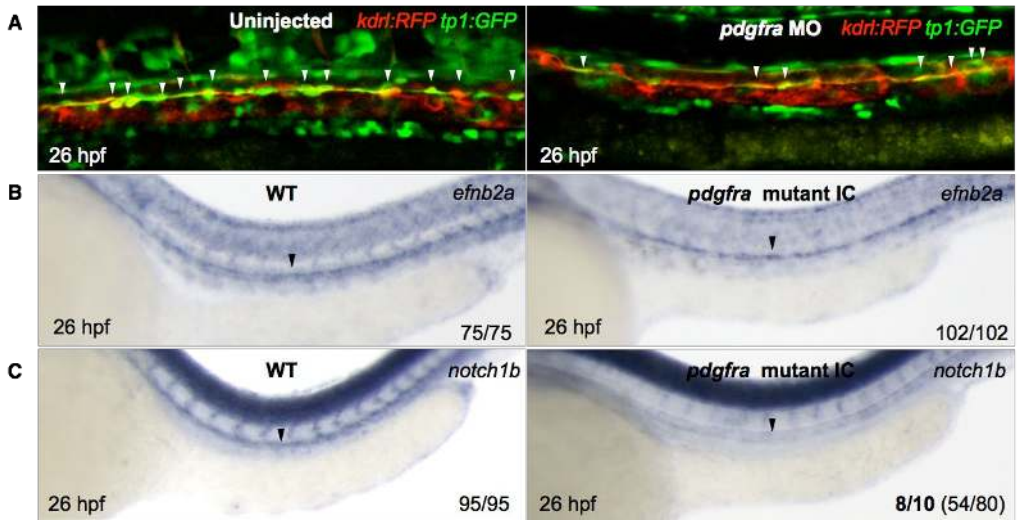

Figure 2-3 Pdgfra is required for Notch1b-mediated signaling in the dorsal aorta

Confocal fluorescence microscopy images of transgene reporter expression in *tp1:GFP; kdrl:RFP* transgenics uninjected or with *pdgfra* morpholino injected at 26 hpf. Arrowheads in *tp1:GFP; kdrl:RFP* embryos indicate double positive cells in the DA. WISH for *runx1* (Aberle et al) and *notch1b* (C) at 26 on wildtype or Pdgfra mutant clutches. Counts in bold indicate number of genotyped Pdgfra mutants with depicted phenotype out of total genotyped embryos, counts in parenthesis indicate number of progeny from adult Pdgfra heterozygote matings that show depicted phenotype out of total sample size.

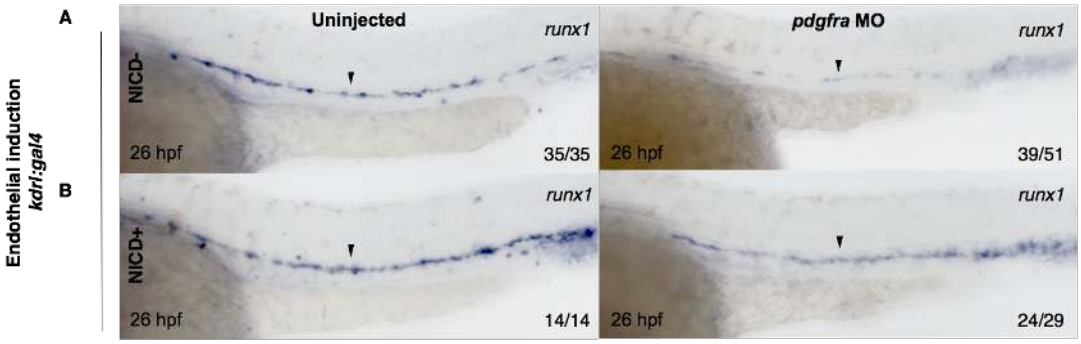

Figure 2-4 Endothelial activation of Notch signaling is sufficient to rescue HSCs in *pdgfra* morphants

WISH for *runx1* in 26 hpf in *kdrl:gal4-mcherry* crossed to *UAS:NICD-myc* transgenic embryos either uninjected or injected with *pdgfra* morpholino, with (A) or without (B) enforced NICD expression. Arrowheads indicate presence or absence of HSCs at the midline.

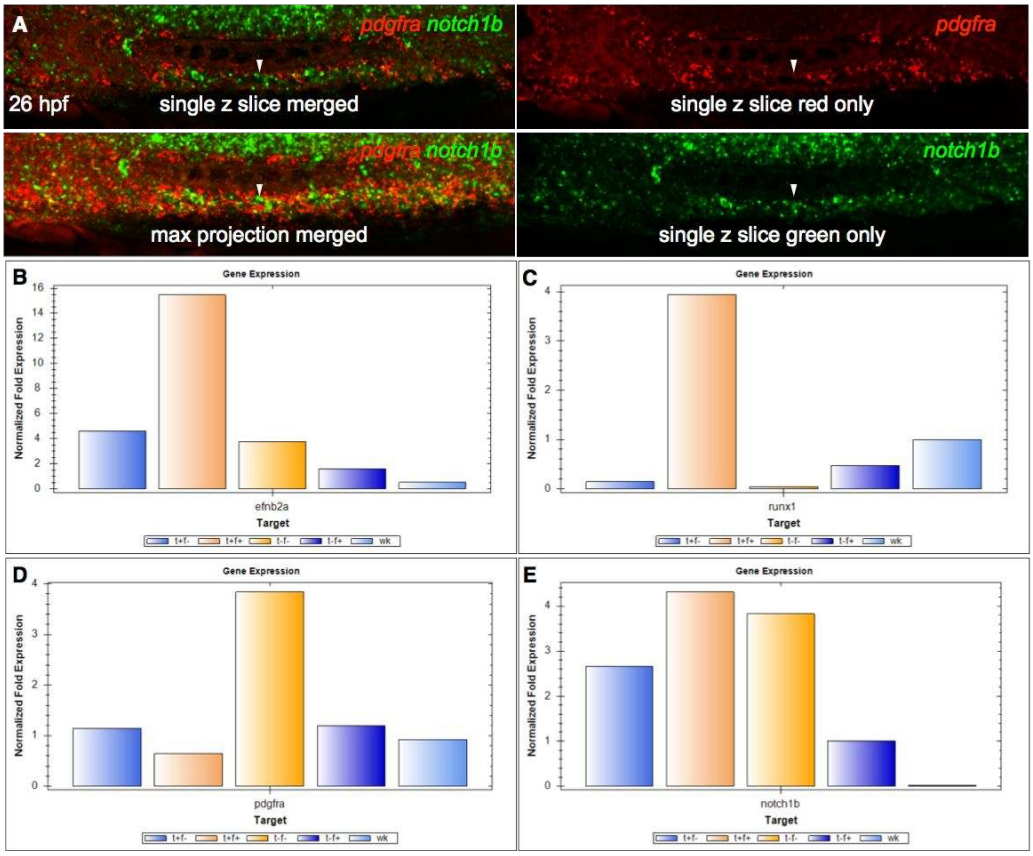

Figure 2-5 Notch1b and Pdgfra are expressed in and around the endothelium

(A) Single plane and max projection confocal images with optical section thickness of 1.47 μm of whole mount two-color FISH of *pdgfra* in red with *notch1b* in green viewed laterally in the endothelium at 26 hpf. Arrowheads denote domains of coexpression. QPCR showing relative expression of *efnb2a* (B), *runx1* (C), *pdgfra* (D), and *notch1b* (E) from sorted *tp1:GFP; kdrl:RFP* transgenics at 26 hpf. Samples names are abbreviated 't' for *tp1:GFP* + or - cells , 'f' for *kdrl:RFP* + or - cells, and wk for whole kidney marrow control tissue.

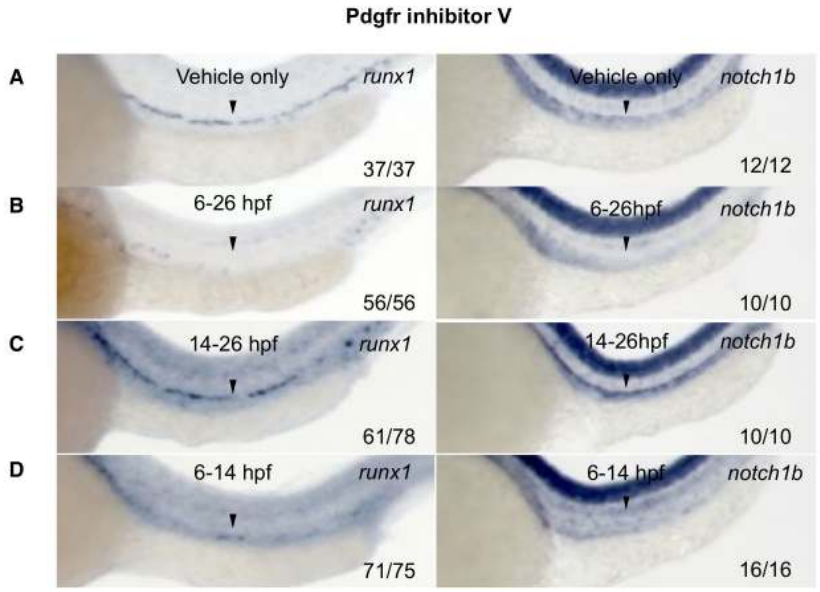

Figure 2-6 Pdgfr signaling is required during mid-somitogenesis for specification of HSCs and Notch1b expression

WISH for *runx1* and *notch1b* in 26 hpf embryos treated with DMSO vehicle (A) or 2uM Pdgfr inhibitor V at 6-26 hpf (B), 14-26 hpf (C), or 6-14 hpf (D). Arrowheads indicate expression in DA.

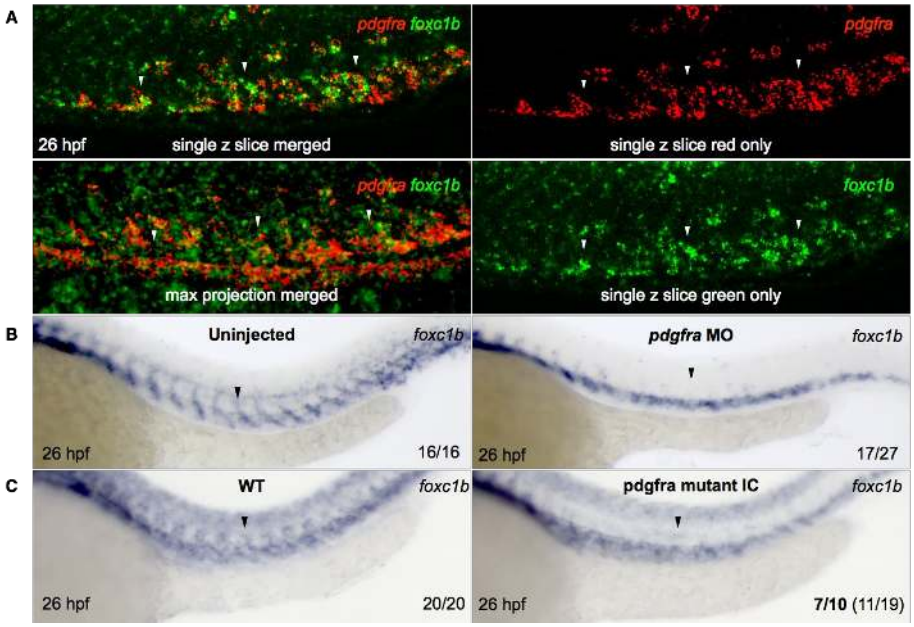

Figure 2-7 Pdgfra is required cell-intrinsically for sclerotome specification

(A) Single plane and max projection confocal images with optical section thickness of 1.47 μm of whole mount two-color FISH of *pdgfra* in red with *foxc1b* in green viewed laterally in the sclerotome at 26 hpf. Arrowheads denote domains of coexpression. WISH *foxc1b* in wild-type and Pdgfra morphant (B) mutant (C) clutch embryos at 26 hpf. Counts in bold indicate number of genotyped Pdgfra mutants with depicted phenotype out of total genotyped embryos, counts in parenthesis indicate number of progeny from adult Pdgfra heterozygote matings that show depicted phenotype out of total sample size.

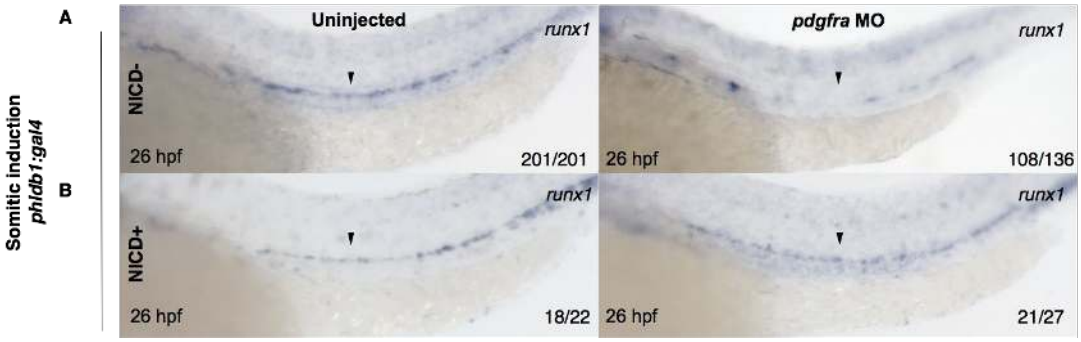

Figure 2-8 Somitic activation of Notch signaling is sufficient to rescue HSCs in *pdgfra* morphants

WISH for *runx1* in 26 hpf in *phldb1:gal4-mcherry* crossed to *UAS:NICD-myc* transgenic embryos either uninjected or injected with *pdgfra* morpholino, with (A) or without (B) enforced NICD expression. Arrowheads indicate presence or absence of HSCs at the midline.

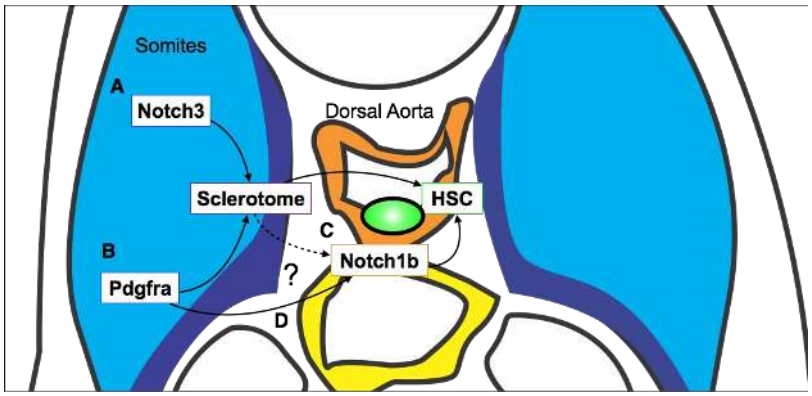

Figure 2-9 Interaction between Pdgf and Notch signaling in HSC specification

Notch3 is required for sclerotome and HSC specification during mid-somitogenesis (A) in parallel with Pdgra (B). Notch1b is required in the endothelium later during vasculogenesis (C) and is dependent on Pdgfra signaling (D).

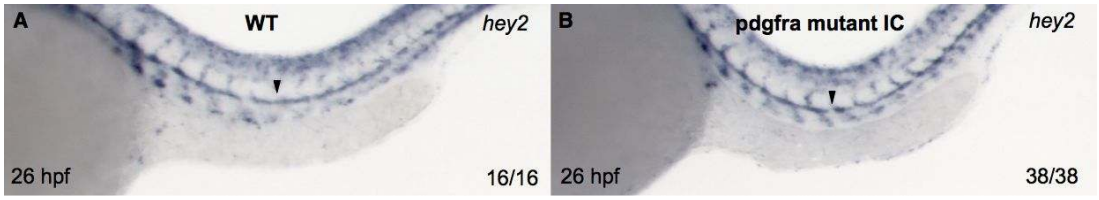

Figure 2-S1 Pdgfra is dispensable for Hey2 expression in the endothelium.

WISH of *hey2* in wildtype (A) and Pdgfra mutant (B) embryos viewed laterally at 26 hpf. Arrowheads denote expression in the endothelium.

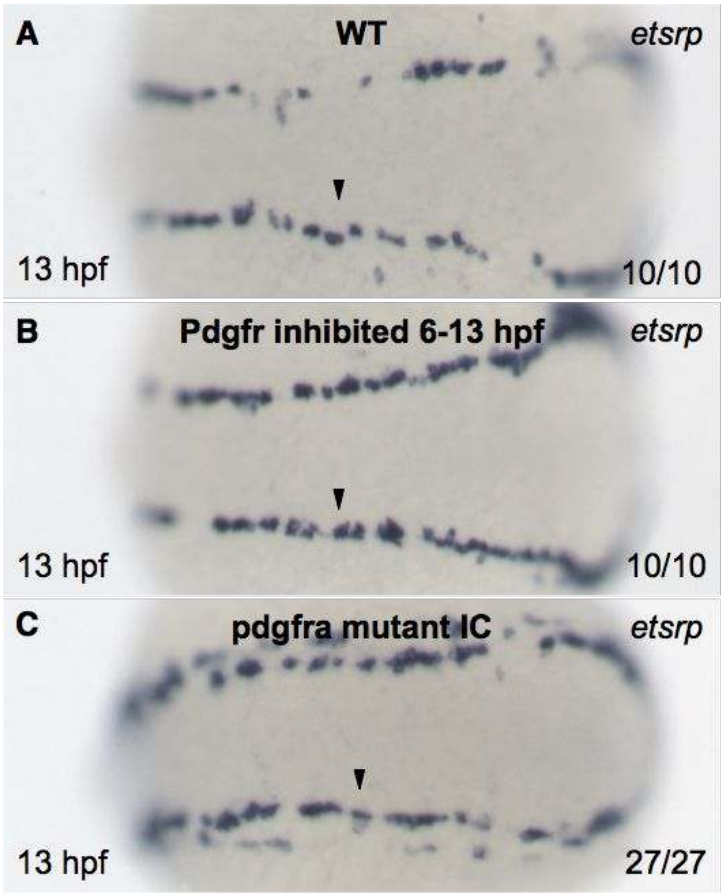

Figure 2-S2 Pdgfra is dispensable for lateral plate mesoderm formation.

WISH of *etsrp* in wildtype (A), Pdgfr inhibitor treated from 6-13 hpf (B), and Pdgfra mutant (C) embryos viewed dorsally at 13 hpf. Arrowheads denote expression in the LPM.

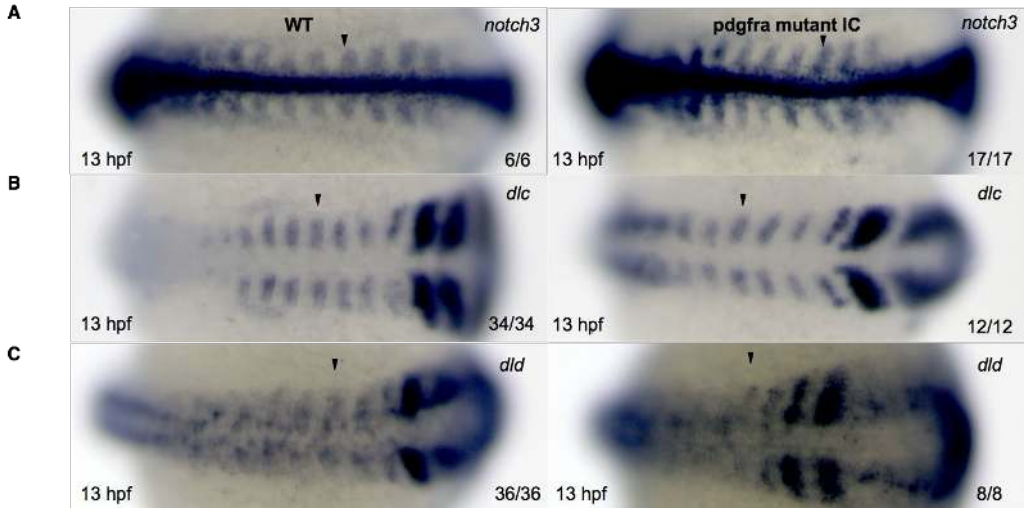

Figure 2-S3 Pdgfra is dispensable for somitic expression of Notch3, Dlc, and Dld.

WISH of *notch3* (A), *dlc* (B), and *dld* (C) in wild-type and Pdgfra mutant embryos viewed dorsally at 13 hpf. Arrowheads denote somite-specific expression of each gene.

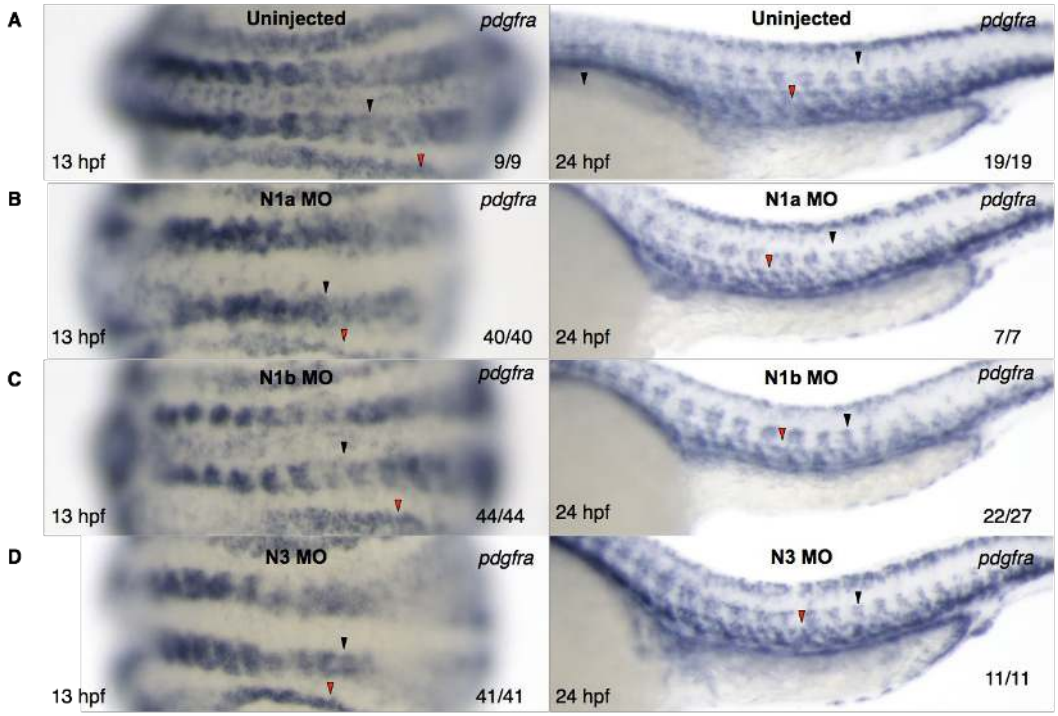

Figure 2-S4 Notch signaling is dispensable for Pdgfra expression

WISH of *pdgfra* in uninjected (A), *notch1a* (B), *notch1b,* or *notch3* morpholino injected embryos viewed dorsally at 13 hpf and laterally at 26 hpf. Arrowheads denote somite-specific expression of each gene. Black arrowheads denote somitic expression, red arrowheads denote lateral plate mesoderm expression at 13 hpf and endothelial expression at 24 hpf.

Acknowledgements

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Author Contribution

A.D.K., J.B., B.W., C.H.M, and D.T. designed all experiments; Whole-mount in situs, whole-mount immunofluorescence, and double fluorescence in situs were performed by A.D.K. and C.H.M; Cell sorting experiments were performed by D.L.S. and A.D.K.; J.B. and D.Y. provided the *pdgfra*^{ref} mutant line; The manuscript was written by A.K. and edited by J.B., B.W., and D.T.

Conflict of Interest

The authors declare that they have no conflicts of interest.

CHAPTER 3

Conclusion and Future directions

Notch receptor and ligand interactions in HSC specification

Previous studies established that Notch signaling is generally required for HSC specification through the function of Mindbomb and RBPj (Burns et al, 2005; Robert-Moreno et al, 2008; Yoon et al, 2008). Analysis of the role of specific Notch receptors and ligands demonstrated that Notch1 is required cell-intrinsically in HSC precursors (Hadland et al, 2004; Robert-Moreno et al, 2008), while Jagged1 is dispensable for arterial specification but required for HSC specification (Robert-Moreno et al, 2008). The fact that Notch1 and Jagged1 perform specific roles with respect to HSC specification hinted at the possibility that other Notch receptors and ligands might perform distinct roles important for HSC specification as well. Although nearly all previous studies analyzing the role of Notch signaling in HSC formation have focused on the role of Notch signaling within HSC precursors, the fact that Notch signaling is also important within the somitic tissues in the embryo has warranted investigation of specific Notch receptor and ligand involvement in the somites. We demonstrated that Dlc and Dld Notch ligands and Notch3 receptor are important for intitiation of the HSC program and are required within the somites. In conservation with mammalian development, Notch2 is dispensable while the Notch1 homologs Notch1a and Notch1b are required cell-intrinsically for HSC emergence in the zebrafish embryo (Clements et al, 2011; Kim et al, 2014b). The fact that three of the four Notch receptors are spatially and temporally significant for HSC specification at specific times and places

raises an immediate question of which ligands activate each receptor. We have identified that Dlc and Dld are required, however which of the remaining five ligands are also important for HSC formation remains in question. Identification of all of the required ligands will enable investigation of which specific receptor and ligand pairings are necessary in each Notch signaling event associated with HSC specification. This knowledge will greatly elucidate our understanding of the multiple roles of Notch signaling in HSC formation, and could inform future studies aimed at recapitulating these biological processes *in vitro*.

Functional promiscuity of required Notch receptors

Several lines of evidence indicate that Notch receptor involvement in HSC specification is functionally promiscuous beyond a role of Notch3 in the somites and Notch1a/b in the endothelium. While loss of Notch3 or Notch1b alone did not affect any markers of arterial specification including *efnb2a* and *dlc*, and loss of Notch1a alone caused defects in aorta markers, combinatorial loss of Notch1b and Notch3 led to severe defects in aortic and HSC specification (Kim et al, 2014a; Quillien et al, 2014). These data indicate that dorsal aorta formation is predominantly dependent on Notch1a activity and can tolerate loss of Notch1b or Notch3 alone but not in combination. This Notch1b/Notch3 signaling event required for aorta specification likely takes place as early as 11 hpf in Notch-responsive cells in the lateral plate mesoderm (LPM) (Quillien et al, 2014), before the requirement of Notch3 in the somites at 14 hpf, and Notch1a and Notch1b in the endothelium at 20 hpf. Thus, there

are likely at least 4 distinct waves of Notch signaling at distinct times and places in the embryo that are required for HSC formation.

Utilization of multiple Notch receptors for specific developmental processes is conserved between mammals and zebrafish. In mouse loss of Notch4 has mild defects on vascular patterning, however compounded loss of Notch1 and Notch4 have more severe vascular defects compared to Notch1 mutants (Krebs et al, 2000), similar to the functional synergy between Notch3 and Notch1b in fish. Why multiple Notch receptors might be used for similar biological processes with varying degrees of activation or dependency is a fascinating question. Perhaps varying usage is simply a result of different levels of availability of receptor, ligand, or modifications that alter activation. Alternatively a precise cocktail of specific Notch receptor activation might tailor a precise transcriptional response. One striking difference between mouse and zebrafish is the role of Notch3 with respect to HSC formation; in mouse, Notch3-deficient mice are viable and show no obvious developmental defects (Krebs et al, 2003). Why Notch3 alone performs an important role in fish but not in mouse for HSC formation is unclear. The presence of Notch4 in mammals might afford loss of function of Notch3 if they can functional compensation occurs. Phylogenetic analysis has predicted that Notch4 evolved from duplication of the Notch3 gene (Kortschak et al, 2001); thus Notch3 may perform a similar role in mammals as it does in zebrafish, but may be functionally synergistic with one or more other Notch receptors. An important question for future studies is to understand if somitic Notch signaling is important for HSC formation in mammals as it is in zebrafish, and which Notch receptors and ligands are involved.

Activation of specific Notch receptors

Distinct functions of each Notch receptor might be explained by specific pairing and activation by specific ligands, but differential regulation of the NICD domains may also contribute to distinct functions. One of the recently appreciated functions of Notch signaling in HSC function is that inactivation is important for HSC emergence and maintenance (Richard et al, 2013). If the final Notch signal that HSC precursors require also needs to be deactivated in order for HSCs to fully mature, we predict based on our studies that the final Notch signal is likely activation of Notch1. Notch1 NICD may be specifically suited to brief activation and subsequent inactivation compared to other NICDs. Consistent with this hypothesis, Notch1 intracellular domain (ICD) is readily degraded by Numb-mediated E3 ubiquitination while Notch3 ICD is not (Beres et al, 2011). Therefore one possible function of Notch3 in somitic Notch signaling is to activate high and/or persistent levels of Notch activation in tissues with high Numb-mediated degradation, while Notch1 may be reserved for a temporary pulse of Notch activation in the endothelium where it can be quickly degraded. One prediction made by this paradigm is that loss of somitic Notch3 might be rescued by inhibiting the function of Numb and allow compensation by activation of other Notch receptors expressed in the somite. Analyzing the temporal kinetics and stability of specific NICDs could elucidate the distinct functions of each Notch receptor in hematopoiesis, and possibly a greater range of biological process.

Careful analysis of the temporal kinetics of each Notch receptor activation will greatly inform when and where Notch signaling events important for HSC formation

are occurring, but will require the generation of receptor-specific reporters of activation. Notch receptor specific antibodies and Notch receptor-promoter driven reporter transgenes have informed which cells have expressed Notch receptors, but are not capable of reporting when the receptors are activated. Previous studies have utilized a combination of Notch receptor promoter-driven reporters alongside a Notch receptor target gene-specific promoter (such as Hes/Her genes) to report activation of Notch receptors (Oh et al, 2013). A more refined reporter of Notch receptor activation would precisely indicate when a Notch receptor is cleaved and NICD is translocated to the nucleus. Fusion of a functionally-inert tag to the NICD domain could potentially nuclear localization as a surrogate marker of receptor activation when detected by high-resolution live imaging. Such a construct would ideally be knocked-into the endogenous locus of a Notch receptor gene, however a more conventional transgenesis approach may be more feasible. Generation of more sophisticated molecular tags capable of being detected in live tissues with minimized off-target effects and temporal accuracy (fast maturing and quickly degrading) will lead to a higher resolution of understanding of Notch signaling and other important cellular processes.

Regulation of Notch inputs in HSC specification

While activation of Notch signaling is known to be important for HSC formation, little is known about the specific regulation of Notch receptors and their activating Notch ligands. Informed by the fact that specific Notch receptors and

ligands have distinct functions in somitic or endothelial tissue compartments, we asked if specific Notch receptors and ligands are regulated by unique inputs. Within somitic tissues we have demonstrated that Notch3 is functionally dependent on *dlc* and *dld* expression by low-dose knockdown synergy experiments, but evidence of direct binding await validation. We demonstrated that expression of *dlc* and *dld* are dependent on expression of non-canonical Wnt ligand Wnt16, therefore somitic Notch signaling requires a signaling cascade of Wnt16, Dlc/Dld, and Notch3 to specify HSCs. Interestingly Dlc but not Dld is regulated specifically by activity of the Fgf receptor Fgfr4 which is itself a target of Wnt16 (Lee et al, 2014), indicating Dlc and Dld have distinct regulatory inputs from each other. Why the somite requires Wnt16, Dlc, Dld, and Notch3 in concert is an intriguing question. The answer to this question can inform approaches for reconstructing a minimal set of signaling inputs required for generate HSCs *in vitro*.

Regulation of cell-intrinsic Notch signaling

Understanding how Notch1 is regulated is key to understanding the transcriptional regulation of HSC formation. The obligate ligand of Notch1 is believed by be Jagged1 due to the fact that Jagged1 mutants display hematopoietic defects. However our studies predict that Jagged1 could activate other Notch receptors required for HSC formation, potentially activating Notch3 in the somites. Zebrafish offer a unique opportunity to finely dissect the regulation of Notch1 due to the presence of two Notch1 homologues Notch1a and Notch1b that are both required cell-

intrinsically for HSC formation and have distinct regulatory inputs. The Notch target gene Hey2 is surprisingly required for endothelial expression of Notch1b and Notch3 in dorsal aorta and HSC specification (Rowlinson & Gering, 2010); therefore, understanding the Notch signaling event that initially activates Hey2 can inform a putative signaling cascade in which Notch regulates itself in HSC formation. Surprisingly inflammation and myeloid cell function is important for regulation of Notch1; the TNF proinflammatory signaling pathway has been shown to regulate activation of Notch1a via Jagged1a in HSC formation through the generation of primitive neutrophils (Espin-Palazon et al, 2014). Dissecting how this complex network of inflammation and myeloid cell recruitment precisely regulates Notch1a/Jagged1a will be an important question for future studies. Therefore understanding to what extent Notch1a and Notch1b divide the role of mammalian Notch1 is an intriguing line of investigation. Due to the fact that aberrant Notch1 activity is commonly associated with leukemia and pancreatic cancer, understanding the distinct functions and regulation of Notch1a and Notch1b may prove insightful to uncovering discrete functional properties of Notch1 in cancer pathogenesis.

Within the endothelial precursors of HSCs, regulation and activation of Notch1 is likely the predominant Notch signaling requirement ultimately leading to activation of genes required for HSC formation including Gata2 and Runx1 (Nakagawa et al, 2006; Robert-Moreno et al, 2008). Intriguingly the Runx1 promoter lacks bindings sites for Notch-responsive elements but contains putative binding elements for Gata2, thus Notch1 may activate Runx1 via Gata2 through an unknown mechanism.

Understanding how Notch1 activates these transcription factors is currently a major question in the field.

Functional promiscuity of Dld in HSC specification

Multiple distinct combinations of specific Notch ligand and receptor pairings might define the functional diversity of Notch signaling, thus understanding the mechanisms that regulate this signaling diversity is an important goal. Our studies demonstrate that somitic Dld is likely to perform at least two roles in HSC formation; to activate somitic Notch3, and activate Notch signaling in migrating LPM as it migrates past the somites to the midline that is potentiated by the Jam integrins (Kobayashi et al, 2014). Functional dependence on Jam-mediated adhesion might be an additional layer of regulation that tailors a unique output of a specific Notch signaling event. Which Notch receptor, if one or more, requires Jam-mediated interaction is an important follow-up question for these studies. The fact that somitic Dld is required for HSC specification may be related to its role in somitic segmentation and polarization (Julich et al, 2005). Therefore a greater understanding of how Notch receptors and ligands perform HSC-related and unrelated biological processes in similar tissues is required.

Pdgf signaling involvement in HSC specification

We identified a novel regulator of HSC specification in the Pdgf signaling pathway. Pdgfra is expressed in lateral plate mesoderm and somitic tissues from 6 to 14 hpf, therefore understanding in which tissues Pdgfra is required in is crucial. Pdgfra was shown to regulate endothelial expression of Notch1b while being dispensable for expression of other required Notch receptors and ligands. Understanding the mechanism by which Pdgfra specifically regulates Notch1b expression is an ongoing line of investigation for the laboratory. In addition to regulating Notch1b, Pdgfra is also required for the sclerotome, which has been implicated in HSC formation. Understanding what immediate downstream molecular cascade that Pdgfra activates that ultimately activates Notch1b expression and sclerotome formation is an important question. The extensive number of downstream kinase pathways Pdgfra is known to activate includes Ras, MAPK, PI3K, AKT, and JNK pathways. Therefore expression analyses studies may be required to identify specific candidates. Comparing relative expression of target transcripts and/or phosphorylated proteins from candidate cell populations such as the LPM or somitic tissues from wildtype to Pdgfra-deficient embryos may prove informative. Thus we have identified one molecular target by which Pdgfra regulates HSC formation, however the mechanism underlying this process and understanding the full extent of Pdgfra requirements in HSC formation is currently unknown.

Role of somites in HSC formation

The fact that Wnt16, Dlc, Dld, Notch3, and Pdgfra are required for somitic patterning of sclerotome and HSC formation suggest that these biological processes may be related. The fact that somitic Notch signaling caused by a loss of Dlc/Dld is reduced in Wnt16 deficient embryos, and that ectopic activation of ithin the somites of Notch3-deficient embryos rescued loss of runx1+ HSCs suggest that somitic Notch signaling is required for HSC formation. The target of this somitic Notch signaling event, though, is currently unknown. Sclerotome formation could be dispensable for HSC formation, but it shares a requirement for somitic Notch signaling along with HSCs. Future studies testing the functional requirement of sclerotome are thus necessary. The fact that Jam integrins are required for LPM precursors of HSCs to migrate past the sclerotome and experience a Dld-mediated Notch signaling event suggest that the sclerotome could be required to facilitate this process. Thus Notch signaling could be required in the somite for effective presentation of Notch ligand to HSC precursors. Additionally, the sclerotome is known to contribute to the vascular smooth muscle cells (VSMCs) of mouse and chick (Pouget et al, 2008; Wasteson et al, 2008). Whether this process occurs in zebrafish and is involved in HSC formation is a key question for future directions. VSMC generation is a poorly understood organogenesis event in zebrafish due to the lack of markers that can sufficiently identify their tissue of origin. This question may require lineage-tracing analysis to retrospectively identify the precursors of VSMCs. VSMCs could provide supportive signals that are required for HSC formation, and/or in maintenance of mature HSCs.

Recent studies have identified that a somitic fraction located medially to the ventrally positioned sclerotome referred to as the endotome is required for HSC formation via a somitic to endothelial migration event (Nguyen et al, 2014). The endotome is required for HSC formation through expression of a required Cxcl12b ligand to HSC precursors. Thus the somites are required for HSC specification in as many as four distinct ways; activation of Notch signaling within the somites and in migrating LPM, presentation of Cxcl12b, and finally somitic secretion of Vegfa needed for vasculogenesis and angiogenesis (Ciau-Uitz et al, 2010). Investigating what domains within the somite perform each of these signaling functions may reveal previously unappreciated functional and cellular heterogeneity within the somites.

Application to translational medicine

The generation of HSCs in a dish has been a highly sought after goal for nearly 50 years, and although our understanding of how this highly complex biological process is continuously expanding and being refined, we have yet to recapitulate this process in a manner sufficient for regenerative medicine. Our studies along with others continue to reveal novel morphogenetic, cellular, signaling, and transcriptional processes required for normal HSC emergence in an intact embryo, suggesting that we have not identified all of the inputs required *in vivo*. A taxing but necessary endeavor for the field is to continuously attempt to utilize the latest and most refined understanding of embryonic HSC formation to attempt generation of HSC-like hematopoiesis from a variety of cellular precursors.

Informed by the work of others in the field and the work described in this dissertation, I propose a progression of incremental cellular programming goals compared to the current goal of recapitulating the entire developmental process involved in HSC formation from pluripotent sources. Recapitulating the terminal process of commitment of arterial endothelium to an HSC-like fate is a suitable initial aim. We have demonstrated that the somites provide multiple supportive cues for HSC formation from arterial endothelium. Therefore the generation of stromal lines derived from embryonic somites that possess a molecular signature suggestive of HSC-supportive capacity is prudent, for example, expression of Wnt16, Dlc, Dld, Notch3, Pdgfra, Jam, Fgfr4, Vegfa, Cxcl12b, etc. In parallel, substrate tissues that will be the target for transformation to HSC fate should begin with bona fide HSC precursor tissues; ideally Notch-responsive arterial endothelium from the AGM region. This approach is the preliminary step for the eventual goal of generating HSCs for regenerative medicine. If HSC-transformation is successful with this initial aim, then progressively more primitive embryonic tissues such as endothelial precursors to artery can be utilized in place of more mature arterial HSC precursors with growth conditions aimed at recapitulating arteriogenesis and hemogenesis. This stepwise approach can inform specific investigation of developmental hematopoiesis in a way that is more synchoronous with the current state of regenerative medicine and mutually beneficial for both fields. The goal of generating HSCs and other stem-cell populations desirable for regenerative medicine is a compelling goal that will drive meaningful advancements in multiple scientific disciplines including biology, chemistry, medicine, engineering, material sciences, and computational biology.

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