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The role of Wnt signaling in hematopoietic stem cell development

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ABSTRACT
Hematopoietic stem cells (HSCs) can self-renew and differentiate into all cell types of the blood. This is therapeutically important as HSC transplants can provide a curative effect for blood cancers and disorders. The process by which HSCs develop has been the subject of extensive research in a variety of model organisms; however, efforts to produce bonafide HSCs from pluripotent precursors capable of long-term multilineage reconstitution have fallen short. Studies in zebrafish, chicken, and mice have been instrumental in guiding efforts to derive HSCs from human pluripotent stem cells and have identified a complex set of molecular signals and cellular interactions mediated by such developmental regulators as fibroblast growth factor, Notch, transforming growth factor beta (TGF\(\beta\)), and Wnt, which collectively promote the stepwise developmental progression toward mature HSCs. Tight temporal and spatial control of these signals is critical to generate the appropriate numbers of HSCs needed for the life of the organism. The role of the Wnt family of signaling proteins in hematopoietic development has been the subject of many studies owing in part to the complex nature of its signaling mechanisms. By integrating cell fate specification with cell polarity establishment, Wnt is uniquely capable of controlling complex biological processes, including at multiple stages of embryonic HSC development, from HSC specification to emergence from the hemogenic epithelium to subsequent expansion. This review highlights key signaling events where specific Wnt signals instruct and guide hematopoietic development in both zebrafish and mice and extend these findings to current efforts of generating HSCs in vitro.

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Introduction
Hematopoietic stem cells (HSCs) are adult stem cells that are capable of self-renewal and giving rise to all terminally differentiated cells of the blood. This property has allowed HSCs to be used as a therapeutic for various blood disorders and cancers by repopulating the patient’s deficient blood system with a full complement of healthy blood cells. Currently, HSCs are harvested from healthy donor bone marrow, peripheral blood, or umbilical cord blood, and most procedures require the donor cells and the patient recipient to be human leukocyte antigen (HLA)-matched. In some cases, the patient’s own HSCs may be harvested and banked for later therapeutic use. Though HSCs have been a viable therapeutic for decades, many patients lack an HLA-matched donor (Hatzimichael and Tuthill 2010, Peters et al. 2010).

The advent of induced pluripotent stem cell (iPSC) technology has made the possible facile derivation of PSCs from patients, thus creating a possible source of autologous HSCs for each patient in need of a transplant (Takahashi et al. 2007). PSCs are, in theory, capable of differentiating into all cells that make up an organism, including HSCs. However, it is currently not possible to generate therapeutically viable HSCs for human patients (reviewed in Slukvin (2013) and Vo and Daley (2015)). A more thorough understanding of the molecular cues that instruct the native development of HSCs will contribute to improving protocols to generate these cells in vitro. This review focuses on the role of the Wnt signaling pathway during HSC development.

Hematopoietic stem cell development in vivo
Hematopoietic development is separated into two phases. The first phase, termed primitive, produces mostly erythrocytes and macrophages that transiently sustain the organism during early development. These cell types arise in the yolk sac in mammals and in the intermediate cell mass/cephalic mesoderm in the zebrafish (reviewed in Davidson and Zon (2004) and Batta et al. (2016)). In the zebrafish, these waves are
temporally and spatially distinct from the definitive waves of hematopoiesis, which give rise first to committed erythromyeloid precursors (EMPs) in the posterior blood island then to HSCs that appear along the floor of the dorsal aorta. HSCs are derived from the mesodermal lineage, the generation of which is dependent on the coordinate regulation of multiple signaling pathways, including Nodal, bone morphogenic protein (BMP), fibroblast growth factor, and Wnt (reviewed in Clements and Traver (2013)). A subset of mesodermal cells, specifically lateral plate mesoderm, migrates laterally past the somites, which provide critical signaling and guidance cues, to the midline of the organism, eventually forming the vasculature (reviewed in Medvinsky et al. (2011)). Cooperation between the VEGF, Hedgehog, and Notch signaling pathways further specify these cells to become either arterial or venous endothelium (Rowlinson and Gering 2010). Specific cells within the floor of the aorta termed hemogenic endothelium undergo an endothelial to hematopoietic transition (EHT) to become HSCs. These cells undergo a change in morphology, transitioning from a flattened endothelial cell to a round hematopoietic cell, and bud from the wall of the aorta (Kissa et al. 2008, Eilken et al. 2009, Bertrand et al. 2010, Kissa and Herbomel 2010, Mizuochi et al. 2012). These nascent HSCs enter circulation and home to the placenta and fetal liver (mice) or the caudal hematopoietic tissue (CHT) (zebrafish), where HSCs that are embedded within the aortic endothelium emerge from the aorta in a process called the endothelial to hematopoietic transition, and enter circulation into the vein (fish) or the aorta (mouse). Relatively few HSCs emerge from the aorta, so their numbers are expanded in a niche that supports proliferation. In fish, this is the caudal hematopoietic tissue (CHT), and in mouse, this is the fetal liver. Eventually, the HSCs seed the adult hematopoietic organs where they will be maintained for the lifetime of the animal (fish: kidney marrow, mouse: bone marrow).

Figure 1. Model organisms used to study embryonic hematopoiesis. Early HSC development can be divided into three phases: specification, emergence, and expansion. These stages are conserved among vertebrates, but the precise anatomical locations where these events take place vary slightly between model organisms. This review focuses on three model systems: zebrafish, mouse, and the human embryonic stem cell in vitro differentiation system. The specification is the process by which developing HSCs receive molecular cues that inform their fate before they emerge. In the zebrafish, these cells arise from the posterior lateral mesoderm (PLM), which migrates beneath the somites to the midline of the embryo to form the vasculature. This process is similar in the mouse embryo, with HSCs deriving from cells of the mesoderm. Emergence in both the zebrafish and the mouse occurs in the aorta (fish), or the aorta - gonad - mesonephros (AGM) region (mouse). HSCs that are embedded within the aortic endothelium emerge from the aorta in a process called the endothelial to hematopoietic transition, and enter circulation into the vein (fish) or the aorta (mouse). Relatively few HSCs emerge from the aorta, so their numbers are expanded in a niche that supports proliferation. In fish, this is the caudal hematopoietic tissue (CHT), and in mouse, this is the fetal liver. Eventually, the HSCs seed the adult hematopoietic organs where they will be maintained for the lifetime of the animal (fish: kidney marrow, mouse: bone marrow).

The Wnt signaling pathway
Wnt signaling is an evolutionarily highly conserved pathway critical for the generation of cell diversity and polarity amongst all metazoan species. Although much
of the published literature distinguishes Wnt signaling into two broad types, the canonical and non-canonical pathways, recent studies suggest a more integrated view where Wnt proteins through their short range signaling nature simultaneously activate “cell fate” (canonical) and “cell polarity” (non-canonical) cascades (reviewed in Loh et al. (2016)). Broadly speaking, both pathways employ the Wnt signaling molecules, their cognate receptors encoded by the Frizzled (Fzd) gene family and the intracellular signaling molecule Dishevelled (Dvl/Dsh). Downstream of these shared signaling units, the two pathways are quite distinct, with the cell fate cascade defined by the signaling components glycogen synthase kinase 3 (GSK3), Axin and adenomatous polyposis coli (APC), and β-catenin. In contrast, the cell polarity cascades act through proteins like Vangl, Celsr, and Prickle to regulate cellular orientation within a group of cells. Over the years, the cell fate pathway has garnered the most attention, yielding important insights into its mode of action and its roles in development and disease.

The key mediator of the cell fate cascade is β-catenin: in the absence of Wnt signal, a destruction complex consisting of Axin, GSK3β, APC, and other proteins promotes phosphorylation of β-catenin, thereby targeting it for ubiquitination and degradation by the proteasome (Aberle et al. 1997). Transcription factors T-cell factor (TCF) and lymphoid enhancer binding factor (LEF) reside in the nucleus bound to regulatory regions of Wnt target genes and to co-repressors, such as Groucho to inhibit transcription (Cavallo et al. 1998). Upon transduction of a Wnt signal through a Fzd receptor complexed with an LRPS/6 co-receptor, Dvl/Dsh and components of the destruction complex are re-localized to the membrane (Bhanot et al. 1996, Yang-Snyder et al. 1996, Holmen et al. 2002), releasing β-catenin from constitutive degradation. Increased cytosolic β-catenin translocates to the nucleus, where it binds TCF and LEF to act as a co-activator to initiate transcription of Wnt target genes (Daniels and Weis 2005) (Figure 2).

Several features ensure tight control of this signaling pathway, which is critical for proper cell fate diversification and specification. First, the signaling range of Wnt proteins is highly restricted, a feature afforded by the covalent attachment of a lipid, thus rendering the protein highly hydrophobic and poorly soluble once
secreted from a cell (Willert et al. 2003, Takada et al. 2006). Second, a host of negative regulators act at multiple levels of the signaling cascade, including on the Wnt proteins themselves (e.g. Sfrp, Notum), the Fzd/LRP receptor complexes (e.g. Rnf43, Dkk), the intracellular signaling cascade (e.g. Axin2, Nkd), and on the transcriptional response (e.g. Inhibitor of β-catenin and T-cell factor (ICAT), Sp5). Several of these negative regulators are target genes of Wnt/β-catenin signaling, thus establishing negative feedback loops that restrict the spatial and temporal response to Wnt signals.

In contrast to this cell fate cascade, which exerts much of its effects through changes in gene expression, the Wnt cell polarity pathway acts independently of β-catenin and regulates complex biological processes, such as planar cell polarity, convergent extension, and cell migration. The study of the Wnt polarity pathway has proven more difficult than the study of the Wnt cell fate pathway, owing in large part to the scarcity of in vitro assays and the need for complex biological systems, such as imaging of explants or whole animals. In addition to this pathway utilizing distinct intracellular effectors, such as Vangl, Celsr, and Prickle, the interactions of which remain poorly understood, this pathway in certain contexts employs non-Fzd receptors, such as Ror1/2 and Ryk. While this review will focus primarily on the cell fate pathway, we will also highlight notable roles of the Wnt cell polarity pathway. It should be stressed that development of HSCs, like many complex biological processes, requires input from both Wnt signaling pathways.

Zebrafish hematopoietic development

As zebrafish HSC precursors develop from cells of the posterior lateral mesoderm, they receive a complement of molecular cues that informs their identity as HSCs; this process is termed specification. Eventually, specialized cells of the endothelium will undergo an EHT and bud off from the endothelium in a process termed emergence, which initiates at 26 h post fertilization (hpf). Next, nascent HSCs move to the CHT where they proliferate during the expansion phase of HSC development. We will discuss the requirement for Wnt signaling during each of these three phases: specification, emergence, and expansion (Figure 1).

The Wnt cell polarity pathway is required for zebrafish HSC specification

To date, a requirement for the Wnt cell fate pathway (−Wnt/β-catenin) in HSC specification in zebrafish has not been demonstrated. However, Wnt16 acting in a β-catenin-independent manner is required in the somites for HSC specification in a non-cell autonomous manner (Clements et al. 2011). Knockdown of wnt16 via injection of an antisense morpholino oligonucleotide decreases HSC marker gene expression at 24 hpf, before HSCs have begun to emerge, indicating a defect in HSC specification. This decrease in HSC number is sustained into later stages of hematopoietic development. Knockdown of wnt16 causes a loss of Notch ligand deltaC and deltaD expression in the somites, and over-expression of deltaC and deltaD in the context of the wnt16 morpholino is sufficient to rescue the loss of HSC phenotype. This Wnt16 signal sets up a Notch3 cue that is not received directly by the HSCs but must be received by somite – adjacent cells between 15 and 17 hpf (Clements et al. 2011, Kim et al. 2014). Knockdown of wnt16 led to a reduction in expression of markers of the sclerotome, a compartment of the somite that has been shown to be important for HSC development, although the exact mechanisms by which it is required is not known (reviewed in Butko et al. (2016)). Rspordin1, an activator of the Wnt pathway, regulates this requirement for Wnt16. Loss of Rspordin1 results in decreased wnt16 expression, and subsequent loss of HSC specification (Genthe and Clements 2017).

Wnt is required for zebrafish HSC emergence

Many lines of evidence support the conclusion that zebrafish HSC emergence is dependent upon Wnt cell fate signaling. Our recent work showed that loss of Wnt9a in the somites caused a decrease in HSC number after initiation of emergence with no discernible negative impact on HSC specification (Grainger et al. 2016). This Wnt signal is unrelated to the somitic requirement for wnt16, as it is temporally distinct and does not impact specification. Interestingly, Wnt9a is required pre-20 hpf, but the HSC phenotype does not occur until much later, around 32 hpf, indicating that Wnt9a establishes a permissive environment for later HSC amplification. Similarly, overexpression of the Wnt antagonist dkk1 causes a decrease in HSCs and progenitors at 36 hpf (as detected by expression of cmyb), which is during the emergence window (Goessling et al. 2009). Overexpression of wnt8, which in this context potently activates the cell fate pathway, caused an increase in HSCs during the emergence window (Goessling et al. 2009). Inhibiting the secretion of all Wnt ligands using a chemical inhibitor of Porcupine (Porcn) (Chen et al. 2009), an enzyme which is required for the lipid modification and subsequent secretion of Wnts, also caused a loss of HSCs during the emergence window (Biechele et al. 2011, Grainger et al. 2016).
Results from manipulation of cytoplasmic components of the Wnt pathway also support the conclusion that Wnt cell fate is required for HSC emergence. Overexpressing axin1 or stabilizing Axin1 protein by chemically inhibiting Tankyrases, which promote degradation of Axin, inhibited the Wnt pathway and resulted in a decrease of cmyb+ cells during HSC emergence (Goessling et al. 2009, Wang et al. 2013). Conversely, using a small molecule to increase the association between Axin and LRP6 to stimulate the Wnt pathway caused an increase in cmyb+ cells during HSC emergence (Wang et al. 2013). Activating the pathway by inhibiting GSK3β with lithium increased the number of flk1/cmyb+ double positive HSCs emerging from the aortic floor (Grainger et al. 2016). Overexpression of a constitutively active β-catenin also increased the number of cmyb+ cells within the HSC emergence window (Grainger et al. 2016). Altogether, these experiments provide strong evidence that cytoplasmic components of the Wnt cell fate pathway are necessary for HSC emergence.

Inhibition of Wnt signaling at the level of target gene activation also demonstrated that Wnt is required for HSC emergence. Expression of a dominant-negative Tcf transgene (dntcf) that lacks the β-catenin binding domain results in cells unable to respond to an extracellular Wnt signal. Downregulation of the Wnt pathway via expression of dntcf resulted in decreased numbers of cmyb+ hematopoietic cells during HSC emergence in multiple studies (Goessling et al. 2009, Grainger et al. 2016). Interestingly, expression of dntcf also caused a decrease in gata1+ primitive blood cells, suggesting Wnt may also play a role in earlier waves of hematopoietic development (Lengerke et al. 2008). Experiments utilizing the dntcf transgene have provided insight into the critical tissue that must receive this canonical Wnt cue. Tissue-specific expression of dntcf in fli1a+ endothelium and, more specifically, gata2b+ hemogenic endothelium was sufficient to recapitulate whole-embryo dntcf expression (Grainger et al. 2016). This suggests that cells of the hemogenic endothelium must receive a critical Wnt cell fate cue to successfully develop and emerge from the aortic endothelium.

**The role of Wnt signaling in zebrafish HSC expansion**

The CHT has long been considered the main site of HSC proliferation in the zebrafish embryo, analogous to the mouse placenta or fetal liver. Recent reports have provided evidence for HSCs undergoing expansion within the aorta prior to emergence and migration to the CHT (Goessling et al. 2009, Grainger et al. 2016). It is not clear whether Wnt signaling plays a role in HSC expansion within the CHT, but the Wnt pathway does have a critical function in the more recently described intra-aortic expansion of cells fated to become HSCs. It is not yet clear whether this proliferation occurs within hemogenic endothelial cells or in nascent HSCs due to a lack of marker genes that differentiate these cell types. The proliferation of intra-aortic hematopoietic cells has been described in mouse development; providing evidence that this aortic expansion event is conserved between species (Boisset et al. 2015). Inhibition of Wnt via overexpression of axin1, dkk1, or dntcf decreased proliferative cells within the aorta as measured by the incorporation of BrdU into dividing cells. Conversely, overexpression of wnt8 to stimulate the Wnt pathway resulted in an increase in proliferation (Goessling et al. 2009). Proliferative cells within the aorta have been shown to be positive for the HSC marker gata2b (Grainger et al. 2016). Upon morpholino-mediated knockdown of wnt9a, gata2b+ HSCs are arrested in the G1 phase of the cell cycle and failed to undergo intra-aortic proliferation. This phenotype is likely due to a reduction in expression of the cell cycle regulator and Wnt target gene myca (the zebrafish homolog of Myc) that occurs when the Wnt pathway is inhibited. The importance of myca is further evidenced by its ability to rescue the HSC defect in wnt9a morphants (Grainger et al. 2016). Interestingly, Goessling et al. (2009) reported that overexpression of negative regulators of Wnt signaling caused both an increase in apoptosis and a decrease in proliferation, while Grainger et al. (2016) did not observe an increase in apoptosis with the overexpression of dntcf, but saw a lack of proliferation consistent with previously published results. These studies provide strong evidence that Wnt cell fate signaling is required for proliferation of developing HSCs within the aorta.

**Mouse hematopoietic development**

The majority of the research on the role of Wnt signaling in mouse hematopoietic development has focused on the emergence and expansion of HSCs. There has been no direct evidence that Wnt signaling plays a role in mouse HSC specification, though Wnt has been implicated in the development of primitive blood and erythromyeloid progenitors in the mouse (Nostro et al. 2008, Frame et al. 2016). We will focus on the role of Wnt in the emergence and expansion of HSCs in the mouse embryo (Figure 1).

**Mouse HSC emergence is regulated by Wnt signaling**

Mouse HSCs emerge directly from aortic endothelium that undergoes an EHT within the aorta–gonad–
mesonephros (AGM) region. Wnt pathway components such as Dishevelled, TCF, and β-catenin are expressed in the AGM around the time of HSC emergence (E10-E12), and nuclear β-catenin is restricted to distinct endothelial cells at the base of intra-aortic hematopoietic clusters, which hints at a possible role for Wnt during HSC emergence (Orelio and Dzierzak 2003, Ruiz-Herguido et al. 2012). This was further investigated using explant culture experiments where the AGM region was dissected from mouse embryos and cultured in vitro. Treating E10.5 AGM explants with a GSK3 inhibitor (SB216763) to activate the Wnt pathway increased HSC emergence as measured by a colony forming cell (CFC) assay and by hematopoietic reconstitution of irradiated recipients. Conversely, inhibiting Wnt with a small molecule that interferes with the β-catenin/TCF complex (PKF-115) caused a decrease in HSCs (Ruiz-Herguido et al. 2012). Tissue-specific loss of Wnt using a conditionally inactivatable β-catenin allele in VE-Cadherin+ endothelial cells caused a significant decrease in HSC emergence, as measured by a CFC assay. Interestingly, loss of Wnt after hematopoietic fate acquisition by inactivating β-catenin in Vav1+ hematopoietic cells had no effect on HSC function (Zhao et al. 2007, Ruiz-Herguido et al. 2012). Together, these data suggest that Wnt is required in endothelial cells during HSC emergence from the aorta, but is dispensable after HSCs have emerged and begun to express mature hematopoietic markers.

**Wnt is required in the fetal liver for HSC function**

After HSCs emerge from the aorta in the mouse embryo they migrate to the placenta and fetal liver, both niches that promote HSC proliferation. Wnt pathway components such as β-catenin and Wnt3a are expressed in the fetal liver at E12.5, a time period when HSC numbers expand (Orelio and Dzierzak 2003, Luis et al. 2010). Loss of Wnt3a by genetic knockout caused early lethality at E12.5 due to many severe developmental phenotypes, but analysis of hematopoiesis in the fetal liver was still possible. Wnt3a−/− embryos displayed a severe reduction in HSC numbers (as defined by the HSC signature LSK+ (Lineage−, Sca1+, c-kit−) Flt3+) in the fetal liver, with the remaining HSCs exhibiting defects in self-renewal and poor long-term reconstitution capacity in wild-type hosts (Luis et al. 2009). This loss of Wnt3a was not compensated by any other Wnt genes expressed in the fetal liver, suggesting that Wnt3a is the primary Wnt regulating fetal liver HSC function (Luis et al. 2010). It is not clear whether the HSC defects seen in the fetal liver of Wnt3a−/− embryos are a result of earlier hematopoietic events gone wrong, such as decreased HSC emergence due to lack of Wnt signaling, or if these experiments represent yet another requirement for canonical Wnt during the developmental journey of an HSC. Ex vivo experiments in which fetal liver cells were co-cultured on the bone marrow stromal cell line OP9 showed that exposure to exogenous Wnt3a affects HSC differentiation into downstream lineages; high Wnt3a arrested T-cell development in vitro and in vivo and increased differentiation into B-cells in vivo (Famili et al. 2015). Together, these data suggest that Wnt3a acting through the cell fate pathway is required in the fetal liver for proper HSC function, including self-renewal and differentiation into downstream blood lineages.

**Mouse hematopoietic development in vitro**

The importance of Wnt signaling in hematopoietic development has been investigated in mouse embryonic stem cells differentiating in vitro to hematopoietic lineages. Activation of the Wnt pathway with exogenous Wnt3a increased the number of hematopoietic cells either by CFC assay or by expression of hematopoietic markers by qPCR (Naito et al. 2006, Goessling et al. 2009). Inhibition of Wnt via the addition of DKK1 decreased hematopoietic output as measured by the expression of hemoglobin y (Hbb-y) (Rai et al. 2012). These results are consistent with the generally positive correlation between Wnt signaling and hematopoietic development. However, this does not seem to be true for all Wnts: Wnt2−/− embryonic stem cells gave rise to an increased number of blast CFCs (BL-CFCs), suggesting that some Wnts, like Wnt2, have a repressive effect on hematopoietic differentiation (Wang et al. 2007). This provides support for the theory that Wnts can have unique functions and may be required in a non-redundant manner for various hematopoietic processes, such as Wnt9a in the zebrafish hemogenic endothelium and Wnt3a in the mouse fetal liver.

**Human hematopoietic development in vitro**

Although derivation of HSCs capable of multilineage engraftment from human pluripotent stem cells (hPSCs) has not been achieved, significant insights have been made on the role of Wnt signaling during hematopoietic development in vitro, which largely mimics development in an organism: cells are first committed to the mesodermal lineage and are further specified toward a specialized type of hemogenic endothelium, which then gives rise to hematopoietic stem and progenitor cells (HSPCs (Figure 3)) (reviewed in Ditadi et al. 2017)). The spatial compartmentalization of in vivo HSC development is non-existent in this in vitro system, and
differences among individual hPSC lines and between differentiation protocols confound comparison of multiple studies, as the timing of developmental stages may vary. However, the requirement for Wnt signaling in the in vitro differentiation system largely mirrors the requirements for Wnt in hematopoietic development in model organisms.

Multiple studies provide evidence that Wnt is required for specification of HSPCs in vitro. Stimulating the pathway using a GSK3β inhibitor (CHIR99021) early in the differentiation protocol promoted the specification of posterior mesoderm that gave rise to hemogenic endothelium (Kitajima et al. 2016). These results are consistent with previously described roles for Wnt during the specification of mesoderm in vivo (reviewed in Clements and Traver (2013)). Wnt also is required for the specification of definitive hematopoiesis at the expense of primitive hematopoiesis as inhibition of Wnt secretion (with the Porcn inhibitor IWP-2) during a mid-early stage of differentiation abrogated T-cell differentiation potential of hematopoietic progenitors. Stimulating the pathway with a GSK3β inhibitor during the same time frame inhibited primitive hematopoiesis and enhanced definitive hematopoiesis, as measured by T-cell potential (Sturgeon et al. 2014). These findings are contradictory to others suggesting that Wnt is required for the development of primitive blood in the mouse (Nostro et al. 2008). However, these distinctions are consistent with differences in the ways that mesoderm is patterned in the human and mouse embryo (reviewed in Ditadi et al. (2017)). Activating the pathway via the addition of Wnt3a or Wnt1 protein throughout the course of differentiation resulted in an increase in HSPCs, and the addition of DKK1 inhibited HSPC production (Woll et al. 2008, Wang and Nakayama 2009, Gertow et al. 2013). These results support the model that Wnt signaling is required for the development of HSPCs, mirroring the requirements identified in vivo.

**Conclusion**

HSCs are capable of giving rise to all cells of the blood. The ability to derive patient-specific HSCs in vitro is of great interest to the scientific and medical communities, as these cells have high therapeutic potential. However, it is still not possible to generate therapy-grade HSCs from pluripotent precursors. A better understanding of signals, including Wnt, that promote the differentiation to HSCs is critical in achieving this goal.

As documented in this review, Wnt signaling influences HSCs at multiple stages and in many systems, at times with varying conclusions as to the role that Wnt plays in the context of HSC biology (Table 1). Depending on the age of the animal or the system used to analyze HSCs, Wnt has been shown to promote the development, expansion, and maintenance of HSCs (reviewed in Lento et al. (2013)). In other contexts, Wnt has been shown to inhibit self-renewal and eliminate the HSC pool (Kirstetter et al. 2006, Scheller et al. 2006).

In the adult system, the dosage of Wnt dictates its effect on the maintenance of HSCs and differentiation into downstream lineages (Luis et al. 2011).

Evidence from the mouse and zebrafish systems indicates that Wnt is required in the endothelium for HSCs to emerge from the aorta, but is dispensable after HSCs have already emerged (Zhao et al. 2007, Ruiz-Herguido et al. 2012, Grainger et al. 2016). Wnt may also be necessary for the embryonic expansion of HSCs (Luis et al. 2009, Grainger et al. 2016). Wnt has also been shown to promote the expansion of adult HSCs in vitro (Reya et al. 2003, Willert et al. 2003). Wnt is required for HSC specification in the human embryonic stem cell differentiation system, suggesting that this requirement

![Figure 3](image-url) Hematopoietic development during in vitro differentiation. The development process is similar between human cells in vitro and cells in vivo; albeit with a lack of spatial separation in vitro. Hematopoietic stem and progenitor cells (HSPCs) are derived from the mesodermal lineage that is further specified to become cells that will contribute to definitive hematopoiesis. These cells are pushed toward an endothelial fate by a growth factor cocktail usually containing VEGF, and eventually become hemogenic endothelium. The emergence process yields HSPCs expressing various hematopoietic marker genes.
<table>
<thead>
<tr>
<th>Gene (perturbed)</th>
<th>Wnt up/ down-regulated</th>
<th>Model system</th>
<th>Phenotype</th>
<th>Tissue</th>
<th>Time</th>
<th>Notes</th>
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<td>Exogenous DKK1</td>
<td>Down</td>
<td>Human (in vitro ESC differentiation)</td>
<td>Decrease in HSPCs</td>
<td>N/A</td>
<td>d0 – endpoint</td>
<td>Decrease in CD34/CD45+ cells, decrease in CFCs</td>
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<tr>
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<td>Up</td>
<td>Human (in vitro ESC differentiation)</td>
<td>Increase in HSPCs</td>
<td>N/A</td>
<td>d0 – endpoint</td>
<td>Increase in CD34/CD45+ cells, decrease in CFCs</td>
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<td>Up</td>
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<td>d0 – endpoint</td>
<td>Wnt3a protein; Increase in HSC markers, EM-CFC colonies in CFC assay</td>
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<td>N/A</td>
<td>d2–3</td>
<td>IWP2; Decreased HSCs as measured by lack of T cell potential</td>
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<td>CHIR99021; increased CFU-mix colonies; increased %CD34+/CD45+</td>
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<td>Wnt2 KO, increased BL-CFC colonies in EB differentiation</td>
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<td>Fetal liver on OP9 stroma</td>
<td>N/A</td>
<td>Co-culture assay, and fetal liver LSK cells transplanted into adult mice</td>
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<td>Acceleration of T cell development</td>
<td>Fetal liver on OP9 stroma</td>
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<td>Co-culture assay, and fetal liver LSK cells transplanted into adult mice</td>
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<td></td>
<td>Exogenous Wnt3a</td>
<td>Up</td>
<td>Mouse (in vitro ESC differentiation)</td>
<td>Increase in HSPCs</td>
<td>N/A</td>
<td>Tx: d4</td>
<td>CFC assay</td>
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<tr>
<td></td>
<td>Exogenous Wnt3a</td>
<td>Up</td>
<td>Mouse (in vitro ESC differentiation)</td>
<td>Increase in HSPCs</td>
<td>N/A</td>
<td>d5–10</td>
<td>Increase in blood markers by qPCR (CD31, CD45, VE-Cad, Bhl-globin)</td>
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<tr>
<td></td>
<td>Exogenous Dkk1</td>
<td>Down</td>
<td>Mouse (in vitro ESC differentiation)</td>
<td>Decrease in primitive blood</td>
<td>N/A</td>
<td>d4–6</td>
<td>Decrease in primitive erythrocyte colonies</td>
</tr>
<tr>
<td></td>
<td>Exogenous DKK1</td>
<td>Down</td>
<td>Mouse (in vitro ESC differentiation)</td>
<td>Decrease in hematopoietic cells</td>
<td>Ubiquitous</td>
<td>d2–4</td>
<td>Decrease in Hbb-y expression by qPCR</td>
</tr>
<tr>
<td>Cytoplasmic GSK3B inhibition</td>
<td>Up</td>
<td>Mouse (AGM explants, E10.5)</td>
<td>Increase in HSPCs</td>
<td>AGM</td>
<td>E10.5</td>
<td>CFC assay, hematopoietic reconstitution assay</td>
<td>Ruiz-Herguido et al. 2012</td>
</tr>
<tr>
<td>Gene (perturbed)</td>
<td>Wnt up/ down-regulated</td>
<td>Model system</td>
<td>Phenotype</td>
<td>Tissue</td>
<td>Time</td>
<td>Notes</td>
<td>References</td>
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<tr>
<td>Stabilized β-catenin overexpression</td>
<td>Up</td>
<td>Mouse (in vitro ESC differentiation)</td>
<td>Increase in primitive blood</td>
<td>N/A</td>
<td>d4–6</td>
<td>Increase in primitive erythrocyte colonies</td>
<td>Nostro et al. 2008</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Down</td>
<td>Mouse (in vivo)</td>
<td>Decrease in EMPs</td>
<td>Gh5:Cre; conditional B-cat</td>
<td>E9.5–10.5</td>
<td>CFC assay, focus on yolk sac</td>
<td>Frame et al. 2016</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Down</td>
<td>Mouse (in vivo)</td>
<td>Decrease in HSCs</td>
<td>AGM (VECad-Cre)</td>
<td>E10.5–11.5</td>
<td>CFC assay; mutants had hematopoietic and vascular defects</td>
<td>Ruiz-Herguido et al. 2012</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Down</td>
<td>Mouse (in vivo)</td>
<td>No effect on HSCs</td>
<td>HSCs (Vav-Cre)</td>
<td>E12.5 onward</td>
<td>Adult analysis; Wnt is required in the endothelium</td>
<td>Ruiz-Herguido et al. 2012</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Down</td>
<td>Mouse (in vivo)</td>
<td>No effect on HSC number</td>
<td>HSCs (Vav-Cre)</td>
<td>E12.5 onward</td>
<td>Decreased HSC function – poor reconstitution capacity</td>
<td>Zhao et al. 2007</td>
</tr>
<tr>
<td>Zebrafish Extracellular</td>
<td>Wnt8 overexpression</td>
<td>Up</td>
<td>Zebrafish (Tg)</td>
<td>Increase in HSCs</td>
<td>Ubiquitous</td>
<td>10 somite stage</td>
<td>hsp:wnt8 (Ba); increase in cmyb+ cells at 36 hpf; Wnt-regulated by PGE2</td>
</tr>
<tr>
<td>Wnt8</td>
<td>Down</td>
<td>Zebrafish (MO and Tg)</td>
<td>Decrease in HSC emergence, specification OK</td>
<td>Somite</td>
<td>pre-20 hpf</td>
<td>Decrease in an aortic expansion of HSCs</td>
<td>Grainger et al. 2016</td>
</tr>
<tr>
<td>Wnt16</td>
<td>Down</td>
<td>Zebrafish (MO)</td>
<td>Lack of HSC specification</td>
<td>Somite</td>
<td>pre-15 hpf</td>
<td>Sets up Notch cue needed for specification</td>
<td>Clements et al. 2011</td>
</tr>
<tr>
<td>dkk1 overexpression</td>
<td>Down</td>
<td>Zebrafish (Tg)</td>
<td>Decrease in HSCs</td>
<td>Ubiquitous</td>
<td>12 somite stage</td>
<td>hsp:dkk1; decrease in cmyb+ cells at 36 hpf; Wnt-regulated by PGE2</td>
<td>Goessling et al. 2009</td>
</tr>
<tr>
<td>Porcupine inhibition (no Wnt secretion)</td>
<td>axin1 overexpression</td>
<td>Down</td>
<td>Zebrafish (drug)</td>
<td>Decrease in HSCs</td>
<td>Ubiquitous</td>
<td>1ss–40 hpf</td>
<td>IWP-2, decrease in flk1/cmyb+ cells</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>increased Axin – Lrp6 interaction</td>
<td>Up</td>
<td>Zebrafish (drug)</td>
<td>Increased HSCs</td>
<td>Ubiquitous</td>
<td>3ss–36 hpf</td>
<td>HLY78, decreased</td>
<td>Wang et al. 2013</td>
</tr>
<tr>
<td>Tankyrase inhibition</td>
<td>Down</td>
<td>Zebrafish (drug)</td>
<td>Decreased HSCs</td>
<td>Ubiquitous</td>
<td>3ss–36 hpf</td>
<td>XAN929, decreased cmyb+ cells</td>
<td>Wang et al. 2013</td>
</tr>
<tr>
<td>Constitutively active β-catenin GSK3β inhibition</td>
<td>Up</td>
<td>Zebrafish (Tg)</td>
<td>Increased HSCs</td>
<td>Endothelium</td>
<td>10–40 hpf</td>
<td>Increase in cmyb+ cells</td>
<td>Grainger et al. 2016</td>
</tr>
<tr>
<td>Nuclear</td>
<td>dntcf overexpression</td>
<td>Down</td>
<td>Zebrafish (Tg)</td>
<td>Lack of primitive blood gata1 expression in PLM</td>
<td>Ubiquitous</td>
<td>8 h</td>
<td>Signals coordinately with BMP to turn on Cdx and Hox genes</td>
</tr>
<tr>
<td>dntcf overexpression</td>
<td>Down</td>
<td>Zebrafish (Tg)</td>
<td>Increase in HSCs</td>
<td>Ubiquitous</td>
<td>13 somite stage</td>
<td>hsp:dntcf, increase in cmyb+ cells at 36 hpf; Wnt-regulated by PGE2</td>
<td>Goessling et al. 2009</td>
</tr>
<tr>
<td>dntcf overexpression</td>
<td>Down</td>
<td>Zebrafish (Tg)</td>
<td>Decrease in HSC emergence, specification OK</td>
<td>Hemogenic endothelium (gata2b:Gah4)</td>
<td>pre-20 hpf</td>
<td>Decrease in an aortic expansion of HSCs</td>
<td>Grainger et al. 2016</td>
</tr>
</tbody>
</table>
for Wnt during HSC development is highly conserved amongst different organisms (Sturgeon et al. 2014).

Although there is clear evidence supporting a role for Wnt during HSC emergence and expansion, we do not yet have a clear understanding of the mechanism by which a Wnt signal acts on HSCs. In the zebrafish, Wnt acts as a proliferative cue for HSCs in the aorta by signaling through myca, a previously described Wnt target (Grainger et al. 2016). However, we do not know whether the downstream response to Wnt is similar in other systems, like in the mouse endothelium. It may also be important to understand the specific ligands that mediate the Wnt signal, as multiple studies have hinted that other Wnts cannot compensate for the loss of critical ligands, and various Wnts affect hematopoietic development in different ways (Wang et al. 2007, Luis et al. 2010, Grainger et al. 2016). This is likely due to a combination of receptor-ligand specificity and differences in spatiotemporal expression of Wnts and Frizzleds. Most differentiation protocols utilize small molecule activators or inhibitors of the Wnt pathway, many of which have off-target effects. Stimulation of the Wnt pathway using the specific molecules that direct HSC development in vivo may improve differentiation protocols to generate HSCs in vitro.

Disclosure statement
The authors declare no conflict of interest.

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CRITICAL REVIEWS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY 423


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