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Wnt9a is a conserved regulator of hematopoietic stem and progenitor cell development

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for the degree Doctor of Philosophy

in

Biomedical Sciences

by

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The Dissertation of Jenna Schug Hicks is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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LIST OF ABBREVIATIONS

HSC: Hematopoietic stem cell

HLA: Human-leukocyte antigen

iPSC: induced pluripotent stem cell

EMP: erythromyeloid precursors

BMP: bone morphogenic protein

FGF: fibroblast growth factor

EHT: endothelial to hematopoietic transition

PLM: posterior lateral mesoderm

AGM: aorta-gonad-mesonephros

CHT: Caudal hematopoietic tissue

hpf: hours post fertilization

CFC: Colony forming cell

BL-CFC: Blast colony forming cell

LSK+: Lineage^{-}, Sca1^{+}, c-kit^{-}, Flt3^{-}

hPSC: human pluripotent stem cell

HSPC: Hematopoietic stem and progenitor cell

MO: Morpholino

FACS: Fluorescence-activated cell sorting

WISH: Whole-mount *in situ* hybridization

hESC: Human embryonic stem cell

EB: Embryoid body

hs: heat shock

HE: Hemogenic endothelium

qRT-PCR: quantitative reverse transcription polymerase chain reaction

Fzd: Frizzled

CHIR: CHIR98014 (Wnt agonist small molecule)

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PUBLICATIONS

Huggins, I., Bos, T., Gaylord, G., Jessen, C., Lonquich, B., Puranen, A., **Richter, J.**, Rossdam, C., Brafman, D., Gaasterland, T., Willert, K. The WNT target SP5 negatively regulates WNT transcriptional programs in human pluripotent stem cells. *Nature Communications* 8, 1034 (2017).

Richter, J., Traver, D., Willert, K. The Role of Wnt Signaling in Hematopoietic Stem Cell Development. *Critical Reviews in Biochemistry and Molecular Biology*. 52(4): 414- 424 (2017).

Grainger, S.*, **Richter, J.***, Espín Palazón, R., Pouget, C., Lonquich, B., Wirth, S., Grassme, K.S., Herzog, W., Swift, M., Weinstein, B., Willert, K., Traver, D. Wnt9a is required for the aortic amplification of nascent hematopoietic stem cells. *Cell Reports* 17, 1595-1606 (2016).

Kumar, N., **Richter, J.**, Cutts, J., Bush, K.T., Trujillo, C., Nigam, S.K., Gaasterland, T., Brafman, D., Willert, K. Generation of an expandable intermediate mesoderm restricted progenitor cell line from human pluripotent stem cells. *ELife* 4:e08413 (2015).

Charbord, P., Pouget, C., Binder, H., Dumont, F., Stik, G., Levy, P., Allain, F., Marchal, C., **Richter, J.**, Uzan, B., Pflumino, F., Letourneur, F., Wirth, H., Dzierzak, E., Traver, D., Jaffredo, T., Durand, C. A developmental systems biology approach to define the molecular framework of the hematopoietic stem cell niche. *Cell Stem Cell* 15(3):376-39 (2014).

Itou, J., Oishi, I., Kawakami, H., Glass, T.J., **Richter, J.**, Johnson, A., Lund, T.C., Kawakami, Y. Migration of cardiomyocytes is essential for heart regeneration in zebrafish. *Development* 139(22): 4133-4142 (2012).

ABSTRACT OF THE DISSERTATION

Wnt9a is a conserved regulator of hematopoietic stem and progenitor cell development

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Hematopoietic stem cells (HSCs) can give rise to all terminally differentiated cells of the blood, and are used in therapeutic approaches to treat hematopoietic cancers and disorders. Patient-specific HSCs derived from pluripotent precursors have long been a goal of the field, but despite tireless efforts it is not yet possible to derive therapy-grade HSCs *in vitro*. Here, I utilize the zebrafish as a model system in which to identify critical molecular cues that are required for normal HSC development. I have identified the Wnt signaling pathway to be critical for the intra-aortic expansion of zebrafish HSCs, and Wnt9a to be the ligand that mediates this signal. In the zebrafish, this requirement is highly specific; loss of Wnt9a cannot be compensated for by the exogenous expression of Wnt9b or Wnt3a. The function of Wnt9a is conserved across species; exogenous expression of

WNT9A during *in vitro* hematopoietic progenitor differentiation improves the efficiency of generating $CD34^{+}/CD45^{+}$ hematopoietic progenitor cells from human embryonic stem cells. My data indicates that Wnt9a is a conserved regulator of hematopoietic stem and progenitor cell development. Inclusion of WNT9A in protocols to derive hematopoietic stem and progenitor cells from pluripotent precursors could more accurately mimic the molecular cues that drive HSC development *in vivo*.

CHAPTER 1: 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 a HLA-matched donor (Hatzimichael and Tuthill 2010, Peters *et al.* 2010).

The advent of induced pluripotent stem cell (iPSC) technology has made possible facile derivation of pluripotent stem cells from patients, thus creating a possible source of autologous HSCs for each patient in need of a transplant (Takahashi *et al.* 2007). Pluripotent stem cells 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, 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.

1.1: 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 (FGF), 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, Chi, *et al.* 2010, Kissa and Herbomel 2010, Mizuochi *et al.* 2012a). These nascent HSCs enter circulation and home to the placenta and fetal liver (mice) or the caudal hematopoietic tissue (zebrafish), where HSCs proliferate before transitioning to the adult niche that maintains the HSC population for the remainder of the lifetime of the animal; the bone marrow in the mouse and the kidney marrow in the zebrafish (Murayama *et al.* 2006, reviewed in Medvinsky *et al.* 2011). The journey of a developing HSC in the model organisms focused on here (mouse and zebrafish) proceeds through similar stages of development: specification, emergence, and expansion before moving to the adult maintenance niche (Figure 1.1). The specific anatomical regions for these events vary between organisms, but the niche functions appear conserved.

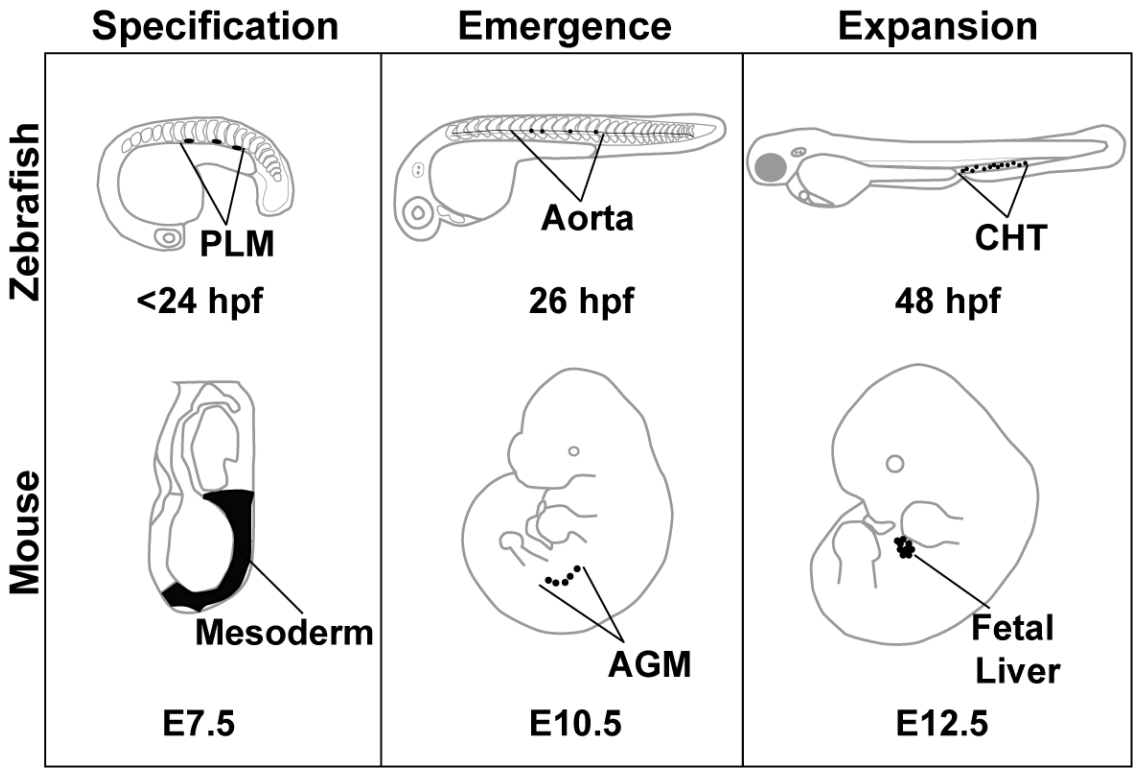

Figure 1.1. Model organisms used to study embryonic hematopoiesis. Early HSC development can be divided into three phases: specification, emergence, and expansion. These stages are conserved among vertebrates, but the precise anatomical locations where these events take place vary slightly between model organisms. 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 migrate 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).

1.2: 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 acts 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 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 LRP5/6 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 1.2).

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. 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.

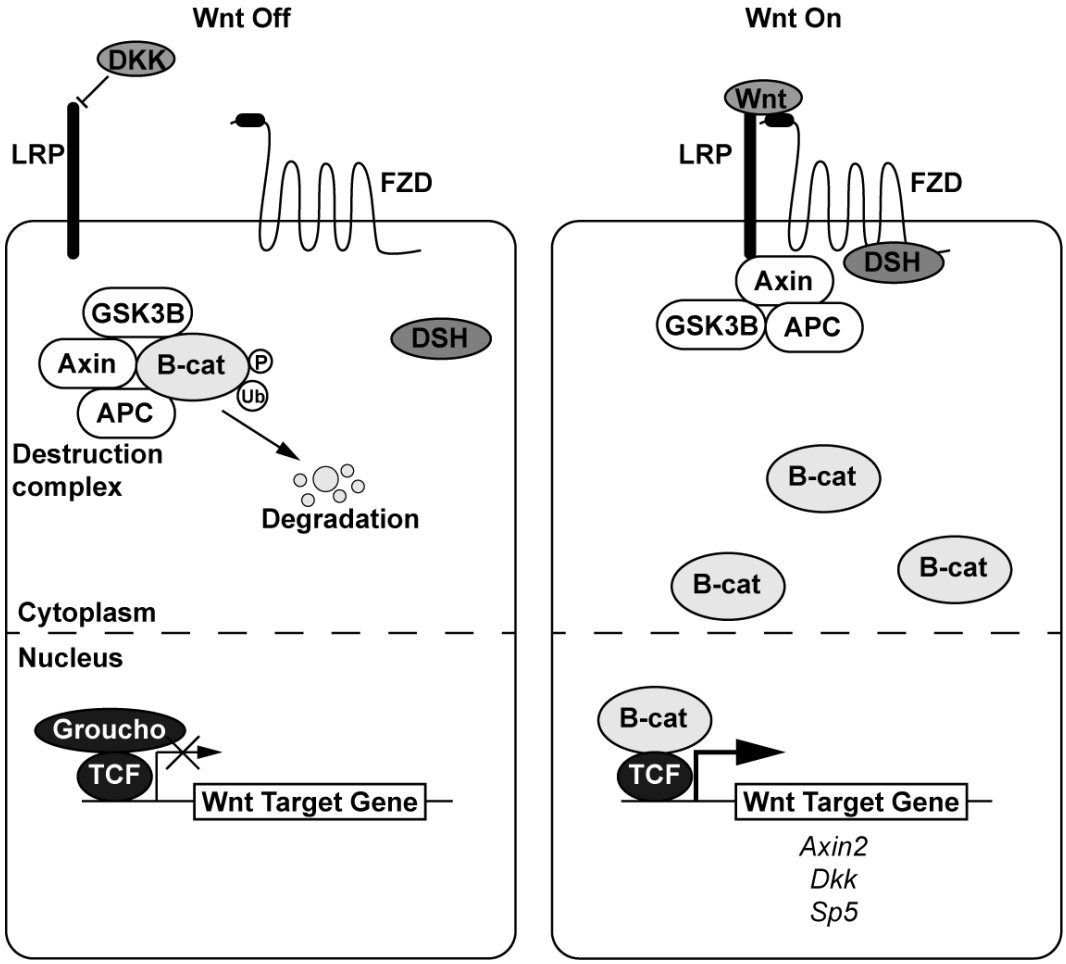

Figure 1.2. The (β–catenin mediated) Wnt signaling pathway. In the absence of a Wnt ligand, a destruction complex phosphorylates β–catenin and targets it for ubiquitination and degradation. The transcription factor TCF is bound to Wnt target genes and acts as a co-repressor with Groucho. When a Wnt ligand is present, it binds a Frizzled receptor and LRP co-receptor which causes the destruction complex to associate away from β–catenin, which builds up in the cytoplasm and translocates to the nucleus to act as a transcriptional co-activator with TCF to initiate transcription of Wnt target genes such as *Axin2, Dkk,* and *Sp5*.

1.3: 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 endothelial to hematopoietic transition and bud off from the endothelium in a process termed emergence, which initiates at 26 hours post fertilization (hpf). Next, nascent HSCs move to the caudal hematopoietic tissue (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 1.1).

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 zabrafish 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.* 2011a). 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 overexpression 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 – 17 hpf (Clements *et al.* 2011a, 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). Rspondin1, an activator of the Wnt pathway, regulates this requirement for Wnt16. Loss of Rspondin1

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 depends 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 and Richter *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 (Chen et al. 2009, Biechele et al. 2011, Grainger and Richter *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, Yin, *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, Yin, *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 and Richter *et al.* 2016). Overexpression of a constitutively active β–catenin also increased the number of *cmyb*+ cells within the HSC emergence window (Grainger and Richter *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 and Richter *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 and Richter *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 caudal hematopoietic tissue 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 caudal hematopoietic tissue (Goessling *et al.* 2009, Grainger and Richter *et al.* 2016). It is not clear whether Wnt signaling plays a role in HSC expansion within the caudal hematopoietic tissue, 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. 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 and Richter *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 and Richter *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 and Richter *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.

1.4: 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 1.1).

Mouse HSC emergence is regulated by Wnt signaling

Mouse HSCs emerge directly from aortic endothelium that undergoes an endothelial to hematopoietic transition 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.* 2012a). 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.* 2012a). Tissue specific loss of Wnt using a conditionally inactivatable β–catenin allele in VE-Cadherin-positive 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 affect on HSC function (Zhao *et al.* 2007, Ruiz-Herguido *et al.* 2012a). 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.* 2009a). 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 general 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 colony-forming cells (BL-CFCs), suggesting that some Wnts, like Wnt2, have a repressive affect 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.

1.5: 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 towards a specialized type of hemogenic endothelium, which then gives rise to hematopoietic stem and progenitor cells (HSPCs (Figure 1.3)) (reviewed in Ditadi *et al.* 2017). The spatial compartmentalization of *in vivo* HSC development is nonexistent in this *in vitro* system, and differences among individual hPSC lines and between differentiation protocols confounds comparison of multiple studies, as 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.* 2015). 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 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 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*.

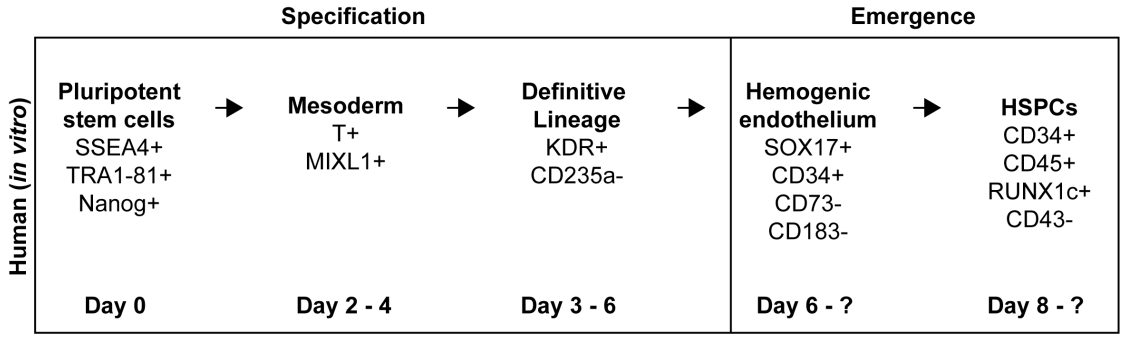

Figure 1.3. Hematopoietic development during *in vitro* differentiation. The development process is similar between human cells *in vitro* and cells *in vivo*; albeit with a lack of spatial separation *in vitro*. Hematopoietic stem and progenitor cells (HSPCs) are derived from the mesodermal lineage that is further specified to become cells that will contribute to definitive hematopoiesis. These cells are pushed towards 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.

1.6: Conclusions

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 1.1). 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 and Richter *et al.* 2016). Wnt may also be necessary for the embryonic expansion of HSCs (Luis *et al.* 2009a, 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 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 and Richter *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 and Richter *et al.* 2016). This is likely due to a combination of receptor-ligand specificity and differences in spatio-temporal 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*.

1.7: Rationale and Specific Aims

HSCs are the adult stem cell population that gives rise to all cells of the blood. This is exploited in therapeutic settings, where HSC transplants are used as a curative treatment for many blood disorders and cancers (Hatzimichael and Tuthill 2010). It would be of great benefit to patients and the medical community if these transplants were not reliant on the supply of HLA-matched HSCs from donors. hPSCs have great potential as a source for patient-matched HSCs. In theory, somatic cells from a patient could be reprogrammed to a pluripotent state (induced Pluripotent Stem Cells, iPSCs), differentiated to HSCs, and transplanted back into the patient. However, it is still not possible to derive a therapy-grade HSC *in vitro*, likely due to an incomplete understanding of the many complex molecular cues that direct HSC development *in vivo*.

I specifically focused on the Wnt signaling pathway as a critical mediator of HSC development. In Chapter 3, I will detail how I used the zebrafish as a model of normal *in vivo* hematopoiesis and identified a critical cue, Wnt9a, which is required for HSC development. The zebrafish is an ideal model in which to study hematopoiesis, as they develop quickly, are easy to image and analyze, and the process of HSC development is highly conserved amongst vertebrates. In Chapter 4, I explain how I translated my zebrafish findings to the human system by modulating expression of WNT9A throughout *in vitro* differentiation of hPSCs to HSPCs.

Chapter 1, in part, was originally published in *Critical Reviews in Biochemistry and Molecular Biology*. Richter, J., Traver, D., Willert, K. The role of Wnt signaling in hematopoietic stem cell development. *Critical Reviews in Biochemistry and Molecular Biology*, 52(4): 414-424 (2017). The dissertation author was the primary author of this paper.

CHAPTER 2: MATERIALS AND METHODS

2.1: Zebrafish Lines and Maintenance

Zebrafish were maintained and propagated according to University of California and local institutional animal care and use committee policies (Protocol S04168). AB*, *Tg(cmyb:eGFP)*^{zf169Tg} (North *et al.* 2007), *Tg(hsp:Gal4)*$^{kca4Tg/+}$ (Lawson *et al.* 2001), *Tg(fli1a:eGFP)* (Lawson and Weinstein 2002), *Tg(kdrl:eGFP)*^{s843} (Jin *et al.* 2005), *Tg(fli1a:Gal4), Tg(kdrl:Cherry-CAAX)*^{y171} (Fujita *et al.* 2011), *Tg(fli1a:EcRF-VP16)* (Swift *et al.* 2014), *Tg(gata2b:KalTA4; UAS:Lifeact:eGFP)* (Butko *et al.* 2015), *Tg(hsp70l:dntcf7l1a)*^{w26} (Lewis *et al.* 2004), *Tg(7X TCF-X.laveis-siamois:eGFP)*^{ia4} (Moro *et al.* 2012), *Tg(Dual FUCCI)* (Sugiyama *et al.* 2009), *Tg(phldb1:KalTA4)* (Distel *et al.* 2009), and *Tg(T2KTp1bglob:hmgb1-mCherry)*^{jh11} (Parsons *et al.* 2009) lines have been previously described. *Tg(UAS-dntcf7l1a-CG2), Tg(UAS: wnt9a)*and *Tg(UAS:myca)* founders were established by injecting 25 pg of the constructs described below with 100 pg of transposase mRNA at the one-cell stage. For simplicity in the text, these lines are referred to with shortforms listed in square brackets: *Tg(cmyb:eGFP)*^{zf169Tg} *[cmyb:eGFP], Tg(kdrl:Cherry-CAAX)*^{y171} *[kdrl:mCherry], Tg(fli1a:eGFP) [fli1a:eGFP], Tg(kdrl:eGFP)*^{s843} *[kdrl:eGFP], Tg(7X TCF-X.laveis-siamois:eGFP)*^{ia4} *[7XTCF:eGFP], Tg(gata2b:KalTA4; UAS:Lifeact:eGFP) [gata2b:eGFP]* and *Tg(hsp70l:dntcf7l1a)*^{w26} *[hsp:dntcf], Tg(UAS-dntcf7l1a-CG2)[UAS:dntcf], Tg(UAS: wnt9a) [UAS: wnt9a], Tg(UAS:myca) [UAS:myca], Tg(phldb1:KalTA4) [phldb:KalTA4*, *Tg(Dual FUCCI) [FUCCI],* and *Tg(T2KTp1bglob:hmgb1-mCherry)*^{jh11} [*TP1:mCherry*].

2.2: Morpholino knockdowns and cDNA rescues

Morpholinos (MOs) for wnt9a were targeted to retain the first intron with sequence 5′-GAAAGAATTGTCCTGCCTACCCGAA-3′, or targeted to block the ATG start codon (wnt9a ATG-MO) with sequence 5′-CCAGGAGAAGGTGTCCATCCAGCAT-3′ from GeneTools (Philomath. OR, USA). A MO for wnt9b was targeted to block the ATG start codon (wnt9b ATG-MO) with sequence

5’-CAGTCCTCGGAAGCCCGGTGCACAT-3’. One-cell stage zebrafish zygotes were injected with 1 ng of wnt9a MO, and retention of the intron was confirmed by PCR. Both ATG-MOs were used at a concentration of 2 ng per injection. Rescue experiments were performed by injecting the indicated amount of plasmid DNA with 1 ng of wnt9a MO.

2.3: Generation of zebrafish mutants

Mutation of the *wnt9a* locus was achieved by injecting 100 ng of Cas9 mRNA (Trilink, San Diego, CA, USA) and 100 ng of short guide RNA (sgRNA) targeting exon 1 (5’-ATTGGGACGGCTAATAGATT- 3’). Mutations were confirmed by sequencing individuals.

2.4: Whole-mount in situ hybridization (WISH)

RNA probe synthesis was carried out according to the manufacturer’s recommendations using the DIG-RNA labeling kit (Roche, Basel, Switzerland). Probes for *dll4, hey2, notch1b*, *msr*, *kdrl, cdh17, cmyb*, and *runx1* and WISH protocols have been previously described (Rowlinson and Gering 2010, Clements *et al.* 2011a, Kobayashi *et al.* 2014). The probe constructs for all *wnt* genes were generous gifts from W. Herzog.

2.5: Fluorescence-activated cell sorting of zebrafish embryos

Zebrafish were dissociated using Liberase TM (Roche) and filtered through an 80-μm filter. Cells were sorted on a BD Influx cell sorter according to standard procedures.

2.6: Flow cytometry and fluorescence-activated cell sorting of cultured cells

Cells were dissociated using TrypLE Express and resuspended in FACS buffer. When indicated, cells were stained with anti-CD34-APC and anti-CD45-PE antibodies (BioLegend, San

Diego, CA, USA). Cells were analyzed by standard means on a BD FACSCanto or a BD LSRFortessa. Cells were sorted on a BD FACSAria II according to standard procedures. Flow cytometry data was analyzed using FlowJo software (FlowJo, Ashland, OR, USA).

2.7: qPCR

RNA and cDNA were synthesized by standard means and qPCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer's recommendations and analyzed using the $2^{-\Delta\Delta Ct}$ method (Schefe *et al.* 2006). Sequences of primers used are shown in Table 1.2.

Table 1.1. Zebrafish gene qPCR primers used in this study.

Target	Fwd (5'-3')	Rev (5'-3')
ef1a	GGAGGCTGCCAACTTCAACGCTC	GCTTCTTGCCAGAACGACGGTCG
wnt1	TTAACTTTGCCCTTTCAGAT	GTACCAGCTGCACATTCTTA
wnt2	AAGACTCGCCTGACTACTGT	GACTCACTCTTGATGTGTCG
wnt2ba	GAGAACTCTCCGGACTACTG	CACCATTTAAACTTGCACTC
wnt2bb	ACGGGACAGGATTTACAGTAGC	TTGTTACAGACCCGACCAGC
wnt3	CAGTGAAGATGCTGAATTTG	CATGTTCTCCAATATGGTCA
wnt3a	GTGTCCCGAGAGTTTGCTGA	GCCCGTGACACTTGCATTTC
wnt4a	TAAAGGACAGTCATCCAACA	GTCTTGACTTCGCAGGAG
wnt4b	TCATGGAGTCCGTGCGAAAG	TTCACGCGTTCCTTGGTTCA
wnt5a	TCTGCCGACTTGAACAGACA	AGAGCAGTGTGTGTGGAGTG
wnt5b	ATGAATCTGCAGAACAATGA	CGTATTTCTCCTTCAGGAAT
wnt7aa	AGATGCACGGGAGATCAAGC	CCGAAACTTGGGGAGAGTGG
wnt7ba	GGATGCACGAGAGATAAAA	CCATGACATTTACATTCCAG
wnt7bb	GAGTTCTCACGGAAGTTTGT	ACATTTACATTCCAGCTTCA
wnt8a	TTCTAAACAATTCGTGGATG	CTTCATTGTCGCTTTTACAG
wnt8b	GCACAACAACGAAGTAGGA	GTTTCCCACTTCTCGAAAC
wnt9a	TGGAGACAACCTCAAATACA	TGATCACCTTCATTCCTACA
wnt9b	AAAGAGGGTCAGCAAGGACC	TGTCTTGAAACGGGGAGAGC
wnt10a	CGCGAGACGTACAGAGACAT	ACCTGCCAACAGGTCTTCAG
wnt10b	CGCTTTTCTAGAGATTGGTT	TGCACTTTCTCCTCATATTG
wnt11	TCAGCTCACCGGATTACT	CACCTCTGTGTAGGCATTAT
wnt16	ATGAAGCAGCATAACAGTGA	GTCTTTACCGCACATGAAC
runx1	ACGGTCCTATAGACGGTCCT	GGATCTGTGAACGCCGTCAG
axin2	GACAAGAGTCACCCAGTGCC	GTACGTGACTACCGTCTCCG

Table 1.2. Human gene qPCR primers used in this study.

Target	Fwd (5'-3')	Rev (5'-3')
ACTB	CATCCGTAAAGACCTCTATGCC	ATGGAGCCACCATCCACA
GAPDH	CCTGCACCACCAACTGCTTA	CCATCACGCCACAGTTTCC
RPL13A	CCTTCCTCCATTGTTGCCCT	TGCACAATTCTCCGAGTGCT
BRY	CAGTGGCAGTCTCAGGTTAAGAAGGA	CGCTACTGCAGGTGTGAGCAA
CD31	TTCCTGACAGTCTCTTGAGTGG	TTTGGCTAGGCGTGGTTCTCAT
CD34	CAATGAGGCCACAACAAACATC	GGTGGTGAACACTGTGCTGATT
VE-Cadherin	ATGCACCTGTGTTTGAGAAAAGC	CCTGGATGCGGTATGAGACTT
SOX17	AGGAAATCCTCAGACTCCTGG	CCCAAACTGTTCAAGTGGCAG
GATA2	AGCCGGCACCTGTTGTGCAA	TGACTTCTCCTGCATGCACT
CD45	CTCTACGCAAAGCTAGGCCA	ACTTGTCCATTCTGAGCAGG
CMYB	GTCACAAATTGACTGTTACAACACCAT	TTCTACTAGATGAGAGGGTGTCTGAGG
RUNX1c	TGGTTTTCGCTCCGAAGGT	CATGAAGCACTGTGGGTACGA
AXIN2	CAGCAGAGGGACAGGAATC	CAGTTTCTTTGGCTCTTTGTG
SP5	GCAAGGTGTACGGGAAGACG	GAAGAGCCAGTTGCACACGA
WNT9A	ACAACCTCGTGGGTGTGAAG	CTCATGGAAAGGCGCCAACT
FZD9	GCCTGTGCTACCGCAAGATA	GTTCTCCAAAGAGGGGTCCG

2.8: Quantifying HSPCs in zebrafish embryos

HSPCs were quantified by counting the number of *kdrl:mCherry; cmyb:eGFP* or *kdrl:mCherry; gata2b:GFP* double positive cells in the floor of the DA in the region above the yolk extension in a 625 μm confocal Z stack encompassing the entire mediolateral segment of the aorta. The number of HSPCs per millimeter was calculated from these data. Confocal images were generated by stacking one to four individual Z slices. When quantifying WISH data, the number of *cmyb*+ cells was counted above the yolk extension.

2.9: Cell lines and culture conditions

All experiments described in this dissertation were approved by a research oversight committee (IRB/ESCRO Protocol #100210, PI Willert). Human embryonic stem cell (hESC) H9 cells were obtained from WiCell (Madison, WI). H9-RUNX1c:GFP; SOX17:mCherry reporter cells were obtained under a Material Transfer Agreement from Dr. Andrew Elefanty (Monash University, Melbourne, Australia) (Ng *et al.* 2016). Cells were maintained in Essential 8 (E8) media (DMEM/F12 supplemented with L-Ascorbic Acid, Selenium, Transferrin, $NaHCO_3$, Insulin, TGFβ1, and FGF2) as described previously (Chen *et al.* 2011). Transgenic cell lines were maintained in 2 ug/mL Puromycin (Thermo Fisher Scientific, Waltham, MA, USA). Cells were passaged every 5 days with TrypLE Express (Thermo Fisher Scientific) and seeded onto Matrigel- (Corning, Corning, NY, USA) coated tissue culture dishes in medium containing 1 uM Rock inhibitor (Y-27632 dihydrochloride; Enzo Life Sciences, Farmingdale, NY, USA). HEK293, and HEK293T cells were cultured in 1× high glucose DMEM, 10% (vol/vol) FBS, and 1% (vol/vol) L-glutamine penicillin/streptomycin. HEK-293 TOP:FLASH cells have been previously generated (Fernandez *et al.* 2014).

2.10: Generation of transgenic and mutant hESC lines

Doxycline-inducible WNT9A and Wnt3a H9 cell lines were generated by co-transfecting PiggyBac transposons (Li *et al.* 2013) encoding the transgene with a plasmid constitutively expressing a hyperactive version of the PiggyBac transposase (Doherty *et al.* 2011, Yusa *et al.* 2011, Burnight *et al.* 2012) using GeneIn transfection reagent (GlobalStem, Rockville, MD, USA) according to manufacturer directions. On the day of transfection, 10% Fetal Bovine Serum (FBS; Peak Serum, Inc., Fort Collins, CO, USA) was added to E8 growth media. Cells were selected for transgene integration with 4 ug/mL Puromycin (Thermo Fisher Scientific). The Wnt reporter cell line was generated by infecting H9 cells with a lentivirus containing a previously cloned lentivector (Addgene Plasmid #24304). Infected cells were sorted for high expression of mCherry using Fluorescence-Activated Cell sorting, and used for differentiation within 10 passages of lentiviral infection. *WNT9A* mutant cells were generated by co-transfection of CMV:Cas9-EGFP plasmid and U6:guide RNA plasmid into H9 cells as written above. Single GFP^+ cells were sorted on a BD Influx cytometer 48 hours after transfection and recultured to isolate clonal mutant lines, which were screened by PCR amplification and sequencing from genomic DNA.

2.11: Hematopoietic differentiation

hESCs were differentiated to HSPCs as described by Ng et al. (Ng *et al.* 2005, 2008). Briefly, on the day before beginning the differentiation, cells were passaged at a high density (approximately 1.5 x 10^5 cells/cm^2) into E8 media with 2.5 uM Rock inhibitor (Y-27632 dihydrochloride) onto Matrigel-coated tissue culture dishes using TrypLE Express. 24 hours later, cells were dissociated using TrypLE Express and resuspended in STEMdiff APEL2 medium (Stemcell Technologies, Vancouver, BC, Canada) supplemented with 40 ng/mL BMP4, 40 ng/mL SCF, 20 ng/mL VEGF (R&D Systems, Minneapolis, MN, USA), 10 ng/mL FGF2 (Peprotech, Rocky Hill, NJ, USA), and 2.5 uM Rock inhibitor (Y-27632 dihydrochloride). 3 x 10^3 cells were seeded per well of a low-attachment

U-bottom 96 well plate in 100 uL of media, and incubated for 7 days in a standard tissue culture incubator. After 7 days, embryoid bodies (EBs) were transferred to gelatin-coated plates and 1 volume of media with growth factors was added. Cells were harvested and analyzed after 14 days of differentiation (unless otherwise indicated). When indicated, CHIR-98014 and C59 (Selleck Chemicals, Houston, TX, USA) were added to the differentiation at a final concentration of 0.1% DMSO. Doxycycline (Sigma-Aldrich, St. Louis, MO, USA) was added to induce transgene expression when indicated.

2.12: Immunofluorescence

Cells were grown on glass coverslips coated with Matrigel, and fixed using 4% paraformaldehyde. Coverslips were washed, blocked and permeabilized with 2% BSA and 0.2% TritonX-100 in PBS. Cells were stained with antigen-sepcific antibodies and fluorescent secondary antibodies. A list of antibodies used in this dissertation can be found in Table 1.4. Coverslips were mounted onto glass slides in mounting medium containing DAPI and imaged on a Ziess AxioImager microscope.

2.13: Western blot

Cells were lysed in buffer containing 1% TritonX-100, 150 mM NaCl, 50 mM Tris, pH 8.0, and protease inhibitors on ice, and centrifuged. Lysate was run on a SDS-PAGE and transferred to nitrocellulose, blotted with antibodies against the indicated target protein, and detected using enhanced chemiluminescence. A list of antibodies used in this dissertation can be found in Table 1.4.

2.14: anti-Wnt9a antibody generation

The anti-Wnt9a antibody was generated against a fusion protein comprised of GST fused to 70 amino acids of the zebrafish Wnt9a protein (zWnt9a, amino acid sequence

LAPFHEIGKQLKQRYETSVKVASSTNEATGEGEISQSRSQSQQPPQPDIPRTPDLLHIEDSPSLE RPHRD). This GST-zWnt9a fusion protein was generated by subcloning the open reading frame encoding the above 70 amino acids of zWnt9a into pGEX4T-1 (GE Healthcare Life Sciences, Marlborough, MA, USA), expressing the protein in BL21 bacteria induced with 1 mM IPTG, and purifying the protein using Glutathione Sepharose beads (GE Healthcare Life Sciences). This purified fusion protein was injected into rabbits at Lampire Biological Laboratories (Pipersville, PA, USA) according to standard practices. Serum from immunized rabbits was depleted of anti-GST antibodies by affinity purification over a GST column, and enriched for anti-Wnt9a antibodies by affinity purification over a GST-Wnt9a column. This Wnt9a antibody is reactive with both zebrafish and human Wnt9a (data not shown). This enriched material was utilized for immunoblotting (see Table 1.4 for dilutions used).

2.15: Luciferase assay

HEK 293-TOP:FLASH cells were lysed in 100 mM K-PO_4 buffer with 0.2% TritonX-100. 100 uL of assay cocktail (25 mM Tris, pH 8.0; 15 mM $MgSO_4$; 10 mM ATP; 65 uM D-luciferin) was added to 20 uL of lysate, and assayed on a Perkin Elmer Envision luminometer (Perkin Elmer, Waltham, MA, USA).

2.16: Blue Sepharose Wnt pull down from conditioned media

Conditioned media was collected 24 hours after indicated treatments, and TritonX-100, Tris-Cl pH 7.5, and NaN_3 were added to final concentrations of 1%, 20 mM, and 0.1%, respectively, and sterile filtered. Blue Sepharose bead slurry (1:1 ratio of PBS:beads) was added to conditioned media in a 1:100 dilution, and rotated at room temperature for 1 hour. The beads were spun down at 1000 x g for 3 minutes, and washed with 1% CHAPS, 150mM KCl, 50mM Tris-Cl pH 7.5 three times. The

washed beads were diluted in 1X protein loading buffer, heated at 95 °C for 5 minutes, then used for Western blotting with the indicated antibodies.

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Chapter 2, in part, has been submitted for publication in *Genes*. Richter J., Elefanty, A., Ng, E., Stanley, E., Traver, D., Willert, K. "WNT9A is a conserved regulator of hematopoietic stem and progenitor cell development." The dissertation author was the primary investigator and author of this paper.

Table 1.3. Antibodies used in this study.

Target Antigen	Conjugation	Purpose	Company	Cat. No.	Dilution used
wnt9a	N/A	Western blot	Lampire Biologicals	Custom	1:2000
wnt9a	N/A	Immunofluorescence	Lampire Biologicals	Custom	1:50
WNT9B	N/A	Western blot	Everest	EB09452	1:1000
WNT9B	N/A	Immunofluorescence	Everest	EB09452	1:100
CD34	APC	Flow cytometry	Biolegend	343608	1:100
CD45	PE	Flow cytometry	Biolegend	304008	1:100
B-Actin	N/A	Western Blot	Sigma Aldrich	A2228	1:10,000
Goat IgG	Alexa Fluor 647	Immunofluorescence	Invitrogen	A-21447	1:200
Rabbit IgG	Alexa Fluor 647	Immunofluorescence	Invitrogen	A32733	1:200
Goat IgG	HRP	Western blot	Life Technologies	A16136	1:10,000
Rabbit IgG	HRP	Western blot	Southern Biotechnology	4050-05	1:8000

CHAPTER 3: THE ROLE OF WNT9A IN ZEBRAFISH HEMATOPOIETIC DEVELOPMENT

3.1: Introduction

Hematopoietic stem cells (HSCs) both self-renew and generate all mature blood cell types throughout the lifespan of the vertebrate organism. Derivation of HSCs *in vitro* would allow patient-specific replacement therapies. Currently, deriving an HSC *in vitro* that is suitable for therapeutic use is not possible. Gaining a more thorough understanding of the molecular cues that direct HSC development *in vivo* will aid in improving protocols to derive HSCs from pluripotent precursors.

HSCs originate from arterial hemogenic endothelium (HE) during vertebrate development, a cell population derived from posterior lateral mesoderm (PLM) in lower vertebrates, which also forms the vascular cord and subsequently the aorta (Fouquet *et al.* 1997, Liao *et al.* 1997, Brown *et al.* 2000, Jin *et al.* 2007, Herbert *et al.* 2009). During the migration of PLM cells, inductive cues from the somites are thought to instruct the fate of HE from shared vascular precursors (Burns *et al.* 2005, Wilkinson *et al.* 2009, Bertrand, Chi, *et al.* 2010, Clements *et al.* 2011b, Clements and Traver 2013, Leung *et al.* 2013, Zhen *et al.* 2013, Kobayashi *et al.* 2014, Butko *et al.* 2015). Hematopoietic stem and progenitor cells (HSPCs) emerge directly from the floor of the dorsal aorta (aorta hereafter) in a process termed the endothelial-to-hematopoietic transition (EHT) (Bertrand, Chi, *et al.* 2010, Kissa and Herbomel 2010). In zebrafish, EHT begins at 26 hours post fertilization (hpf), and the number of emerging HSPCs peaks around 36 hpf (Bertrand, Chi, *et al.* 2010, Kissa and Herbomel 2010). Following this transition, HSPCs enter circulation through the posterior cardinal vein and migrate to the caudal hematopoietic tissue in zebrafish (CHT; analogous to the placenta/fetal liver in mammals) for secondary amplification (Murayama *et al.* 2006, Tamplin *et al.* 2015) and finally to the kidney (akin to the mammalian bone marrow), where they reside for the remainder of adult life (Jagannathan-Bogdan and Zon 2013). Although the anatomical location of these sites varies among organisms, HSC

fate is likely instructed by conserved developmental cues. Understanding the inductive signals that instruct HSC fate from mesoderm will be essential to the eventual derivation of HSCs from pluripotent precursors.

Wnt signaling is vital for the maintenance and development of stem cell populations in many organ systems, including the intestine, skin and liver (Clevers and Nusse 2012). *Wnt* genes encode lipid-modified, secreted growth factors that initiate signaling cascades, including the Wnt/b-catenin pathway (commonly referred to as the "canonical" Wnt pathway). Upon Wnt binding its cognate receptor encoded by a *Frizzled* (*Fzd*) gene, the b-catenin protein becomes stabilized and enters the nucleus where it interacts with the Lymphoid enhancer binding factor/T-cell factor (LEF/TCF) transcription factors to drive expression of Wnt target genes to regulate a variety of developmental processes.

The role of Wnt function in HSPC development and function remains poorly understood, in part due to conflicting reports. For example: loss of Wnt function depleted the HSPC pool (Zhao *et al.* 2007, Fleming *et al.* 2008, Goessling *et al.* 2009, Luis *et al.* 2009a), and activation of Wnt signaling resulted in increased HSPC number (Reya *et al.* 2003, Willert *et al.* 2003, Baba *et al.* 2005, Malhotra *et al.* 2008, Goessling *et al.* 2009). In contrast, others have observed a depletion of the progenitor pool upon b-catenin overexpression (Kirstetter *et al.* 2006, Scheller *et al.* 2006), which may relate to dosage-dependent effects of Wnt signaling on different populations of blood cells (Luis *et al.* 2011). While these studies establish that Wnt is essential for the function of adult HSPCs, what is lacking is a clear understanding of Wnt function during the embryonic development of HSPCs. In particular, it is unclear at what stage(s) of HSPC development Wnt is critical. Furthermore, the identification of a specific Wnt ligand regulating HSPC development remains elusive. Here, we demonstrate that Wnt signaling is required prior to formation of the aorta. We further show that the *Wnt* gene *wnt9a* is expressed in relevant spatio-temporal domains and that HSPCs are depleted following loss-of-function of *wnt9a;* this loss of function cannot be rescued with ectopic expression of other Wnt genes. This

Wnt9a cue drives an early aortic amplification of HSPCs, which occurs after HSPC emergence begins. This proliferative event is mediated, at least in part, through regulation of *myc-a* (also known as *c-myc*).

3.2: Results

Hematopoietic stem cell numbers are affected by Wnt signaling

Using zebrafish, in which complex developmental processes can be readily observed and dissected, we examined the requirement for Wnt signaling during HSPC development. We observed robust Wnt reporter activity in the floor of the dorsal aorta at 26 hpf (during HSPC emergence) using double transgenic *7X TCF:eGFP* (Moro *et al.* 2012); *kdrl:mCherry* (Bertrand, Chi, *et al.* 2010) embryos, which express eGFP from a Wnt responsive sequence and membrane-bound mCherry in the vasculature (Figure 3.1), indicating that endothelial cells have received a Wnt cue.

To monitor the effect of Wnt/b-catenin modulation on HSPCs, we used LiCl, which activates Wnt/b-catenin signaling through inhibition of GSK3b, and IWP-L6, which inhibits Porcn, an essential regulator of Wnt ligand maturation and secretion (Kadowaki *et al.* 1996, Komekado *et al.* 2007). As previously established, dosages of 0.15M LiCl or 1.5mM IWP-L6 did not alter overall embryonic morphogenesis or vasculature, as visualized by *kdrl:mCherry* expression (Figure 3.2a), but were able to activate or inhibit Wnt signaling, respectively (van de Water *et al.* 2001, Wang, Moon, *et al.* 2013), as measured by expression of the Wnt target gene *axin2* (Jho *et al.* 2002) (Figure 3.2b).

HSPCs can be identified as *kdrl:mCherry*; *cmyb:eGFP* double positive cells in the floor of the aorta (Bertrand, Chi, *et al.* 2010). To determine if there was an overall function for Wnt leading to HSPC emergence, we treated larvae from 10 hpf to 36 hpf to activate [LiCl] or inhibit [IWP] Wnt and observed emerging HSPCs at 36 hpf, when their numbers peak (Bertrand, Chi, *et al.* 2010, Kissa and Herbomel 2010). By doing so, we observed a 2-fold decrease and a 1.5-fold increase in HSPC number after Wnt inhibition [IWP] or activation [LiCl], respectively (Figure 3.3a-c). These effects were confirmed with reverse transcription quantitative PCR (qPCR) for the hematopoietic marker *cmyb* (Figure 3.3d), indicating that Wnt signaling regulates HSPC number.

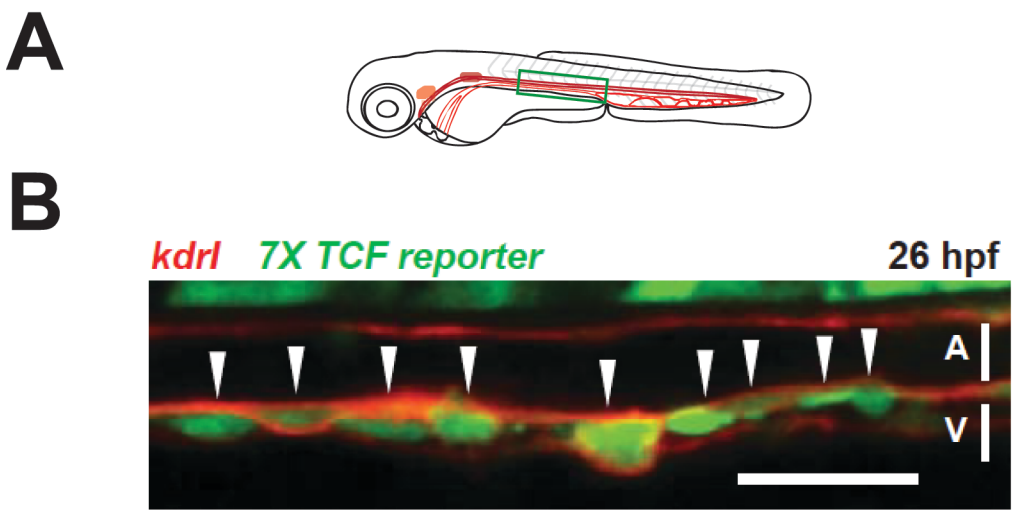

Figure 3.1. Endothelial cells receive a Wnt cue. Confocal imaging of the region of zebrafish vasculature shown in the green box in (A). *kdrl:mCherry; 7XTCF:eGFP* reporter transgenic embryos at 26 hpf. Endothelial cells that have received a Wnt cue are double positive and indicated with a white arrowhead. A=aorta, V=vein. Scale bar = 30 um.

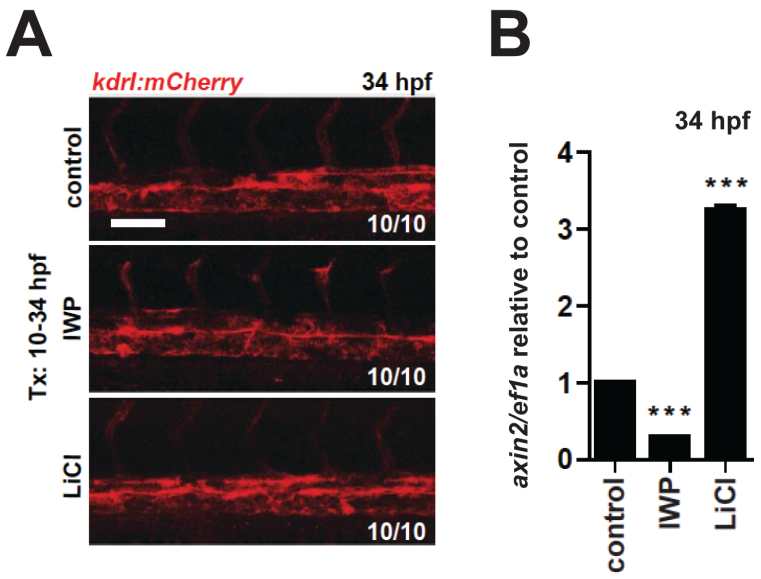

Figure 3.2. Small molecule modulators of the Wnt pathway do not impact endothelial cell development. (A) Confocal imaging of 34 hpf *kdrl:mCherry* transgenic fish treated with 1.5 μM IWP-L6 or 0.15M LiCl. Images are representative of 10 individual animals. The dosages used did not alter overall morphology or vasculature as measured by expression of the vascular marker *kdrl:mCherry*. (B) Trunk and tail tissue from wildtype AB* fish under the same treatment regimen were examined; an effect on Wnt signaling was confirmed at 34 hpf by qPCR for expression of the Wnt target gene *axin2* (n=15). HSPCs were examined by qPCR at 34 hpf for marker *cmyb* (C) (n=20) in dissected trunks and tails of larvae. ***=p<0.001. Scale bar = 30 um.

Credit: Imaging and qPCR performed by Stephanie Grainger, PhD.

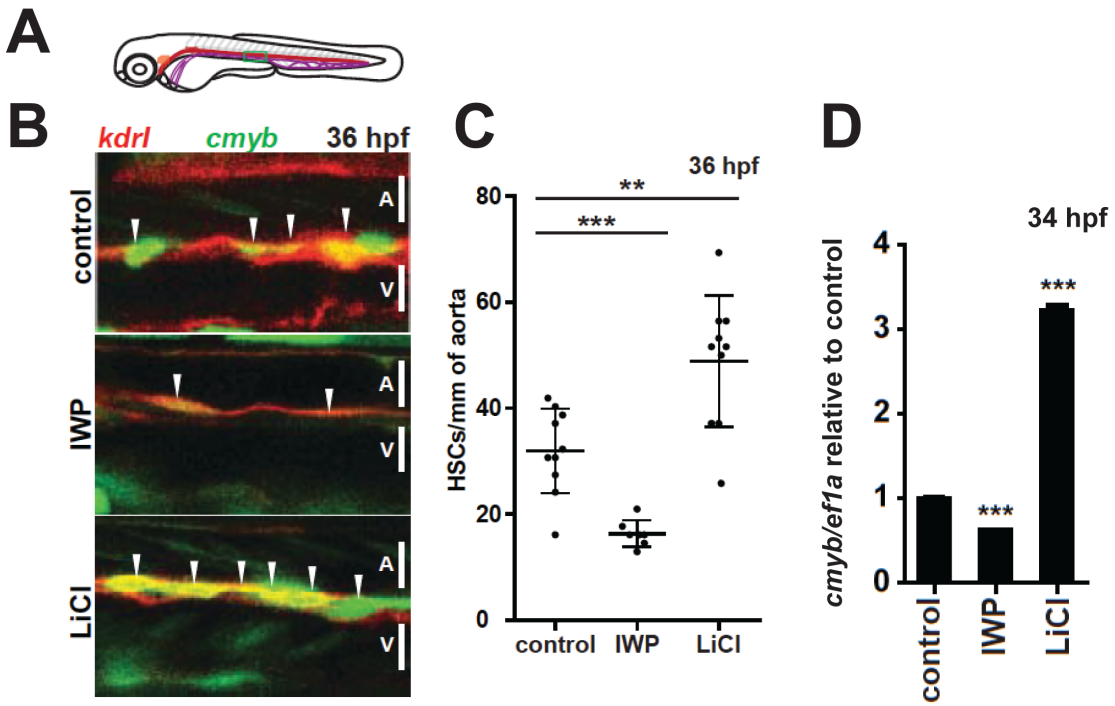

Figure 3.3. Wnt signaling regulates HSC numbers. The region of 36 hpf *kdrl:mCherry; cmyb:eGFP* embryos depicted in (A) was imaged on a confocal microscope following treatment with IWP or LiCl. Double-labeled HSCs (white arrowhead) are quantified in (C). A=aorta, V=vein. (D) The effect on the HSC population was confirmed by qPCR for HSC marker *cmyb* at 34 hpf. ***=p<0.001, **=p<0.01.

Credit: HSC enumeration and qPCR were performed by Stephanie Grainger, PhD.

Wnt is required transiently prior to 20 hpf

To identify the window in HSPC development when Wnt is required, we used *hsp:dntcf* transgenic animals, which carry a dominant negative version of *tcf* (*dntcf*), the expression of which leads to rapid inhibition of Wnt signaling (Figure 3.4a-b) (Lewis *et al.* 2004, Clevers and Nusse 2012). We performed heat shocks each hour spanning the window prior to HSPC emergence (13-26 hpf), followed by analysis of *cmyb* expression at 40 hpf by whole-mount *in situ* hybridization (WISH) (Kissa *et al.* 2008). Heat shock before 19 hpf resulted in a profound loss of *cmyb* expression in the aorta at 40 hpf, whereas heat shock at 20 hpf or later had no effect (Figure 3.5). Since the *dntcf* effect on *axin2* expression occurs acutely and is long-lasting (Figure 3.4a-b), these results suggested that the role for Wnt in HSPC development occurs prior to 20 hpf.

Specification, when HSCs acquire identity cues, occurs as mesodermal cells migrate to the midline underneath the somites to form the aorta and vein (Kobayashi *et al.* 2014) (Fig. 1I), and can be monitored with early expression of HSPC markers such as *runx1*. The expression of *runx1* at 26 hpf was unaffected following the drug treatment regime (Figure 3.6a) (Burns *et al.* 2005); *dntcf* expression at 13 hpf also did not affect *runx1* or *cmyb* expression at 29 hpf (Figure 3.6b-c). These results indicate that Wnt signaling positively regulates the number of emerging HSPCs after specification.

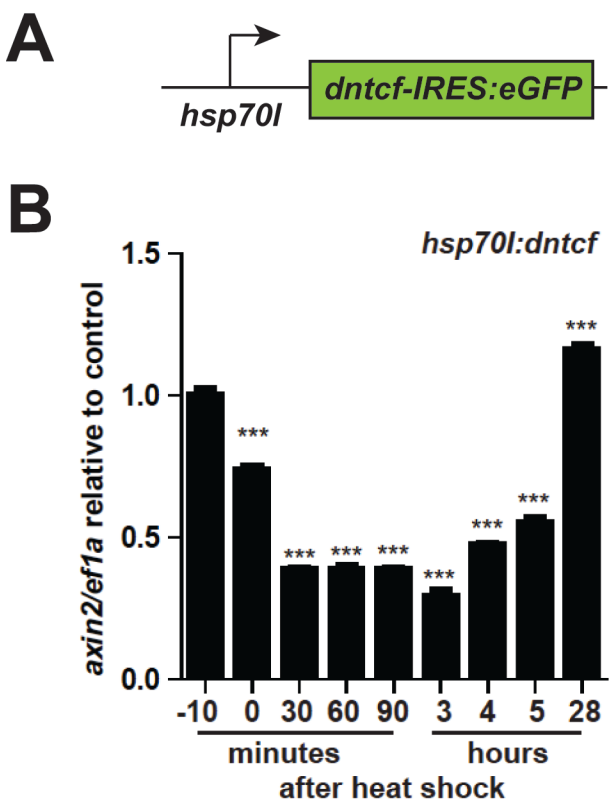

Figure 3.4. The *dntcf* transgene rapidly downregulates Wnt signaling. (A) Schematic of the heat shock-inducible *dntcf* transgene. (B) *hsp:dntcf* fish were heat shocked and compared to AB* for *axin2* by qPCR to assess Wnt activity following *dntcf* induction (n=20 at each time point). ***=p<0.001

Credit: qPCR was performed by Stephanie Grainger, PhD.

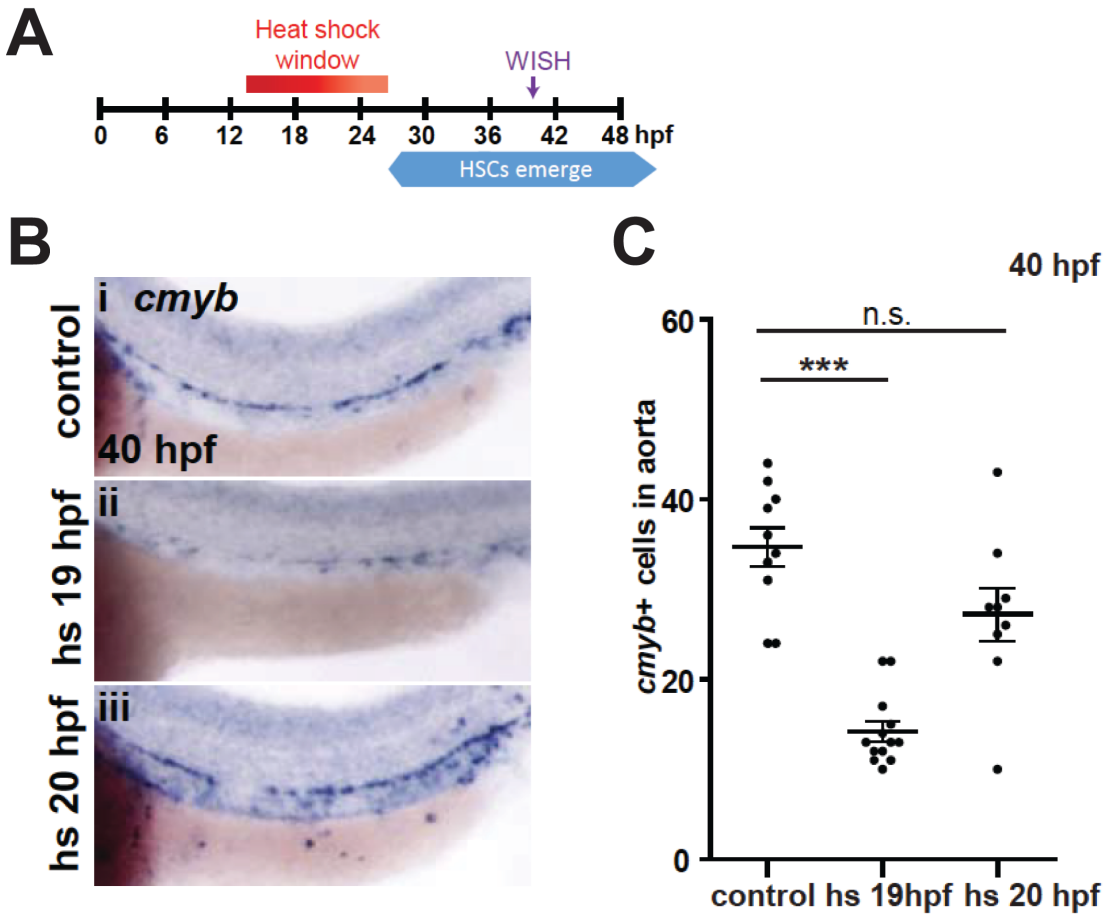

Figure 3.5. Wnt signaling is required for HSC development prior to 20 hpf. (A) Schematic of heat shock regimen. (B) *hsp:dntcf* fish were heat shocked at 16.5 hpf, pools were fixed every hour from 23 to 36 hpf, and they were analyzed for *cmyb* expression by WISH. Cells that are positive for *cmyb* expression are quantified in (C). ***=p<0.001, n.s.=Not significant.

Credit: Experiment performed by Stephanie Grainger, PhD.

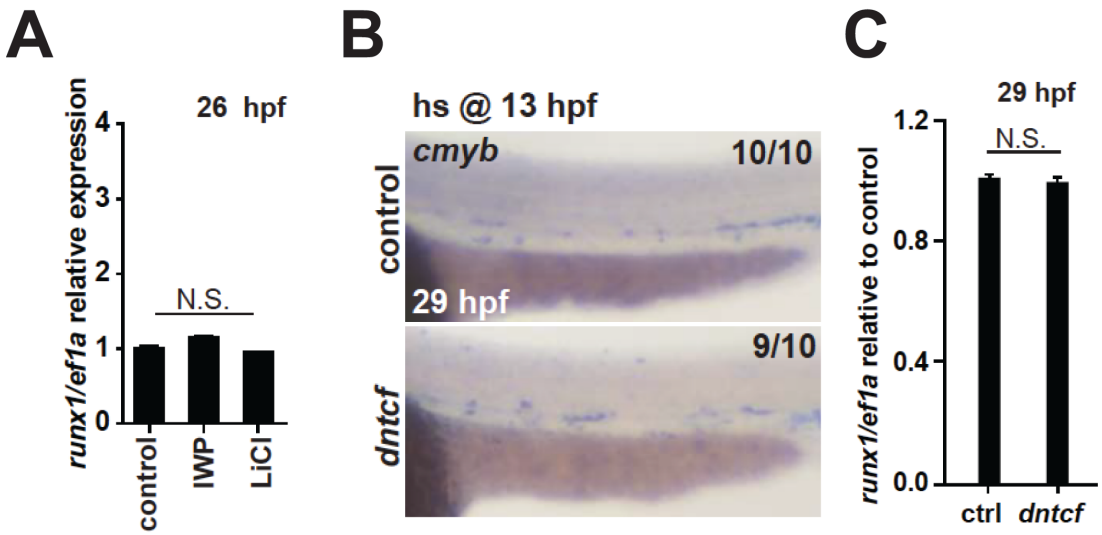

Figure 3.6. Modulation of Wnt signaling does not affect HSC specification. (A) Treatment with small molecules to inhibit [IWP] or activate [LiCl] does not affect HSC specification, as measured by qPCR for expression of marker gene *runx1*. Inhibition of Wnt signaling via expression of the *dntcf* transgene also does not impact *runx1* expression at 29 hpf by WISH (B) or qPCR (C). N.S.=Not significant.

Credit: Experiment performed by Stephanie Grainger, PhD.

The Wnt cue is required in cells of the hemogenic endothelium

To test whether the Wnt signal is received by endothelial cells, we generated Upstream Activating Sequence (UAS):dntcf transgenic animals to drive the previously reported dntcf transgene expression with Gal4 in the vasculature (Figure 3.7a). Similar to the ubiquitous induction of the hsp:dntcf transgene, endothelial-specific (using fli1a:Gal4) Wnt inhibition resulted in a loss of HSPCs at 40 hpf (Figure 3.7b-c), indicating that the Wnt signal acts upon vascular cells to influence HSPC development.

To determine if the Wnt cue is required broadly in endothelial cells, or more specifically in HE, we drove dntcf using a gata2b:Gal4 driver (Figure 3.7d), which is expressed in the earliest known population of HE (the transient population of endothelial precursors that can differentiate into HSCs) (Butko *et al.* 2015). Similar to ubiquitous hsp:dntcf and endothelial-specific transgenes, Wnt inhibition in gata2b^{+} cells resulted in a loss of HSPCs at 40 hpf (Figure 3.7e-f), indicating that the Wnt signal acts upon HE to influence HSPC development.

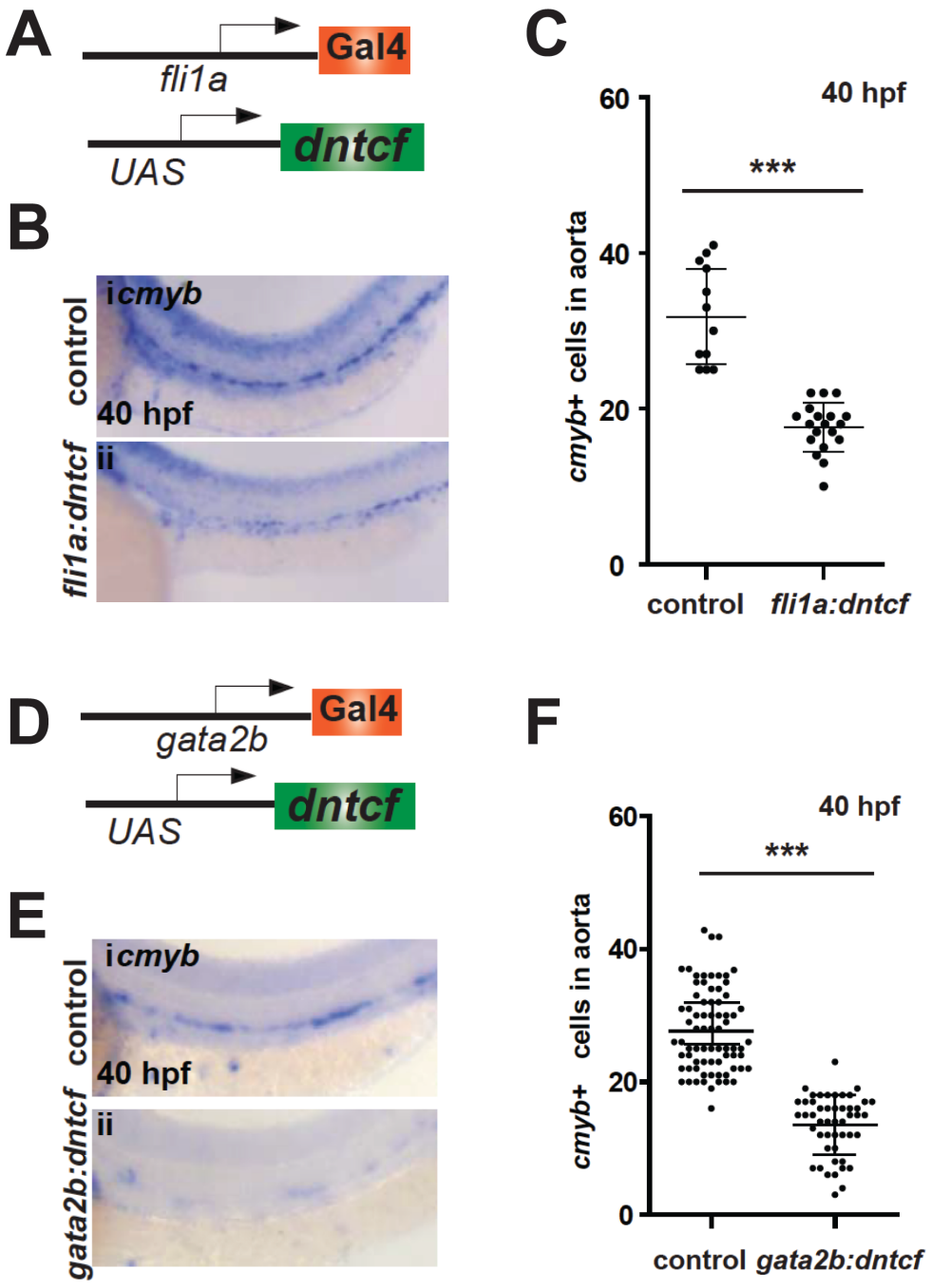

Figure 3.7. The Wnt signal is required in cells of the hemogenic endothelium. (A) Schematic of transgenic elements used for endothelial specific expression of *dntcf* in endothelial cells. (B) WISH for *cmyb* at 40hpf in control and in *fli1a:Gal4; UAS:dntcf,* fish. (C) Quantitation of *cmyb*+ cells from B. (D) Schematic of transgenic elements used for endothelial specific expression of *dntcf* in the HE. E. WISH for *cmyb* at 40hpf in control and in *gata2b:Gal4; UAS:dntcf,* fish. (F) Quantitation of *cmyb*+ cells from E. ***P<0.001.

Credit: Experiment performed by Stephanie Grainger, PhD.

Wnt9a is required for HSPC development

The data presented above establish an essential role for Wnt signaling in HSPC development; we next aimed to find the Wnt driving this process. We have previously demonstrated that the ventral somite signals to neighboring endothelial precursors during HSPC migration to the embryonic midline (Clements *et al.* 2011b, Kobayashi *et al.* 2014). The timing of the Wnt requirement in HSPC development suggested the somite as a possible source of Wnts. To identify candidate *wnt* gene(s), we surveyed expression of 21 *wnt* genes by qPCR in *myf5-GFP* positive (somitic) cells (Chen *et al.* 2007) prior to (16.5 hpf) and after (20 hpf). We identified *wnt9a* as our prime candidate because it is expressed at 16.5 hpf, and downregulated at 20 hpf (Figure 3.8a-c). By WISH, *wnt9a* transcript is detected in the posterior somites at 16.5 hpf; in regions consistent with vasculature at 19 hpf; and in the vasculature and the caudal hematopoietic tissue (CHT) by 28 hpf (Figure 3.9a-c).

To determine the effect of loss of *wnt9a* on HSPC development, we used a splice-blocking morpholino (MO) to knock down *wnt9a* expression in embryos (Figure 3.10a-b). Upon injection into embryos, this MO decreased Wnt signaling, as demonstrated by reduction in *axin2* expression and decrease in *7XTCF:eGFP* reporter signal (Figure 3.10c-d). We observed a two-fold decrease in *kdrl:mCherry*; *cmyb:eGFP* HSPCs emerging from the floor of the dorsal aorta at 36 hpf using this MO and a second MO designed to block translation of *wnt9a* (ATG-MO) (Figure 3.11). This phenotype was specific to the hematopoietic system, as the vasculature, aorta and pronephros were properly specified in MO-injected embryos (Figure 3.12). MO knockdown of the closely related *wnt9b* gene did not affect HSPCs, suggesting that *wnt9a* is specifically required for HSPC development (Figure 3.11). To further determine whether or not Wnt9a specifically instructs HSPC development, we performed co-injections of cDNA (to circumvent early lethality) encoding either *wnt9a*, *wnt9b*, or *wnt3a* with the *wnt9a* splice-blocking MO. Ectopic expression of *wnt9a* rescued HSPC numbers in *wnt9a* morphants (Figure 3.11). In contrast, ectopic expression of *wnt9b* or *wnt3a* did not rescue the *wnt9a* MO effect on

HSPC numbers (Figure 3.11). These data suggest that Wnt9a specifically drives HSPC development, and that loss of *wnt9a* cannot be rescued by overexpression of other *wnt* genes.

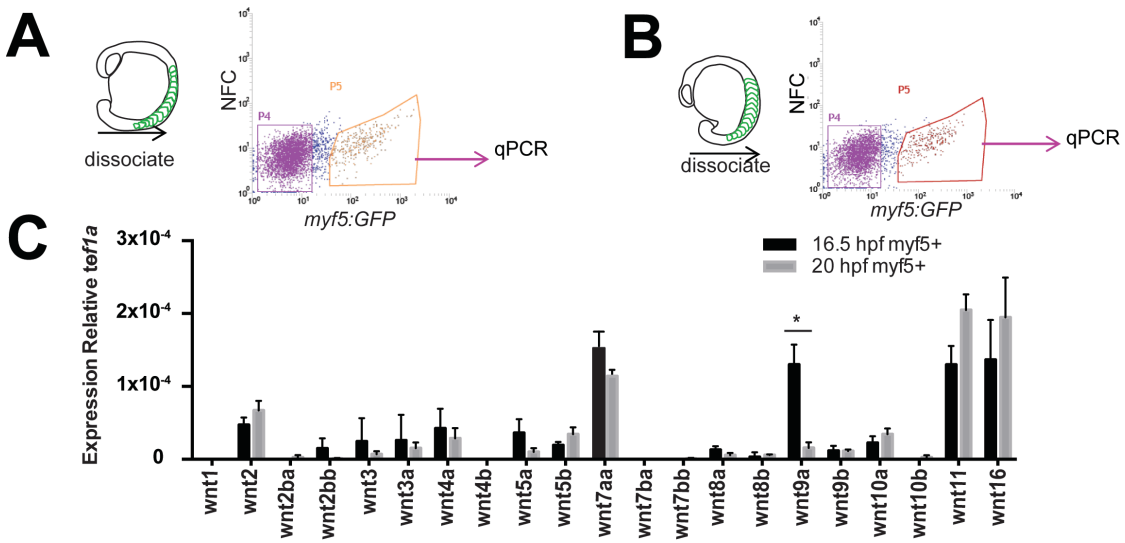

Figure 3.8. Identification of *wnt9a* as the candidate Wnt. Somitic cells were sorted from *myf5:GFP* embryos at 16.5 (A) or 20 (B) hpf, and profiled for *wnt* gene expression by qPCR (C). *wnt9a* is highly expressed at 16.5 hpf and is downregulated by 20 hpf. *=p<0.05.

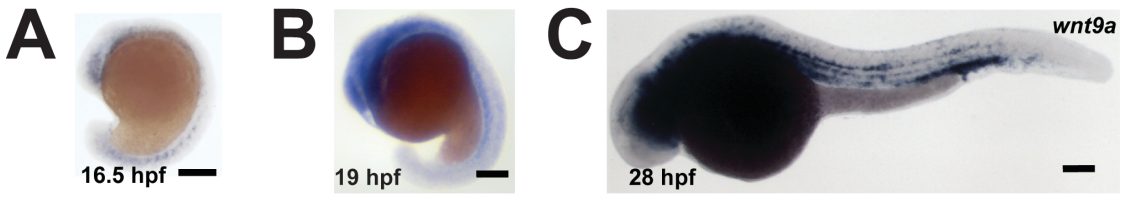

Figure 3.9. Expression domains of *wnt9a*. WISH for *wnt9a* at 16.5 (A), 19 (B), and 28 (C) hpf. *Wnt9a* is expressed in the somites at 16.5 hpf, the vasculature at 16.5 hpf, and prominently in the head and CHT at 28 hpf. Scale bar = 0.2 mm.

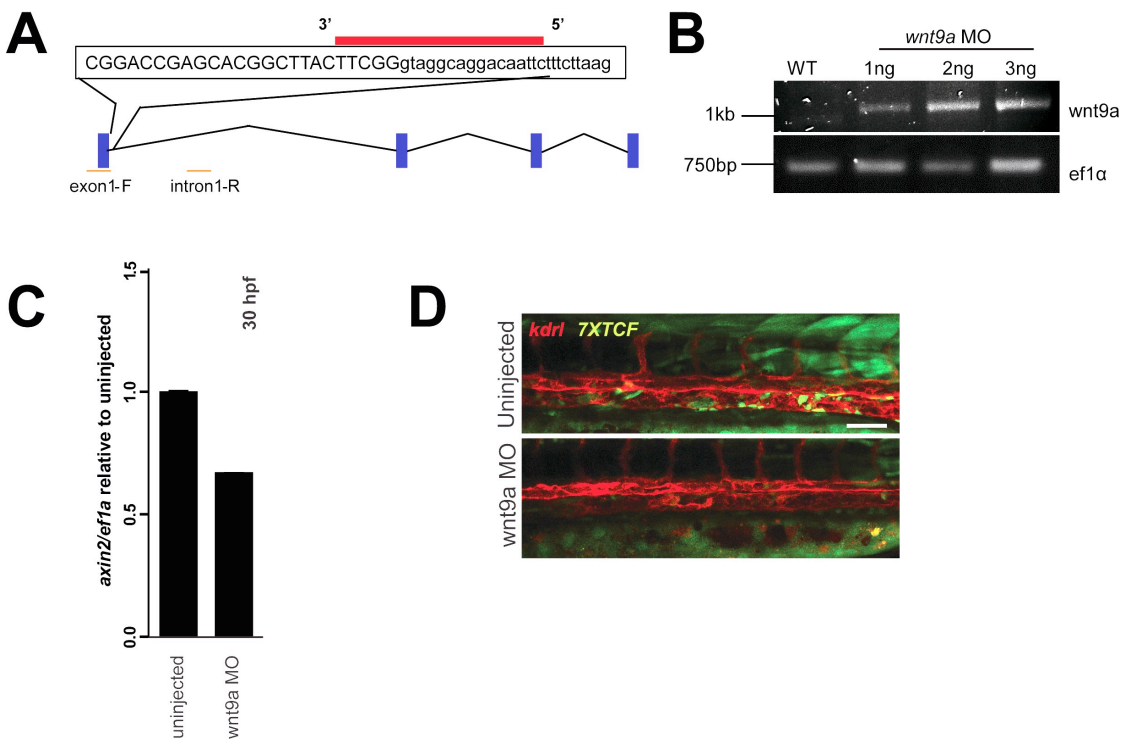

Figure 3.10. The *wnt9a* MO knocks down *wnt9a* expression and decreases Wnt signaling. Knockdown of wnt9a was achieved with a MO (A). Intron retention was verified by PCR (n=10 at each dosage) (B). Loss of canonical Wnt activity was verified by qPCR for *axin2* on whole embryos (n=30) at 30 hpf (C), and visualization of *7XTCF:eGFP* reporter expression at 24 hpf. Scale bar=30 um.

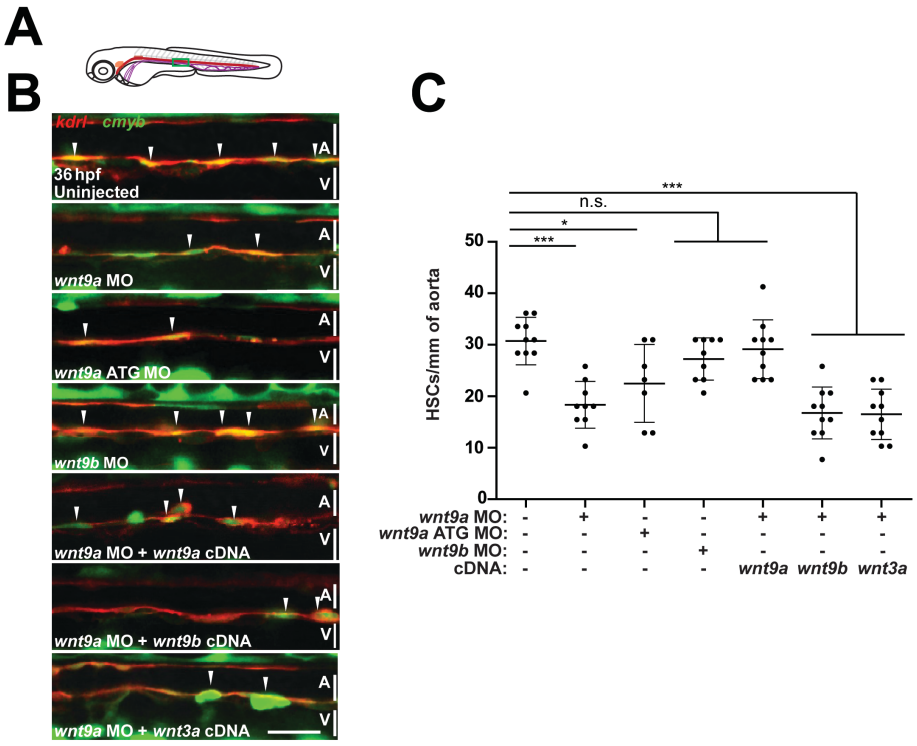

Figure 3.11. Loss of *wnt9a* results in loss of HSCs. The region of 36 hpf *kdrl:mCherry;cmyb:eGFP* zebrafish embryos shown in (A) was imaged on a confocal microscope after injection with the indicated MO and/or cDNA. Double-labeled HSC (white arrowheads) are quantified in (C). A=aorta, V=vein. Scale bar =30 um. *=p<0.05, ***=p<0.001, n.s.=Not significant.

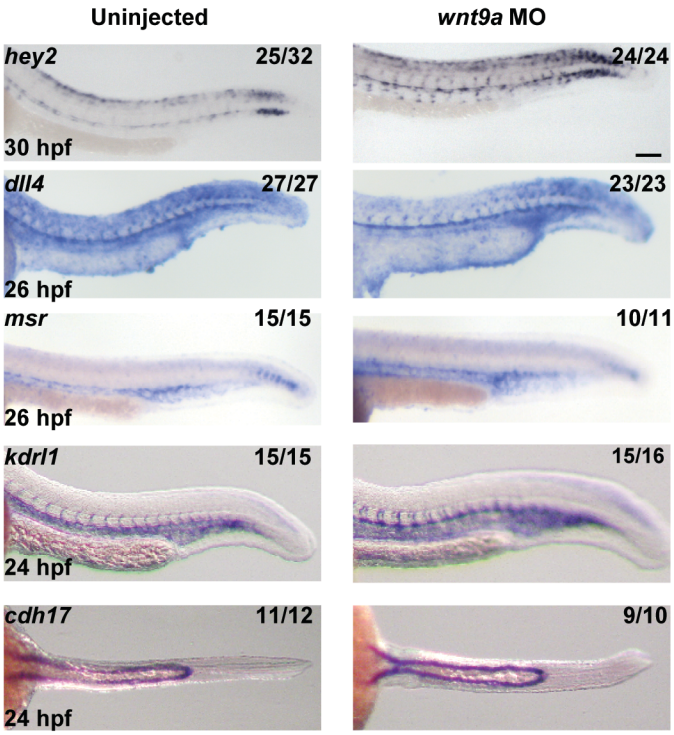

Figure 3.12. Loss of *wnt9a* does not affect the aorta, vasculature, or pronephros. Uninjected control or *wnt9a* morphant embryos were analyzed by WISH for markers of the aorta (*hey2,* 30 hpf; *dll4,* 26 hpf); vein (*msr,* 26 hpf), vasculature (*kdrl,* 24 hpf), and pronephros (*cdh17,* 24 hpf). Scale bar = 30 um.

Credit: Claire Pouget, PhD performed the hey2 *WISH, and Stephanie Grainger, PhD performed the* dll4 *WISH.*

The loss of HSPCs in *wnt9a* morphants persisted to later stages of embryonic hematopoiesis: at 4 days post-fertilization, we observed a dose-dependent loss of *cmyb*$^{+}$ cells in the CHT, indicating a substantial decline in the total number of HSPCs (Figure 3.13). Similar to enforced expression of *dntcf*, *wnt9a* knockdown had no effect on *runx1* expression at 26 hpf, indicating that loss of *wnt9a* does not impact HSC specification (Figure 3.14). Finally, since recent reports have indicated that MOs may have non-specific effects (van Impel *et al.* 2014, Schulte-Merker and Stainier 2014, Kok *et al.* 2015), we generated a *wnt9a* knockout allele via CRISPR/Cas9 technology. The location of the mutation is targeted to a similar region as the splice-blocking MO, at the 3' end of the first exon. We confirmed that genetic mutation of *wnt9a* also results in loss of *cmyb*$^{+}$ cells at 36 hpf (Figure 3.15). We confirmed that somitic *wnt9a* is required for HSPC emergence by injecting a *UAS:wnt9a* cDNA construct into fish with a somitic gal4 driver (*phldb1:gal4*), in the context of the *wnt9a* MO, and found that somitic overexpression of *wnt9a* was sufficient to partially rescue the loss of HSPCs (Figure 3.16). Taken together, these results indicate a defect in the emergence of HSPCs following loss of *wnt9a*.

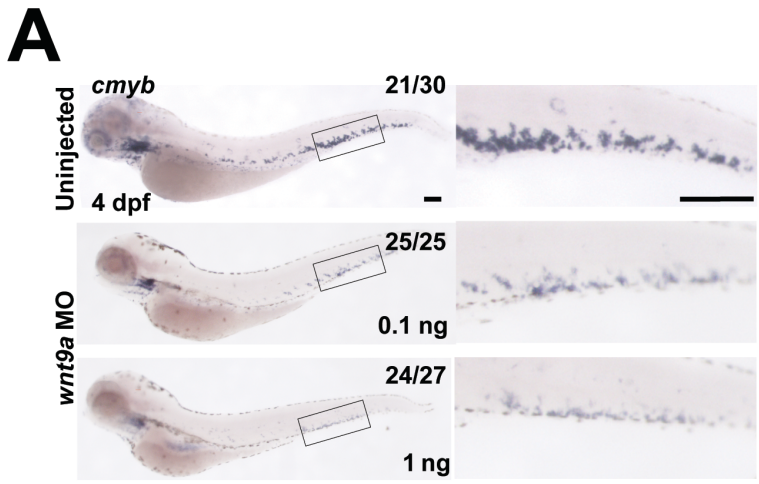

Figure 3.13. Loss of *wnt9a* reduces HSC numbers during later stages of HSC development. Uninjected or *wnt9a* MO injected embryos were analyzed for *cmyb* expression by WISH at 4 dpf. Scale bar = 30 um.

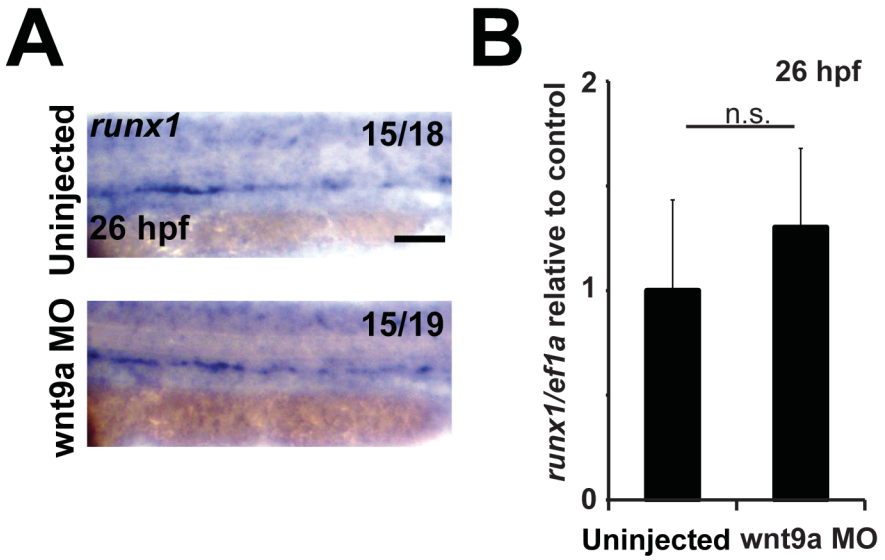

Figure 3.14. HSC specification is unaffected in *wnt9a* morphants. HSC specification was measured in uninjected control and *wnt9a* morphant embryos at 26 hpf by WISH (A) or qPCR (B) for early HSC marker *runx1*. Scale bar = 30 um, n.s.=Not significant.

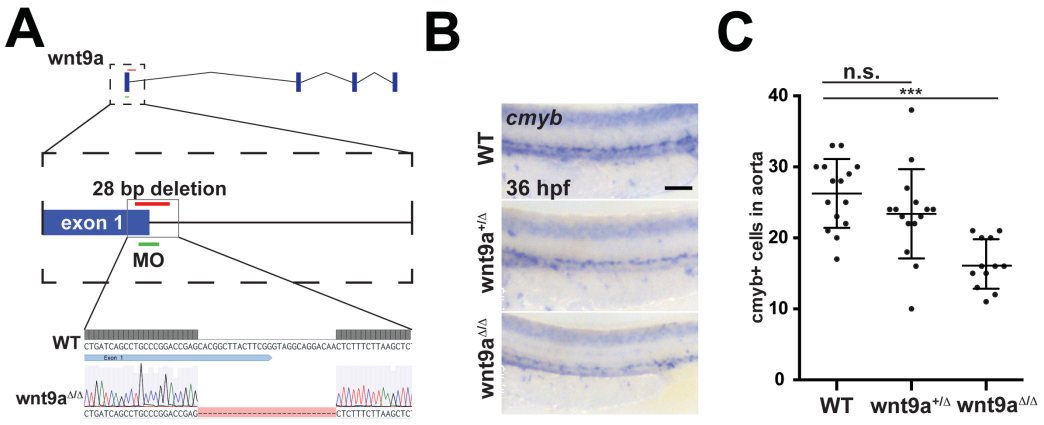

Figure 3.15. Genetic mutation of *wnt9a* results in loss of HSCs. A 28 base-pair deletion of the first exon of *wnt9a* was generated using CRISPR/Cas9 (A). Analysis of *cmyb* expression by WISH at 36 hpf in WT, wnt9a heterozygotes, and wnt9a homozygous mutants (B, *cmyb*+ cells quantified in C). Scale bar = 30 um. ***=p<0.001, n.s.=Not significant.

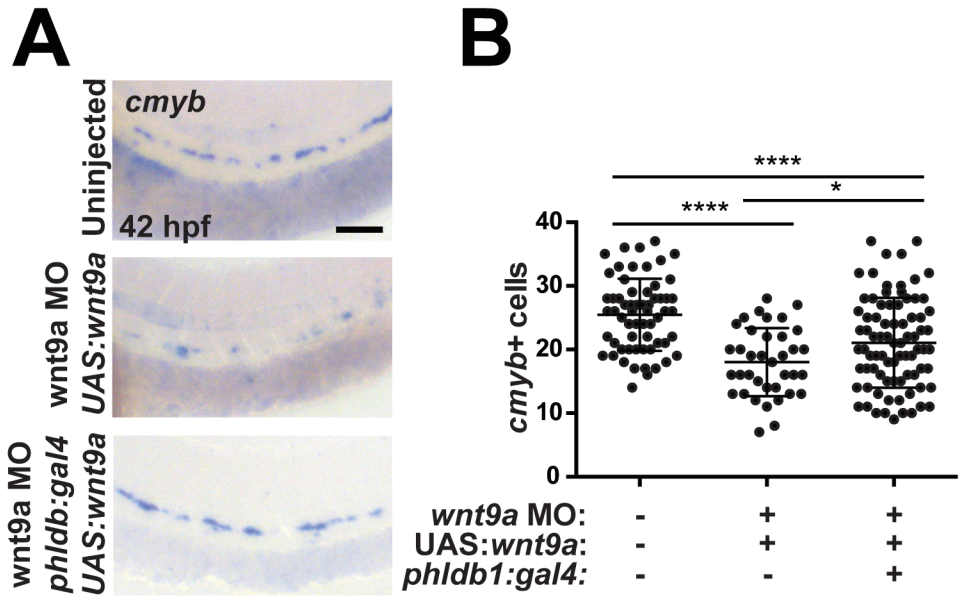

Figure 3.16. Somitic *wnt9a* is required for HSC emergence. (A) Examination of HSPC marker *cmyb* at 42 hpf by WISH in *wnt9a* morphants in the presence or absence of somitic *wnt9a*, compared to uninjected controls. (B) Quantification of cmyb+ cells from (A). *P<0.05, ****P<0.0001, n.s.=not significant.

Hematopoietic precursors expand in the aorta

Next, we aimed to determine the mechanism underlying HSPC reduction upon loss of Wnt function. HSCs arise from hemogenic cells in the aorta, enter circulation and seed the CHT, where they are proliferate and differentiate before migrating to the adult hematopoietic tissues (Murayama *et al.* 2006). The observation that diminished Wnt signaling led to a decrease in aortic HSPCs could suggest that HSPCs also undergo an expansion in the aorta. HSPCs can also be detected using *kdrl:mCherry; gata2b:GFP* double transgenic animals (Butko *et al.* 2015). To determine the extent to which HSPCs proliferate in the aorta, we treated *kdrl:mCherry; gata2b:GFP* animals from 26–35 hpf with 5-Fluorouracil, which selectively kills cells that have undergone DNA synthesis (Heidelberger *et al.* 1957). This resulted in a 2.5-fold reduction in the average number of HSPCs at 36 hpf (Figure 3.17), confirming a requirement for amplification of nascent HSPCs in the aorta.

Wnt9a drives HSPC proliferation in the aorta through myca

The loss of HSPCs independent of apoptosis and the concomitant requirement for proliferation in the aorta suggested that in the absence of a Wnt cue, HSPCs may be G1 arrested; the FUCCI fish identifies G1 phase cells with the red fluorescent protein mCherry (Sugiyama *et al.* 2009, Bouldin and Kimelman 2014). Endothelial cells in the G1 phase can therefore be sorted from *kdrl:GFP; FUCCI* morphant fish and compared to the same population from uninjected controls. We sorted G1-phase endothelial cells (*kdrl:GFP; FUCCI*) from morphant and uninjected fish. At 28 hpf, G1 cells from *wnt9a* MO injected fish had robust expression of *gata2b*, while we were unable to detect *gata2b* transcripts in the uninjected sample; *gata2b* expression in unsorted fish was similar in morphant and control fish, suggesting that HSPCs are G1 arrested in the absence of *wnt9a* (Figure 3.18).

Entrance to the replicative S phase of the cell cycle is governed by a series of cellular events requiring D-class cyclins and their cofactors, cdk2 and cdk4 (Bertoli *et al.* 2013), which we predicted to be decreased in Wnt reduced animals. Consistent with this model, we observed a profound loss of

cyclinD2b and *cdk4,* and a small, but significant, decrease in *cyclinD1* and *cdk2* in Wnt suppressed (*dntcf*$^{+}$) endothelial cells at 30 hpf (Figure 3.19). The transcriptional regulator and context-specific Wnt target gene *myca*, which acts upstream of these cell cycle regulators (Mateyak *et al.* 1999), was also down regulated in *dntcf*$^{+}$ endothelial cells (Figure 3.19). Introducing *myca* mRNA in *wnt9a* morphants was sufficient to rescue the loss of *cmyb*$^{+}$ cells at 40 hpf (Figure 3.20), indicating that at least a portion of Wnt9a function in HSPCs proceeds through *myca*. Finally, expressing *myca* expression under control of the *gata2b* promoter (in *gata2b:KalTA4; UAS:myca*) was sufficient to rescue the loss of *wnt9a* in morphants (Figure 3.20), further supporting this requirement in the HE. Notch has also been previously shown to interact with *myca* (Kumano *et al.* 2003, Burns *et al.* 2005, Palomero *et al.* 2006, Bertrand, Cisson, *et al.* 2010, Kim *et al.* 2014, Bigas *et al.* n.d.). To investigate possible co-regulation of *myca* by Notch and Wnt9a we imaged *TP1:mCherry* (Notch reporter)*;kdrl:eGFP* embryos injected with *wnt9a* MO, and found no change in Notch activation in response to loss of *wnt9a* (Figure 3.21). This suggests that this requirement for *myca* in the aortic proliferation of HSPCs is independent of Notch. Taken altogether, our results indicate that Wnt9a drives an early amplification of HSPCs upstream of the cell cycle regulator *myca* in the HE (Figure 3.22).

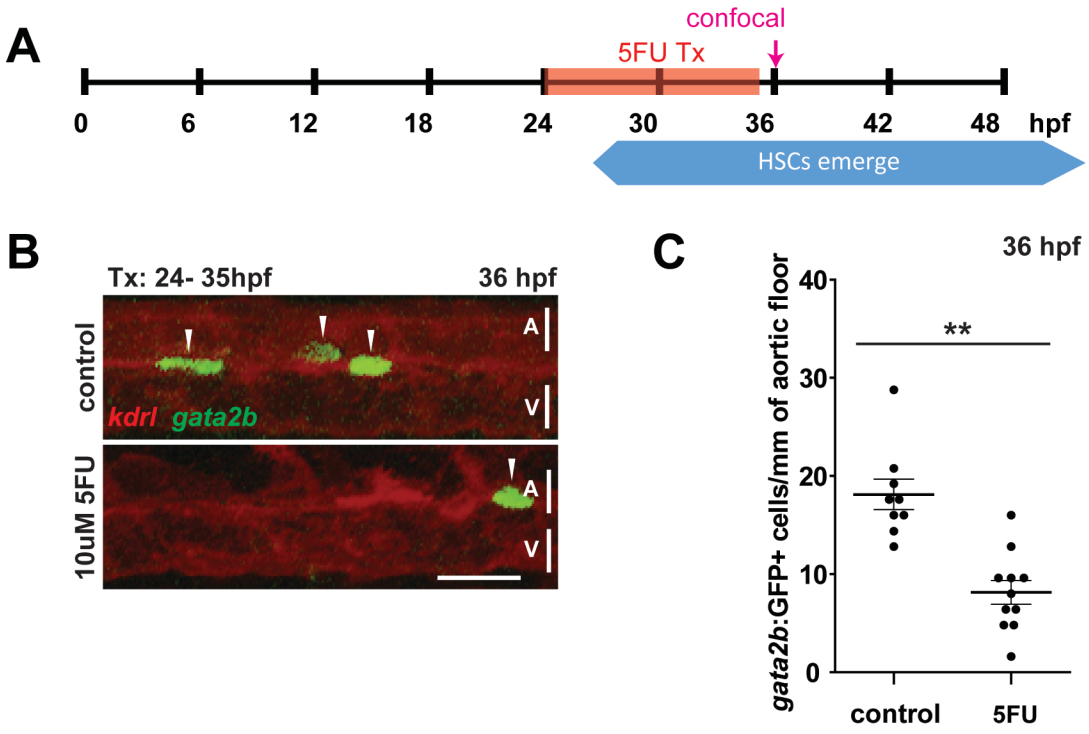

Figure 3.17. HSPCs proliferate in the aorta. Transgenic *gata2b:GFP;kdrl:mCherry* fish were treated with 10 μM 5′ fluorouracil (5FU) from 24 to 35 hpf (A), confocal imaged at 36 hpf (B), and *gata2b*+ cells quantified (C). **=p<0.01.

Credit: Experiment performed by Stephanie Grainger, PhD.

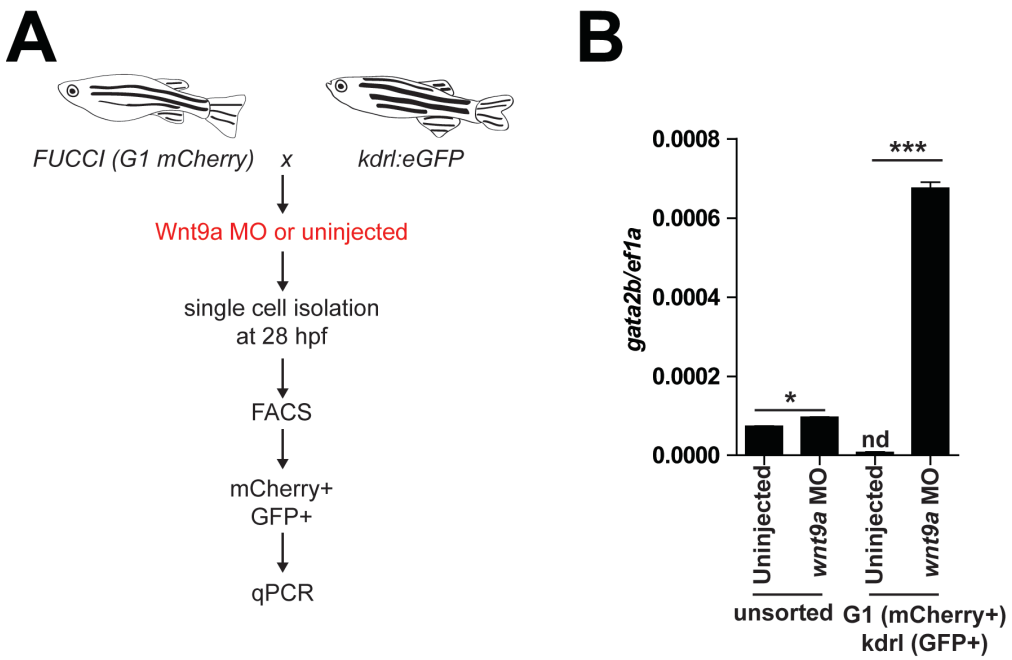

Figure 3.18. Loss of *wnt9a* results in G1 arrest in HSPCs. G1 arrested (mCherry+) endothelial cells (GFP+) from *wnt9a* morphant and control fish (n = 100 embryos per condition) were collected by FACS at 28 hpf (A) and compared by qPCR for *gata2b* (B). *=p<0.05, ***=p<0.001, nd=Not detected.

Credit: Experiment performed by Stephanie Grainger, PhD.

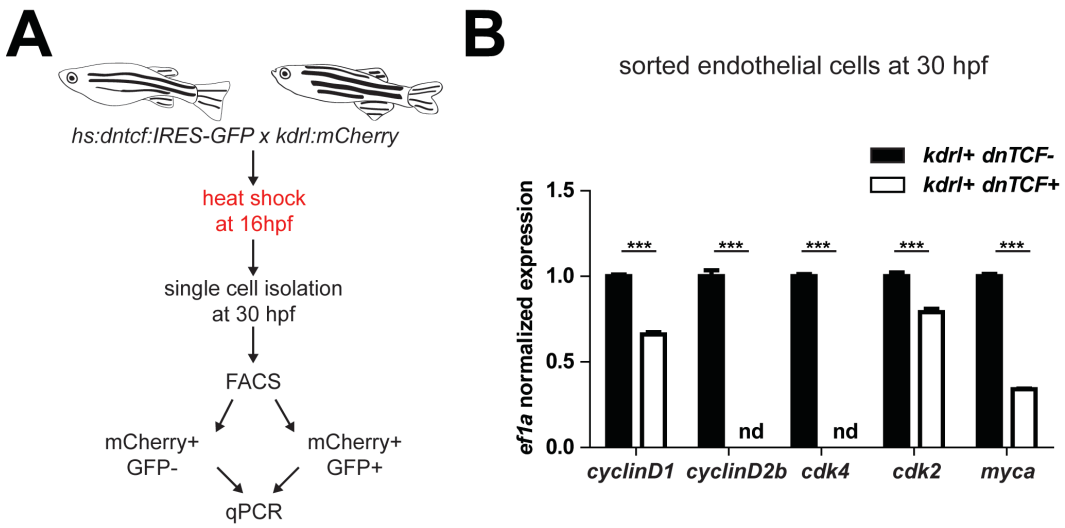

Figure 3.19. Wnt9a affects endothelial *gata2b*+ G1-S cell cycle progression. (A) Endothelial cells (mCherry+) were collected by FACS from *hs:dntcf:IRES-GFP; kdrl:mCherry* fish at 30 hpf after heat shock at 16 hpf (n = 100 embryos per condition). (B) Wnt inhibited (dntcf;GFP+) cells were compared to control (GFP−) cells by qPCR after being sorted by FACS. *=p< 0.05; ***=p < 0.001; n.s.=Not significant.

Credit: Experiment performed by Stephanie Grainger, PhD.

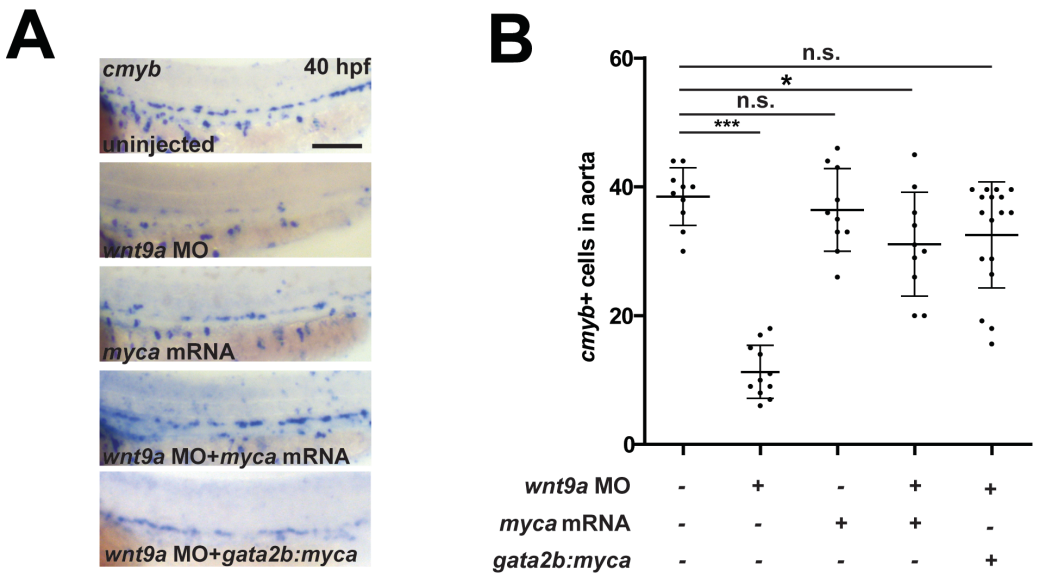

Figure 3.20. Wnt9a signals through *myca* to affect HSPC proliferation. AB* fish were injected with wnt9a MO, *myca* mRNA, or both; *phldb4:Gal4* fish were injected with *wnt9a* MO, *UAS;myca* plasmid, and transposase mRNA, fixed at 40 hpf, analyzed by WISH for *cmyb* (C), and quantified in (D). *=p<0.05, ***=p<0.001, n.s.=Not significant. Scale bar = 100 um.

Credit: Experiment performed by Stephanie Grainger, PhD.

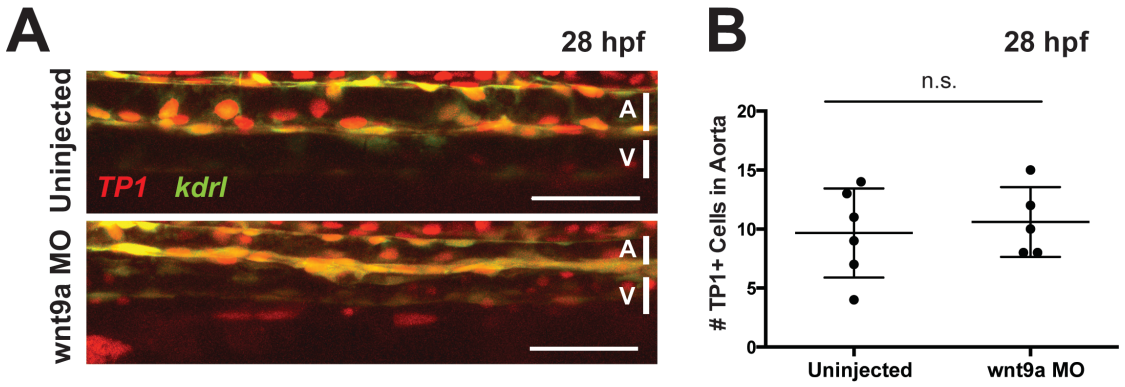

Figure 3.21. Notch activation is unaffected in *wnt9a* morphants. (A) Confocal imaging of control or *wnt9a* MO injected *TP1:mCherry (Notch reporter);kdrl:eGFP* embryos at 28 hpf. The number of TP1:mCherry+ endothelial cells in the floor of the aorta are quantified in (B). A=aorta, V=vein. Scale bar = 50 um. n.s.=Not significant.

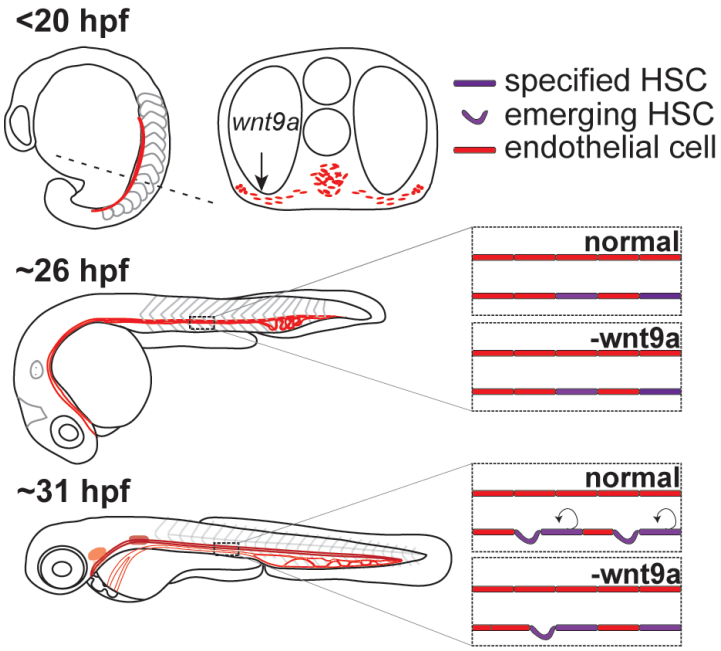

Figure 3.22. An early Wnt9a cue is required for later hematopoietic stem cell amplification. Prior to 20 hpf, ingressing cells of the posterior lateral mesoderm travel beneath the somites. Inductive cues direct from the somite instruct the fate of these cells, some of which are destined to become HE, and later, HSPCs. Wnt9a is expressed in the somite at this stage. By 26 hpf, the aorta has formed and HSPCs have begun to emerge. In the absence of Wnt9a, this early emergence is unaffected, indicating the HSPC fate specification has occurred properly. By 31 hpf, normal HSPCs undergo an expansion event, whereas those in Wnt9a-deficient animals do not.

Credit: Image generated by Stephanie Grainger, PhD.

3.1: Discussion

Wnt signaling regulates multiple stages of hematopoiesis, but requirements for the specific molecules that mediate these signals are not well understood. This study sought to understand the regulation of specific *Wnt* genes that instruct early hematopoietic development. We found that a single *Wnt* gene, *wnt9a*, mediates a critical Wnt signal that is received by the endothelium prior to 20 hpf for HSPC emergence in the zebrafish. The Wnt9a signal stimulates a previously unrecognized HSPC amplification event in the aorta that is mediated through activation of *myca,* a Wnt regulated gene, and important regulator of cell proliferation.

Previous work has demonstrated an early Wnt signaling requirement for the production of long-term HSCs in mouse and zebrafish (Goessling *et al*. 2009, Ruiz-Herguido *et al*. 2012a), however, the identity of Wnt signaling components, particularly the Wnts has remained unclear. We demonstrate here a unique requirement for the somitically expressed *wnt9a* in HSPC development. Knockdown of *wnt9a* causes a decrease in *cmyb*$^{+}$ hematopoietic precursors at 36 hpf, consistent with previous global knockdown of Wnt function in mouse and zebrafish (Goessling *et al*. 2009, Luis *et al*. 2009a, 2011, Ruiz-Herguido *et al*. 2012a). Extending these studies, we have found that loss of *wnt9a* expression does not cause a defect in HSPC specification, as evidenced by normal *runx1* expression in the aorta at 26 hpf. This is also in contrast to other factors known to affect HSPC development at the level of specification (Clements *et al*. 2011a, Espín-Palazón *et al*. 2014, Kim *et al*. 2014, Kobayashi *et al*. 2014, Lee *et al*. 2014, Pouget *et al*. 2014, Butko *et al*. 2015). For example, Wnt16 acts through a non-canonical Wnt pathway upstream of Notch signaling to specify HSPC identity (Clements *et al*. 2011a), a process that is mediated by an interaction between the ventral somite and migrating vascular precursors (Kobayashi *et al*. 2014).

Loss of *wnt9a* cannot be compensated for by overexpression of other *Wnt* genes, suggesting that the requirement for Wnt9a in HSPC development is specific. This specificity is surprising since in many experimental settings individual Wnt proteins produce similar effects and are often

interchangeable. Our current understanding of specific Wnt interactions with their cognate receptors is quite limited and largely restricted to *in vitro* studies. For example, Wingless (the Drosophila Wnt1 ortholog) interacts with the cysteine rich domains (CRD) of both Fz and Dfz2 (two Drosophila *Fzd* proteins that act redundantly in establishing segment polarity in the embryo), but with ten-fold lower affinity for Fz than for Dfz2 (Rulifson *et al*. 2000). Determining specificities of Wnts for their receptors is confounded by the large number of Wnts and Wnt receptors involved: the mammalian genome contains 19 *Wnt* and 10 *Fzd* genes and the zebrafish genome contains 20+ *wnt* and 14 *fzd* genes. A recent study analyzed the interactions of four Wnt proteins with six Fzd CRDs and found a significant range in binding affinities amongst individual pairs (Dijksterhuis *et al*. 2015). Identifying the Fzd(s) and co-receptors expressed in the pre-HE during HSPC development will be informative in studying the interaction of Wnt9a with these potential receptors.

Furthermore, Wnt9a is well conserved amongst vertebrates (Curtin *et al*. 2011, Kamel *et al*. 2013) and is expressed in mouse HSPCs (Wu *et al*. 2012), indicating possible conservation of function during hematopoietic development. In this context it is worth noting that in both zebrafish and mammals the *wnt9a* gene is syntenic to *wnt3a* (Nusse 2001), suggesting coordinate regulation of these two *wnt* genes. Importantly, Wnt3a has been implicated in HSC self-renewal in the mouse (Willert *et al*. 2003, Luis *et al*. 2009a), but does not appear to have a role in zebrafish hematopoiesis (Buckles *et al*. 2004, Thorpe *et al*. 2005, Clements *et al*. 2009). A role for Wnt9a in mammalian hematopoietic development has not yet been addressed.

Interestingly, zebrafish embryos that are deficient for Wnt signaling do not show a hematopoietic phenotype until 30 hpf, which is 10 hours removed from the time that the signal is required. This delayed effect of a Wnt signal may be related to the concept of cellular memory put forth by Vincent and colleagues, who proposed that earlier signaling events allow persistent expression of relevant target genes (Alexandre *et al*. 2014). Our data indicate that during this time, cells of the HE are primed for amplification in the dorsal aorta. Upon loss of Wnt signaling via global

pathway inhibition or *wnt9a* knockdown these emerging HSPCs are arrested in the G1 phase of the cell cycle, causing a decrease in proliferating HSPCs and overall HSPC numbers. This regulation of the cell cycle is mediated, at least in part, by *myca* (the zebrafish homolog of mammalian *C-MYC*), which among other targets, controls transcription of D-class cyclin genes and their associated cyclin-dependent kinases (Hanson *et al*. 1994, Amati *et al*. 1998, Mateyak *et al*. 1999). These genes are downregulated when Wnt is inhibited, and loss of HSPCs in this context can be rescued with *myca*. Our findings are consistent with reports showing that MYC expression is vital to maintaining HSPC numbers and function (Laurenti *et al*. 2008, Delgado and León 2010), and studies using MYC as a factor to facilitate reprogramming to HSPC fate (Riddell *et al*. 2014), and also that MYC is a context-dependent Wnt target (Kolligs *et al*. 1999, Muncan *et al*. 2006, Sansom *et al*. 2007, Cole *et al*. 2010). Although it appears that Myca operates downstream of the Wnt9a cue, further investigation will be required to determine the nature of the timing delay between 20 and 31 hpf, since both *myca* transcript and protein are known to be tightly controlled, with estimated half-lives of 20-30 minutes or less (McCormack *et al*. 1984, Rabbitts *et al*. 1985). Interestingly, Myca and Notch1 (which is critical to HSC fate specification) have been previously shown to interact (Kumano *et al*. 2003, Burns *et al*. 2005, Palomero *et al*. 2006, Bertrand, Chi, *et al*. 2010, Kim *et al*. 2014, Bigas *et al*. n.d.). However, we could not detect any differences in Notch reporter expression in *wnt9a* morphants (Figure 3.21), suggesting that this process occurs independently of Notch signaling. This suggests that tight regulation of cell proliferation by the Wnt signaling pathway through myca is critical for proper hematopoietic development.

Our findings show a unique role for Wnt9a in zebrafish HSPC development. This signal is received by cells of the HE as they ingress to the midline to form the vascular cord prior to 20 hpf. The Wnt9a signal instructs HSPC emergence, but not HSPC specification via priming HSPCs for later aortic amplification. The specific Wnts necessary to differentiate human pluripotent stem cells to hematopoietic or other lineages are often unknown or unused in protocols. Instead, global small

molecule pathway activators or inhibitors are often favored due to their widespread availability and inexpensive nature. Our data indicate that the specific molecules that mediate a specific signal during *in vivo* development may provide more precise developmental instruction than small molecules with non-specific effects on signaling pathways, especially since differences in Wnt requirements could also be reflective of precise timing and ligand requirements. Understanding the precise identity of these instructive signals and their temporal regulation is critical in improving differentiation protocols to develop HSCs *in vitro*, which one day could be used as a therapeutic alternative to bone-marrow transplants.

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CHAPTER 4: WNT9A IS A CONSERVED REGULATOR OF HEMATOPOIETIC STEM AND PROGENITOR CELL DEVELOPMENT

4.1: Introduction

Hematopoietic stem cells (HSCs) are the multipotent stem cell population that gives rise to all cells of the blood. This property is exploited for therapeutic use; HSC transplants from bone marrow or peripheral blood are commonly used to treat hematopoietic cancers and disorders (Hatzimichael and Tuthill 2010, Peters *et al*. 2010). Induced pluripotent stem cell technology has made possible the derivation of patient-matched pluripotent cells that theoretically are capable of differentiating into HSCs (Takahashi *et al*. 2007, Slukvin 2013, Vo and Daley 2015). These cells represent a potential source of HSCs for each patient in need of a transplant, which would be a significant advance in the field of regenerative medicine. However, it is not yet possible to derive therapy-grade HSCs *in vitro* from pluripotent precursors (Slukvin 2013, Vo and Daley 2015, Ditadi *et al*. 2017). Current protocols yield hematopoietic cells with limited repopulation capacity, subpar ability to differentiate to all blood lineages, or utilize enforced expression of hematopoietic transcription factors to induce hematopoietic fate (Kyba *et al*. 2002, Wang *et al*. 2005, p. 4, Ledran *et al*. 2008, Lu *et al*. 2009, Doulatov *et al*. 2013, Riddell *et al*. 2014, Sandler *et al*. 2014, Ditadi *et al*. 2017, Sugimura *et al*. 2017). Thus, many researchers are turning to vertebrate model organisms such as the mouse and the zebrafish to identify critical cues that instruct HSC identity *in vivo*, with hopes that these signals can be leveraged to generate hematopoietic stem and progenitor cells from pluripotent precursors *in vitro*.

Multiple studies have identified specific molecules required for HSC development in the zebrafish; these have shown great translational potential for increasing the functionality and viability of human HSCs derived from umbilical cord blood (Goessling *et al*. 2009, 2011, Cortes *et al*. 2016). Therefore, many of the signals that govern HSC development are highly conserved across vertebrate

species. However, it remains to be seen if this is true of many other signaling cues that have been identified as critical regulators of hematopoietic development in model organisms. Furthermore, how these regulators impact on human hematopoietic development during *in vitro* differentiation from pluripotent precursors remains unknown.

The molecular cues governing embryonic HSC development are highly conserved amongst vertebrates, though the anatomical sites of hematopoiesis can vary between organisms (Clements and Traver 2013). In all vertebrate organisms, HSCs develop from a specialized population of hemogenic endothelium derived from the mesodermal lineage *in vivo* and this is recapitulated *in vitro* in human cells (Turpen *et al*. 1981, Kissa *et al*. 2008, Eilken *et al*. 2009, Bertrand, Chi, *et al*. 2010, Boisset *et al*. 2010, Kissa and Herbomel 2010, Mizuochi *et al*. 2012b, Boisset *et al*. 2015, Ditadi *et al*. 2015). *In vivo*, these cells originate as lateral populations of mesoderm that migrate to the midline underneath the somites to eventually form the vasculature, which contains the hemogenic endothelium (Fouquet *et al*. 1997, Jin *et al*. 2005, Herbert *et al*. 2009, Medvinsky *et al*. 2011). The somites provide many inductive cues to migrating endothelial cells to instruct the specification of hematopoietic endothelium (Burns *et al*. 2005, Bertrand, Chi, *et al*. 2010, Clements *et al*. 2011b, Kobayashi *et al*. 2014, Butko *et al*. 2015). We and others have recently identified Wnt/β-catenin signaling to be one such molecular cue that is required for the development of HSCs in the zebrafish (Goessling *et al*. 2009, Grainger *et al*. 2016).

Wnt/β-catenin signaling has been implicated in directing hematopoietic stem and progenitor cell development both *in vivo* and *in vitro* (Luis *et al*. 2010, 2011, Ruiz-Herguido *et al*. 2012b, Sturgeon *et al*. 2014, Richter *et al*. 2017). Wnt ligands are lipid-modified, secreted growth factors that bind to Frizzled (Fzd) receptors to activate intracellular signaling cascades (Clevers and Nusse 2012). Upon Wnt-Fzd binding, a protein complex that targets β-catenin for proteasomal degradation is dissociated, stabilizing β-catenin, and allowing its translocation to the nucleus, where it interacts with co-activators to initiate expression of target genes. In zebrafish HSC development, Wnt/β-catenin

signaling is required in a temporally restricted manner during early endothelial cell migration, after which it is dispensable (Grainger *et al*. 2016). We have shown that Wnt9a is the key mediator of this critical Wnt signal. Wnt9a is secreted by the somites and received by adjacent hemogenic endothelial cells, which later proliferate in response to the activation of the Wnt target gene *myca* (Grainger *et al*. 2016).

Utilizing the *in vitro* hematopoietic differentiation system as a model for human hematopoietic development, we show that (1) Wnt positively regulates differentiation of human progenitors; (2) WNT9A impacts *in vitro* human hematopoiesis in a time-dependent manner; (3) WNT9A has a dose-dependent effect on hematopoietic differentiation; and (4) *in vitro* differentiation dynamics in response to WNT9A mimic the paracrine nature of the Wnt9a signal observed in the zebrafish.

4.2: Results

Wnt/β-catenin signaling regulates in vitro *development of human hematopoietic progenitor cells*

We have previously identified that a β-catenin mediated Wnt signal is required in the zebrafish during a tightly regulated time window as endothelial cells derived from the posterior lateral mesoderm migrate underneath the somites (Grainger *et al*. 2016). After this time window, Wnt signaling is dispensable for HSC development. Hematopoietic progenitor cells can be derived from human embryonic stem cells, which can be used as an *in vitro* model of human hematopoietic development (Ng *et al*. 2005, 2008). This process closely mimics how hematopoietic cells develop in an organism as pluripotent cells progress through mesodermal and endothelial cell fates before a subset are further specified towards hematopoietic progenitor cells (Figure 4.1); the corresponding time window for the Wnt requirement *in vitro* is approximately between day 2 and day 4 of differentiation, according to expression of marker genes for mesoderm (*BRY, WNT3*) and endothelium (*CD31/PECAM*) (Figure 4.1). We inhibited Wnt signaling using a small molecule inhibitor of Wnt secretion (C59, (Proffitt *et al*. 2013)) during multiple stages of differentiation (Figure 4.2), and analyzed hematopoietic differentiation by flow cytometry for a *RUNX1c:GFP* transgenic reporter, which marks hematopoietic stem and progenitor cells (Ng *et al*. 2016). Only Wnt inhibition from days 2-4 significantly decreased differentiation efficiency (Figure 4.2), suggesting that as in the zebrafish, Wnt signaling is required during a narrow time window during human *in vitro* hematopoietic development.

We next sought to determine if ectopic activation of Wnt signaling during this time window could boost hematopoietic differentiation efficiency by treating cells with the Wnt activator CHIR98014 (CHIR) (Chen *et al*. 2014, Naujok *et al*. 2014), during days 2-4 of differentiation (Figure 4.3). Consistent with Wnt inhibition and previous reports (Sturgeon *et al*. 2014, Ng *et al*. 2016), we found that activating Wnt during days 2-4 increased the efficiency of differentiation to RUNX1c:GFP^{+} cells. Consistent with the role of Wnt in stem cell maintenance and differentiation, we also observed a

dose-specific effect, where both low and high doses of CHIR were suboptimal to deriving hematopoietic progenitors (Figure 4.3) (Luis *et al*. 2011). Taken together, these data indicated that Wnt signaling is required in a time- and dose- dependent manner during *in vitro* development of human pluripotent stem cells to hematopoietic progenitors.

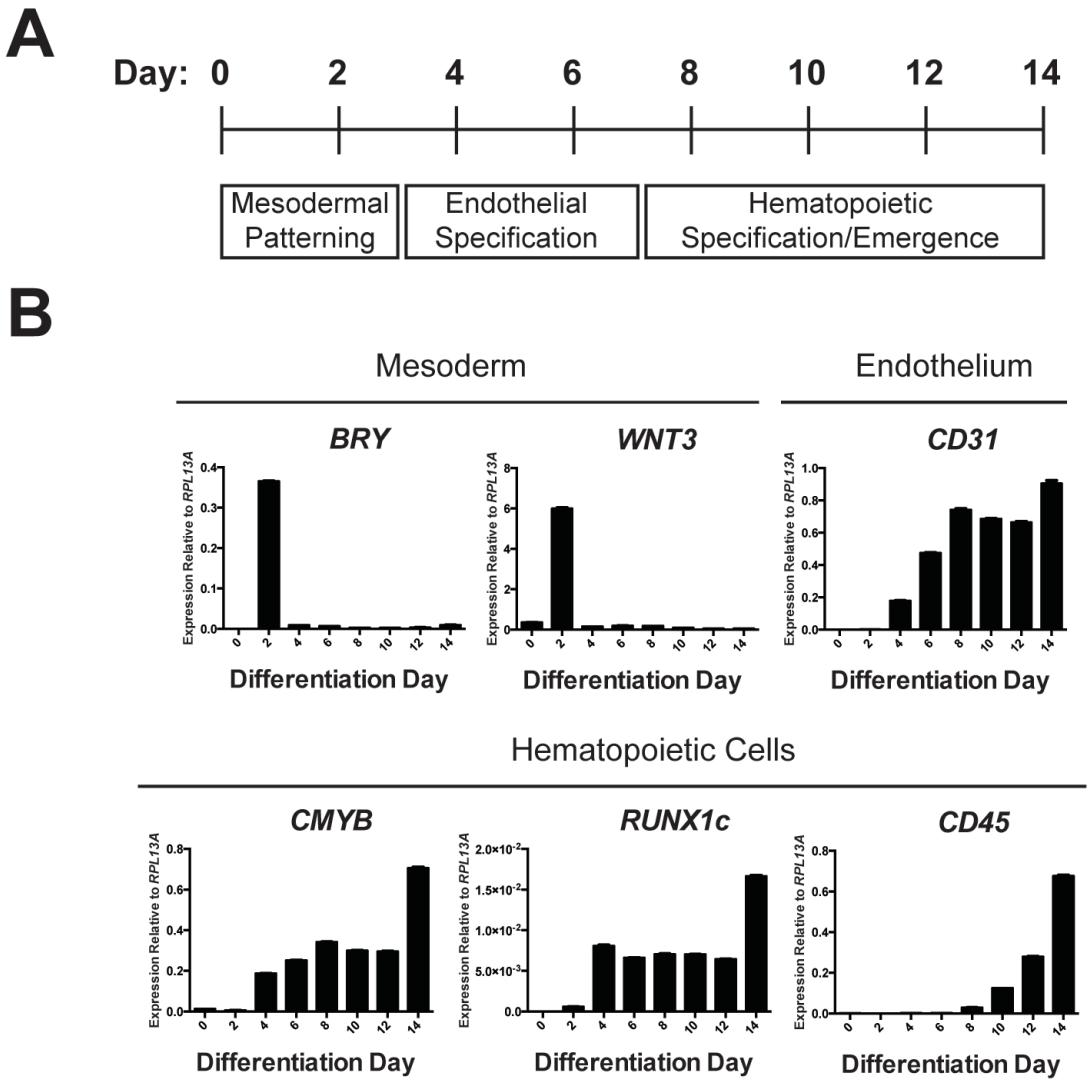

Figure 4.1. ***In vitro*** **differentiation of hematopoietic progenitor cells models** ***in vivo*** **HSC development.** (A) Schematic of differentiation protocol. (B) Reverse transcription quantitative PCR (RT-qPCR) analysis of mesoderm, endothelial, and hematopoietic marker gene expression throughout the differentiation protocol. Error bars represent standard deviation.

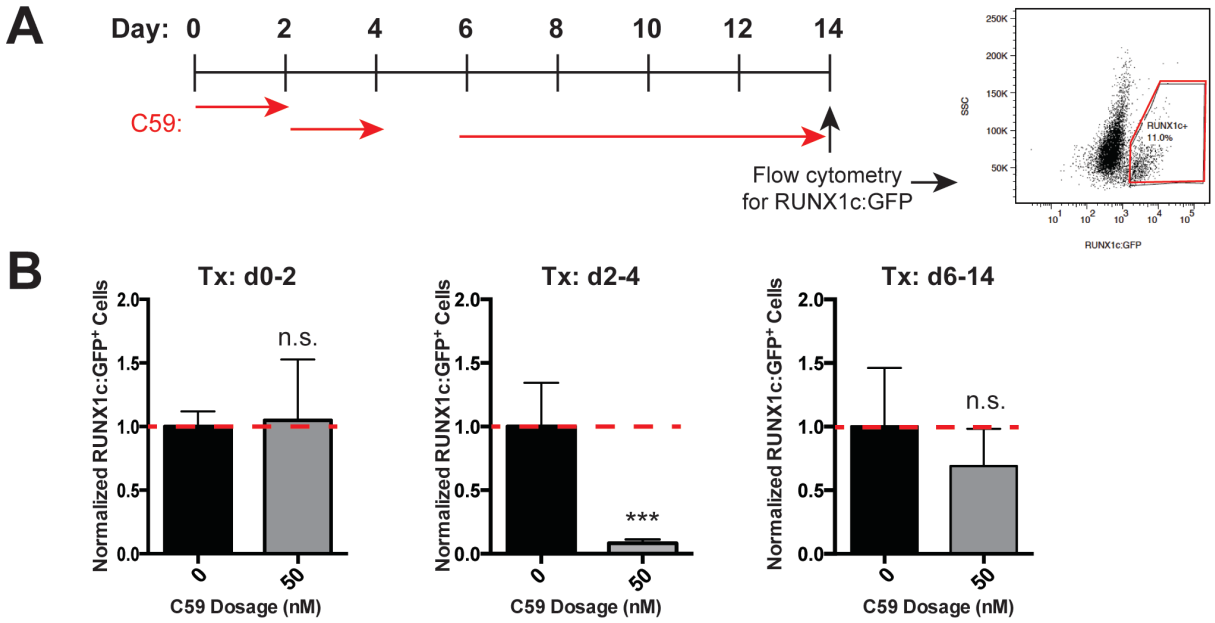

Figure 4.2. Wnt inhibition decreases hematopoietic progenitor differentiation efficiency in a time-dependent manner. (A) Schematic of C59 treatment windows and RUNX1c:GFP reporter analysis by flow cytometry at day 14. (B) RUNX1c:GFP^{+} cells normalized to control (0 nM C59, 0.1% DMSO treated) cells. Tx = treatment. Data shown are representative of three biological replicates. Error bars represent standard deviation; *=p<0.05, **=p<0.01, ***=p<0.001, n.s.=Not significant.

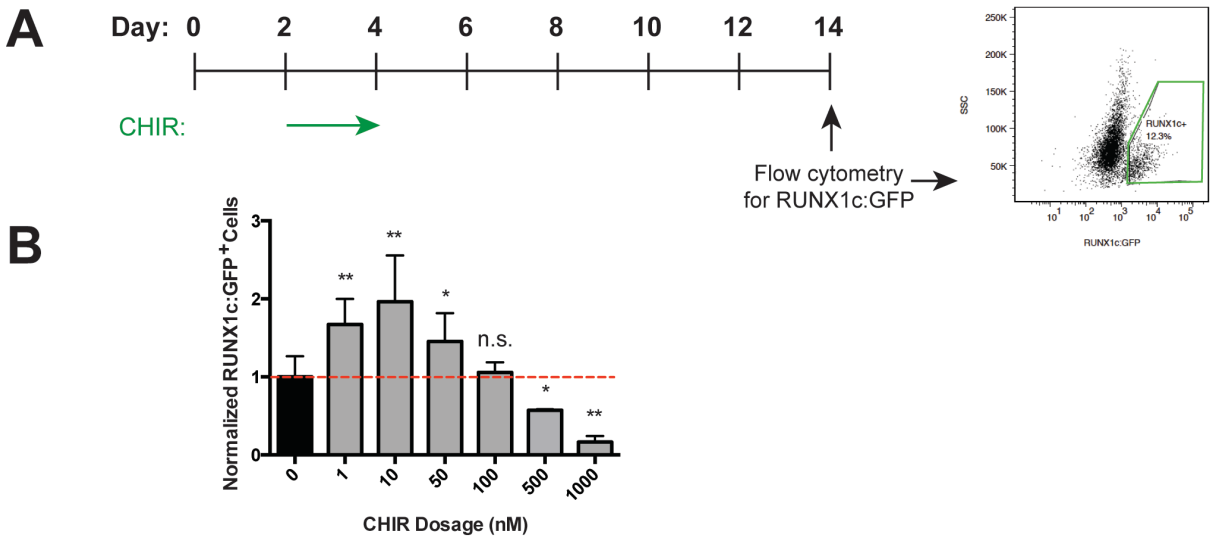

Figure 4.3. Wnt activation increases hematopoietic progenitor differentiation efficiency in a dose-dependent manner. (A) Schematic of CHIR98014 (CHIR) treatment window and RUNX1c:GFP reporter analysis at day 14. (B) RUNX1c:GFP^{+} cells normalized to control (0 nM CHIR, 0.1% DMSO treated) cells. Data shown are representative of three biological replicates. Error bars represent standard deviation; *=p<0.05, **=p<0.01, ***=p<0.001, n.s.=Not significant.

WNT9A increases the efficiency of hematopoietic progenitor differentiation in a time-dependent manner

We previously identified Wnt9a to be specifically required during zebrafish HSC development (Grainger *et al.* 2016). To determine to what extent the requirement for this Wnt protein is conserved during human hematopoietic development, we generated an hESC cell line (H9) carrying a *WNT9A* transgene under the control of a doxycycline-inducible promoter, and a constitutive promoter driving Citrine expression to monitor silencing of the transgene (Figure 2a-b). Upon expression of WNT9A during various time windows of differentiation, we determined that induction of WNT9A from day 0-2 resulted in an increase in $CD34^{+}/CD45^{+}$ hematopoietic progenitors, while other timepoints did not (Figure 2c-e). These data show that WNT9A impacts human hematopoietic progenitor development in a temporally regulated way.

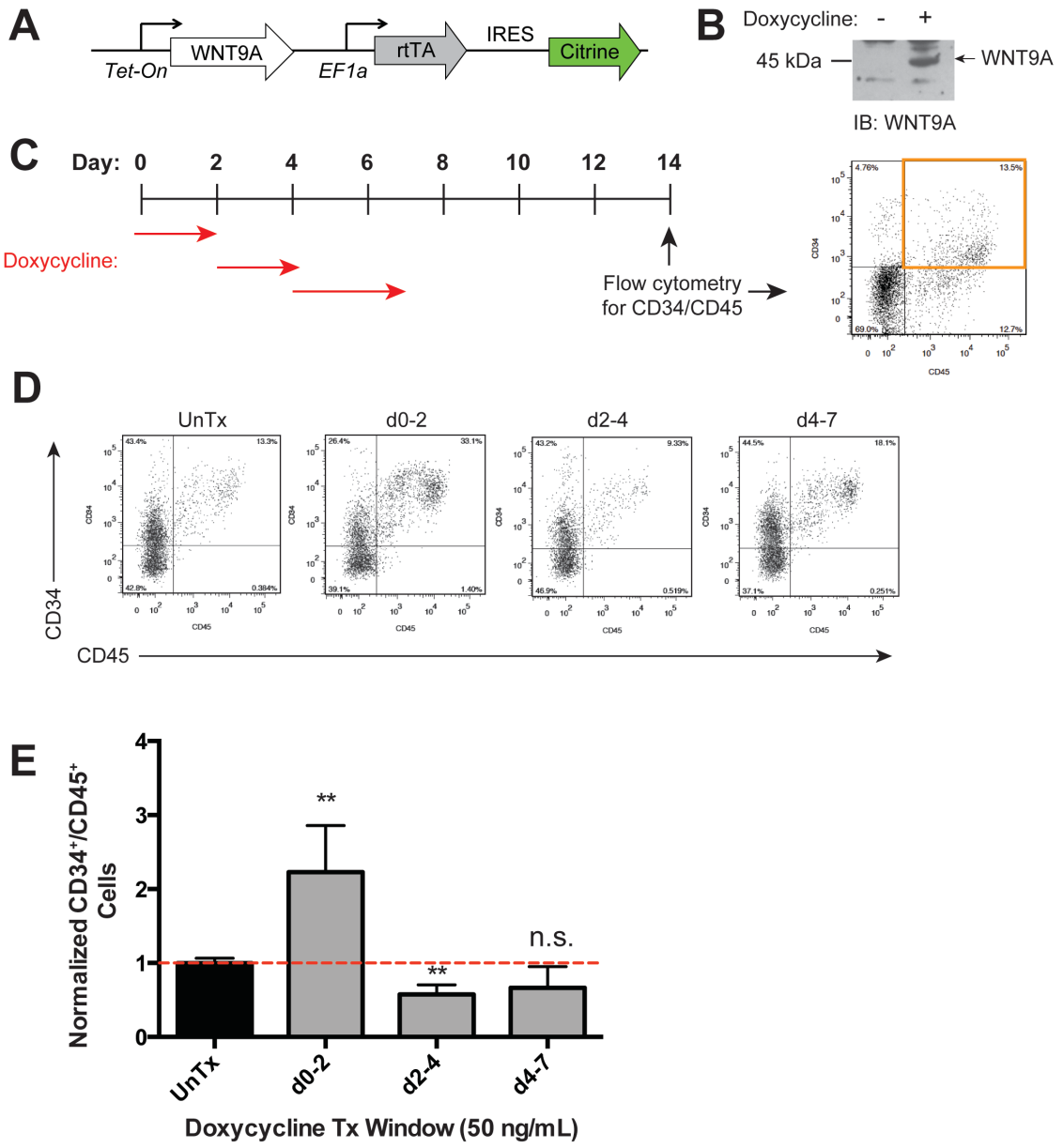

Figure 4.4 WNT9A increases the efficiency of hematopoietic progenitor differentiation in a time-dependent manner. (A) Schematic of inducible WNT9A transgene; (B) Immunoblot depicting WNT9A protein expression in response to doxycycline treatment; (C) Schematic of doxycycline treatment (Tx) to induce WNT9A expression, and readout of hematopoietic progenitor differentiation by flow cytometry for CD34 and CD45 for the data shown in (D and E); (D) Representative flow cytometry plots for data graphed in (E). (E) Percentage of $CD34^+/CD45^+$ cells normalized to untreated control. Data represent average of 3 independent biological replicates; **=p<0.01, n.s.=Not significant. Error bars represent standard deviation.

WNT9A stimulates hematopoietic differentiation in a dose-dependent fashion

The above data indicate that there is an optimal level of Wnt activation that results in maximal increases in differentiation efficiency (Figure 4.3). We assessed the dose-dependent effect of WNT9A during days 0-2 of differentiation (Figure 4.5) and found that while all doses tested resulted in an increase in hematopoietic differentiation efficiency, a maximal effect was reached with a moderate dosage of doxycycline (50 ng/mL), which leads to sub-maximal levels of WNT9A protein (Figure 3c-e), consistent with the previously observed ideal level of Wnt signaling (Figure 4.3) (Luis *et al.* 2011). This may be, in part, due to a secretion blockade that occurs at high doses of doxycycline. Maximal secretion and downstream activation of the Wnt pathway, as measured by expression of the context-specific Wnt target gene *SP5,* is reached at 250 ng/mL doxycycline treatment (Figure 4.6). However, the maximal effect on differentiation efficiency still does not occur at the WNT9A dosage that induces maximal Wnt pathway activation. Taken together, our data suggest that WNT9A can modulate *in vitro* human hematopoietic development in a time- and dose- dependent manner.

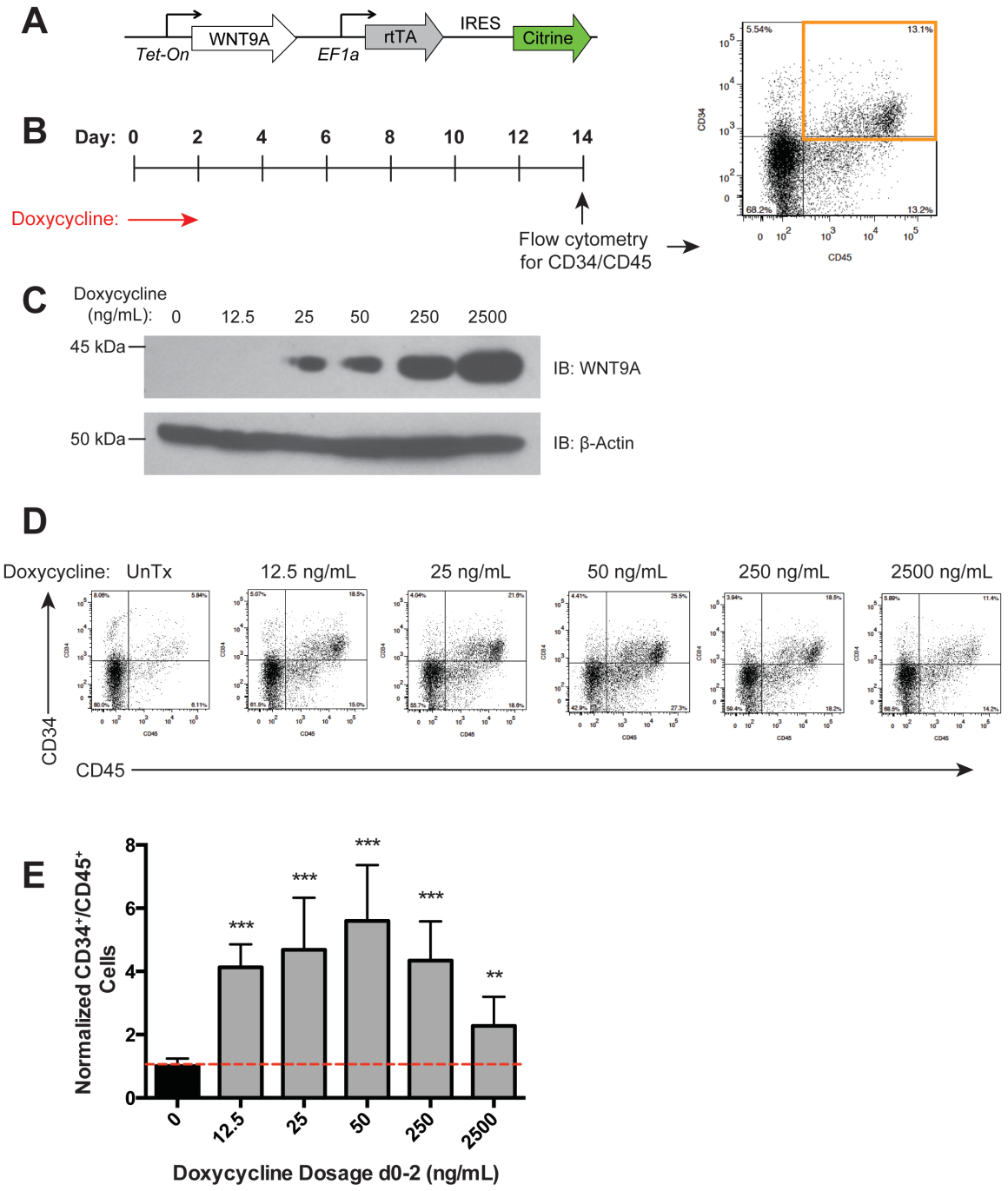

Figure 4.5. WNT9A has a dose-dependent effect on hematopoietic differentiation efficiency. (A) Schematic of doxycycline-inducible *WNT9A* transgene. (B) Schematic of doxycycline induction of WNT9A expression and flow cytometry readout for the data shown in (D and E); (C) Immunoblot (IB) showing increasing WNT9A protein expression in response to the dosages of doxycycline shown in (C). Top: WNT9A, Bottom: β-Actin loading control. (D) Representative flow cytometry plots for data graphed in (E). (E) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to untreated control. Data represent average of 3 biological replicates. **=p<0.01, ***=p<0.001, n.s.=Not significant. Error bars represent standard deviation.

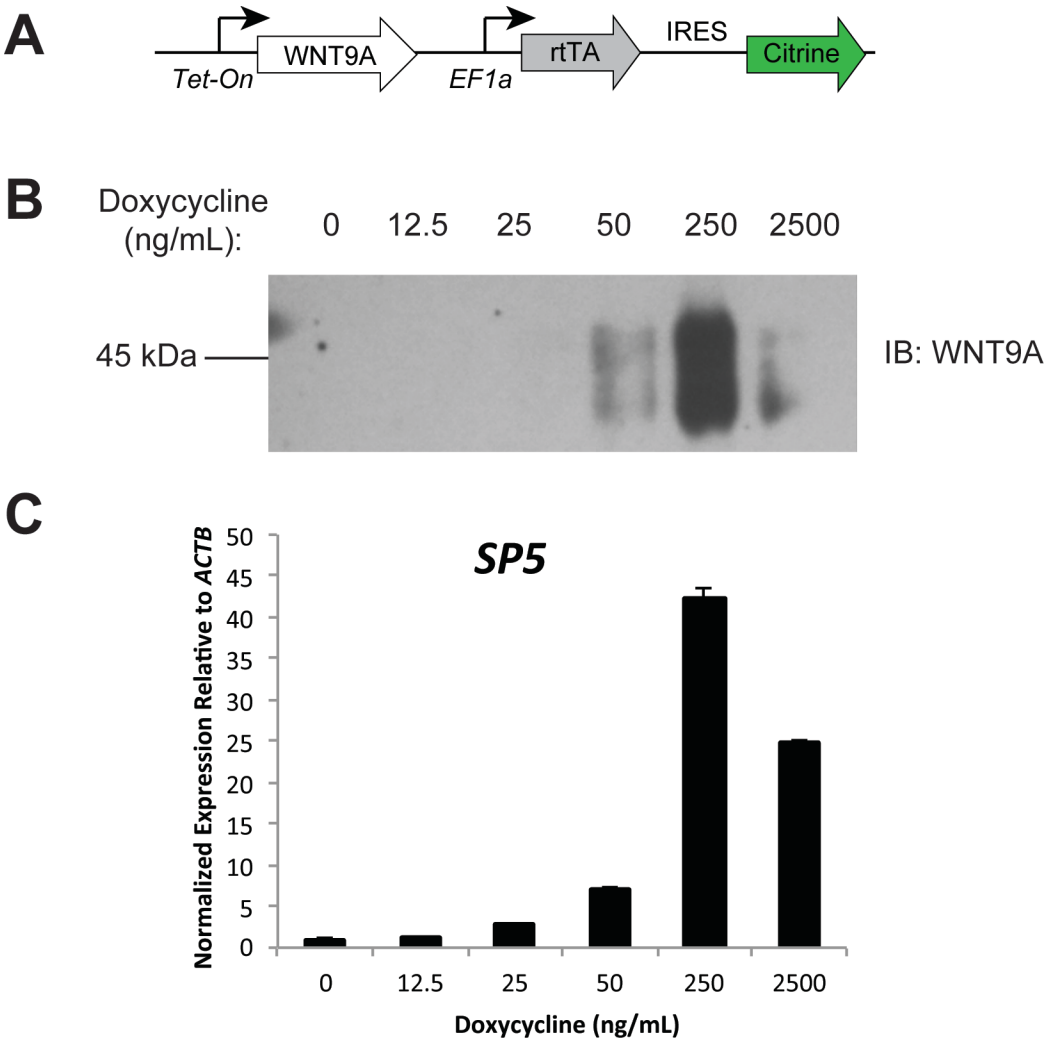

Figure 4.6. Inducible-WNT9A hESCs do not secrete WNT9A efficiently at high doses of doxycycline. (A) Schematic of inducible WNT9A (iWNT9A) transgene. (B) Immunoblot (IB) for WNT9A in Blue Sepharose-pulled down conditioned media from hESCs treated with the indicated doses of doxycycline. (C) RT-qPCR for Wnt target gene *SP5* in cells treated with the indicated dose of doxycycline for 24 hours. Expression values are shown as fold change, normalized to untreated (0 ng/mL doxycycline) cells. Error bars represent standard deviation.

Wnt ligands exhibit varied effects on hematopoietic progenitor differentiation

Loss of Wnt9a results in decreased numbers of HSCs *in vivo*, and this loss cannot be compensated for by exogenous expression of Wnt9b or Wnt3a (Grainger *et al.* 2016). This suggests that the requirement for Wnt9a in hematopoietic development is specific. We sought to model this *in vitro* by overexpressing human WNT9B and mouse Wnt3a, which is more active than human WNT3A, during days 0-2 of differentiation (Figure 4.7, Figure 4.8). Surprisingly, we observed a 17-fold increase in hematopoietic progenitor differentiation when a low dose (12.5 ng/mL doxycycline) of WNT9B was added, and increasing doses of WNT9B decreased this effect (Figure 4.7). Wnt3a addition had a more modest impact on differentiation outcome; a moderate dose of doxycycline (25 ng/mL) resulted in a 3-fold increase in hematopoietic progenitor differentiation efficiency, and high doses of Wnt3a had an inhibitory effect on differentiation (Figure 4.8). This is in line with previous reports showing that Wnt3a can improve *in vitro* derivation of hematopoietic progenitor cells (Goessling *et al.* 2009, Wang and Nakayama 2009, Gertow *et al.* 2013), and that high levels of Wnt activation can negatively impact hematopoietic progenitor numbers (Luis *et al.* 2011). However, these findings are divergent from our previous studies in the zebrafish, which indicated that Wnt9b and Wnt3a had no role in zebrafish hematopoietic development (Grainger *et al.* 2016). These data may indicate that Wnt ligands act differently to instruct hematopoietic progenitor fate.

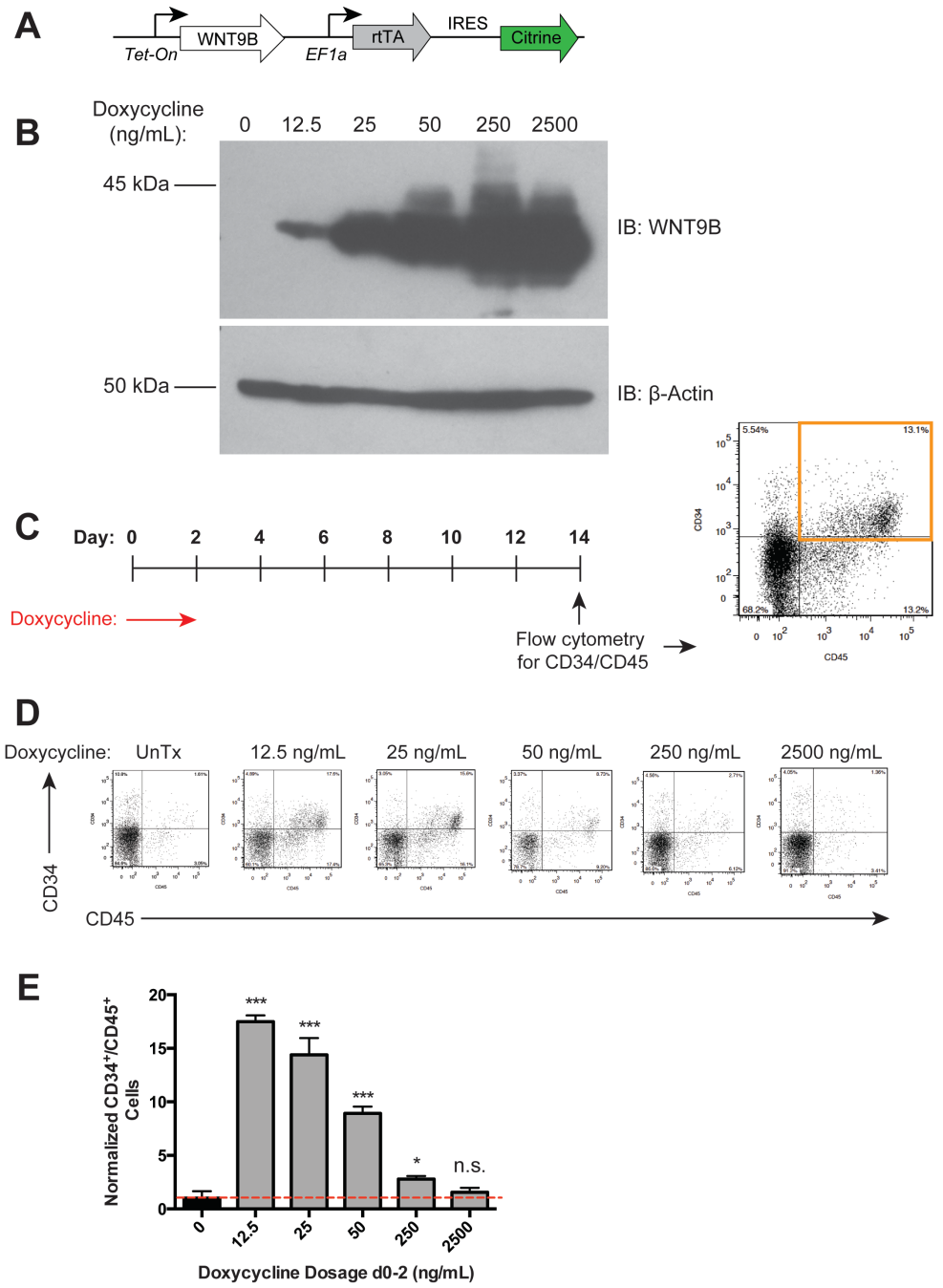

Figure 4.7. WNT9B has a dose-dependent effect on hematopoietic differentiation efficiency. (A) Schematic of doxycycline-inducible *WNT9B* transgene. (B) Immunoblot (IB) showing increasing WNT9B protein expression in response to the dosages of doxycycline shown in (D). Top: WNT9B, Bottom: β-Actin loading control. (C) Schematic of doxycycline induction of WNT9B expression and flow cytometry readout for the data shown in (D and E); (D) Representative flow cytometry plots for data graphed in (E). (E) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to untreated control. Data are representative of 2 biological replicates. *=p<0.05, ***=p<0.001, n.s.=Not significant. Error bars represent standard deviation.

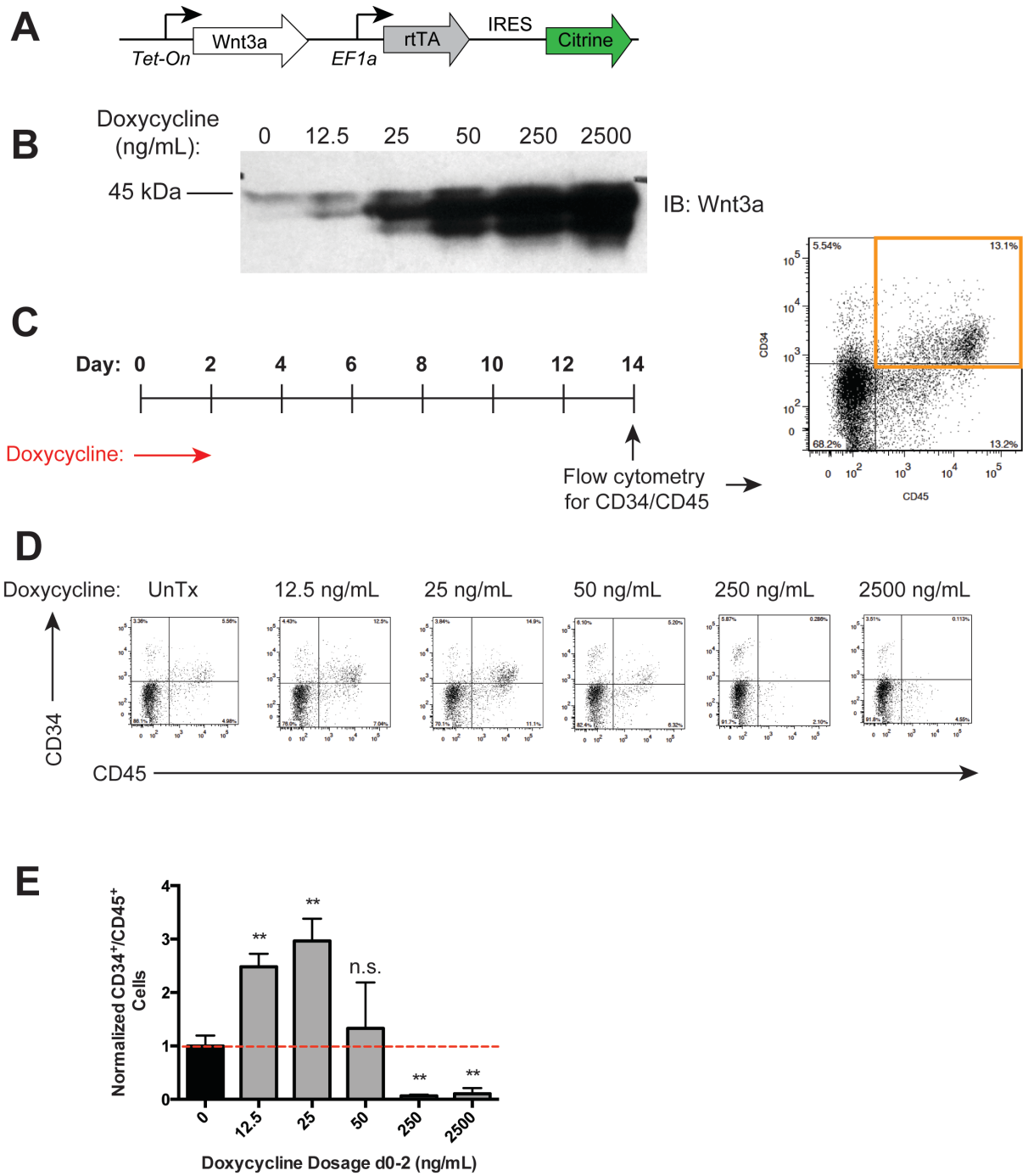

Figure 4.8. Wnt3a has a dose-dependent effect on hematopoietic differentiation efficiency. (A) Schematic of doxycycline-inducible *Wnt3a* transgene. (B) Immunoblot (IB) showing increasing Wnt3a protein expression in response to the dosages of doxycycline shown in (D) (C) Schematic of doxycycline induction of Wnt3a expression and flow cytometry readout for the data shown in (D and E); (D) Representative flow cytometry plots for data graphed in (E). (E) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to untreated control. Data are representative of 2 biological replicates. **=$p<0.01$, n.s.=Not significant. Error bars represent standard deviation.

WNT9A stimulates hematopoietic differentiation in a paracrine manner

In the zebrafish embryo, WNT9A is secreted by the somites and received by the developing hemogenic endothelium in a paracrine manner (Grainger *et al.* 2016). To model this, we used the inducible WNT9A hESC line shown in Figures 2 and 3 (constitutively labeled with Citrine), and co-cultured these cells with a hESC line harboring a *TOP:GFP;SV40:mCherry* transgene (constitutively labeled with mCherry) (Figure 4.9). This cell line will express GFP after receiving a β-catenin-mediated Wnt cue, and can be distinguished from the WNT9A producing cells by mCherry expression, allowing for analysis of (1) All cells [black bars], (2) WNT9A-secreting cells [Citrine$^+$, green bars], (3) non-WNT9A-secreting, non-Wnt-receiving cells [mCherry$^+$, red bars], and (4) Wnt-receiving cells [GFP$^+$/mCherry$^+$, yellow bars] (Figure 4.9). When WNT9A is expressed, differentiation efficiency is increased in all cell populations (Figure 4.9). By analyzing hematopoietic markers in sorted CD34$^+$ endothelial cells within each of the above populations, we found that the Wnt-receiving cells are biased to differentiate to hematopoietic cells as early as day 5 of the differentiation since Wnt-receiving endothelial cells are enriched for expression of the hematopoietic marker genes *GATA2, CMYB,* and *CD45* (Figure 4.10). Taken together, these data indicate that during *in vitro* hematopoietic differentiation, cells must receive a Wnt signal to efficiently differentiate to hematopoietic progenitors. Receiving a WNT9A signal increases this differentiation efficiency. Interestingly, Wnt-receiving cells that receive a WNT9A signal differentiate more efficiently than cells that receive endogenous Wnt cues. Altogether, this suggests that, like in the zebrafish, there is a requirement for specific Wnt ligands during *in vitro* differentiation of hematopoietic progenitors.

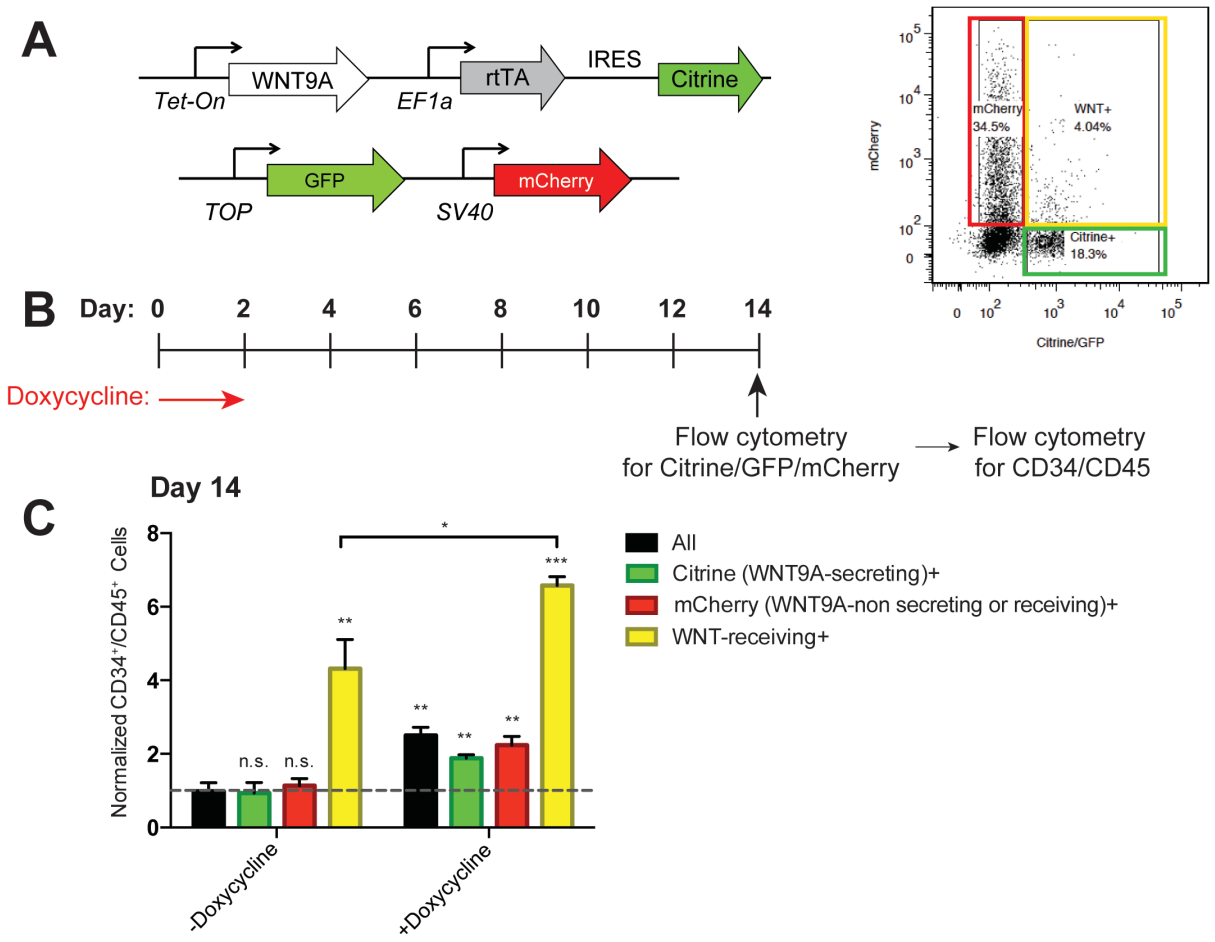

Figure 4.9. WNT9A signals in a paracrine fashion to instruct differentiation to hematopoietic progenitor cells. (A) Schematic of inducible WNT9A and *TOP (Tcf optimal promoter):GFP;SV40:mCherry* transgenes and flow cytometry gating strategy; (B) Schematic of doxycycline induction of WNT9A expression, and readout of hematopoietic progenitor differentiation by flow cytometry for GFP/mCherry expression, then subsequent CD34 and CD45 expression for the data shown in (C); (C) Percentage of $CD34^{+}/CD45^{+}$cells in the indicated cell populations normalized to all cells in the untreated control. Data are representative of 3 biological replicates. *=p<0.05, **=p<0.01, ***=p<0.001, n.s.=Not significant. Error bars represent standard deviation.

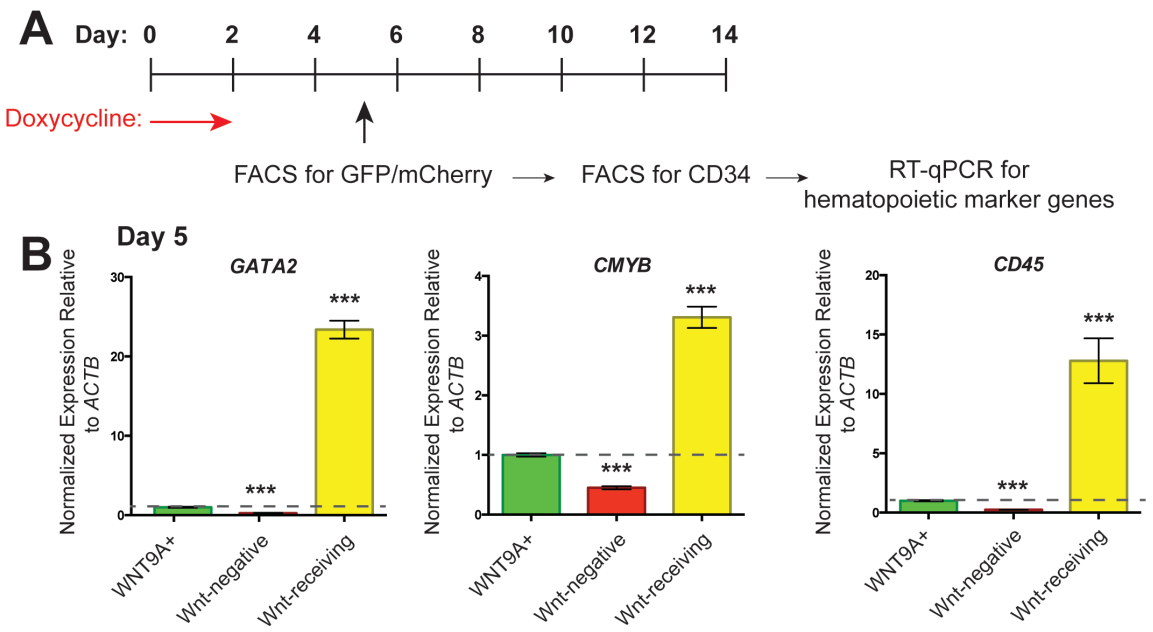

Figure 4.10. Wnt-receiving cells are biased toward hematopoietic progenitor fate by day 5. (A) Schematic of doxycycline induction of WNT9A expression and isolation of CD34^{+} endothelial cells within the indicated cell populations for data shown in (B); (B) RT-qPCR for hematopoietic markers *GATA2*, *CMYB*, and *CD45* in CD34^{+} endothelial cells from the indicated cell populations. Expression values are shown as fold change, normalized to WNT9A-secreting cells (Citrine^{+}, green bar). Data represent average of 3 technical replicates; ***=p<0.001. Error bars represent standard deviation.

Loss of WNT9A inhibits hematopoietic progenitor differentiation

Loss of zebrafish *wnt9a* via morpholino knockdown resulted in loss of HSCs. To determine if WNT9A is similarly required in human hematopoietic progenitor development, we utilized CRISPR/Cas9 to generate a genomic mutation in WNT9A in hESCs. We isolated two mutant clones with an indel mutation in exon 4 of WNT9A, resulting in an early STOP codon and a truncated protein (Figure 4.11). WNT9A mutant cells did not exhibit a defect in ability to differentiate to hematopoietic progenitor cells compared to wild-type H9 hESCs (Figure 4.11). However, *WNT9A* is expressed at very low levels throughout differentiation, so it is possible that loss of a low-abundance protein does not have drastic effects on differentiation (Figure 4.12). This lack of impact on hematopoietic differentiation could also indicate that, unlike in the zebrafish embryo, WNT9A is not required for the differentiation of hematopoietic progenitor cells, though exogenous WNT9A can boost *in vitro* hematopoietic development. These results could also suggest that loss of WNT9A is being compensated for by overexpression of other Wnts, such as WNT9B, which can also improve hematopoietic differentiation efficiency in our system (Figure 4.7). It has been previously reported in the zebrafish that transient gene knockdowns can be subject to less genetic compensation than constitutive genetic mutations (Rossi *et al*. 2015). In light of this, we also utilized a transient shRNA knockdown approach to reduce *WNT9A* levels during differentiation. WNT9A knockdown resulted in a 2-fold decrease in hematopoietic progenitor differentiation efficiency compared to a control shRNA knockdown (Figure 4.13).

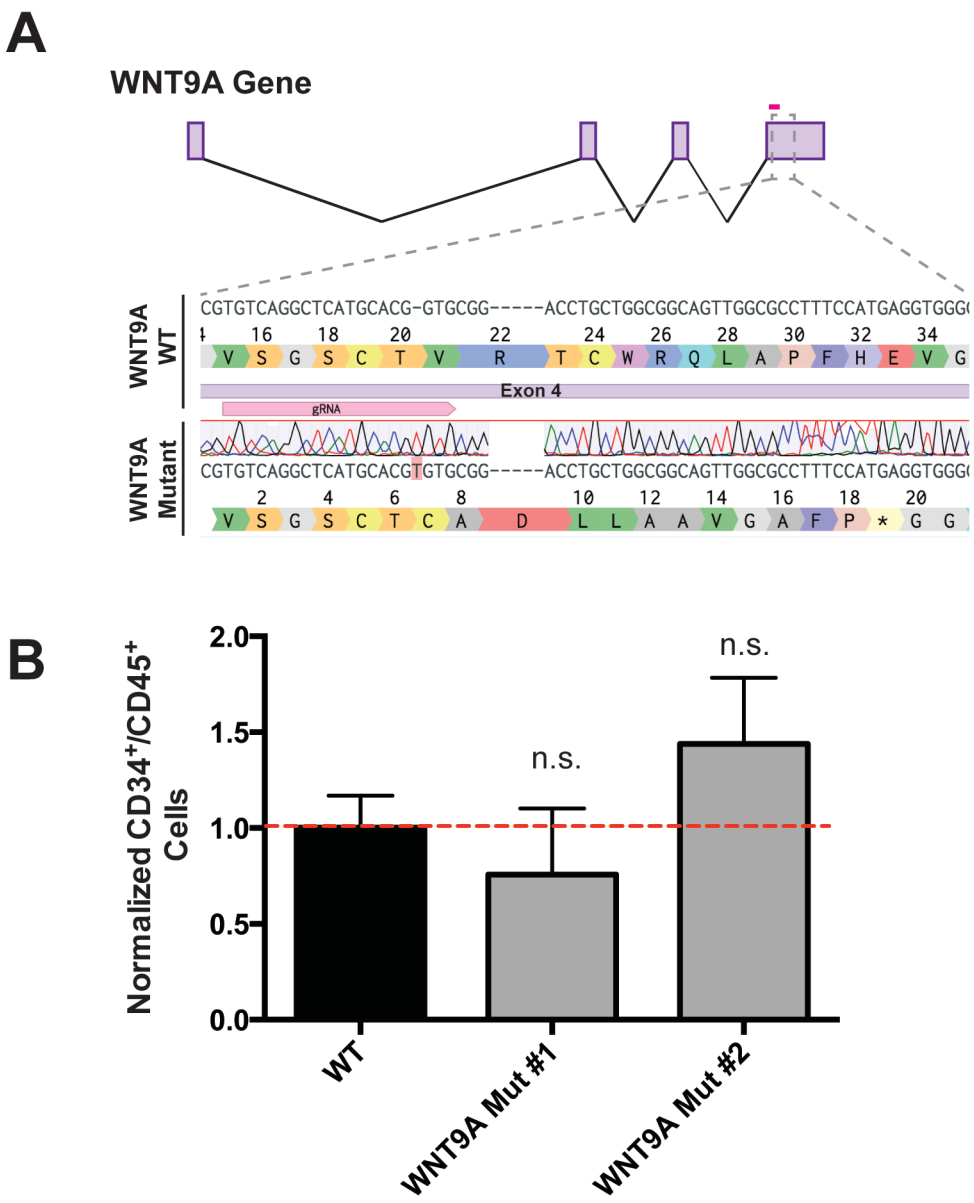

Figure 4.11. WNT9A mutant cells do not exhibit a deficiency in ability to differentiate to hematopoietic progenitors. (A) Top: Schematic of the *WNT9A* gene and CRISPR/Cas9 targeting strategy (purple = exons; pink = guide RNA); Bottom: wild-type (WT) *WNT9A* sequence and mutant *WNT9A* sequence (both Mutant #1 and #2 have the same mutation shown here). (B) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to untreated control. Error bars represent standard deviation. n.s = Not significant.

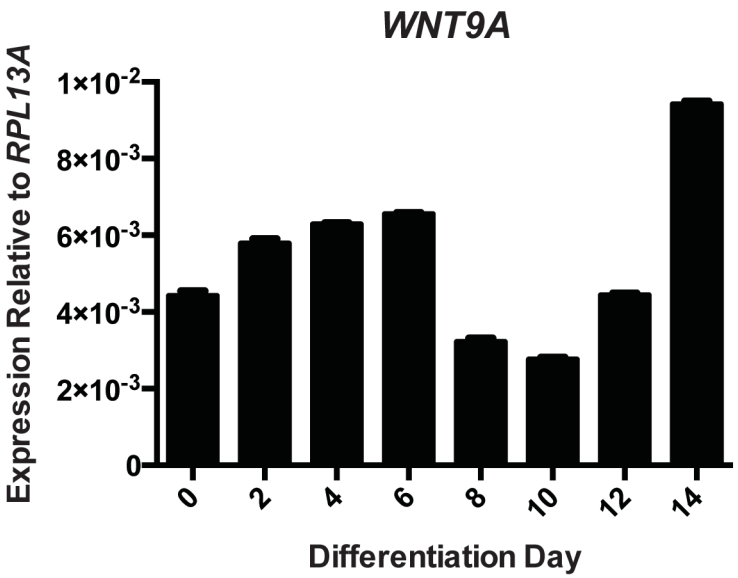

Figure 4.12. Endogenous *WNT9A* is expressed at low levels during hematopoietic differentiation. RT-qPCR for *WNT9A* at the indicated days of hematopoietic differentiation. Expression values are shown relative to *RPL13A*. Error bars indicate standard deviation.

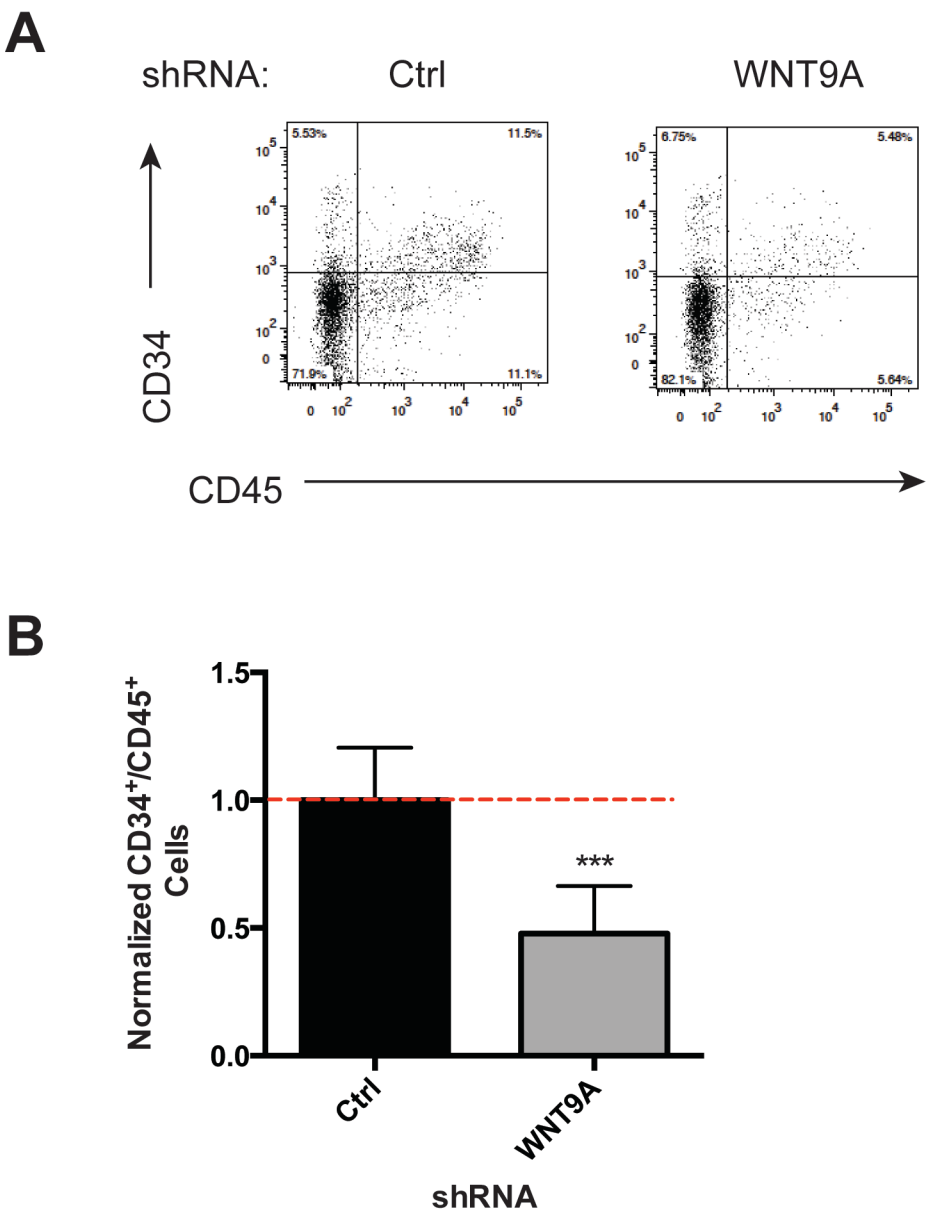

Figure 4.13. WNT9A knockdown decreases hematopoietic progenitor differentiation efficiency. (A) Representative flow cytometry plots for the data shown in (B). (B) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to control. Data are representative of 2 biological replicates. Error bars represent standard deviation. *** = $p<0.001$.

4.3: Discussion

Wnt signaling has been shown to regulate adult and developmental hematopoiesis *in vivo*, as well as during *in vitro* differentiation of hematopoietic progenitor cells (reviewed in Richter *et al.* 2017). We recently identified Wnt9a as a specific mediator of a critical Wnt signal required for zebrafish HSC development (Grainger *et al.* 2016). In this study, we sought to extend our findings to human cells and determine to what extent WNT9A can regulate the differentiation of human hematopoietic progenitors from pluripotent precursors. We found that, as in the zebrafish embryo, Wnt/β-catenin signaling positively regulates human hematopoietic differentiation. Similarly, WNT9A improves differentiation efficiency in a time- and dose- dependent manner. We also show that the paracrine nature of the Wnt9a signal identified in the zebrafish occurs in the *in vitro* hematopoietic differentiation. This is the first report describing a role for WNT9A in mammalian hematopoiesis.

We show that Wnt/β-catenin stimulation improves human hematopoietic progenitor differentiation efficiency, which is consistent with other reports (Woll *et al.* 2008, Wang and Nakayama 2009, Sturgeon *et al.* 2014, Kitajima *et al.* 2015, Ng *et al.* 2016). These studies have described an *in vitro* requirement for Wnt/β-catenin signaling to properly instruct mesodermal fate commitment, first to primitive streak-like mesoderm (Sumi *et al.* 2008), and then further to hematopoietic mesoderm (Wang and Nakayama 2009, Sturgeon *et al.* 2014, Ng *et al.* 2016). This requirement for Wnt/β-catenin signaling is stage specific; we found that inhibition of Wnt secretion negatively impacted hematopoietic only during certain windows of differentiation. The Wnt pathway is known to have temporally restricted effects on hematopoietic differentiation *in vitro*; Wnt stimulation early typically stimulates hematopoietic differentiation, and later Wnt stimulation results in differentiation to mesenchymal cells (Gertow *et al.* 2013, Sturgeon *et al.* 2014, Ng *et al.* 2016) and cells restricted to intermediate mesoderm (Kumar *et al.* 2015), which contributes to the urogenital system, and not to the hematopoietic system. The effect of Wnt activation on *in vitro* hematopoietic progenitor differentiation is dose dependent, which was previously described *in vivo* during adult

hematopoiesis using an allelic series of APC mutations (Luis *et al.* 2011). Fine-tuning of the level of Wnt activation during *in vitro* hematopoietic differentiation protocols may be critical for maximizing the number and functionality of hematopoietic progenitors derived from pluripotent precursors.

Here, we specifically modulate expression of WNT9A, which results in a 5-fold increase in $CD34^{+}/CD45^{+}$ hematopoietic progenitor cells. In contrast, stimulation of the Wnt pathway using a small molecule activator [CHIR98014], or Wnt3a results in only a 2-fold increase in $CD34^{+}/CD45^{+}$ cells. Interestingly, WNT9B treatment results in a 17-fold increase in $CD34^{+}/CD45^{+}$ cells, which is unexpected since Wnt9b is not sufficient to rescue loss of Wnt9a in the zebrafish model. This WNT9B effect may be due to its ability to signal with many FZD receptors. In this *in vitro* system, addition of a specific ligand affords a larger increase in efficiency than ligand-independent activation of the pathway. This may be, in part, due to off-target effects that small molecules, such as this potent GSK3 inhibitor, can have on other cellular processes; it is possible that these off-target effects slightly inhibit hematopoietic differentiation. If this is the case, stimulation with a specific Wnt ligand may activate the Wnt/β-catenin signaling cascade more uniquely and potently. Expression of other Wnts, including Wnt1, Wnt5, WNT3, and WNT3A have been modulated during *in vitro* hematopoietic differentiation with varying effects on differentiation outcome, suggesting that specific Wnts may act non-redundantly during differentiation (Woll *et al.* 2008, Wang and Nakayama 2009, Gertow *et al.* 2013). This has also been described during *in vivo* hematopoietic stem cell development (Luis *et al.* 2009b, 2010, Grainger *et al.* 2016). This may be due to differential Wnt/Fzd binding affinities and activation of subsequent Fzd-specific gene programs (Li *et al.* 2004, Carmon and Loose 2010, Dijksterhuis *et al.* 2015, Voloshanenko *et al.* 2017). Investigation into which ligand(s) most efficiently instruct hematopoietic progenitor fate *in vitro* may further improve current differentiation protocols.

Unlike in the zebrafish, loss of *WNT9A* in this *in vitro* model of human hematopoietic development does not negatively impact hematopoietic progenitor cells. This could be, in part, due to genetic compensation by upregulation of other Wnt genes. Exogenous expression of other Wnts, like

WNT9B and Wnt3a, resulted in varying degrees of increase in differentiation efficiency, and upregulation of these genes could result in a lack of defect in the WNT9A mutant cells. Additionally, since *WNT9A* is not highly expressed during differentiation, loss of already minimal levels of WNT9A signaling may not have a negative impact on differentiation outcome. It has been reported in the zebrafish that compensation that may arise in genetic knockouts does not always occur in transient knockdowns, such as morpholinos (Rossi *et al.* 2015). Knockdown of *WNT9A* using shRNAs or other transient knockdown methods may result in less compensation.

In the zebrafish, Wnt9a is secreted by the somites and received by neighboring cells of the hemogenic endothelium in a paracrine fashion (Grainger *et al.* 2016). This signaling mechanism also holds true *in vitro;* it is the cells that receive a Wnt signal, not the cells that secrete WNT9A, that differentiate most efficiently to hematopoietic progenitors. This is somewhat surprising, since the spatial architecture that is characteristic of a complex zebrafish embryo does not exist during *in vitro* hematopoietic differentiation; a small population of WNT9A-secreting cells is not a limiting factor *in vitro* as it may be during zebrafish development. Interestingly, Wnt9a has also been described to signal in a paracrine fashion during zebrafish palate morphogenesis and chick hepatic endothelium development (Matsumoto *et al.* 2008, Rochard *et al.* 2016). This may indicate that secretion of WNT9A precludes a cell from responding to the Wnt signal in an autocrine manner, and thus largely signals in a paracrine fashion. This differentiation protocol utilizing a co-culture method to study Wnt-secreting and Wnt-receiving cells could prove to be a useful platform in future experiments to dissect the mechanisms of paracrine versus autocrine Wnt signaling, and to better understand the mechanics of Wnt ligand secretion.

Our data indicate that signals identified as regulators of hematopoietic development in the zebrafish can be translated to impact human hematopoietic progenitor cell during *in vitro* models of development. Wnt9a, which is required for zebrafish HSC development, can be modulated to increase the efficiency of $CD34^+/CD45^+$ hematopoietic cell differentiation from human pluripotent stem cells.

These findings represent an important initial step in translating our knowledge of molecular cues governing organismal hematopoiesis to protocols for instructing HSC differentiation *in vitro*.

Chapter 4, in part, has been submitted for publication in *Genes*. Richter J., Elefanty, A., Ng, E., Stanley, E., Traver, D., Willert, K. "WNT9A is a conserved regulator of hematopoietic stem and progenitor cell development." The dissertation author was the primary investigator and author of this paper.

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

5.1: Conclusions

The ability to derive therapy-grade HSCs *in vitro* from pluripotent precursors has been elusive for decades (Ditadi *et al*. 2017). Current protocols using timed induction of growth factors or stromal feeder cells to derive hematopoietic stem and progenitor cells *in vitro* result in cells that are immature, have not been shown to sufficiently repopulate a recipient animal, or exhibit defects in differentiation to downstream blood lineages (Ledran *et al*. 2008, Lu *et al*. 2009, Gori *et al*. 2015). Some protocols utilize enforced expression of hematopoietic transcription factors to induce hematopoietic progenitor fate, but the resulting cells are still not suitable for therapeutic applications (Doulatov *et al*. 2013, Riddell *et al*. 2014, Sandler *et al*. 2014). In this study, I utilized the zebrafish as a model for normal *in vivo* HSC development to identify a critical molecular cue that governs this process, then validated that this cue also impacts mammalian hematopoiesis using an *in vitro* differentiation protocol as a model for human hematopoietic progenitor development.

Here, I show that Wnt/β-catenin signaling is required in a tightly controlled time window. In the zebrafish, this window occurs prior to 20 hpf, and prior to 4 days of differentiation in an *in vitro* model of human hematopoietic development. This early timing is consistent with prior studies in the mouse, zebrafish, and other *in vitro* differentiation protocols (Lengerke *et al*. 2008, Goessling *et al*. 2009, Ruiz-Herguido *et al*. 2012b, Gertow *et al*. 2013, Sturgeon *et al*. 2014, Ditadi *et al*. 2015, Ng *et al*. 2016). Interestingly, this requirement is 10 hours prior to the onset of a hematopoietic defect in the zebrafish. This early Wnt cue primes HSCs for later amplification in the aorta, mediated at least in part by Myca, which is downregulated upon loss of Wnt9a (Grainger *et al*. 2016). It is not yet known what mechanism accounts for the delay between Wnt9a requirement and the resulting phenotype. It is possible that this early Wnt9a signal mediates epigenetic changes that allow Myca access to open chromatin, resulting in later expression of D-class cyclins and their cofactors, Cdk2 and Cdk4. It is not clear whether this Wnt cue also results in later proliferation of hematopoietic progenitor cells *in vitro*.

It is worth noting that the time when Wnt stimulation or inhibition can impact differentiation outcome (days 2-4) is relatively removed from the time point of my? analysis (day 14), though expression of hematopoietic progenitor markers at earlier time points have not been thoroughly analyzed.

We identified Wnt9a as the ligand that mediates this critical Wnt cue in the zebrafish embryo. Loss of Wnt9a results in fewer HSCs that emerge from the aorta. WNT9A overexpression during the *in vitro* differentiation of human hematopoietic progenitors resulted in a 5-fold increase in differentiation efficiency, which suggests that the importance of this molecular cue is conserved across species. WNT9A overexpression impacts *in vitro* differentiation efficiency in a dose-dependent manner; low or high doses of WNT9A result in sub-maximal increases in differentiation of hematopoietic progenitor cells. This is consistent with global Wnt/β-catenin pathway activation with CHIR, and with previously published work in the adult mouse, which showed that moderate levels of Wnt signaling were optimal for maintenance of the HSC pool (Luis *et al.* 2011). These results raise the possibility that HSCs are similarly sensitive to the level of Wnt signaling during development; further experiments analyzing the effects of Wnt9a overexpression on zebrafish HSCs could provide evidence for this.

Wnt ligands have often been thought to signal promiscuously through any available Frizzled (Fzd) receptor, and that ligand specific phenotypes can largely be attributed to differences in spatial and temporal availability. Our results indicate that this is not the case; loss of Wnt9a cannot be compensated for by overexpression of Wnt9b or Wnt3a, which also signal in a β-catenin-dependent manner. However, this specificity is not as clear-cut in the *in vitro* model; overexpression of WNT9B and Wnt3a also resulted in an increase in hematopoietic progenitor differentiation efficiency. WNT9B proved more potent than WNT9A in improving differentiation efficiency. This may be due to differences in the ability of each Wnt to signal through the Fzd receptors that are expressed during differentiation. Preliminary data from our lab indicates that Wnt9b can signal through more Fzd receptors than Wnt9a, which is in line with published work (Voloshanenko *et al.* 2017).

Overexpression of the correct Fzd receptor in concert with Wnt9a could result in greater increases in differentiation efficiency. Overall, our data indicate that Wnt ligands act differently on hematopoietic progenitor development, and addition of the correct Wnt ligand to *in vitro* hematopoietic differentiation protocols could better mimic *in vitro* development.

Wnt9a is secreted by the somites and received by neighboring hemogenic endothelial cells during zebrafish development. This paracrine signaling structure is modeled in the *in vitro* differentiation system; it is the cells that receive the Wnt signal, not the cells that make and secrete WNT9A, that differentiate most efficiently to hematopoietic progenitor cells. Wnt9a has also been reported to signal in a largely paracrine manner in other settings, such as zebrafish palate morphogenesis, mouse cranial skeletal development, and development of chick hepatic endothelium (Matsumoto *et al.* 2008, Zhou *et al.* 2009, Rochard *et al.* 2016). This may indicate that Wnt9a signaling is biased towards paracrine mechanisms, as opposed to other ligands such as Wnt3, which has been shown to signal in either paracrine and autocrine fashions (Sato *et al.* 2011, Voloshanenko *et al.* 2013, Kato *et al.* 2014). The mechanism that instructs whether a Wnt will signal in an autocrine or paracrine fashion is not understood. Wnt9a signaling during HSC development could serve as a model to dissect the mechanisms of paracrine and autocrine Wnt signaling.

Addition of WNT9A to *in vitro* protocols for deriving hematopoietic progenitor cells from pluripotent precursor cells improves differentiation efficiency. However, downstream analysis of hematopoietic progenitor function has not been completed. Further experimentation to determine to what extent these hematopoietic progenitors are capable of differentiating into specific blood lineages. In the zebrafish, a Wnt9a signal results in *myca* activation and subsequent cell proliferation; in the *in vitro* setting, this could be a double-edged sword. Proliferation of hematopoietic progenitors would be beneficial in deriving greater numbers of cells for further experiments or transplantation into animals, but excessive proliferation could lead to cancerous phenotypes in recipient animals. Fine-tuning of the

dosage of WNT9A for not only optimal differentiation efficiency, but also downstream applications will be critical in implementing this cue into current differentiation protocols.

My study has utilized the zebrafish as a model of developmental hematopoiesis, and identified that a critical Wnt cue is required prior to 20 hpf in developing hemogenic endothelial cells. I identified Wnt9a as the unique mediator of this signal, and showed that exogenous expression of WNT9A during the differentiation of human embryonic stem cells to hematopoietic progenitor cells increased differentiation efficiency. The findings of my study improve our understanding of the necessary cues required for *in vivo* HSC development. My results also show that such cues can be translated to *in vitro* differentiation systems to improve established protocols for deriving hematopoietic progenitor cells from pluripotent precursors.

5.2: Future Directions

My study has elucidated a conserved role for Wnt9a in zebrafish HSC development and *in vitro* human hematopoietic progenitor development. However, many open questions remain. In this section, I will discuss open lines of inqury and potential experiments to address these questions.

How does WNT9A affect the functionality of in vitro*-derived hematopoietic progenitors?*

Here, I have shown that WNT9A addition to the *in vitro* differentiation of human embryonic stem cells results in greater numbers of hematopoietic progenitor cells, as measured by $CD34^+/CD45^+$ cells. My data provide evidence that this WNT9A signal impacts the progenitor population at least at the level of marker expression, but it remains to be seen how WNT9A affects the function of these *in vitro*-derived hematopoietic cells. To determine if WNT9A is acting in this system on multilineage progenitors or HSC-like cells, as opposed to lineage-restricted progenitors, which may also share this $CD34^+/CD45^+$ signature (Doulatov *et al*. 2013), analysis of additional surface markers should be done. For example, surface markers such as CD49f and CD90 are expressed on HSC-like cells, but not lineage-restricted progenitors. Our zebrafish data indicates that the Wnt9a signal acts on HSCs, not downstream progenitors, but the *in vitro* differentiation system is an imperfect model of hematopoietic stem cell development, so it is possible that the mechanism of this signal may be slightly different in this model.

Many protocols to derive hematopoietic stem and progenitor cells *in vitro* result in hematopoietic cells that express many surface markers indicative of HSC fate, but they have a limited capacity to properly differentiate into downstream blood lineages, or have limited repopulation ability (Kyba *et al*. 2002, Wang *et al*. 2005, p. 4, Ledran *et al*. 2008, Lu *et al*. 2009, Doulatov *et al*. 2013, Riddell *et al*. 2014, Sandler *et al*. 2014, Ditadi *et al*. 2017, Sugimura *et al*. 2017). I hypothesize that the addition of WNT9A to the differentiation protocol to more accurately mimic *in vivo* HSC developmental cues will result in an increase in functionality of the resulting hematopoietic

progenitors. To test whether WNT9A improves hematpoietic progenitor function, I propose two experimental approaches. The first approach utilizes methylcellulose culture medium supplemented with hematopoietic cytokines (SCF, IL-3, EPO, G-SCF, GM-SCF) to analyze the ability of the *in vitro*-derived hematopoietic progenitor cells to differentiate into downstream blood lineages (Wognum *et al*. 2013). I propose to differentiate inducible WNT9A hESCs with and without doxycycline from day 0-2, sort out $CD34^{+}/CD45^{+}$ cells, replate them into methylcellulose, and analyze their ability to differentiate into downstream blood lineages. My model predicts that addition of WNT9A will stimulate later proliferation of hematopoietic progenitor cells, so I hypothesize that cells treated with WNT9A will form more colonies in this assay. It will be important to determine whether the types of colonies formed are similar to or different from control cells (i.e. without doxycycline). For the second approach, I propose isolating $CD34^{+}/CD45^{+}$ cells treated with or without WNT9A as above, transplanting them into irradiated mouse recipients, and analyzing the degree of engraftment (Cheng *et al*. 2013). These experiments will provide important information regarding the functionality of these *in vitro* derived cells, which is necessary to reach the long-term goal of deriving a transplantable HSC *in vitro*.

Paracrine vs. autocrine Wnt signaling

Wnt9a is secreted by the somites and received by the nearby migrating hemogenic endothelial cells during zebrafish HSC development (Grainger *et al.* 2016). The paracrine structure of this signal is also modeled during *in vitro* hematopoietic progenitor development; cells that receive the Wnt signal, not cells that secrete WNT9A, differentiate most efficiently towards the hematopoietic lineage (Figure 4.9, Figure 4.10). Though many Wnts have been shown to signal in both paracrine and autocrine fashions (i.e. Wnt3; (Sato *et al.* 2011, Voloshanenko *et al.* 2013, Kato *et al.* 2014)), Wnt9a has been primarily reported to signal in a paracrine context (Matsumoto *et al.* 2008, Zhou *et al.* 2009, Rochard *et al.* 2016). The mechanism by which Wnts signal in a paracrine or autocrine fashion is not

well understood. The cell lines that I have developed in the context of the *in vitro* hematopoietic differentiation may begin to provide insight into the mechanics of Wnt secretion and paracrine Wnt signaling.

Unlike in the zebrafish, where Wnt9a is uniquely required for HSC development (Grainger *et al.* 2016), multiple Wnts can improve hematopoietic progenitor differentiation in the *in vitro* model of human hematopoietic development, albeit to varying degrees of efficiency (Figure 4.5, Figure 4.7, Figure 4.8). This could be exploited to study whether all Wnts act in a paracrine fashion to improve hematopoietic progenitor differentiation efficiency. I hypothesize that while Wnt9a signals in a primarily paracrine manner, other Wnts, such as Wnt3a, can signal in both a paracrine and autocrine manner. This can be tested during the *in vitro* hematopoietic differentiation protocol by co-culturing cells expressing either Wnt3a or WNT9A with Wnt-reporter cells (as in Figure 4.9). If Wnt3a can signal equally well in a paracrine or autocrine fashion, I would expect to see no significant difference in differentiation efficiency between the Wnt-receiving population with or without Wnt3a. It may also be informative to activate WNT9A signaling by adding purified WNT9A protein to the differentiation media, instead of utilizing the doxycycline-inducible expression system. This paracrine nature of the WNT9A signal may act more like a juxtacrine signal that is highly dependent on cell context, which would be absent if purified protein is added directly to the media. These experiments will provide useful insight into the mechanism of different Wnt signals during hematopoietic differentiation.

The mechanism determining whether different Wnt ligands signal in a paracrine or autocrine context is not well understood during hematopoietic progenitor differentiation, or more broadly in other contexts. Characterizing the effects of Wnt signaling in both the ligand-secreting and ligand-receiving cell populations could further our understanding of how Wnts signal. I hypothesize that Wnt3a, which can signal in a paracrine or autocrine manner, and Wnt9a, which has been primarily described to signal in paracrine manner, stimulate differential responses in the cells that receive the Wnt signal, but also in the cells that secrete the Wnt ligand. A co-culture system similar to the one

described in Figure 4.9, using pluripotent cells harboring inducible Wnt transgenes mixed with Wnt reporter cells enables facile analysis of the Wnt-producing and the Wnt-receiving cells. These cells can be analyzed by RNA-seq to determine differences in transcriptional profiles in paracrine or autocrine Wnt settings.

Identifying the Wnt9a receptor

This study has identified Wnt9a as the ligand that mediates the critical Wnt signal that is required for HSC development. Data from the zebrafish indicates that overexpression of other Wnt ligands cannot compensate for the loss of Wnt9a, which may indicate very stringent ligand-receptor specificity between Wnt9a and its cognate Fzd receptor. However, we have yet to show which Fzd receptor Wnt9a signals through. Preliminary data from our lab indicates that zebrafish Wnt9a binds and signals through Fzd9b; they interact synergistically in *in vitro* signaling assays, and knockdown of *fzd9b* in the zebrafish phenocopies loss of *wnt9a*, resulting in fewer HSCs (data not shown). To determine if human WNT9A also signals with FZD9, I overexpressed WNT9A or each FZD alone or in combination in cells harboring a *TOP (TCF Optimal Promoter)*:*FLASH* transgene. This cell line will express Luciferase in response to a Wnt/β-catenin signal, which can be read out as light. WNT9A alone minimally activates this Wnt reporter, but signals synergistically with FZD9, the human homolog of Fzd9b (Figure 5.1). This indicates that this WNT9A-FZD9 signaling axis may be conserved among species. Interestingly, Wnt9a and Fzd9 have been previously shown to interact in other developmental settings, such as the chick hepatic endothelium development, providing further evidence that this may be a highly conserved signaling mechanism (Matsumoto *et al*. 2008). However, overexpression experiments in artificial *in vitro* settings can often be fraught with false interactions that are merely artifacts of the experimental system, so it is important to validate that FZD9 signals with WNT9A in a more biologically relevant setting. To determine if FZD9, like WNT9A, is important for hematopoietic differentiation of hESCs *in vitro,* I performed an shRNA knockdown of

FZD9 and analyzed the effect of differentiation outcome by flow for CD34 and CD45. FZD9 knockdown resulted in a 4-fold reduction as compared to a control shRNA knockdown (Figure 5.2). In comparison, WNT9A knockdown resulted in a 2-fold decrease in differentiation efficiency (Figure 4.13). These differences in the severity of differentiation inhibition may be indicative of differences in expression levels; FZD9 is expressed slightly more than WNT9A. This could also be reflective of how this ligand-receptor pair would signal *in vivo*: Wnt9a is secreted by the somites, and received by the hemogenic endothelium (which presumably expresses Fzd9b). In this model, Fzd9b/FZD9 would be required in a cell-autonomous manner, and so loss of this factor is predicted to have the more drastic effect on hematopoietic development.

Preliminary loss of function data indicates a role for a WNT9A/FZD9 signaling axis in hematopoietic stem and progenitor cell development in both the zebrafish and our *in vitro* model of human hematopoietic development. As a next step, I propose to overexpress WNT9A and FZD9 alone or in combination during days 0-2 of differentiation (as in Figure 4.5) and analyze the effect on hematopoietic differentiation outcome by flow cytometry for CD34 and CD45. I predict that overexpression of WNT9A and FZD9 together will result in a synergistic effect on differentiation efficiency. To further show that the interaction between WNT9A and FZD9 is specific, WNT9A overexpression could be combined with FZD9 or control knockdowns during hematopoietic differentiation. If WNT9A-FZD9 signaling is highly specific, and WNT9A is unable to signal through other Fzd receptors, I predict that knockdown of FZD9 will abrogate the effect of adding exogenous WNT9A during differentiation. The experiments outlined in this section will give further insight into the mechanism of WNT9A signaling during hematopoietic development.

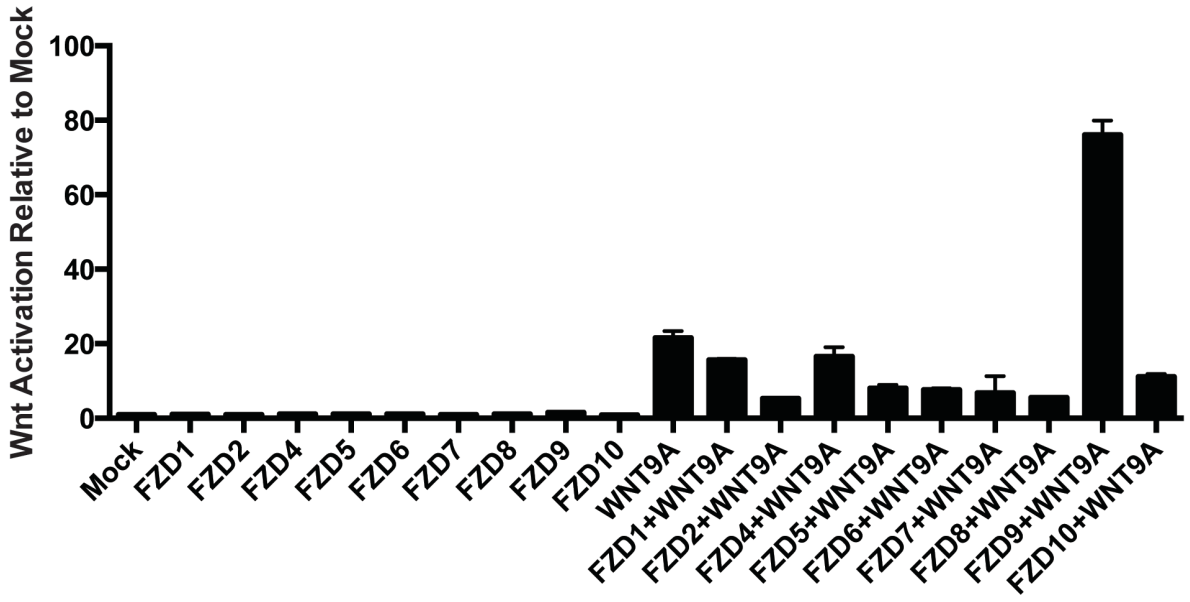

Figure 5.1. WNT9A signals synergistically with FZD9. TOP:FLASH Wnt reporter assay. HEK293-TOP:FLASH cells were transfected with WNT9A, FZD, or both and assayed after 48 hours for Luciferase activity. Values are shown as fold activation relative to a mock transfected control. Error bars represent standard deviation.

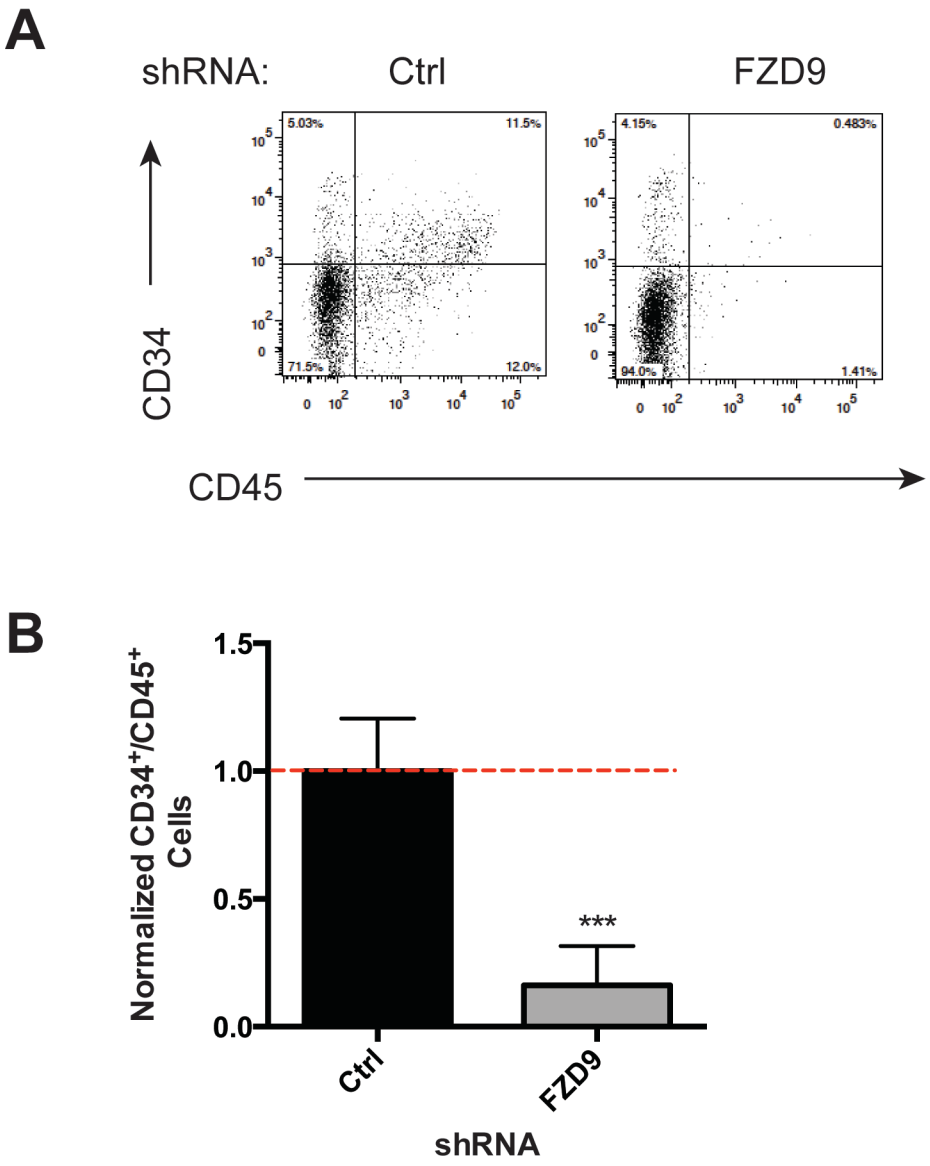

Figure 5.2. FZD9 knockdown decreases hematopoietic progenitor differentiation efficiency. (A) Representative flow cytometry plots for the data shown in (B). (B) Percentage of $CD34^{+}/CD45^{+}$ cells normalized to control. Data are representative of 2 biological replicates. Error bars represent standard deviation. *** = $p<0.001$.

Elucidating a role for Wnt9a in adult hematopoiesis

My study has established a role for Wnt9a during developmental hematopoiesis in both zebrafish *in vivo* and a human *in vitro* model system. Though Wnt9a is a critical regulator of early HSC development in the zebrafish, loss of Wnt9a does not result in a complete ablation of the HSCs. These few HSCs that remain appear to adequately contribute to developmental hematopoiesis. *wnt9a* mutant fish (described in Figure 3.15) exhibit normal *rag1* expression in the thymus at 5 dpf, indicating that there is no defect in the development of T cells, which derive from definitive HSCs (Figure 5.3). Although these early HSCs can populate maintenance niches such as the thymus, *wnt9a* mutant fish are not completely healthy for the remainder of their lifespan. Preliminary data indicates that this early requirement for Wnt9a can have long-lasting effects in the zebrafish; *wnt9a* mutant fish progressively die off over time, and have a maximal lifespan of approximately 6 months (Figure 5.3). This contrasts with the average lifespan of zebrafish: 3 years (Gilbert *et al.* 2014). Interestingly, *wnt9a* mutant fish do not all die off at the same time, which would suggest the inability to complete a specific developmental event or stage, but instead gradually die off. This could be indicative of anemia, leading to poor health and overall sickliness, which could be a product of decreased numbers of HSCs during early development. To determine if this is the case, future experiments could be done on juvenile and adult fish to examine peripheral blood for hematologic abnormalities via histological analysis (i.e. defects in erythrocyte maturation or erythrocyte number). Additional analysis could be performed on the adult kidney, the site of adult hematopoiesis in the zebrafish (analogous to the mammalian bone marrow) to examine the degree of maintenance of the five major blood lineages: erythrocytes, eosinophils, myeloid cells, lymphocytes, and precursor cells. This analysis can be easily performed by visualizing forward- and side-scatter profiles on a flow cytometer (Boatman et al. 2013). Analysis of the adult kidney marrow may give an indication of whether there is abnormal HSC differentiation to downstream blood lineages, which could be contributing to the early death phenotype seen in adult *wnt9a* mutants. It could also be useful to analyze the effect of *wnt9a*

overexpression on adult hematopoiesis; these results may illuminate potential problems with utilizing WNT9A in protocols to derive hematopoietic progenitors *in vitro* for later use in transplantations into recipient organisms.

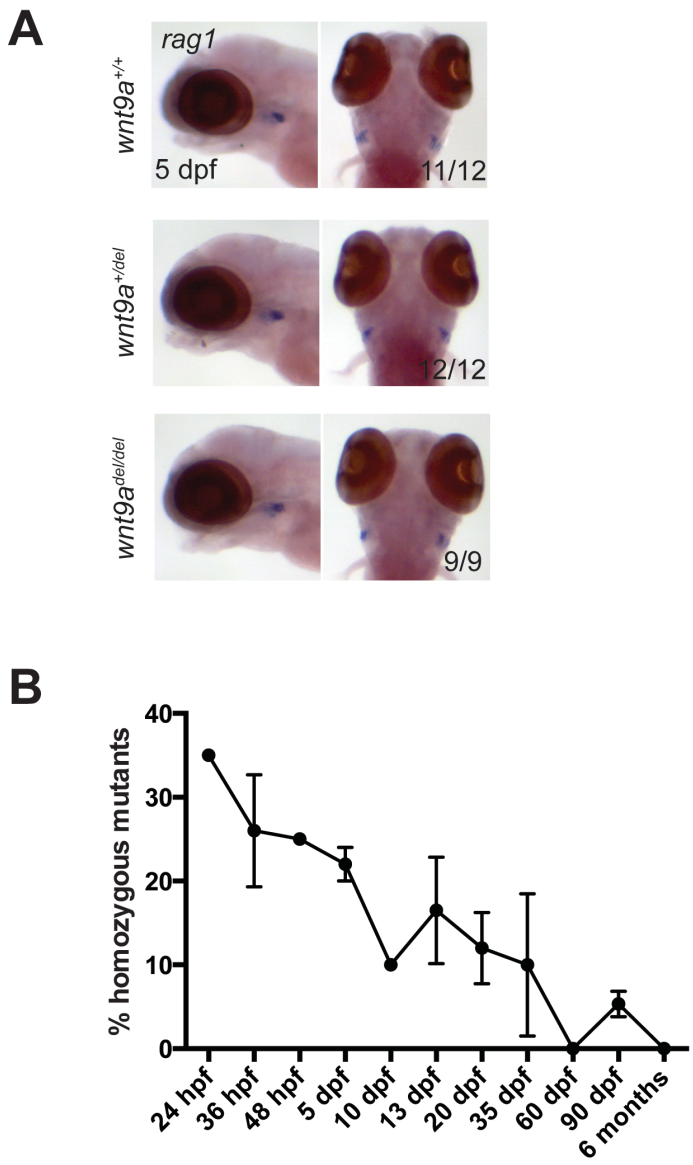

Figure 5.3. Early loss of *wnt9a* has long-lasting effects on zebrafish health. (A) *In situ* hybridization for the T-cell marker *rag1* at 5 dpf. Embryos are from a heterozygote incross, resulting progeny are grouped into wild-type (*wnt9a*$^{+/+}$; top), heterozygous (*wnt9a*$^{+/del}$; middle), and homozygous mutant (*wnt9a*$^{del/del}$; bottom) siblings. (B) Embryos from a *wnt9a*$^{+/del}$ heterozygote incross were raised normally and tracked over time to analyze the lifespan of *wnt9a*$^{del/del}$ homozygous mutant fish. At the indicated time points, 20 embryos were removed from the tank and genotyped. Plotted are the % of the genotyped embryos that were homozygous mutants. Mendelian genotype ratios predict that 25% of the fish would be homozygous mutants. Error bars represent standard deviation.

CHAPTER 6: REFERENCES

Aberle, H., Bauer, A., Stappert, J., Kispert, A., and Kemler, R., 1997. β-catenin is a target for the ubiquitin–proteasome pathway. *The EMBO Journal*, 16 (13), 3797–3804.

Alexandre, C., Baena-Lopez, A., and Vincent, J.-P., 2014. Patterning and growth control by membrane-tethered Wingless. *Nature*, 505 (7482), 180–185.

Amati, B., Alevizopoulos, K., and Vlach, J., 1998. Myc and the cell cycle. *Frontiers in Bioscience: A Journal and Virtual Library*, 3, d250-268.

Baba, Y., Garrett, K.P., and Kincade, P.W., 2005. Constitutively active beta-catenin confers multilineage differentiation potential on lymphoid and myeloid progenitors. *Immunity*, 23 (6), 599–609.

Batta, K., Menegatti, S., Garcia-Alegria, E., Florkowska, M., Lacaud, G., and Kouskoff, V., 2016. Concise Review: Recent Advances in the In Vitro Derivation of Blood Cell Populations. *STEM CELLS Translational Medicine*, 5 (10), 1330–1337.

Bertoli, C., Skotheim, J.M., and de Bruin, R.A.M., 2013. Control of cell cycle transcription during G1 and S phases. *Nature Reviews. Molecular Cell Biology*, 14 (8), 518–528.

Bertrand, J.Y., Chi, N.C., Santoso, B., Teng, S., Stainier, D.Y.R., and Traver, D., 2010. Haematopoietic stem cells derive directly from aortic endothelium during development. *Nature*, 464 (7285), 108–111.

Bertrand, J.Y., Cisson, J.L., Stachura, D.L., and Traver, D., 2010. Notch signaling distinguishes 2 waves of definitive hematopoiesis in the zebrafish embryo. *Blood*, 115 (14), 2777–2783.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.-C., Wang, Y., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R., 1996. A new member of the frizzled family from Drosophila functions as a Wingless receptor. *Nature*, 382 (6588), 225–230.

Biechele, S., Cox, B.J., and Rossant, J., 2011. Porcupine homolog is required for canonical Wnt signaling and gastrulation in mouse embryos. *Developmental Biology*, 355 (2), 275–285.

Bigas, A., Guiu, J., and Gama-Norton, L., n.d. Notch and Wnt signaling in the emergence of hematopoietic stem cells. *Blood Cells, Molecules, and Diseases*.

Boatman, S., Barrett, F., Satishchandran, S., Jing, L., Shestopalov, I., and Zon, L.I., 2013. Assaying Hematopoiesis Using Zebrafish. *Blood cells, molecules & diseases*, 51 (4).

Boisset, J.-C., van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C., 2010. In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. *Nature*, 464 (7285), 116–120.

Boisset, J.-C., Clapes, T., Klaus, A., Papazian, N., Onderwater, J., Mommaas-Kienhuis, M., Cupedo, T., and Robin, C., 2015. Progressive maturation toward hematopoietic stem cells in the mouse embryo aorta. *Blood*, 125 (3), 465–469.

Bouldin, C.M. and Kimelman, D., 2014. Dual fucci: a new transgenic line for studying the cell cycle from embryos to adults. *Zebrafish*, 11 (2), 182–183.

Brown, L.A., Rodaway, A.R.F., Schilling, T.F., Jowett, T., Ingham, P.W., Patient, R.K., and Sharrocks, A.D., 2000. Insights into early vasculogenesis revealed by expression of the ETS-domain transcription factor Fli-1 in wild-type and mutant zebrafish embryos. *Mechanisms of Development*, 90 (2), 237–252.

Buckles, G.R., Thorpe, C.J., Ramel, M.-C., and Lekven, A.C., 2004. Combinatorial Wnt control of zebrafish midbrain-hindbrain boundary formation. *Mechanisms of Development*, 121 (5), 437–447.

Burnight, E.R., Staber, J.M., Korsakov, P., Li, X., Brett, B.T., Scheetz, T.E., Craig, N.L., and McCray, P.B., 2012. A Hyperactive Transposase Promotes Persistent Gene Transfer of a piggyBac DNA Transposon. *Molecular Therapy - Nucleic Acids*, 1, e50.

Burns, C.E., Traver, D., Mayhall, E., Shepard, J.L., and Zon, L.I., 2005. Hematopoietic stem cell fate is established by the Notch–Runx pathway. *Genes & Development*, 19 (19), 2331–2342.

Butko, E., Distel, M., Pouget, C., Weijts, B., Kobayashi, I., Ng, K., Mosimann, C., Poulain, F.E., McPherson, A., Ni, C.-W., Stachura, D.L., Cid, N.D., Espín-Palazón, R., Lawson, N.D., Dorsky, R., Clements, W.K., and Traver, D., 2015. Gata2b is a restricted early regulator of hemogenic endothelium in the zebrafish embryo. *Development*, 142 (6), 1050–1061.

Butko, E., Pouget, C., and Traver, D., 2016. Complex regulation of HSC emergence by the Notch signaling pathway. *Developmental Biology*, 409 (1), 129–138.

Carmon, K.S. and Loose, D.S., 2010. Development of a Bioassay for Detection of Wnt-Binding Affinities for Individual Frizzled Receptors. *Analytical biochemistry*, 401 (2), 288–294.

Cavallo, R.A., Cox, R.T., Moline, M.M., Roose, J., Polevoy, G.A., Clevers, H., Peifer, M., and Bejsovec, A., 1998. Drosophila Tcf and Groucho interact to repress Wingless signalling activity. *Nature*, 395 (6702), 604–608.

Chen, B., Dodge, M.E., Tang, W., Lu, J., Ma, Z., Fan, C.-W., Wei, S., Hao, W., Kilgore, J., Williams, N.S., Roth, M.G., Amatruda, J.F., Chen, C., and Lum, L., 2009. Small molecule-mediated disruption of Wnt-dependent signaling in tissue regeneration and cancer. *Nature Chemical Biology*, 5 (2), 100–107.

Chen, E.Y., DeRan, M.T., Ignatius, M.S., Grandinetti, K.B., Clagg, R., McCarthy, K.M., Lobbardi, R.M., Brockmann, J., Keller, C., Wu, X., and Langenau, D.M., 2014. Glycogen synthase kinase 3 inhibitors induce the canonical WNT/β-catenin pathway to suppress growth and self-renewal in embryonal rhabdomyosarcoma. *Proceedings of the National Academy of Sciences of the United States of America*, 111 (14), 5349–5354.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., Wagner, R., Lee, G.O., Antosiewicz-Bourget, J., Teng, J.M.C., and Thomson, J.A., 2011. Chemically defined conditions for human iPSC derivation and culture. *Nature Methods*, 8 (5), 424–429.

Chen, Y.-H., Wang, Y.-H., Chang, M.-Y., Lin, C.-Y., Weng, C.-W., Westerfield, M., and Tsai, H.-J., 2007. Multiple upstream modules regulate zebrafish myf5 expression. *BMC developmental biology*, 7, 1.

Cheng, H., Liang, P.H., and Cheng, T., 2013. Mouse hematopoietic stem cell transplantation. *Methods in Molecular Biology (Clifton, N.J.)*, 976, 25–35.

Clements, W.K., Kim, A.D., Ong, K.G., Moore, J.C., Lawson, N.D., and Traver, D., 2011a. A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. *Nature*, 474 (7350), 220–224.

Clements, W.K., Kim, A.D., Ong, K.G., Moore, J.C., Lawson, N.D., and Traver, D., 2011b. A somitic Wnt16/Notch pathway specifies haematopoietic stem cells. *Nature*, 474 (7350), 220–224.

Clements, W.K., Ong, K.G., and Traver, D., 2009. Zebrafish wnt3 is expressed in developing neural tissue. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, 238 (7), 1788–1795.

Clements, W.K. and Traver, D., 2013. Signalling pathways that control vertebrate haematopoietic stem cell specification. *Nature Reviews Immunology*, 13 (5), 336–348.

Clevers, H. and Nusse, R., 2012. Wnt/β-catenin signaling and disease. *Cell*, 149 (6), 1192–1205.

Cole, A.M., Myant, K., Reed, K.R., Ridgway, R.A., Athineos, D., Van den Brink, G.R., Muncan, V., Clevers, H., Clarke, A.R., Sicinski, P., and Sansom, O.J., 2010. Cyclin D2-cyclin-dependent kinase 4/6 is required for efficient proliferation and tumorigenesis following Apc loss. *Cancer Research*, 70 (20), 8149–8158.

Cortes, M., Chen, M.J., Stachura, D.L., Liu, S.Y., Kwan, W., Wright, F., Vo, L.T., Theodore, L.N., Esain, V., Frost, I.M., Schlaeger, T.M., Goessling, W., Daley, G.Q., and North, T.E., 2016. Developmental Vitamin D Availability Impacts Hematopoietic Stem Cell Production. *Cell reports*, 17 (2), 458–468.

Curtin, E., Hickey, G., Kamel, G., Davidson, A.J., and Liao, E.C., 2011. Zebrafish wnt9a is expressed in pharyngeal ectoderm and is required for palate and lower jaw development. *Mechanisms of Development*, 128 (1–2), 104–115.

Daniels, D.L. and Weis, W.I., 2005. Beta-catenin directly displaces Groucho/TLE repressors from Tcf/Lef in Wnt-mediated transcription activation. *Nature Structural & Molecular Biology*, 12 (4), 364–371.

Davidson, A.J. and Zon, L.I., 2004. The 'definitive' (and 'primitive') guide to zebrafish hematopoiesis. *Oncogene*, 23 (43), 7233–7246.

Delgado, M.D. and León, J., 2010. Myc Roles in Hematopoiesis and Leukemia. *Genes & Cancer*, 1 (6), 605–616.

Dijksterhuis, J.P., Baljinnyam, B., Stanger, K., Sercan, H.O., Ji, Y., Andres, O., Rubin, J.S., Hannoush, R.N., and Schulte, G., 2015. Systematic mapping of WNT-FZD protein interactions reveals functional selectivity by distinct WNT-FZD pairs. *The Journal of Biological Chemistry*, 290 (11), 6789–6798.

Distel, M., Wullimann, M.F., and Köster, R.W., 2009. Optimized Gal4 genetics for permanent gene expression mapping in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (32), 13365–13370.

Ditadi, A., Sturgeon, C.M., and Keller, G., 2017. A view of human haematopoietic development from the Petri dish. *Nature Reviews Molecular Cell Biology*, 18 (1), 56–67.

Ditadi, A., Sturgeon, C.M., Tober, J., Awong, G., Kennedy, M., Yzaguirre, A.D., Azzola, L., Ng, E.S., Stanley, E.G., French, D.L., Cheng, X., Gadue, P., Speck, N.A., Elefanty, A.G., and Keller, G., 2015. Human definitive haemogenic endothelium and arterial vascular endothelium represent distinct lineages. *Nature Cell Biology*, 17 (5), 580–591.

Doherty, J.E., Huye, L.E., Yusa, K., Zhou, L., Craig, N.L., and Wilson, M.H., 2011. Hyperactive piggyBac Gene Transfer in Human Cells and In Vivo. *Human Gene Therapy*, 23 (3), 311–320.

Doulatov, S., Vo, L.T., Chou, S.S., Kim, P.G., Arora, N., Li, H., Hadland, B.K., Bernstein, I.D., Collins, J.J., Zon, L.I., and Daley, G.Q., 2013. Induction of multipotential hematopoietic progenitors from human pluripotent stem cells via respecification of lineage-restricted precursors. *Cell Stem Cell*, 13 (4), 459–470.

Eilken, H.M., Nishikawa, S.-I., and Schroeder, T., 2009. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature*, 457 (7231), 896–900.

Espín-Palazón, R., Stachura, D.L., Campbell, C.A., García-Moreno, D., Del Cid, N., Kim, A.D., Candel, S., Meseguer, J., Mulero, V., and Traver, D., 2014. Proinflammatory signaling regulates hematopoietic stem cell emergence. *Cell*, 159 (5), 1070–1085.

Famili, F., Naber, B.A.E., Vloemans, S., de Haas, E.F.E., Tiemessen, M.M., and Staal, F.J.T., 2015. Discrete roles of canonical and non-canonical Wnt signaling in hematopoiesis and lymphopoiesis. *Cell Death & Disease*, 6 (11), e1981.

Fernandez, A., Huggins, I.J., Perna, L., Brafman, D., Lu, D., Yao, S., Gaasterland, T., Carson, D.A., and Willert, K., 2014. The WNT receptor FZD7 is required for maintenance of the pluripotent state in human embryonic stem cells. *Proceedings of the National Academy of Sciences*, 111 (4), 1409–1414.

Fleming, H.E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K.M., Kronenberg, H.M., and Scadden, D.T., 2008. Wnt Signaling in the Niche Enforces Hematopoietic Stem Cell Quiescence and Is Necessary to Preserve Self-Renewal In Vivo. *Cell Stem Cell*, 2 (3), 274–283.

Fouquet, B., Weinstein, B.M., Serluca, F.C., and Fishman, M.C., 1997. Vessel Patterning in the Embryo of the Zebrafish: Guidance by Notochord. *Developmental Biology*, 183 (1), 37–48.

Frame, J.M., Fegan, K.H., Conway, S.J., McGrath, K.E., and Palis, J., 2016. Definitive Hematopoiesis in the Yolk Sac Emerges from Wnt-Responsive Hemogenic Endothelium Independently of Circulation and Arterial Identity. *STEM CELLS*, 34 (2), 431–444.

Fujita, M., Cha, Y.R., Pham, V.N., Sakurai, A., Roman, B.L., Gutkind, J.S., and Weinstein, B.M., 2011. Assembly and patterning of the vascular network of the vertebrate hindbrain. *Development*, 138 (9), 1705–1715.

Genthe, J.R. and Clements, W.K., 2017. R-spondin-1 is required for specification of hematopoietic stem cells through Wnt16 and Vegfa signaling pathways. *Development*, dev.139956.

Gertow, K., Hirst, C.E., Yu, Q.C., Ng, E.S., Pereira, L.A., Davis, R.P., Stanley, E.G., and Elefanty, A.G., 2013. WNT3A Promotes Hematopoietic or Mesenchymal Differentiation from hESCs Depending on the Time of Exposure. *Stem Cell Reports*, 1 (1), 53–65.

Gilbert, M.J.H., Zerulla, T.C., and Tierney, K.B., 2014. Zebrafish (Danio rerio) as a model for the study of aging and exercise: Physical ability and trainability decrease with age. *Experimental Gerontology*, 50 (Supplement C), 106–113.

Goessling, W., Allen, R.S., Guan, X., Jin, P., Uchida, N., Dovey, M., Harris, J.M., Metzger, M.E., Bonifacino, A.C., Stroncek, D., Stegner, J., Armant, M., Schlaeger, T., Tisdale, J.F., Zon, L.I., Donahue, R.E., and North, T.E., 2011. Prostaglandin E2 Enhances Human Cord Blood Stem Cell Xenotransplants and Shows Long-Term Safety in Preclinical Nonhuman Primate Transplant Models. *Cell Stem Cell*, 8 (4), 445–458.

Goessling, W., North, T.E., Loewer, S., Lord, A.M., Lee, S., Stoick-Cooper, C.L., Weidinger, G., Puder, M., Daley, G.Q., Moon, R.T., and Zon, L.I., 2009. Genetic Interaction of PGE2 and Wnt Signaling Regulates Developmental Specification of Stem Cells and Regeneration. *Cell*, 136 (6), 1136–1147.

Gori, J.L., Butler, J.M., Chan, Y.-Y., Chandrasekaran, D., Poulos, M.G., Ginsberg, M., Nolan, D.J., Elemento, O., Wood, B.L., Adair, J.E., Rafii, S., and Kiem, H.-P., 2015. Vascular niche promotes hematopoietic multipotent progenitor formation from pluripotent stem cells. *The Journal of Clinical Investigation*, 125 (3), 1243–1254.

Grainger, S., Richter, J., Palazón, R.E., Pouget, C., Lonquich, B., Wirth, S., Grassme, K.S., Herzog, W., Swift, M.R., Weinstein, B.M., Traver, D., and Willert, K., 2016. Wnt9a Is Required for the Aortic Amplification of Nascent Hematopoietic Stem Cells. *Cell Reports*, 17 (6), 1595–1606.

Hanson, K.D., Shichiri, M., Follansbee, M.R., and Sedivy, J.M., 1994. Effects of c-myc expression on cell cycle progression. *Molecular and Cellular Biology*, 14 (9), 5748–5755.

Hatzimichael, E. and Tuthill, M., 2010. Hematopoietic stem cell transplantation. *Stem Cells and Cloning : Advances and Applications*, 3, 105–117.

Heidelberger, C., Chaudhuri, N.K., Danneberg, P., Mooren, D., Griesbach, L., Duschinsky, R., Schnitzer, R.J., Pleven, E., and Scheiner, J., 1957. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature*, 179 (4561), 663–666.

Herbert, S.P., Huisken, J., Kim, T.N., Feldman, M.E., Houseman, B.T., Wang, R.A., Shokat, K.M., and Stainier, D.Y.R., 2009. Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. *Science (New York, N.Y.)*, 326 (5950), 294–298.

Holmen, S.L., Salic, A., Zylstra, C.R., Kirschner, M.W., and Williams, B.O., 2002. A Novel Set of Wnt-Frizzled Fusion Proteins Identifies Receptor Components That Activate β-Catenin-dependent Signaling. *Journal of Biological Chemistry*, 277 (38), 34727–34735.

van Impel, A., Zhao, Z., Hermkens, D.M.A., Roukens, M.G., Fischer, J.C., Peterson-Maduro, J., Duckers, H., Ober, E.A., Ingham, P.W., and Schulte-Merker, S., 2014. Divergence of zebrafish and mouse lymphatic cell fate specification pathways. *Development (Cambridge, England)*, 141 (6), 1228–1238.

Jagannathan-Bogdan, M. and Zon, L.I., 2013. Hematopoiesis. *Development (Cambridge, England)*, 140 (12), 2463–2467.

Jho, E., Zhang, T., Domon, C., Joo, C.-K., Freund, J.-N., and Costantini, F., 2002. Wnt/beta-catenin/Tcf signaling induces the transcription of Axin2, a negative regulator of the signaling pathway. *Molecular and Cellular Biology*, 22 (4), 1172–1183.

Jin, H., Xu, J., and Wen, Z., 2007. Migratory path of definitive hematopoietic stem/progenitor cells during zebrafish development. *Blood*, 109 (12), 5208–5214.

Jin, S.-W., Beis, D., Mitchell, T., Chen, J.-N., and Stainier, D.Y.R., 2005. Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development*, 132 (23), 5199–5209.

Kadowaki, T., Wilder, E., Klingensmith, J., Zachary, K., and Perrimon, N., 1996. The segment polarity gene porcupine encodes a putative multitransmembrane protein involved in Wingless processing. *Genes & Development*, 10 (24), 3116–3128.

Kamel, G., Hoyos, T., Rochard, L., Dougherty, M., Kong, Y., Tse, W., Shubinets, V., Grimaldi, M., and Liao, E.C., 2013. Requirement for frzb and fzd7a in cranial neural crest convergence and extension mechanisms during zebrafish palate and jaw morphogenesis. *Developmental biology*, 381 (2), 423–433.

Kato, S., Hayakawa, Y., Sakurai, H., Saiki, I., and Yokoyama, S., 2014. Mesenchymal-transitioned cancer cells instigate the invasion of epithelial cancer cells through secretion of WNT3 and WNT5B. *Cancer Science*, 105 (3), 281–289.

Kim, A.D., Melick, C.H., Clements, W.K., Stachura, D.L., Distel, M., Panáková, D., MacRae, C., Mork, L.A., Crump, J.G., and Traver, D., 2014. Discrete Notch signaling requirements in the specification of hematopoietic stem cells. *The EMBO journal*, 33 (20), 2363–2373.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E.W., and Nerlov, C., 2006. Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. *Nature Immunology*, 7 (10), 1048–1056.

Kissa, K. and Herbomel, P., 2010. Blood stem cells emerge from aortic endothelium by a novel type of cell transition. *Nature*, 464 (7285), 112–115.

Kissa, K., Murayama, E., Zapata, A., Cortés, A., Perret, E., Machu, C., and Herbomel, P., 2008. Live imaging of emerging hematopoietic stem cells and early thymus colonization. *Blood*, 111 (3), 1147–1156.

Kitajima, K., Nakajima, M., Kanokoda, M., Kyba, M., Dandapat, A., Tolar, J., Saito, M.K., Toyoda, M., Umezawa, A., and Hara, T., 2015. GSK3β inhibition activates the CDX/HOX pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells. *Experimental Hematology*.

Kobayashi, I., Kobayashi-Sun, J., Kim, A.D., Pouget, C., Fujita, N., Suda, T., and Traver, D., 2014. Jam1a-Jam2a interactions regulate haematopoietic stem cell fate through Notch signalling. *Nature*, 512 (7514), 319–323.

Kok, F.O., Shin, M., Ni, C.-W., Gupta, A., Grosse, A.S., van Impel, A., Kirchmaier, B.C., Peterson-Maduro, J., Kourkoulis, G., Male, I., DeSantis, D.F., Sheppard-Tindell, S., Ebarasi, L., Betsholtz, C., Schulte-Merker, S., Wolfe, S.A., and Lawson, N.D., 2015. Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. *Developmental Cell*, 32 (1), 97–108.

Kolligs, F.T., Hu, G., Dang, C.V., and Fearon, E.R., 1999. Neoplastic transformation of RK3E by mutant beta-catenin requires deregulation of Tcf/Lef transcription but not activation of c-myc expression. *Molecular and Cellular Biology*, 19 (8), 5696–5706.

Komekado, H., Yamamoto, H., Chiba, T., and Kikuchi, A., 2007. Glycosylation and palmitoylation of Wnt-3a are coupled to produce an active form of Wnt-3a. *Genes to Cells: Devoted to Molecular & Cellular Mechanisms*, 12 (4), 521–534.

Kumano, K., Chiba, S., Kunisato, A., Sata, M., Saito, T., Nakagami-Yamaguchi, E., Yamaguchi, T., Masuda, S., Shimizu, K., Takahashi, T., Ogawa, S., Hamada, Y., and Hirai, H., 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity*, 18 (5), 699–711.

Kumar, N., Richter, J., Cutts, J., Bush, K.T., Trujillo, C., Nigam, S.K., Gaasterland, T., Brafman, D., and Willert, K., 2015. Generation of an expandable intermediate mesoderm restricted progenitor cell line from human pluripotent stem cells. *eLife*, 4, e08413.

Kyba, M., Perlingeiro, R.C.R., and Daley, G.Q., 2002. HoxB4 Confers Definitive Lymphoid-Myeloid Engraftment Potential on Embryonic Stem Cell and Yolk Sac Hematopoietic Progenitors. *Cell*, 109 (1), 29–37.

Laurenti, E., Varnum-Finney, B., Wilson, A., Ferrero, I., Blanco-Bose, W.E., Ehninger, A., Knoepfler, P.S., Cheng, P.-F., MacDonald, H.R., Eisenman, R.N., Bernstein, I.D., and Trumpp, A., 2008. Hematopoietic Stem Cell Function and Survival Depend on c-Myc and N-Myc Activity. *Cell stem cell*, 3 (6), 611–624.

Lawson, N.D., Scheer, N., Pham, V.N., Kim, C.H., Chitnis, A.B., Campos-Ortega, J.A., and Weinstein, B.M., 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development (Cambridge, England)*, 128 (19), 3675–3683.

Lawson, N.D. and Weinstein, B.M., 2002. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. *Developmental Biology*, 248 (2), 307–318.

Ledran, M.H., Krassowska, A., Armstrong, L., Dimmick, I., Renström, J., Lang, R., Yung, S., Santibanez-Coref, M., Dzierzak, E., Stojkovic, M., Oostendorp, R.A.J., Forrester, L., and Lako, M., 2008. Efficient Hematopoietic Differentiation of Human Embryonic Stem Cells on Stromal Cells Derived from Hematopoietic Niches. *Cell Stem Cell*, 3 (1), 85–98.

Lee, Y., Manegold, J.E., Kim, A.D., Pouget, C., Stachura, D.L., Clements, W.K., and Traver, D., 2014. FGF signalling specifies haematopoietic stem cells through its regulation of somitic Notch signalling. *Nature Communications*, 5.

Lengerke, C., Schmitt, S., Bowman, T.V., Jang, I.H., Maouche-Chretien, L., McKinney-Freeman, S., Davidson, A.J., Hammerschmidt, M., Rentzsch, F., Green, J.B.A., Zon, L.I., and Daley, G.Q., 2008. BMP and Wnt Specify Hematopoietic Fate by Activation of the Cdx-Hox Pathway. *Cell Stem Cell*, 2 (1), 72–82.

Lento, W., Congdon, K., Voermans, C., Kritzik, M., and Reya, T., 2013. Wnt Signaling in Normal and Malignant Hematopoiesis. *Cold Spring Harbor Perspectives in Biology*, 5 (2), a008011.

Leung, A., Ciau-Uitz, A., Pinheiro, P., Monteiro, R., Zuo, J., Vyas, P., Patient, R., and Porcher, C., 2013. Uncoupling VEGFA functions in arteriogenesis and hematopoietic stem cell specification. *Developmental Cell*, 24 (2), 144–158.

Lewis, J.L., Bonner, J., Modrell, M., Ragland, J.W., Moon, R.T., Dorsky, R.I., and Raible, D.W., 2004. Reiterated Wnt signaling during zebrafish neural crest. *Development*, 131 (6), 1299–1308.

Li, H., Malbon, C.C., and Wang, H.-Y., 2004. Gene profiling of Frizzled-1 and Frizzled-2 signaling: expression of G-protein-coupled receptor chimeras in mouse F9 teratocarcinoma embryonal cells. *Molecular Pharmacology*, 65 (1), 45–55.

Li, X., Burnight, E.R., Cooney, A.L., Malani, N., Brady, T., Sander, J.D., Staber, J., Wheelan, S.J., Joung, J.K., McCray, P.B., Bushman, F.D., Sinn, P.L., and Craig, N.L., 2013. piggyBac transposase tools for genome engineering. *Proceedings of the National Academy of Sciences*, 110 (25), E2279–E2287.

Liao, W., Bisgrove, B.W., Sawyer, H., Hug, B., Bell, B., Peters, K., Grunwald, D.J., and Stainier, D.Y., 1997. The zebrafish gene cloche acts upstream of a flk-1 homologue to regulate endothelial cell differentiation. *Development (Cambridge, England)*, 124 (2), 381–389.

Loh, K.M., van Amerongen, R., and Nusse, R., 2016. Generating Cellular Diversity and Spatial Form: Wnt Signaling and the Evolution of Multicellular Animals. *Developmental Cell*, 38 (6), 643–655.

Lu, M., Kardel, M.D., O'Connor, M.D., and Eaves, C.J., 2009. Enhanced generation of hematopoietic cells from human hepatocarcinoma cell-stimulated human embryonic and induced pluripotent stem cells. *Experimental Hematology*, 37 (8), 924–936.

Luis, T.C., Naber, B.A.E., Fibbe, W.E., Dongen, J.J.M. van, and Staal, F.J.T., 2010. Wnt3a nonredundantly controls hematopoietic stem cell function and its deficiency results in complete absence of canonical Wnt signaling. *Blood*, 116 (3), 496–497.

Luis, T.C., Naber, B.A.E., Roozen, P.P.C., Brugman, M.H., de Haas, E.F.E., Ghazvini, M., Fibbe, W.E., van Dongen, J.J.M., Fodde, R., and Staal, F.J.T., 2011. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. *Cell stem cell*, 9 (4), 345–356.

Luis, T.C., Weerkamp, F., Naber, B.A.E., Baert, M.R.M., de Haas, E.F.E., Nikolic, T., Heuvelmans, S., De Krijger, R.R., van Dongen, J.J.M., and Staal, F.J.T., 2009a. Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood*, 113 (3), 546–554.

Luis, T.C., Weerkamp, F., Naber, B.A.E., Baert, M.R.M., de Haas, E.F.E., Nikolic, T., Heuvelmans, S., De Krijger, R.R., van Dongen, J.J.M., and Staal, F.J.T., 2009b. Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood*, 113 (3), 546–554.

Malhotra, S., Baba, Y., Garrett, K.P., Staal, F.J.T., Gerstein, R., and Kincade, P.W., 2008. Contrasting responses of lymphoid progenitors to canonical and noncanonical Wnt signals. *Journal of Immunology (Baltimore, Md.: 1950)*, 181 (6), 3955–3964.

Mateyak, M.K., Obaya, A.J., and Sedivy, J.M., 1999. c-Myc regulates cyclin D-Cdk4 and -Cdk6 activity but affects cell cycle progression at multiple independent points. *Molecular and Cellular Biology*, 19 (7), 4672–4683.

Matsumoto, K., Miki, R., Nakayama, M., Tatsumi, N., and Yokouchi, Y., 2008. Wnt9a secreted from the walls of hepatic sinusoids is essential for morphogenesis, proliferation, and glycogen accumulation of chick hepatic epithelium. *Developmental Biology*, 319 (2), 234–247.

McCormack, J.E., Pepe, V.H., Kent, R.B., Dean, M., Marshak-Rothstein, A., and Sonenshein, G.E., 1984. Specific regulation of c-myc oncogene expression in a murine B-cell lymphoma. *Proceedings of the National Academy of Sciences of the United States of America*, 81 (17), 5546–5550.

Medvinsky, A., Rybtsov, S., and Taoudi, S., 2011. Embryonic origin of the adult hematopoietic system: advances and questions. *Development*, 138 (6), 1017–1031.

Mizuochi, C., Fraser, S.T., Biasch, K., Horio, Y., Kikushige, Y., Tani, K., Akashi, K., Tavian, M., and Sugiyama, D., 2012a. Intra-Aortic Clusters Undergo Endothelial to Hematopoietic Phenotypic Transition during Early Embryogenesis. *PLoS ONE*, 7 (4), e35763.

Mizuochi, C., Fraser, S.T., Biasch, K., Horio, Y., Kikushige, Y., Tani, K., Akashi, K., Tavian, M., and Sugiyama, D., 2012b. Intra-aortic clusters undergo endothelial to hematopoietic phenotypic transition during early embryogenesis. *PloS One*, 7 (4), e35763.

Moro, E., Ozhan-Kizil, G., Mongera, A., Beis, D., Wierzbicki, C., Young, R.M., Bournele, D., Domenichini, A., Valdivia, L.E., Lum, L., Chen, C., Amatruda, J.F., Tiso, N., Weidinger, G., and Argenton, F., 2012. In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. *Developmental Biology*, 366 (2), 327–340.

Muncan, V., Sansom, O.J., Tertoolen, L., Phesse, T.J., Begthel, H., Sancho, E., Cole, A.M., Gregorieff, A., de Alboran, I.M., Clevers, H., and Clarke, A.R., 2006. Rapid loss of intestinal crypts upon conditional deletion of the Wnt/Tcf-4 target gene c-Myc. *Molecular and Cellular Biology*, 26 (22), 8418–8426.

Murayama, E., Kissa, K., Zapata, A., Mordelet, E., Briolat, V., Lin, H.-F., Handin, R.I., and Herbomel, P., 2006. Tracing Hematopoietic Precursor Migration to Successive Hematopoietic Organs during Zebrafish Development. *Immunity*, 25 (6), 963–975.

Naito, A.T., Shiojima, I., Akazawa, H., Hidaka, K., Morisaki, T., Kikuchi, A., and Komuro, I., 2006. Developmental stage-specific biphasic roles of Wnt/β-catenin signaling in cardiomyogenesis and hematopoiesis. *Proceedings of the National Academy of Sciences*, 103 (52), 19812–19817.

Naujok, O., Lentes, J., Diekmann, U., Davenport, C., and Lenzen, S., 2014. Cytotoxicity and activation of the Wnt/beta-catenin pathway in mouse embryonic stem cells treated with four GSK3 inhibitors. *BMC Research Notes*, 7, 273.

Ng, E.S., Azzola, L., Bruveris, F.F., Calvanese, V., Phipson, B., Vlahos, K., Hirst, C., Jokubaitis, V.J., Yu, Q.C., Maksimovic, J., Liebscher, S., Januar, V., Zhang, Z., Williams, B., Conscience, A., Durnall, J., Jackson, S., Costa, M., Elliott, D., Haylock, D.N., Nilsson, S.K., Saffery, R., Schenke-Layland, K., Oshlack, A., Mikkola, H.K.A., Stanley, E.G., and Elefanty, A.G., 2016. Differentiation of human embryonic stem cells to HOXA+ hemogenic vasculature that resembles the aorta-gonad-mesonephros. *Nature Biotechnology*, 34 (11), 1168–1179.

Ng, E.S., Davis, R., Stanley, E.G., and Elefanty, A.G., 2008. A protocol describing the use of a recombinant protein-based, animal product-free medium (APEL) for human embryonic stem cell differentiation as spin embryoid bodies. *Nature Protocols*, 3 (5), 768–776.

Ng, E.S., Davis, R.P., Azzola, L., Stanley, E.G., and Elefanty, A.G., 2005. Forced aggregation of defined numbers of human embryonic stem cells into embryoid bodies fosters robust, reproducible hematopoietic differentiation. *Blood*, 106 (5), 1601–1603.

North, T.E., Goessling, W., Walkley, C.R., Lengerke, C., Kopani, K.R., Lord, A.M., Weber, G.J., Bowman, T.V., Jang, I.-H., Grosser, T., Fitzgerald, G.A., Daley, G.Q., Orkin, S.H., and Zon, L.I., 2007. Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis. *Nature*, 447 (7147), 1007–1011.

Nostro, M.C., Cheng, X., Keller, G.M., and Gadue, P., 2008. Wnt, Activin, and BMP Signaling Regulate Distinct Stages in the Developmental Pathway from Embryonic Stem Cells to Blood. *Cell Stem Cell*, 2 (1), 60–71.

Nusse, R., 2001. An ancient cluster of Wnt paralogues. *Trends in genetics: TIG*, 17 (8), 443.

Orelio, C. and Dzierzak, E., 2003. Identification of 2 novel genes developmentally regulated in the mouse aorta-gonad-mesonephros region. *Blood*, 101 (6), 2246–2249.

Palomero, T., Lim, W.K., Odom, D.T., Sulis, M.L., Real, P.J., Margolin, A., Barnes, K.C., O'Neil, J., Neuberg, D., Weng, A.P., Aster, J.C., Sigaux, F., Soulier, J., Look, A.T., Young, R.A., Califano, A., and Ferrando, A.A., 2006. NOTCH1 directly regulates c-MYC and activates a feed-forward-loop transcriptional network promoting leukemic cell growth. *Proceedings of the National Academy of Sciences of the United States of America*, 103 (48), 18261–18266.

Parsons, M.J., Pisharath, H., Yusuff, S., Moore, J.C., Siekmann, A.F., Lawson, N., and Leach, S.D., 2009. Notch-responsive cells initiate the secondary transition in larval zebrafish pancreas. *Mechanisms of Development*, 126 (10), 898–912.

Peters, A., Burridge, P.W., Pryzhkova, M.V., Levine, M.A., Park, T.-S., Roxbury, C., Yuan, X., Peault, B., and Zambidis, E.T., 2010. Challenges and strategies for generating therapeutic patient-specific hemangioblasts and hematopoietic stem cells from human pluripotent stem cells. *The International Journal of Developmental Biology*, 54 (6–7), 965–990.

Pouget, C., Peterkin, T., Simões, F.C., Lee, Y., Traver, D., and Patient, R., 2014. FGF signalling restricts haematopoietic stem cell specification via modulation of the BMP pathway. *Nature Communications*, 5.

Proffitt, K.D., Madan, B., Ke, Z., Pendharkar, V., Ding, L., Lee, M.A., Hannoush, R.N., and Virshup, D.M., 2013. Pharmacological inhibition of the Wnt acyltransferase PORCN prevents growth of WNT-driven mammary cancer. *Cancer Research*, 73 (2), 502–507.

Rabbitts, P.H., Watson, J.V., Lamond, A., Forster, A., Stinson, M.A., Evan, G., Fischer, W., Atherton, E., Sheppard, R., and Rabbitts, T.H., 1985. Metabolism of c-myc gene products: c-myc mRNA and protein expression in the cell cycle. *The EMBO journal*, 4 (8), 2009–2015.

Rai, M., Walthall, J.M., Hu, J., and Hatzopoulos, A.K., 2012. Continuous antagonism by Dkk1 counter activates canonical Wnt signaling and promotes cardiomyocyte differentiation of embryonic stem cells. *Stem Cells and Development*, 21 (1), 54–66.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L., 2003. A role for Wnt signalling in self-renewal of haematopoietic stem cells. *Nature*, 423 (6938), 409–414.

Richter, J., Traver, D., and Willert, K., 2017. The role of Wnt signaling in hematopoietic stem cell development. *Critical Reviews in Biochemistry and Molecular Biology*, 52 (4), 414–424.

Riddell, J., Gazit, R., Garrison, B.S., Guo, G., Saadatpour, A., Mandal, P.K., Ebina, W., Volchkov, P., Yuan, G.-C., Orkin, S.H., and Rossi, D.J., 2014. Reprogramming Committed Murine Blood Cells to Induced Hematopoietic Stem Cells with Defined Factors. *Cell*, 158 (1), 226.

Rochard, L., Monica, S.D., Ling, I.T.C., Kong, Y., Roberson, S., Harland, R., Halpern, M., and Liao, E.C., 2016. Roles of Wnt pathway genes wls, wnt9a, wnt5b, frzb and gpc4 in regulating convergent-extension during zebrafish palate morphogenesis. *Development (Cambridge, England)*, 143 (14), 2541–2547.

Rossi, A., Kontarakis, Z., Gerri, C., Nolte, H., Hölper, S., Krüger, M., and Stainier, D.Y.R., 2015. Genetic compensation induced by deleterious mutations but not gene knockdowns. *Nature*, 524 (7564), nature14580.

Rowlinson, J.M. and Gering, M., 2010. Hey2 acts upstream of Notch in hematopoietic stem cell specification in zebrafish embryos. *Blood*, 116 (12), 2046–2056.

Ruiz-Herguido, C., Guiu, J., D'Altri, T., Inglés-Esteve, J., Dzierzak, E., Espinosa, L., and Bigas, A., 2012a. Hematopoietic stem cell development requires transient Wnt/β-catenin activity. *The Journal of Experimental Medicine*, 209 (8), 1457–1468.

Ruiz-Herguido, C., Guiu, J., D'Altri, T., Inglés-Esteve, J., Dzierzak, E., Espinosa, L., and Bigas, A., 2012b. Hematopoietic stem cell development requires transient Wnt/β-catenin activity. *The Journal of Experimental Medicine*, 209 (8), 1457–1468.

Rulifson, E.J., Wu, C.H., and Nusse, R., 2000. Pathway specificity by the bifunctional receptor frizzled is determined by affinity for wingless. *Molecular Cell*, 6 (1), 117–126.

Sandler, V.M., Lis, R., Liu, Y., Kedem, A., James, D., Elemento, O., Butler, J.M., Scandura, J.M., and Rafii, S., 2014. Reprogramming human endothelial cells to haematopoietic cells requires vascular induction. *Nature*, 511 (7509), 312–318.

Sansom, O.J., Meniel, V.S., Muncan, V., Phesse, T.J., Wilkins, J.A., Reed, K.R., Vass, J.K., Athineos, D., Clevers, H., and Clarke, A.R., 2007. Myc deletion rescues Apc deficiency in the small intestine. *Nature*, 446 (7136), 676–679.

Sato, T., van Es, J.H., Snippert, H.J., Stange, D.E., Vries, R.G., van den Born, M., Barker, N., Shroyer, N.F., van de Wetering, M., and Clevers, H., 2011. Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. *Nature*, 469 (7330), 415–418.

Schefe, J.H., Lehmann, K.E., Buschmann, I.R., Unger, T., and Funke-Kaiser, H., 2006. Quantitative real-time RT-PCR data analysis: current concepts and the novel 'gene expression's CT difference' formula. *Journal of Molecular Medicine*, 84 (11), 901–910.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A., 2006. Hematopoietic stem cell and multilineage defects generated by constitutive β-catenin activation. *Nature Immunology*, 7 (10), 1037–1047.

Schulte-Merker, S. and Stainier, D.Y.R., 2014. Out with the old, in with the new: reassessing morpholino knockdowns in light of genome editing technology. *Development*, 141 (16), 3103–3104.

Slukvin, I.I., 2013. Hematopoietic specification from human pluripotent stem cells: current advances and challenges toward de novo generation of hematopoietic stem cells. *Blood*, 122 (25), 4035–4046.

Sturgeon, C.M., Ditadi, A., Awong, G., Kennedy, M., and Keller, G., 2014. Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nature Biotechnology*, 32 (6), 554–561.

Sugimura, R., Jha, D.K., Han, A., Soria-Valles, C., da Rocha, E.L., Lu, Y.-F., Goettel, J.A., Serrao, E., Rowe, R.G., Malleshaiah, M., Wong, I., Sousa, P., Zhu, T.N., Ditadi, A., Keller, G., Engelman, A.N., Snapper, S.B., Doulatov, S., and Daley, G.Q., 2017. Haematopoietic stem and progenitor cells from human pluripotent stem cells. *Nature*, 545 (7655), 432–438.

Sugiyama, M., Sakaue-Sawano, A., Iimura, T., Fukami, K., Kitaguchi, T., Kawakami, K., Okamoto, H., Higashijima, S., and Miyawaki, A., 2009. Illuminating cell-cycle progression in the developing zebrafish embryo. *Proceedings of the National Academy of Sciences of the United States of America*, 106 (49), 20812–20817.

Sumi, T., Tsuneyoshi, N., Nakatsuji, N., and Suemori, H., 2008. Defining early lineage specification of human embryonic stem cells by the orchestrated balance of canonical Wnt/beta-catenin, Activin/Nodal and BMP signaling. *Development (Cambridge, England)*, 135 (17), 2969–2979.

Swift, M.R., Pham, V.N., Castranova, D., Bell, K., Poole, R.J., and Weinstein, B.M., 2014. SoxF factors and Notch regulate nr2f2 gene expression during venous differentiation in zebrafish. *Developmental Biology*, 390 (2), 116–125.

Takada, R., Satomi, Y., Kurata, T., Ueno, N., Norioka, S., Kondoh, H., Takao, T., and Takada, S., 2006. Monounsaturated fatty acid modification of Wnt protein: its role in Wnt secretion. *Developmental Cell*, 11 (6), 791–801.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S., 2007. Induction of Pluripotent Stem Cells from Adult Human Fibroblasts by Defined Factors. *Cell*, 131 (5), 861–872.

Tamplin, O.J., Durand, E.M., Carr, L.A., Childs, S.J., Hagedorn, E.J., Li, P., Yzaguirre, A.D., Speck, N.A., and Zon, L.I., 2015. Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular niche. *Cell*, 160 (1–2), 241–252.

Thorpe, C.J., Weidinger, G., and Moon, R.T., 2005. Wnt/beta-catenin regulation of the Sp1-related transcription factor sp5l promotes tail development in zebrafish. *Development (Cambridge, England)*, 132 (8), 1763–1772.

Turpen, J.B., Knudson, C.M., and Hoefen, P.S., 1981. The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: Localization of a prospective stem cell compartment. *Developmental Biology*, 85 (1), 99–112.

Vo, L.T. and Daley, G.Q., 2015. De novo generation of HSCs from somatic and pluripotent stem cell sources. *Blood*, 125 (17), 2641–2648.

Voloshanenko, O., Erdmann, G., Dubash, T.D., Augustin, I., Metzig, M., Moffa, G., Hundsrucker, C., Kerr, G., Sandmann, T., Anchang, B., Demir, K., Boehm, C., Leible, S., Ball, C.R., Glimm, H., Spang, R., and Boutros, M., 2013. Wnt secretion is required to maintain high levels of Wnt activity in colon cancer cells. *Nature Communications*, 4, ncomms3610.

Voloshanenko, O., Gmach, P., Winter, J., Kranz, D., and Boutros, M., 2017. Mapping of Wnt-Frizzled interactions by multiplex CRISPR targeting of receptor gene families. *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*.

Wang, H., Gilner, J.B., Bautch, V.L., Wang, D.-Z., Wainwright, B.J., Kirby, S.L., and Patterson, C., 2007. Wnt2 coordinates the commitment of mesoderm to hematopoietic, endothelial, and cardiac lineages in embryoid bodies. *The Journal of Biological Chemistry*, 282 (1), 782–791.

Wang, L., Menendez, P., Shojaei, F., Li, L., Mazurier, F., Dick, J.E., Cerdan, C., Levac, K., and Bhatia, M., 2005. Generation of hematopoietic repopulating cells from human embryonic stem cells independent of ectopic HOXB4 expression. *The Journal of Experimental Medicine*, 201 (10), 1603–1614.

Wang, S., Yin, J., Chen, D., Nie, F., Song, X., Fei, C., Miao, H., Jing, C., Ma, W., Wang, L., Xie, S., Li, C., Zeng, R., Pan, W., Hao, X., and Li, L., 2013. Small-molecule modulation of Wnt signaling via modulating the Axin-LRP5/6 interaction. *Nature Chemical Biology*, 9 (9), 579–585.

Wang, X., Moon, J., Dodge, M.E., Pan, X., Zhang, L., Hanson, J.M., Tuladhar, R., Ma, Z., Shi, H., Williams, N.S., Amatruda, J.F., Carroll, T.J., Lum, L., and Chen, C., 2013. The development of highly potent inhibitors for porcupine. *Journal of Medicinal Chemistry*, 56 (6), 2700–2704.

Wang, Y. and Nakayama, N., 2009. WNT and BMP signaling are both required for hematopoietic cell development from human ES cells. *Stem Cell Research*, 3 (2–3), 113–125.

van de Water, S., van de Wetering, M., Joore, J., Esseling, J., Bink, R., Clevers, H., and Zivkovic, D., 2001. Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development (Cambridge, England)*, 128 (20), 3877–3888.

Wilkinson, R.N., Pouget, C., Gering, M., Russell, A.J., Davies, S.G., Kimelman, D., and Patient, R., 2009. Hedgehog and Bmp polarize hematopoietic stem cell emergence in the zebrafish dorsal aorta. *Developmental Cell*, 16 (6), 909–916.

Willert, K., Brown, J.D., Danenberg, E., Duncan, A.W., Weissman, I.L., Reya, T., Yates, J.R., and Nusse, R., 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. *Nature*, 423 (6938), 448–452.

Wognum, B., Yuan, N., Lai, B., and Miller, C.L., 2013. Colony forming cell assays for human hematopoietic progenitor cells. *Methods in Molecular Biology (Clifton, N.J.)*, 946, 267–283.

Woll, P.S., Morris, J.K., Painschab, M.S., Marcus, R.K., Kohn, A.D., Biechele, T.L., Moon, R.T., and Kaufman, D.S., 2008. Wnt signaling promotes hematoendothelial cell development from human embryonic stem cells. *Blood*, 111 (1), 122–131.

Wu, J.Q., Seay, M., Schulz, V.P., Hariharan, M., Tuck, D., Lian, J., Du, J., Shi, M., Ye, Z., Gerstein, M., Snyder, M.P., and Weissman, S., 2012. Tcf7 Is an Important Regulator of the Switch of Self-Renewal and Differentiation in a Multipotential Hematopoietic Cell Line. *PLoS Genet*, 8 (3), e1002565.

Yang-Snyder, J., Miller, J.R., Brown, J.D., Lai, C.J., and Moon, R.T., 1996. A frizzled homolog functions in a vertebrate Wnt signaling pathway. *Current biology: CB*, 6 (10), 1302–1306.

Yusa, K., Zhou, L., Li, M.A., Bradley, A., and Craig, N.L., 2011. A hyperactive piggyBac transposase for mammalian applications. *Proceedings of the National Academy of Sciences*, 108 (4), 1531–1536.

Zhao, C., Blum, J., Chen, A., Kwon, H.Y., Jung, S.H., Cook, J.M., Lagoo, A., and Reya, T., 2007. Loss of β-Catenin Impairs the Renewal of Normal and CML Stem Cells In Vivo. *Cancer Cell*, 12 (6), 528–541.

Zhen, F., Lan, Y., Yan, B., Zhang, W., and Wen, Z., 2013. Hemogenic endothelium specification and hematopoietic stem cell maintenance employ distinct Scl isoforms. *Development (Cambridge, England)*, 140 (19), 3977–3985.

Zhou, H., Mak, W., Kalak, R., Street, J., Fong-Yee, C., Zheng, Y., Dunstan, C.R., and Seibel, M.J., 2009. Glucocorticoid-dependent Wnt signaling by mature osteoblasts is a key regulator of cranial skeletal development in mice. *Development (Cambridge, England)*, 136 (3), 427–436.