Metabolic Regulation of Hematopoietic Stem Cell Production and Maintenance

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Abstract

Metabolic disorders are a leading cause of morbidity and mortality, with gestational diabetes impacting embryogenesis. Intriguingly, children born to diabetic mothers have a higher risk of developing childhood leukemia, suggesting increased blood sugar concentrations may have lasting hematological impact. Hematopoietic stem cells (HSCs), born during embryogenesis, are capable of both self-renewal and differentiation into mature blood cell types for the life of an organism; yet, the impact of glucose elevation on hematopoietic system development is unclear. We recently showed transient glucose elevation elicited dose-dependent effects on HSCs through metabolic stimulation and subsequent ROS-mediated induction of Hypoxia Inducible Factor-1α (Hif1α). Platelet Derived Growth Factor-B (pdgfb), a Hif1α-target, and its receptor, pdgfrb, were significantly upregulated in response to metabolic induction. Morpholino (MO) knockdown of pdgfrb blocked HSC elevation by Hif1α-stimulation as determined by in situ hybridization (WISH) for conserved HSC markers runx1 and cmyb; similar results were observed for the pan-PDGF inhibitor AG1295 and PDGFRβ-selective modifier DMPQ. Notably, overexpression of pdgfb enhanced runx1 expression in the AGM at 36hpf and cmyb in the CHT at 48hpf. A qRT-PCR survey of PDGF-B/PDGFRβ regulatory targets revealed a significant increase in IL-6 and its receptor (IL-6R). MO-mediated knockdown of il6 antagonized effects of pdgfb overexpression, while epistatic analysis indicated function downstream of Hif1α. Together, these findings define a Hif1α-regulated signaling axis acting
via PDGFRβ and IL-6/IL-6R to control HSPC production. In contrast to responses to moderate flux in metabolic rate, chronic embryonic glucose elevation caused hematologic abnormalities, including elevated erythrocyte and myeloid cell numbers, with decreased lymphoid production, as seen by WISH, qPCR, and FACS. This is due in part to Hif1α-mediated transcriptional regulation, as determined by exposure to the Hif1 antagonist YC1. Interestingly, ablation of islet cell-mediated insulin production antagonized myeloid lineage dysregulation. Further, phosphorylation of FOXOs1/3/4, downstream targets of insulin signaling, was increased in glucose treated embryos. Microarray analysis of CD41:GFP+ HSCs from embryos exposed to chronic glucose elevation identified a number of putative pathways contributing to lineage dysregulation. Together, these studies indicate both acute and chronic alterations in metabolic state affect HSCs and may further explain immunological phenotypes associated with gestational diabetes.
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Chapter 1:

Introduction
**Introduction**

**Hematopoietic Stem Cells**

Hematopoietic stem cells (HSCs) have the ability to both self-renew and differentiate into all the mature blood cell lineages, including erythroid, myeloid, and lymphoid cells, and thereby reconstitute the entire blood system for the lifetime of the host (Figure 1.1). Since mature blood cells are predominantly short-lived, HSCs serve to continuously provide more differentiated progenitors while properly maintaining the HSC pool size by precisely balancing self-renewal and differentiation (Seita and Weissman, 2010). The concept of self-renewing multipotent HSCs originated in the 1960s through the work of Till and McCulloch, who demonstrated that the blood system of irradiated mice could be reestablished by intravenously injected bone marrow cells and further demonstrated the clonal nature of spleen colonies derived from transplanted mouse marrow cells (McCulloch and Till, 1960; Till and Mc, 1961; Weissman and Shizuru, 2008). Since then, the concept of self-renewing multipotent HSCs, the cells at the top of the hierarchy of the ‘blood lineage tree’, has become the archetype in the field of stem cell biology and more importantly has led to the development of new therapeutic methods to treat blood related disease, such as leukemia and bone marrow failure (Myers and Davies, 2009).
Figure 1.1
The hematopoietic stem cell (HSC) hierarchy showing that the HSC gives rise to all the lineages in the blood system, including erythroid, myeloid, and lymphoid cells. (Adapted from Trista North).
Hematopoiesis in vertebrates

In vertebrates, hematopoiesis occurs in two distinct waves and is accompanied by shifting sites of production and colonization (Cumano and Godin, 2007) (Figure 1.2). The first wave, termed primitive, begins at embryonic day 7.5 (E7.5) in the extraembryonic yolk sac blood island in mammalian embryos. The primitive wave produces mostly erythroid cells, and is thought to be a transient mechanism to provide the embryo with oxygen as passive diffusion becomes inadequate. Although there is still a debate about whether the yolk sac can contribute to definitive hematopoiesis, it is generally believed that primitive and definitive hematopoiesis have separate origins in mammals (Zhang et al., 2013). The definitive wave, beginning at E10.5, produces the long-term repopulating hematopoietic cells that can give rise to erythroid, myeloid, and lymphoid cells. Definitive HSCs are initially generated during embryogenesis in vertebrates from hemogenic endothelium in the ventral wall dorsal aorta, within an intraembryonic termed the aorta-gonad-mesonephros (AGM) in mammals (Bertrand et al., 2010; Chen and Zon, 2009; Dzierzak and Speck, 2008; Kissa and Herbomel, 2010). During human gestation, a similar process occurs with HSCs emerging directly from the AGM from days 27 to 40 of gestation (Tavian and Peault, 2005). Once formed, the HSCs and progenitors that arise from the AGM will migrate to and colonize subsequent sites of definitive hematopoiesis including, the fetal liver, thymus, and spleen where they undergo further maturation and expansion (Mikkola and Orkin, 2006; Orkin and Zon, 2008). Eventually, induced by several signaling pathways and chemokines, the HSCs will lodge in the bone marrow at by E14.5, the site which will serve as the hematopoietic organ for the lifetime of the organism (Zhang et al., 2013). The murine placenta has been more recently shown to harbor a large pool of multi-potential progenitors and HSCs during midgestation, indicating that the placenta potentially has an important role in contributing
Figure 1.2
The sites of HSC production and maintenance change throughout embryonic development, but is conserved across vertebrate species. HSCs arise in the AGM and migrate to subsequent sites of hematopoiesis before reaching the bone marrow (human and mouse) or kidney marrow (zebrafish), the sites of adult hematopoiesis. (Adapted from Trista North).
to the establishment of HSCs (Alvarez-Silva et al., 2003; Mikkola et al., 2005; Ottersbach and Dzierzak, 2005). Studies in the mouse embryos have likewise demonstrated that the yolk sac contributes a second wave of hematopoiesis that consists of erythro-myeloid progenitors (EMP) that gives rise to erythroid, megakaryocyte, myeloid, and multipotent progenitors which contribute to hematopoietic progenitors that are later found in the fetal liver prior to definitive HSC formation (Chen et al., 2011; Palis et al., 1999). Recent lineage tracing experiments have also indicated that the yolk sac contributes to fetal liver myelopoiesis (Gomez Perdiguero et al., 2015); however, it is not clear which sites give rise to HSCs that eventually end up in the BM.

**Runx1 is a Critical Regulator of HSC Development**

Runx-related transcription factor 1 (Runx1), also known as Acute Myeloid Leukemia 1 (AML1) or Core Binding Factor α2 (CBFα2), is a DNA binding subunit of the core binding factor transcription factor family (together with Runx2 and Runx3) that forms a heterodimer with its non-DNA binding partner CBFβ (Growney et al., 2005). Runx1 is often a target of genetic alterations, including translocation, deletion and duplication, associated with human leukemias (Kalev-Zylinska et al., 2002; Koh et al., 2013). The importance of Runx1 in hematopoietic development was demonstrated by mouse knockout studies. Deletion of *Runx1* in mice leads to an absence of definitive hematopoiesis, central nervous system hemorrhaging, and embryonic lethality by E11.5-12.5 (Wang et al., 1996). Runx1 is expressed in the AGM at E10.5 and was found to be required for the formation of the first intra-aortic hematopoietic clusters (North et al., 1999) (Figure 1.3). Sorted cells from the AGM or fetal liver that were Runx1⁺, but not those that were Runx1⁻, were capable of adult hematopoietic reconstitution upon transplantation (North et al., 2002). Recent research has elucidated that Runx1 is required to control endothelial to
Figure 1.3
At E10.5, Runx1 (indicated in blue) begins to be expressed in the endothelial cells of the AGM and Umbilical and Vitelline Arteries, marking the first HSCs. (Adapted from Trista North).
hematopoietic transition (EHT) thus allowing definitive HSC production (Chen et al., 2009). The essential role of Runx1 in hematopoiesis is highly conserved across species, in both vertebrates and invertebrates (Kalev-Zylinska et al., 2002; Lebestky et al., 2000; Waltzer et al., 2003).

**Zebrafish as a Developmental Model Organism**

Zebrafish have emerged as an advantageous model system for the study of hematopoiesis for many reasons. First, due to their embryonic transparency and external fertilization, the whole process of embryogenesis can be visualized directly, including differentiation and migration of individual cells in vivo (Zhang et al., 2013). Second, high fecundity and rapid growth allows for large-scale forward genetic and/or chemical screens and genetic manipulation (Jing and Zon, 2011). Importantly, zebrafish exhibit strong evolutionary conservation with mammals for both hematopoietic regulatory genes and cell types (Carroll and North, 2014), ensuring its significant research value.

**Hematopoiesis is Highly Conserved in the Zebrafish Model**

In zebrafish, primitive hematopoiesis occurs between 12-24 hours post fertilization (hpf) in ventral mesoderm derived tissues in the intraembryonic regions called anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM), which will form the intermediate cell mass (ICM) (Detrich et al., 1995). (Figure 1.4) The ALM gives rise to primitive myelocytes, while the PLM generates the primitive erythrocytes. A transient population of hematopoietic cells called erythromyeloid progenitors (EMPs) that have myeloid and erythroid but not
Figure 1.4
The sites of hematopoiesis in the zebrafish: primitive erythrocytes in the zebrafish arise in the Inner Cell Mass (ICM). The first definitive HSCs are born in the Aorta-Gonad-Mesonephros (AGM), and subsequently migrate to the Caudal Hematopoietic Tissue (CHT), followed by the thymus and kidney marrow, which serve as sites of hematopoiesis in the adult. (Adapted from Trista North and David Traver).
lymphoid potential have also been described to arise before and independent of HSC specification (Bertrand et al., 2007). Definitive hematopoiesis initiates at 24 hpf with the specification of HSCs in the zebrafish AGM region (Zhang et al., 2013). Similar to the mouse, Runx1 is required for definite hematopoiesis in the zebrafish, indicating conservation of the factors regulating hematopoiesis (Kalev-Zylinska et al., 2002). Definitive HSC production, marked by runx1 and cmyb expression, appears robustly by 30hpf in the AGM (Orkin and Zon, 2008) derived from the ventral wall of the dorsal aorta through the process of EHT as in the mouse (Bertrand et al., 2010; Kissa and Herbomel, 2010). HSCs then colonize the caudal hematopoietic tissue (CHT), an intermediate site of hematopoiesis presumed to be equivalent to the mammalian fetal liver (Murayama et al., 2006). HSCs subsequently migrate to the thymus by 3 days post fertilization (dpf) to initiate lymphopoiesis. By 4 dpf, HSCs can be found in the pronephros (kidney marrow), the zebrafish equivalent of the mammalian bone marrow, where they will sustain lifelong hematopoiesis (Paik and Zon, 2010).

In addition to Runx1, cMyb is another transcription factor required for HSC self-renewal (Greig et al., 2010). cMyb belongs to a proto-oncogene family of transcription factors and in the mouse, its absence leads to embryonic death due to lack of hepatic erythropoiesis (Mucenski et al., 1991), indicating its primary role in definitive hematopoiesis. cMyb, through interaction with p300, controls the proliferation and differentiation of hematopoietic stem and progenitor cells (HSPCs) (Sandberg et al., 2005). cMyb is also essential for myeloid differentiation (Sakamoto et al., 2006). In the zebrafish, cmyb is coexpressed with runx1 in the AGM at 36 hpf and labels the HSC population; its expression is dependent on Runx1, suggesting a transcriptional hierarchy (Soza-Ried et al., 2010; Thompson et al., 1998). HSC maturation, including the ability to migrate and home to the niche, is indicated by the expression of itga2b (also known as CD41) (Bertrand
et al., 2008; Lin et al., 2005), first shown to be required for developmental HSC function in the mouse (Mikkola et al., 2003). In all, the conservation in zebrafish of the above mentioned genes involved in mouse definitive hematopoietic formation has made the zebrafish a valuable model for the study developmental hematopoiesis.

**Hematopoietic Stem Cells arise from an Endothelial to Hematopoietic Transition**

The endothelial origin of hematopoietic cells was postulated in the 1920s when hematopoietic-like clusters were first described in the vessels of many vertebrate models by histological observations (Swiers et al., 2013). However, whether these cells within the AGM region were present due to *de novo* generation or a result of migration of cells originating from another site of hematopoiesis was unknown. In the mid 1970s, chick-quail embryo grafting experiments demonstrated that in the avian species, the HSCs of the adult blood system arise from the intraembryonic embryo body, not from the extraembryonic yolk sac, which at the time was thought to give rise to the definitive HSCs (Dieterlen-Lievre, 1975; Dzierzak and Medvinsky, 2008); similar findings were then made for mammals using the mouse model (Dzierzak and Medvinsky, 2008). These studies led to the search for the intraembryonic source of HSCs in the body of the vertebrate embryo. The first *in vivo* support for the endothelial origin of HSCs came from experiments in the chick embryo involving retroviral lineage tracing of endothelium (Jaffredo et al., 2000; Jaffredo et al., 1998). Subsequent studies showed correlations between Runx1 expression and hematopoietic cluster formation in mouse (North et al., 1999), and absence of transplantable HSC activity with loss of “hemogenic” endothelial Runx1 function (North et al., 2002). This was been corroborated by later murine studies, in which conditional deletion of Runx1 using an endothelial specific Cre failed to produce functional HSCs (Chen et
al., 2009). Studies in zebrafish have helped to definitively prove that HSC development requires a transition through a hemogenic endothelial intermediate. Time lapse imaging of embryos expressing the \textit{flk1} transgene were utilized to visualize the budding and egress of HSCs from the endothelium into the vasculature (Kissa and Herbomel, 2010). The EHT phenomenon was similarly shown by Bertrand et al. using \textit{cmyb:eGFP;kdrl:mCherry} double transgenic embryos marking nascent HSCs. \textit{Kdrl:mCherry} cells displaying typical flattened morphology were observed to transform into spherical shapes, forming buds that extended into the lumen of the DA (Bertrand et al., 2010). This was the first visualization of the process of HSC initiation and provided direct \textit{in vivo} evidence that HSCs arise from the endothelium through an intermediary endothelial population deemed the hemogenic endothelium (\textbf{Figure 1.5}). Visualization of the dynamic emergence of hematopoietic cells via EHT was later demonstrated in the mouse using live embryos slices (Boisset et al., 2010). The ability to visualize the EHT process in the zebrafish embryos has also further elucidated the role of Runx1 in hematopoietic development. Zebrafish embryos with morpholino (MO) mediated \textit{runx1} knockdown initiated EHT very poorly, and in the few embryos that could induce EHT, the hemogenic endothelial cells broke into pieces, thereby aborting the process of HSC formation (Kissa and Herbomel, 2010). Thus together with the murine data, these zebrafish studies indicate that Runx1 is required for EHT and definitive HSC formation across vertebrate species.

\textbf{Genetic Manipulation in the Zebrafish Model}

\textbf{Mutagenesis screens}

In addition to the strong conservation of factors regulating HSC development between the zebrafish and other vertebrate systems, the zebrafish is highly amenable to genetic manipulation,
Figure 1.5.
Cross section of the E10.5 AGM with a magnified view of the ventral wall of the dorsal aorta (boxed in red), showing the hematopoietic cell (red) budding out from the hemogenic endothelium (pink). Non-hemogenic endothelium is labeled in light gray. From (Swiers et al., 2013).
such as forward genetic screens (Jing and Zon, 2011). Zebrafish embryos can survive without red blood cells by passive diffusion of oxygen for the first week, allowing for the identification of mutations that would be otherwise be difficult to study in mice due to embryonic lethality (Rombough and Drader, 2009). One of the most common methods to introduce mutations into the germline of zebrafish involves the use of N-ethyl N-nitrourea (ENU) (Martin et al., 2011; Weinstein et al., 1996). Mutants generated by this method to have helped elucidate the genetic pathways that regulate HSCs as well as other hematopoietic lineages (Carradice and Lieschke, 2008; Shafizadeh and Paw, 2004). Among the many erythropoiesis mutants identified include vlad tepes (vlt), which harbors a mutation in gata1, the master regulator of erythropoiesis (Lyons et al., 2002). Furthermore, many mutants model human diseases including the erythroid mutant weisssherbst (weh), which is a hypochromic anemic mutant with a mutation in a previously unknown iron transporter, ferroportin1 (Donovan et al., 2000).

Other examples of other important mutants discovered via screening approaches include the mutant in stem cell leukemia 1/ T-cell acute lymphocytic leukemia 1 (scl1/tal1), which was found through an ENU screen to identify embryos with abnormal vascular development (Habeck et al., 2002), revealing a severe reduction in endothelial alkaline phosphatase activity at 4 dpf. In addition, the mutant embryos exhibit an absence of the primitive erythroid (gata1) and myeloid (spi1 (formerly pu.1)) lineages as well as the formation of HSCs (runx1) (Bussmann et al., 2007). These fish also display defects in endocardial precursor migration and subsequent failure of endocardial development. The defects found in these mutants have provided further support for the existence of a common vascular-hematopoietic precursor cell.
One of the most enigmatic mutants isolated in the large-scale ENU screens of the 1990s is cloche (Liao et al., 1997). The mutant cloche (clo) is characterized by gross cardiac edema and an enlarged atrium due to endocardial loss, giving it its name, which is French for “bell”. It has not been conclusively cloned in the 16 years since the publication of its initial description due to the mutation’s telomeric location on chromosome 13 (Xiong et al., 2008). However, part of the cloche mutant phenotype appears to be phenocopied by a mutation in lycat1, which is located in the region of the deletion region, and lycat mRNA partially rescues the blood lineage in clo mutants (Xiong et al., 2008). cloche mutants exhibit disrupted differentiation of both endothelial and hematopoietic structures, with almost complete loss of expression of tal1, lmo2, gata1, mpx, l-plastin, and kdrl (Liao et al., 1997; Stainier et al., 1995). The effect of Cloche was shown to have both cell-autonomous and non cell-autonomous features in blastula transplantation studies (Parker and Stainier, 1999). In contrast, other mesodermal organs do not show any defects, further indicating that Cloche might play a role in hemangioblast formation.

Other methodologies, referred to as TILLING (Targeted Induced Local Lesions in Genomes) have taken advantage of the ease of ENU mutagenesis, combined with recent advances in sequencing and cloning of the zebrafish genome to identify specific mutations in hematopoietic regulatory factors of interest (Stemple, 2004); for example, the von Hippel-Lindau tumor suppressor (VHL) mutant was identified through TILLING (van Rooijen et al., 2009). The mutants develop polycythemia, recapitulating the clinical manifestation of VHL-associated Chuvash polycythemia, thereby offering the first in vivo system to study the progression of this disease (Jing and Zon, 2011). VHL plays a critical role in regulating the function of Hypoxia Inducible Factor-1α (Hif1α), which we recently showed was essential for developmental HSPC formation in zebrafish (Harris et al., 2013).
High throughout reverse-genetics screen

Retroviral mutagenesis, whereby gene expression and/or function is disrupted by random integration of retroviral DNA into the genome, has also identified many blood mutants (Amsterdam et al., 1999; Gaiano et al., 1996). These insertional screens have isolated essential genes for HSC emergence, including *tbx*, *plegamma*, and *mib* (Burns et al., 2009; Burns et al., 2005). Analysis of zebrafish mutants from these screens have furthermore aided in the identification and sequential organization of a hierarchy of factors required for HSC emergence in the developing vertebrate embryo including VEGFAa, *notch1*, *hdac1*, and *runx1* (Burns et al., 2009; Burns et al., 2005).

Screens to identify modifiers of hematopoietic phenotypes have also been performed using zebrafish. Recently, a large-scale *in vivo* reverse genetic screen utilizing an Antisense oligonucleotide morpholino (MO)-based “knockdown” approach targeting chromatin factors was conducted to identify chromatin factors with specific regulatory roles in hematopoiesis (Huang et al., 2013). This study led to the identification of multiple chromatin factor complexes required for developmental haematopoiesis, including BAF–PBAF and ISWI (chromatin remodeling), SET1 (histone methylation—activation), and PRC1–2 (histone methylation—repression) (Huang et al., 2013). These families of epigenetic regulators were found to impact either primitive or definitive hematopoiesis, or both, providing insight into the role of epigenetic regulation in establishing hematopoietic commitment and expansion (Huang et al., 2013).

Genetic modifier screens, which are routinely used in invertebrates for identifying genes that suppress or enhance the function of the gene of interest have also been adapted for use in the zebrafish model (Jing and Zon, 2011). One such genetic modifier screen looked for suppressors of the *moonshine* mutant, which has defects in erythropoiesis; this screen resulted in the
identification of transcriptional elongation as a regulator of cell fate (Bai et al., 2010). These studies will be useful for identifying novel regulatory factors that modify known hematopoietic phenotypes and potentially reveal new therapeutic targets in the zebrafish.

Recent technologies for genetic manipulation in the zebrafish

Zebrafish are also amenable to use of a variety of inducible promoter-driven transgenes including: heat shock inducible promoters (Halloran et al., 2000), the cre-lox system (Hans et al., 2009; Thummel et al., 2005), and the Gal4;UAS system (Scheer and Campos-Ortega, 1999), that enable induction of gene expression at a specific time or location. Tissue-specific ablation is also possible by driving expression of nitroreductase under a cell type specific promoter, followed by treatment with metronidazole during a chosen timepoint of interest (Curado et al., 2008). Several techniques have been developed in zebrafish to track progeny of a given cell of interest. Photoconvertible caged fluorescent compounds have been utilized in zebrafish embryos to lineage trace, or track the migration and cell fate of individual hematopoietic cells (Jin et al., 2007; Vogeli et al., 2006; Warga et al., 2009).

More recent technologies, including zinc finger nucleases, TALENS zinc-finger nuclease (ZFN)-mediated mutation and the CRISPR/CAS system, have been established to enable the generation of specific, germline-transmissible stable strains carrying mutations at specific gene loci (Doyon et al., 2008; Hwang et al., 2013; Meng et al., 2008; Sander et al., 2011). These techniques have made it possible to generate full knockouts (null phenotypes) relatively quickly and efficiently, enabling better assessment of genetic null phenotypes in the embryo as well as the adult. A CRISPR/Cas9 vector system has also recently been created for tissue specific gene disruption, which will prove useful for studying gene function in specific tissues of origin.
(Ablain et al., 2015). This approach can be utilized to address cell autonomy in loss-of-function studies, as well as to avoid the embryonic lethality associated with the global knockout of certain genes, which complicates the analysis of their functions in vivo.

**Molecular regulation of hematopoiesis**

During the process of HSC emergence, many signaling pathways, among them Wnt, Hedgehog, Notch, and BMP, have been identified and demonstrated to be critical at different developmental stages of HSC formation, from specification and proliferation to maintenance (Zhang et al., 2013). Studies in zebrafish have demonstrated that BMP and Hedgehog, which function ventrally and dorsally respectively in the AGM region, are required for the polarization of the dorsal aorta and determination of hematopoietic specification fate in the ventral wall of the DA (Wilkinson et al., 2009). As HSCs arise from endothelial cells, correct establishment of the artery has been found to be important for HSC formation. Hedgehog signaling has been established as a key regulator of the essential signaling cascade responsible for vascular patterning and the subsequent generation of hemogenic endothelium (Kim et al., 2014a). Epistasis experiments in the zebrafish have established that Hedgehog signaling is genetically upstream of vascular endothelial growth factor (VEGF) signaling, which controls Notch activation in the endothelium (Lawson et al., 2002). During arterial and blood stem cell development, Notch is a key regulator, acting downstream of VEGF, Sonic hedgehog (Shh) and Gridlock (Kumano et al., 2003). The Hedgehog-VEGF-Notch signaling pathway, has been shown to be essential for the formation of definitive, but not primitive, HSCs and the DA (Carroll and North, 2014; Hsia and Zon, 2005).
The characterization of the hematopoietic defects in the *mindbomb (mib)* mutant confirmed the role of Notch in HSC formation (Burns et al., 2005), initially described in the mouse (Kumano et al., 2003). The *mindbomb* mutant was, as the name implies, first described for its neural defects. It carries a defect in the gene for ubiquitin E3 ligase, which is required for the processing of the Notch ligands Delta and Jagged. While dispensible for primitive hematopoiesis, activation of Notch was shown to be critical for definitive hematopoiesis. In both mice and zebrafish, Notch signaling functions genetically upstream of *runx1* (Burns et al., 2005; Nakagawa et al., 2006), and hematopoietic stem cell fate is established by the Notch-Runx1 pathway (Burns et al., 2005). Studies by Burns et al, by demonstrating that overexpression of NICD increases HSC markers but does not alter arterial markers, demonstrated that artery identity and HSC specification can be uncoupled in vivo, suggesting that Notch signaling acts through separate pathways to regulate induction of each cell fate (Burns et al., 2005). The other well characterized downstream effector of Notch signaling in developmental hematopoiesis is the hematopoietic transcription factor *Gata2*. In zebrafish, two orthologs of the mammalian *Gata2* gene (*gata2a* and *gata2b*) have been identified (Butko et al., 2015). While *Gata2a* broadly regulates the vascular system, *Gata2b* seems to be specifically required for HEC and the generation of functional HSCs through activation of zebrafish *runx1* (Butko et al., 2015; Kanz et al., 2016). *Runx1*, which as mentioned above is essential for HSC generation, does not appear to be a direct molecular target of Notch (Gering and Patient, 2010) but is rather induced through binding of *Gata2* in an RBPjκ dependent manner (Robert- Moreno et al., 2005).

Many studies have shown that Notch1 is a key regulator of hemogenic endothelial cells (Robert-Moreno et al., 2005). *Scl* was found downstream of the Hh-Notch axis to regulate distinct stages of the endothelial-to-hematopoietic transition in differentiating murine ES cells,
midgestation murine embryos, and developing zebrafish embryos (Kim et al., 2013; Lancrin et al., 2009). It is increasingly clear that many factors converge on Notch signaling, and that Notch plays a critical role during EHT. A G-protein coupled receptor (GPR) family member gpr183 has been recently shown to modulate EHT via regulation of Notch1 (Zhang et al., 2015). Furthermore, other studies indicated that biomechanical forces, which have been shown to promote blood development (North et al., 2009), induce Notch signaling as well (Diaz et al., 2015). Recently, several other key downstream regulators of Notch signaling in developmental hematopoiesis have been identified, including Cdca7 and Foxc2. Foxc2 transcription factor was identified as a chief candidate for mediating Notch1 signaling in a study in which induction of Notch1 activity was found to increase the hemogenic endothelial population within differentiating cultures of ESCs and enhanced their hematopoietic potential (Jang et al., 2015). In the setting of Notch induction, morpholino knockdown of the zebrafish orthologs foxc1a and foxc1b markedly reduced cmyb positive hematopoietic progenitors, demonstrating that Notch signaling promotes hematopoietic specification through the Foxc2 transcription factor (Jang et al., 2015). Finally, a Nuclear receptor corepressors (Ncor2), which exert transcriptional repression by coordinating with histone deacetylases, were shown to negatively regulate Notch signaling to tightly control HSC emergence, indicating that different Notches may play various roles at multiple phases of HSC development (Wei et al., 2014).

Besides Notch signaling, Wnt signaling is thought to be essential for both embryonic and definitive hematopoiesis (Bigas et al., 2013). As reviewed by Bigas and colleagues, Wnt pathway elements are found in the AGM region and are important for both vascular and arterial development in the embryo (Bigas et al., 2013). In addition, recently non-canonical wnt16 signaling has been identified as playing an important role in HSC emergence in zebrafish during
somitogenesis (Clements et al., 2011). Wnt16 was found to induce expression of \textit{dlc} and \textit{dld} during somitogenesis thereby establishing competence of mesodermal tissue for the emergence of HSCs in the AGM via regulation of Notch pathway components (Clements et al., 2011). In line with these studies, FGF signaling, acting through \textit{fgfr4}, has been found to mediate the signal-transduction pathway between \textit{wnt16} and \textit{dlc} in zebrafish to regulate HSC specification (Lee et al., 2014). These findings have unveiled an interaction between Wnt and Notch signaling for hematopoietic emergence; furthermore, these studies have indicated that Notch activation is required in the mesodermal precursors to the endothelial cell population at a much earlier developmental stage than previously recognized for HSC fate determination, thereby highlighting the essential and diverse functional role of Notch signaling in HSC emergence.

**In Vivo High-Throughput Small Molecule Screening in the Zebrafish**

In recent years, chemical screening approaches have also yielded great insight into the biology of hematopoietic development. Chemical screening strategies are particularly useful in that, unlike mutation, knockdown, or over expression analysis, the timing and duration of exposure can be altered to directly impact the population of interest during hematopoietic development. Screening can be conducted on wild-type (WT) fish scored for alterations in developmental expression patterns or lineage-specific hematopoietic reporters, or it can be used to modify or rescue alterations in the hematopoietic program caused by mutation or transgenesis. The first hematopoietic screen used the \textit{Tg(gata1:GFP)} line to identify modifiers of primitive hematopoiesis (Shafizadeh et al., 2004); subsequent screens have revealed other novel modifiers of both primitive and definitive hematopoiesis (North et al., 2007; Paik et al., 2010). One screen hit that has shown direct therapeutic potential across species is Prostaglandin E2. In particular,
exposure to exogenous PGE2, the most ubiquitous prostanoid produced during development (Grosser et al., 2002), significantly enhanced Runx1+ HSCs in zebrafish and mice; in contrast, inhibitors of the synthesizing enzymes cyclooxygenase 1 and 2, as well as MO knockdown of these enzymes or the G-protein coupled receptors ptger2 and ptger4 resulted in greatly reduced HSC number (North et al., 2007). In a separate study, a role for PGE2 signaling was also identified specifically in lymphoid development (Villablanca et al., 2007). In ensuing analyses, through the use of multiple inducible transgenic fish to modify Wnt signaling, PGE2 was found to affect HSC formation by modulating Wnt activity via cAMP-mediated activation of PKA (Goessling et al., 2009). These findings and subsequent preclinical studies (Goessling et al., 2011) led to the first FDA-approved clinical trial originated from a chemical screen in the zebrafish, evaluating the use of PGE2 in patients undergoing hematopoietic stem cell transplants with cord blood (Cutler et al., 2013). Studies using an embryonic stem cell differentiation system have further implicated cAMP-PKA in the differentiation into vascular and hematopoietic progenitors via recruitment of the transcriptional activator cAMP response element-binding protein (CREB) to the Etv2 promoter (Yamamizu et al., 2012). The subsequent activation of Etv2 was found to be required for the up-regulation of vascular growth factor receptors and hematopoietic transcription factors including Flk1, Tie2, Scl/Tal1, and Gata2 (Yamamizu et al., 2012). Recently, the cannabinoid receptor CNR2 has also been discovered to regulate HSPC production and function in part by regulating PGE2 synthesis and signaling via prostaglandin endoperoxide synthase 2 (Ptgs2) upregulation, underscoring the role of PGE2 as an established regulator of HSCs across vertebrate species and revealing that HSPC regulation can be modulated by biolipid signaling molecules converging onto a common regulatory factor (Esain et al., 2015).
Chemical screening approach also resulted in the identification of several groups of compounds that had the common ability to regulate blood flow by affecting heart rate, contractility, or vascular diameter of the developing embryo (North et al., 2009). Similarly, Adamo and colleagues showed that mimicking blood flow in vitro induced hematopoietic commitment in differentiating murine embryonic stem cells (Adamo et al., 2009). Recently, blood flow was found to stimulate mechanosensors that trigger calcium flux which upregulated expression of the COX2 gene, \( Ptgs2 \) (Diaz et al., 2015). Subsequent increases in PGE2 biosynthesis and cAMP-PKA activity, induced by blood flow, were necessary for expansion of nascent HSCs and progenitors. Phosphorylation of CREB was associated with transcription of genes containing the cAMP response element. WSS (wall shear stress) and dmPGE2 were each capable of promoting long-term multilineage hematopoietic reconstitution and lymphoid lineage potential from the PSp and AGM respectively (Diaz et al., 2015). Sheer stress was found to activate the PKA-CREB pathway in VE-cadherin cells which promotes AGM HSC emergence (Kim et al., 2015). Blood flow was also shown to induce adenosine release, which was found to be important for HSC emergence (Jing et al., 2015). Recently, Nuclear hormone receptors like estrogen or vitamin D, also identified through chemical screens, were demonstrated to be involved in HSPC formation by regulating the formation of the hemogenic vascular niche (Carroll et al., 2014; Cortes et al., 2015). These studies highlight the importance of integrating mechanical forces and other environmental factors to ensure proper hematopoiesis, and indicate the complex regulation of factors influencing HSC development.
**Inflammatory signals in HSC emergence**

Several recent studies have linked HSC emergence with pro-inflammatory signaling, which appears to operate, at least in part, via the Notch pathway (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014). Espin et al. demonstrated that induction of Tumor necrosis factor α (TNFα), which is required for embryonic blood vessel development, is required for the specification and emergence of HSCs through activation of Notch and NF-κB (Espin-Palazon et al., 2014). Sawamiphak et al. found that the pro-inflammatory cytokine interferon-γ and its receptor *crfb17* also influences EHT and HSC specification in fish and function downstream of Notch signaling (Sawamiphak et al., 2014). A similar role for IFN signaling, as well as other downstream cytokine signaling was found by Li et al. during zebrafish HSC production (Li et al., 2014). Importantly, in that study murine embryos lacking interferon-γ (IFN-γ) were also shown to have significantly fewer AGM HSPCs, suggesting conservation of function across species (Kanz et al., 2016; Li et al., 2014). Interestingly, several of these studies indicate that non-cell autonomous signaling between primitive myeloid populations and the ECs is required for HSPC induction and expansion (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014), suggesting they are the source of inflammatory cytokines for HSC emergence. Together, these data point out the molecular complexity of Notch activation in ECs and/or HSCs during HSC generation. Further studies will be required to explore whether the unexpected importance of inflammatory signaling in HSC emergence can be utilized to enhance *in vitro* HSC specification from pluripotent stem cells or HSC expansion.
Physiological regulation of hematopoietic stem cell development

In accordance with the importance of extrinsic factors in regulating hematopoiesis, HSCs are well established to reside in hypoxic regions of the bone marrow with low oxygen tension, as assessed by Hoeschst33342 perfusion from blood vessels (Parmar et al., 2007; Winkler et al., 2010). LT-HSCs are furthermore positive for pimonidazole staining, a hypoxia indicator, in vivo (Simsek et al., 2010; Takubo et al., 2010). The period during which HSCs emerge coincides with fluctuations in nutrient availability and energy. We recently published a study showing heightened metabolism can act as an inductive trigger for production of Runx1+ HSCs during embryogenesis through metabolism mediated ROS production and subsequent stabilization of Hypoxia Inducible Factor (HIF) levels in vivo (Harris et al., 2013) (Figure 1.6). Exposure of zebrafish embryos to 1% D-glucose increased the number of transplantable HSCs generated in the DA without systemic alterations to growth or vascular development (Figure 1.7) (Ciau-Uitz et al., 2014; Harris et al., 2013). Furthermore, cell proliferation was increased only in the AGM, indicating that the number of HSCs generated in the embryo depends on hemogenic endothelium nutrition and metabolic status (Ciau-Uitz et al., 2014; Harris et al., 2013). This effect was found to be mediated through glucose intake, as well as increased glycolysis and oxidative phosphorylation, as knockdown of the glucose transporter GLUT1 as well as regulators of glycolysis and oxidative phosphorylation all significantly reduced the numbers of Runx1 and cmyb expressing cells and mitigated the effect of glucose (Harris et al., 2013). Increased glucose intake increases glycolysis and oxidative phosphorylation, which produces reactive oxygen species (ROS), which in turn stabilize Hif1α (Figure 1.8 A) (Harris et al., 2013; Pan et al., 2007). The role of Hif1α as the mediator of the glucose response was confirmed by the loss of runx1 expression in the DA of hif1α morphants (Figure 1.8 B-D). Finally, the enhancement of
In a targeted screen for environmental modulators of runx1+ HSC development, glucose was identified as a positive regulator of HSCs (Adapted from Trista North).
Figure 1.7
Glucose enhances HSC formation in the zebrafish embryo (Adapted from Trista North; (Harris et al., 2013))
Figure 1.8 (Adapted from Trista North; (Harris et al., 2013))
(A) Schematic of Hif1α regulation and select downstream targets
(B and C) Hif1α is the mediator of the glucose response
(D) Hif1α protein level is upregulated with increased metabolic stimulation including glucose
(E) Hif1α target genes are upregulated with acute glucose treatment
HSC production was shown to be dependent on Runx1, as the number of cmyb expressing cells did not increase when runx1 morphants were treated with glucose (Harris et al., 2013). In more recent studies, Adenosine, a purinergic signaling molecule involved in biochemical processes such as energy transfer, was found to regulate HSPC emergence by regulating Scl-mediated hematopoietic commitment from endothelium (Jing et al., 2015), further highlighting a role for metabolic signaling in HSC development, and warranting additional studies into the mechanism of how metabolic alterations can affect EHT and/or HSC production.

**Hif1α Hypoxic Response and Hematopoiesis**

In vertebrates, the Hypoxia Inducible Factor (HIF) complex functions as the master regulator of the adaptive response to low oxygen levels, or hypoxia. HIF is a bHLH-PAS-type transcriptional regulator. HIF1 is a transcription factor composed of the oxygen-responsive Hif1α subunit, and the constitutively expressed Hif1β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (Wang et al., 1995). Under normoxia, Hif1α is hydroxylated at proline (Pro) 402 and/or 564 in the O₂-dependent degradation (ODD) domain by HIF prolyl hydroxylases (Phds) and targeted for degradation by the von Hippel-Lindau tumour suppressor protein (VHL) (Semenza, 2007; Simon and Keith, 2008). VHL recruits the Elongin C/Elongin B/Cullin2/E3-ubiquitin-ligase complex, leading to ubiquitination and subsequent proteasomal degradation of Hif1α. In the autosomal-dominant hereditary disorder von Hippel Lindau disease, VHL is mutated, impairing the ubiquitin proteasome pathway resulting in overstabilized Hif1α protein, which causes disease symptoms such as polycythemia (Ang et al., 2002). Hypoxia inhibits Hif1α degradation, allowing it to interact with Hif1β to bind to hypoxia response elements (HREs) to activate transcription of hypoxic responsive genes,
which affect a wide range of cellular processes including, enhanced glucose metabolism, erythropoiesis, and angiogenesis (Simon and Keith, 2008).

Hypoxia has recently been identified as playing a role in adult HSC quiescence in the murine bone marrow (Takubo et al., 2010). Deletion of Hif1α was found to decrease quiescent HSC numbers, and serial transplantation of Hif1α−/− HSCs resulted in loss of long term reconstitution (Takubo et al., 2010). As indicated above, we recently revealed a role for metabolism/ROS induced Hif1α in HSC induction during zebrafish development (Harris et al., 2013). Hif1α has subsequently been identified as a regulator of HSC development in hypoxic sites of the mouse embryo (Imanirad et al., 2014). Pimonidazole staining in the E10 mouse embryos indicated that the endothelium of the DA and intra-aortic clusters is also hypoxic, and deletion of Hif1α in VE-cadherin-expressing cells reduced the number of intra-aortic clusters (Imanirad et al., 2014). However, some phenotypic HSCs were still generated, indicating that not all HSCs are dependent of Hif1α function or that compensatory regulation may exist (Imanirad et al., 2014).

**Hif1 target genes**

Hif1α is considered a master regulator of metabolism because it regulates both glycolysis and mitochondrial respiration, specifically by mediating a switch from oxidative to glycolytic metabolism (Semenza, 2009). The role of HIF on cellular metabolism and proliferation has been well studied in contexts of cancer (Semenza, 2009). Recently, LT- HSCs in the bone marrow were found to express Hif1α intrinsically and utilize glycolysis instead of mitochondrial oxidative phosphorylation; this low mitochondrial potential was associated with Hif1α levels (Simsek et al., 2010; Suda et al., 2011). Thus, it is clear that in both the bone marrow and AGM
niches, in addition to sensing oxygenation levels, Hif1α mediates the metabolic response to glucose and, in the AGM, controls the number of emerging HSCs (Ciau-Uitz et al., 2014).

The HIF complex is essential for embryogenesis, as Hif1α−/− and Hif1b−/− mice die at e9.5 to e10.0, prior to the onset of definitive hematopoiesis, with prominent vascular defects and severe developmental delay (Iyer et al., 1998; Kotch et al., 1999). One of the classic Hif1α targets induced by hypoxia is VEGFA, which is involved in angiogenesis to assist in promoting and increasing oxygen delivery to hypoxic regions (Forsythe et al., 1996). Recently, VEGFA, a factor required in a cell-intrinsic manner for HSC maintenance, was shown to contain hypoxia binding elements in its regulatory regions that, when mutated, decreased VEGFA expression levels and impaired HSC function (Rehn et al., 2011). Hif1β has also been implicated in hematopoiesis; Hif1b deletion resulted in embryos that lacked blood-filled vitelline vessels, suggesting a deficiency in hematopoiesis in addition to previously seen vascular defects (Adelman et al., 1999; Maltepe et al., 1997). Hematopoietic colony formation assays using Arnt−/− E9.5 yolk sac also yielded a significant decrease in the number of colony-forming-unit progenitors, including CFU-E and CFU-GM, compared to their sibling controls (Adelman et al., 1999). Explants from Arnt−/− embryos also exhibited non-cell autonomous defects in vascular and hematopoietic colony formation in vitro, which could be rescued by addition of VEGF (Ramirez-Bergeron et al., 2006). In our previous studies, we saw a number of Hif1α target genes were upregulated by acute glucose exposure (Figure 1.8 E) and also found a role for VEGFA downstream of hif1a MO-mediated reductions in runx/myb HSCs; however while the vegfαa-MO likewise decreased runxl/cmyb expression, it failed to completely block the effect of glucose, suggesting the existence of coordinate upregulation of several hematopoietic factors by Hif1α to impact HSC development downstream of glucose metabolism (Harris et al., 2013). It is
currently unknown what other signaling factors function downstream of Hif1α to influence EHT and/or HSPC production in the vertebrate embryo.

**The Role for PDGF signaling in hemopoiesis**

Platelet derived Growth Factors (PDGFs) belong to family of growth factors that are well established in their role as mitogens for mesenchymal cells (Betsholtz et al., 2001). In both mouse and humans, the PDGF signaling network consists of four ligands, PDGF-A, B, and the more recently discovered -C, and-D, which signal through two protein tyrosine kinase receptors, PDGFRα and PDGFRβ (Hoch and Soriano, 2003). All PDGF ligands are secreted as disulfide-linked homodimers, while PDGF-A and PDGF-B can also form heterodimers with each other (Hoch and Soriano, 2003). The PDGFR receptors also function as homo and heterodimers, and *in vitro* assays have demonstrated that the ligands have different affinities for the α and β receptors (Heldin and Westermark, 1999; Hoch and Soriano, 2003; Li et al., 2000). The PDGFRα receptor can bind all ligand chains, while the PDGFRβ receptor specifically binds the PDGF-B ligand (Betsholtz et al., 2001; Seifert et al., 1989). Each receptor is composed of five immunoglobulin repeats in the extracellular ligand-binding domain, a single spanning transmembrane domain, and a split tyrosine kinase domain in the cytoplasmic region (Demoulin and Montano-Almendras, 2012). Binding of PDGFs to its receptors leads to dimerization and activation of the receptor tyrosine kinases leading to the initiation of cytoplasmic signal transduction pathways including the Phosphatidylinositol 3-kinase (PI3K), Ras-MAPK (mitogen activated protein kinase), Extracellular Signal-regulated kinase (ERK), and Proto-oncogene Src tyrosine-protein kinases, which affect migration, proliferation, survival, and differentiation of PDGF responsive cell types (Rolny et al., 2006).
Knockout mice for the genes that encode two ligands, PDGF-A and PDGF-B, and both receptors, PDGFRα and PDGFRβ have revealed that the two receptors play distinct roles in development and disease formation (Demoulin and Montano-Almendras, 2012; Rosenkranz and Kazlauskas, 1999). PDGF-A and PDGFRα have been found to be more broadly required during embryogenesis, and play essential roles in many contexts, including central nervous system, neural crest and organ development (Bostrom et al., 1996; Fruttiger et al., 1996; Karlsson et al., 2000; Soriano, 1997). PDGF-B and PDGFRβ have important roles in vascular remodeling during later stages of development and in adult angiogenesis through the recruitment of PDGFRβ expressing pericytes (Rolny et al., 2006) and have been implicated in blood vessel formation during embryogenesis (Leveen et al., 1994; Wiens et al., 2010). Previous studies have shown that PDGF-B expression is regulated by hypoxia and Hif1α in cardiovascular and neuronal cell types as well as breast cancer cells (Kelly et al., 2003; Schito et al., 2012; Zhang et al., 2003); Furthermore, PDGF-B has also been documented to be a direct target of Hif1α in experiments using HeLa cells (Schito et al., 2012)

*PDGF-B or PDGFRb* murine knockout embryos die at birth of cardiovascular and kidney dysfunction and organ specific hemorrhages, as numerous vessels lack or are incompletely covered by mural cells (Leveen et al., 1994; Soriano, 1994). However, the direct role of PDGFR signaling in vertebrate hematopoiesis is less clear. *PDGF-B or PDGFRb* knockout embryos also exhibit both anemia and thrombocytopenia, but this is thought to be secondary to other organ defects, such as abnormal development of the placenta labyrinth, which likely produces metabolic stress leading to hypocellularity of the fetal liver (Leveen et al., 1994; Soriano, 1994). Previous *in vitro* and *in vivo* studies have suggested a role of PDGF-B in erythropoiesis (Dainiak et al., 1983; Kaminski et al., 2001; Keutzer and Sytkowski, 1995) and megakaryocytopoiesis
(Yang et al., 1995), but stimulation required the presence of adherent stromal cells. In studies of mixed marrow cultures, PDGF-B was found to stimulate primitive hematopoietic precursors, such as colony-forming units containing granulocytes, erythroid cells, macrophages, megakaryocytes (CFU-GEMM) and lineage-restricted hematopoietic progenitors (Cashman et al., 1990; Yan et al., 1993). However, PDGF-B has been shown to act on platelets during wound healing as well as stimulate marrow macrophages to release Interleukin-1 (IL-1B), and a neutralizing antibody against IL-1B was found to attenuate the stimulatory effect of PDGF-B on hematopoietic progenitors (Yan et al., 1993). PDGF-B thus likely indirectly stimulates hematopoietic precursors through the release of growth and differentiation factors from stromal cells and/or marrow macrophages (Kaminski et al., 2001). In line with these in vitro studies, IL-1B has recently been shown to increase HSC proliferation and differentiation both in ex vivo AGM explant cultures and in vivo (Orelio et al., 2008). Recent work using human umbilical cord blood cells has also revealed that addition of PDGF could enhance the expansion of erythroid, myeloid, and megakaryocyte progenitors including CFU-GM, CFU-GEMM, and BFU-E/CFU-E from CD34+ cells, although it has been unclear whether expansion is a result of direct stimulation or secondary to autologous effects on stromal supporting cells (Su et al., 2002).

To address the basis of the hematopoietic defects of the PDGF-B and PDGFRβ knockout mice, hematopoietic chimeras were generated by transplanting mutant fetal liver cells into lethally irradiated recipient mice; hematopoiesis in the irradiated wild-type mice could be reconstituted by grafting fetal liver cells from PDGF-B or PDGFRβ-deficient mice (Kaminski et al., 2001). These findings indicate that under normal circumstances, neither the release of PDGF from the hematopoietic cells, nor the response of PDGFRβ-expressing hematopoietic cells to
PDGF, such as the induction of IL-1 in receptor expressing macrophages, is required for normal hematopoiesis in vivo (Kaminski et al., 2001; Yan et al., 1994), indicative of a non-cell autonomous function of PDGF in hematopoiesis. Interestingly, overexpression of PDGF-B in murine hematopoietic cells was found to induce a lethal myeloproliferative syndrome in vivo (Yan et al., 1994), and PDGFRb chromosomal translocation have been associated with myeloproliferative neoplasms, indicating PDGF-B signaling may function cell intrinsically in malignant hematopoiesis and disease progression (Demoulin and Montano-Almendras, 2012; Kelly and Gilliland, 2002).

Recently, PDGF-B signaling in the trophoblasts was found to be a key component of the unique placental hematopoietic microenvironment that protects HSPCs from premature differentiation (Chhabra et al., 2012). In the PDGF-B-/- placentas, the placental labyrinth vasculature became a permissive environment for the proliferation and differentiation of definitive erythroid precursors through upregulation of erythropoietin (Epo). This defect was not due to an intrinsic requirement for PDGF-B signaling in hematopoietic cells, as conditional knockout of PDGFRb in hematopoietic cells using Tie2:Cre did not lead to the hematological abnormalities or ectopic definitive erythropoiesis found in either the PDGF-B or PDGFRb knockout embryos (Chhabra et al., 2012). This study further highlights a non-cell autonomous role of PDGF-B in enhancing hematopoiesis by stimulating stromal/niche cells to produce additional factors that act directly on hematopoietic progenitors; furthermore these observations implicate PDGF-B signaling in the regulation of the balance between maintenance and differentiation of hematopoietic cells (Chhabra et al., 2012)

Interestingly, recent studies have revealed proteins related to mammalian PDGFs in invertebrates have been found to play important roles during the development of invertebrate
hematopoietic system (Cho et al., 2002). In *Drosophila melanogaster*, there are three PDGF/VEGF ligands (PVF1, PVF2, and PVF3) and a single receptor (PVR) (Duchek et al., 2001). PVR signaling in *Drosophila* is essential for the embryonic migration of hemocytes, the precursors of the fly blood cell lineage, as well as promote their survival and maintenance (Bruckner et al., 2004; Cho et al., 2002; Mondal et al., 2014), making it intriguing to explore whether these mechanisms of action are conserved in vertebrate hematopoietic development.

PDGF-B is known to stimulate the synthesis of various interleukins to affect cell functions such as proliferation and migration in other systems including osteoblasts and smooth muscle cells (Franchimont et al., 1999). Interleukin 6 (IL-6) is a prominent cytokine produced during infectious disease and is needed in the systemic acute-phase reaction (Moshage, 1997). *In vitro*, IL-6, acting in synergy with IL-3, enhances the formation of multilineage blast cell colonies as well as the proliferation of stem cells, whereas *IL-6* deficient mice show a reduction in the number of hematopoietic primitive clonal progenitor cells (Bernad et al., 1994). IL-6 is among the growth factors including Flt3 ligand, Stem Cell Factor (SCF), and thrombopoietin (Tpo), that have been shown to enhance the proliferation of early hematopoietic stem and progenitor cells (Henschler et al., 1994; Zandstra et al., 1997), making it an intriguing target for Hif1α-mediated PDGF regulation and subsequent impact on HSPC development.
Chapter 2:

HIF1α-induced PDGF signaling promotes HSC production via IL-6 activation

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SL, VE, WK, IF, and SYL performed embryo exposures, MO injections, qPCR, and *in situ* hybridizations. SL and MC conducted FACS. SL and VE conducted fluorescence microscopy. SL, VE and TEN designed experiments and evaluated results.
**Introduction**

Hematopoietic stem cells (HSCs) possess the unique ability to both self-renew and differentiate into all the mature blood cell lineages and thereby reconstitute the entire blood system for a lifetime. In vertebrates, HSCs are initially generated during embryogenesis from specialized hemogenic endothelium in the ventral wall of the dorsal aorta, within an intraembryonic region termed the aorta-gonad-mesonephros (AGM) in mammals (Bertrand et al., 2010; Chen and Zon, 2009; Dzierzak and Speck, 2008; Kissa and Herbomel, 2010). Runx1 is a transcription factor that is required for the production of definitive HSCs (North et al., 2002), controlling endothelial-to-hematopoietic transition (EHT) (Chen et al., 2009). We previously determined that heightened metabolism can act as an inductive trigger for production of Runx1+ HSCs during embryogenesis through metabolism-mediated ROS production and subsequent stabilization of Hypoxia inducible factor 1α (Hif1α) levels *in vivo* (Harris et al., 2013).

The HIF complex functions as the master regulator of the adaptive response to low oxygen levels, or hypoxia. Under normoxia, Hif-1α is targeted for degradation by the von Hippel-Lindau tumour suppressor protein (VHL). Hypoxia inhibits Hif1α degradation, allowing it to interact with Hif1β to activate transcription of hypoxia responsive genes, which affect a wide range of cellular processes including enhanced glucose metabolism, erythropoiesis, and angiogenesis (Simon and Keith, 2008). Our prior studies indicated Hif1α was necessary for HSC production in zebrafish embryos (Harris et al., 2013). While loss of Hif1α is embryonic lethal prior to the formation of definitive HSCs in the mouse, more recent analysis using conditional loss of Hif1α function confirms it likewise regulates HSC development in the mammalian embryo (Imanirad et al., 2014). However, despite apparent conservation of function,
it is currently unknown what functions downstream of Hif1α-regulation to influence EHT and/or embryonic HSPC production.

Platelet Derived Growth Factors (PDGFs) belong to family of regulatory factors that control cell growth and proliferation. PDGFs act through protein tyrosine kinase receptors, PDGFRα and PDGFRβ: binding of PDGFs to its receptors leads to dimerization and activation of the receptor tyrosine kinase activity, leading to the initiation of downstream cytoplasmic signal transduction pathways including the Phosphatidylinositol 3-kinase (PI3K), Extracellular Signal-regulated kinase (ERK), and Proto-oncogene tyrosine-protein kinase Src to affect migration, proliferation, and differentiation of PDGF responsive cell types (Rolny et al., 2006). Previous studies have shown that PDGF-B expression is regulated by hypoxia in cardiovascular and neuronal cell types as well as breast cancer cells (Kelly et al., 2003; Schito et al., 2012; Zhang et al., 2003); PDGF-B was documented to be a direct target of Hif1α in experiments using HeLa cells (Schito et al., 2012). While PDGF-B has an established role in vascular remodeling during later stages of development and in adult angiogenesis through the recruitment of PDGFRβ expressing pericytes (Rolny et al., 2006), its direct impact on hematopoiesis is less clear.

Previous in vitro and in vivo studies have suggested a role of PDGF-B in erythropoiesis (Dainiak et al., 1983; Kaminski et al., 2001; Keutzer and Sytkowski, 1995) and megakaryocytopoiesis (Yang et al., 1995), however stimulation required the presence of adherent stromal cells. In mixed marrow cultures, culturing cells with recombinant PDGF-B enhanced colony formation of primitive hematopoietic precursors, but it remains unclear whether PDGF directly affects hematopoietic function or indirectly stimulates HSPCs through the release of growth and differentiation factors from stromal cells (Kaminski et al., 2001). PDGF has been
shown to act on platelets during wound healing as well as stimulate marrow macrophages to release Interleukin-1 (IL-1β) (Yan et al., 1993); this may explain some of the recently reported stimulatory effects of PDGF on hematopoietic progenitors as IL-1β has been shown to increase HSC proliferation and differentiation in vitro and in vivo (Brugger et al., 1993; Orelio et al., 2008). PDGF-B or PDGFRβ knockout embryos die at birth of cardiovascular dysfunction and organ specific hemorrhages, exhibiting both anemia and thrombocytopenia, which are currently thought to be secondary to defects in other organs, including the heart, placenta, vasculature and liver (Leveen et al., 1994; Soriano, 1994). Interestingly, irradiated wild-type mice can be reconstituted up to 4-12 months by grafting PDGF-B or PDGFRβ-deficient hematopoietic cells (Kaminski et al., 2001), suggesting a non-cell autonomous function of PDGF-B signaling in adult HSC regulation. Recently, PDGF-B signaling in trophoblasts was found to be a key component of the unique placental microenvironment that protects HSPCs from premature differentiation toward the erythroid lineage (Chhabra et al., 2012), indicating it may have additional functions during embryonic development.

PDGF/PDGFR signaling is known to stimulate the synthesis of various interleukins including IL-1β and Interleukin 6 (IL-6) to affect cell functions such as proliferation and migration in osteoblasts and smooth muscle cells (Franchimont et al., 1999). IL-6 is a prominent pro-inflammatory cytokine produced during the immune response to infection (Moshage, 1997) which acts by binding to the receptor complex made of the selective IL-6R (CD126) and the common signal transduction component gp130 (CD130). In vitro, IL-6, acting in synergy with IL-3, enhances the formation of multi-lineage blast cell colonies (Ikebuchi et al., 1987). In contrast, IL-6 deficient mice show a reduction in the number of primitive hematopoietic colony forming progenitor cells, as well as decreased long term reconstituting stem cell potential in
transplantation assays (Bernad et al., 1994). IL-6 is among the growth factors including Flt3, SCF, and Thrombopoietin that have been shown to enhance the proliferation of primitive hematopoietic stem and progenitor cells in vitro (Henschler et al., 1994; Zandstra et al., 1997), making it an intriguing target for Hif1α-mediated regulation of developmental HSPC production.

Here, we demonstrate a role for PDGF-B/PDGFRβ signaling downstream of Hif1α in mediating the effects on HSPC production during embryonic hematopoietic development. Inhibiting PDGFRβ signaling in the presence of chemical or genetic Hif1α stabilization attenuated its ability to induce HSPC formation. In contrast, overexpression of pdgfb robustly increased HSPC production, even in the absence of HIF1α function. The effect of PDGF-B/PDGFRβ was found to be mediated by IL-6/IL-6R activity, whereby loss could block PDGF-induced HSPC expansion. Finally, IL-6 expression was both induced by and determined to function downstream of Hif1α stabilization. Together these findings uncover a signaling axis through PDGF-B/PDGFRβ that regulates the scale of definitive HSPC formation via inflammatory signals in response to Hif1α stimulation.

Results

Hif1α stabilization enhances HSPC production via up-regulation of PDGF-B) signaling

We had previously identified platelet-derived growth factor (pdgf) as a potential target of Hif1α relevant to HSPC production during embryogenesis (Harris et al., 2013). To assess whether PDGF signaling functions downstream of Hif1α in HSPC development, we conducted modified epistasis experiments. Embryos were exposed to the pan-PDGF receptor inhibitor, AG1295 (10μM), in the presence or absence of the known Hif1α agonist CoCl₂ (500μM) during the period of HSPC specification from 12-36hpf. Embryos exposed to CoCl₂ exhibited
qualitatively stronger expression of runx1; cmyb in the AGM compared to age-match sibling controls as visualized by whole mount in situ hybridization (WISH), consistent with our prior analysis (Harris et al., 2013) (Figure 2.1 A,B). In contrast, the majority of embryos exposed to AG1295 displayed reduced runx1; cmyb expression in the AGM (Figure 2.1 A,B). This effect was confirmed and quantified by flow cytometry using the Tg(-6.0itga2b(CD41):eGFP line, previously shown to mark HSPCs with in vivo repopulating potential (Harris et al., 2013), whereby embryos exposed to AG1296 (2.5µM) exhibited a 0.58-fold reduction (p<0.01) in CD41⁺; Gata⁻ HSCs (Figure 2.1 C). Furthermore, exposure to AG1295 blocked the CoCl₂-mediated increase in the distribution of embryos with strong expression of HSPC markers runx1 and cmyb (Figure 2.1 A,B) suggesting that Hif1α influences HSPC development in part through PDGFR signaling.

To determine which PDGF family members are the primary targets of Hif1α in this context, embryos were exposed to CoCl₂ during HSPC specification and PDGF-related gene expression was examined by whole embryo RT-qPCR. Embryos exposed to CoCl₂ exhibited significantly increased expression of pdgfb (p≤0.05) and pdgfrb (p<0.05); notably, pdgfa expression did not change, indicating that the regulatory effect is specific to pdgfb (Figure 2.1 D). As confirmation of regulatory specificity, embryos exposed to DMOG (75µM) a prolyl hydroxylase inhibitor that leads to Hif1α stabilization and increased runx1 expression, also exhibited increased expression of pdgfb (p<0.01) and pdgfrb (p<0.05) by RT-qPCR (Figure 2.2 A). WISH analysis indicated that pdgfrb is expressed in the AGM region at 36hpf, consistent with prior observations (Wang et al., 2014); furthermore, increased expression of pdgfrb was observed in the AGM of embryos exposed to CoCl₂ (Figure 2.2 A,B). RT-qPCR analysis of sorted cell populations from Tg(flk1:dsRed;cmyb:GFP) embryos at 36hpf confirmed that pdgfb...
Figure 2.1

(A) Embryonic exposure to the pan-PDGFR inhibitor AG1295 (10μM) (during HSC formation (12-36hpf) decreased runx1;cmyb WISH expression in the AGM and blocked the increase in runx1;cmyb expression due to exposure to Hif1α agonist (CoCl2, 500μM).

(B) Qualitative phenotypic distribution of embryos from panel 1A scored with low, medium or high runx1;cmyb expression in the AGM (n≥20 condition x 3 replicate clutches).

(C) FACS analysis confirmed the inhibition of PDGFR using the pan-PDGFR inhibitor AG1296 (2.5μM) decreased CD41+gata1- HSPCs at 36hpf (0.58-fold fold decrease, **p<0.01, two-tailed t-test, n≥5 replicates/condition).

(D) RT-qPCR analysis showed that pdgfb and pdgfrb were significantly upregulated over baseline in CoCl2 treated embryos but no effect was observed for pdgfa (tx 12-36hpf) (*p<0.05, two-tailed t-test, n≥3).
Figure 2.2

(A) qPCR analysis showed pdgfrb and pdgfb are upregulated with exposure to the Hif1α agonist DMOG (75uM), but pdgfa is not changed (*p≤0.05, **p≤0.01, two-tailed t-test, n≥3).

(B) WISH analysis of wild type embryos at 36hpf showed pdgfrb was expressed throughout the trunk of the embryo, and enriched in the AGM region at the time of HSC specification; pdgfrb expression was upregulated by CoCl2 (n=20/condition)

(C) qPCR analysis using FACS-sorted populations from Tg(flk1:dsRed;cmyb:egfp) embryos at 48hpf showed that pdgfb and pdgfrb are expressed in both the vasculature (Flk1:dsRed+ ; cMyb:GFP-) and HSCs (Flk1:dsRed+ ; cMyb:GFP+) (normalized to tbp).

(D) qPCR analysis of sorted populations showed that pdgfb was upregulated by CoCl2 in the endothelium (Flk1:dsRed+ ; cMyb:GFP-), while pdgfrb was upregulated in both the endothelium (Flk1:dsRed+ ; cMyb:GFP-) and HSCs (Flk1:dsRed+ ; cMyb:GFP+).
and pdgfrb are expressed in both Flk1⁺;cMyb⁻ endothelium and Flk1⁺;cMyb⁺ HSPCs (Figure 2.2 C), with each also found in the negative fraction. To identify which cell types may be responding to Hif1α regulation through PDGFRβ signaling, we compared expression levels of pdgfb and pdgfrb in sorted cell populations from flk1:dsRed;cmymb:GFP embryos that had been treated from 12-36hpf with DMSO or CoCl₂. As expected, the control gene, the established Hif1α target erythropoietin receptor (epor) was robustly induced following treatment. RT-qPCR analysis showed that expression of pdgfrb was increased in all sorted fractions (Figure 2.2 D); interestingly, pdgfb expression was increased specifically in the Flk⁻cMyb⁻ endothelial population, similar to that seen for runx1, suggesting that this population may be responsible for the increase in hemogenic endothelial commitment and HSPC production seen with Hif1α stimulation (Harris et al., 2013). Together these data suggest Hif1α stabilization increases runx1;cmymb HSPC production via the up-regulation of pdgfb and pdgfrb expression.

**PDGFRβ signaling is required downstream of Hif1α to control AGM HSPC production**

We next sought to confirm our hypothesis that Hif1α increases AGM HSPC production via the up-regulation of PDGFRβ signaling. Embryos exposed to the PDGFRβ-selective inhibitor DMPQ (10μM) during HSC specification (18-36hpf) exhibited no gross alteration in runx1;cmymb⁺ HSPCs compared to DMSO control (Figure 2.3 A,B); DMPQ treatment (12-24hpf) also had no effect on hemogenic niche formation as indicated by vascular flkl and ephrinb2a WISH (Figure 2.4 A). Significantly, however, in the presence of DMPQ, exposure to CoCl₂ was no longer able to increase the proportion of embryos exhibiting strong runx1;cmyb expression (Figure 2.3 A,B), suggesting PDGFRβ is a relevant downstream regulator. Similar findings were
Figure 2.3

(A) Embryonic exposure to the PDGFRβ-selective inhibitor DMPQ (10µM) during HSC formation (tx 18-36hpf) blocked the increase in runx1; cmyb expression in the AGM observed following CoCl₂ exposure.

(B) Qualitative phenotypic distribution of embryos from panel 2.3A (n values as in 2.1B above).

(C) Morpholino (MO) knockdown of vhl increased runx1; cmyb WISH expression in the AGM at 36hpf, while co-injection of pdgfrb MO blocked this increase.

(D) Qualitative phenotypic distribution of embryos from panel 2.3D (n≥20 condition x 2 replicate clutches).
Figure 2.4

(A) WISH analysis of flk1 and ephrinb2a expression in the vasculature of DMPQ-treated embryos showed no impact of treatment compared to controls (n ≥20/condition x 2 replicate clutches).

(B) No impact was seen on VE-cadherin or flk1/fli1 WISH following injection of pdgfrb MO (n ≥20/condition x 2 replicate clutches).

(C) Morpholino knockdown of pdgfrb attenuated the increase in runx1; cmyb expression in the AGM of embryos exposed to DMOG (75μM).

(D) Qualitative phenotypic distribution of embryos from panel 2.4C.
obtained by WISH analysis using DMOG and a previously published pdgfrb MO (Wiens et al., 2010): no gross vascular defects, as assessed by VE-cadherin and fli1/flk1 were observed with pdgfrb MO at the dose utilized in this study, consistent with the recently reported phenotype of pdgfrb mutants (Kok et al., 2015) (Figure 2.4 B); however, with pdgfrb knockdown, DMOG was no longer able to increase the fraction of embryos showing strong AGM HSPC marker expression (Figure 2.4 C,D). The requirement of PDGFRβ activity downstream of Hif1α was further validated using a previously published MO targeting von hippel-lindau (VHL), an E3 ubiquitin ligase that normally targets hydroxylated Hif1α to the proteasome for degradation in normoxic conditions (Harris et al., 2013). Consistent with stabilized Hif1α levels in the absence of VHL function and our prior observations (Harris et al., 2013), vhl embryos had increased runx1;cmyb expression in the AGM. This effect was also blocked in pdgfrb morphants (Figure 2.3 C,D), indicating that pdgfrb expression is required downstream of endogenous Hif1α to increase AGM HSPC number.

**pdgfb overexpression increases HSPC production in the absence of Hif1α**

To determine if PDGF signaling plays an instructive role in embryonic HSPC production in the AGM, we overexpressed murine pdgfb mRNA in the zebrafish embryo. Embryos injected with 25ng/μL pdgfb mRNA exhibited enhanced runx1;cmyb expression (Figure 2.5 A,B) and increased Flk1⁺;cMyb⁺ HSPC counts in the AGM (Uninjected: 6±1.5 cells/AGM, pdgfb mRNA: 10.4±2.2, n≥15/condition, p≤0.0001) (Figure 2.6 A,B). Furthermore, this effect was sustained at 48hpf as assessed by cmyb expression in the CHT (Figure 2.5 D,E) and quantified by Flk1⁺;cMyb⁺ flow cytometry (1.54-fold increase vs. control, p≤0.0001) (Figure 2.5 C). Analysis of embryos expressing Tg(-6.0itga2b(CD41):eGFP also displayed a significant increase in
Figure 2.5

(A) Overexpression of pdgfb by mRNA injection enhanced runx1;cmyb expression in the AGM by WISH at 36hpf.
(B) Qualitative phenotypic distribution of embryos from panel 2.5A (n value as in 2.1B).
(C) FACS analysis confirmed pdgfb overexpression increased Flk1:dsRed⁺cMyb:GFP⁺ HSPCs at 48hpf (1.54 fold increase, ***p≤0.0001, two-tailed t test, n≥5 replicates/condition).
(D) Overexpression of pdgfb increased cmyb expression in the CHT at 48hpf.
(E) Qualitative phenotypic distribution of embryos from panel 2.5D (n value as in 2.1B).
(F) Representative images of cd41:egfp embryos correlating to panel 2.5G.
(G) Absolute cell counts in the CHT of Tg(cd41:egfp) embryos indicated the number of Cd41:GFP⁺ HSPCs was increased at 48hpf in embryos injected with pdgfb mRNA (uninjected: 13.1±7.4 cells/CHT, pdgfb mRNA: 18.9±9.8, n>35/condition, two-tailed t-test, **p≤0.0053).
(H) Overexpression of pdgfb rescued reductions in runx1;cmyb expression in hif1a morphants.
(I) Qualitative phenotypic distribution of embryos from panel 2.5H.
Figure 2.5 (Continued)

A. Control  pdgfb mRNA  runx1;cmyb (36hpf)

B. runx1;cmyb expression

C. FACS (48hpf)

D. Control  pdgfb mRNA  cmyb (48hpf)

E. cmyb expression

F. Control  pdgfb mRNA  CD41:eGFP at 48hpf

G. Cell count

H. Control  hif1a MO  runx1;cmyb

I. runx1;cmyb expression
Figure 2.6

(A) *In vivo* imaging of Tg(flk1:dsRed;cmYb:eGFP) embryos indicated the number of Flk1:dsRed⁺;cMyb:GFP⁺ HSCPs (arrowheads) was increased in the AGM at 36hpf in *pdgfb* mRNA-injected embryos.

(B) Absolute counts of Flk1:dsRed;cmYb:eGFP HSCPs from embryos in panel 2.6A (uninj: 6±1.5, *pdgfb* mRNA: 10.4±2.2; ***p≤0.0001, two-tailed t-test, n≥15/condition).

(C) WISH analysis of *myb* expression at 48hpf following ectopic activation of PDGFRβ signaling using hsp70:ca-pdgfrb induced at 27 hpf (37°C for 1 hour).

(D) Qualitative phenotypic distribution of embryos from panel 2.6C, (n≥15/condition x 2 replicate clutches).
CD41:GFP+ HSPC counts in the CHT following murine pdgfb injection (Uninjected: 13.1±7.4 cells/CHT, pdgfb mRNA: 18.9±9.8, n≥35/condition, p≤0.0053) (Figure 2.5 F,G). These data show that PDGF-B receptor signaling is functionally conserved across vertebrate species and is sufficient to increase HSC induction from the hemogenic endothelium. To assess whether increased PDGF-B signaling can rescue the effect of reduced Hif1α activity on AGM HSC specification a modified epistasis experiment was conducted. Consistent with our prior observations (Harris et al., 2013), runx1;cmyb expression was reduced in the AGM of hif1α morphants. Interestingly, overexpression of pdgfb mRNA in hif1α morphant embryos enhanced the proportion of embryos showing strong runx1;cmyb beyond WT levels, suggesting PDGFRβ signaling is sufficient to increase AGM HSC production in the absence of Hif1α (Figure 2.5 H,I). To further confirm the role of PDGF-B/PDGFRβ signaling in HSPC regulation, we utilized a heat-shock inducible gain-of-function approach using embryos carrying a constitutively-active murine pdgfrb transgene (hsp70:ca-pdgfrb). Heat shock induction of ca-pdgfrb at 27hpf resulted in increased cmyb expression in the AGM at 48hpf compared to heat-shocked uninjected sibling controls (Figure 2.6 C,D). Collectively, these studies suggest that PDGF-B/PDGFRβ signaling is up-regulated downstream of Hif1α to act as an instructive signal to increase AGM HSPC production.

**IL-6 is required downstream of PDGFRβ to stimulate AGM HSPC expansion**

To begin to understand the mechanism by which PDGF-B/PDGFRβ signaling regulates HSPC formation, we examined previously identified PDGF-B regulatory targets known to impact vascular and/or hematopoietic cell types (Wu et al., 2008). The expression of il1b, il6, mmp2 and mmp9 was analyzed in 36hpf embryos injected with pdgfb mRNA: il6 was the most
**Figure 2.7**

(A) qPCR analysis showed *il6* and its receptor and co-receptor, *il6R* and *gp130*, are upregulated in *pdgfb* mRNA-injected embryos (*p*<0.05, **p**<0.01, two-tailed t-test, n≥3).

(B) Morpholino knockdown of *il6* blocked the ability of *pdgfb* mRNA to increase *runx1; cmyb* expression in the AGM.

(C) Qualitative phenotypic distribution of embryos from panel 2.7B (n value as in 2.1B).

(D) In vivo imaging of Tg(*flk1:dsRed; cmyb:egfp*) embryos indicated the number of Flk1:dsRed⁺; cMyb:GFP⁺ HSPCs (arrowheads (yellow)) was increased in the AGM at 36hpf in *pdgfb* mRNA injected embryos; however this effect was blocked in *il6* morphants (n≥15/condition x 2 replicate clutches).

(E) Absolute counts of Flk1:dsRed⁺; cMyb:GFP HSCs from embryos in panel 4D (**p**<0.0005, *p*<0.05, two-tailed t-test, n≥15/condition).

(F) FACS analysis of Flk1:dsRed⁺; cMyb:GFP⁺ HSPCs at 48hpf showed overexpression of *il6* significantly increases HSPCs (1.49-fold vs uninjected control, *p*≤0.02, two-tailed t-test, n≥5 replicates/condition).

(G) Absolute cell counts of PH3⁺ cells in AGM of *pdgfb* and *il6* mRNA-injected embryos (*p*<0.05, **p**<0.01, two-tailed t-test, n≥10/condition).
strongly up-regulated gene (4.6-fold change; \( p \leq 0.05 \)) (Figure 2.7 A and Figure 2.8 A). In addition, \( pdgfb \) mRNA injection also increased the expression of the \( il6 \) receptor (\( il6r \)) and its co-receptor, \( gp130 \) (\( il6st \)), suggesting that IL-6 signaling could be a functionally relevant target of PDGF-B/PDGFR\( \beta \) in regulation of embryonic HSPC production (Figure 2.7 A). RT-qPCR analysis of sorted cell fractions of wild-type embryos at 36hpf showed that \( il6r \) and \( gp130 \) are enriched in Flk1\(^+\);cMyb\(^-\) endothelium and Flk1\(^+\);cMyb\(^+\) HSCs (Figure 2.8 B). Modified epistasis analysis demonstrated that MO-mediated knockdown of \( il6 \) only modestly impacted \( runx1 \) expression in the AGM at 36hpf (Figure 2.7 B,C), consistent with its low baseline level of expression (Figure 2.8 B). However, \( pdgfb \) overexpression no longer increased \( runx1;cmyb \) expression in the AGM in the absence of IL-6 function mediated either by MO knockdown (Figure 2.7 B,C) or chemical inhibition of IL-6R/gp130 with SC-144 (Figure 2.8 C, D). These findings were confirmed and quantified by Flk1\(^+\);cMyb\(^-\) cell counts in the AGM (Uninjected: 2.9±1.2 cells/AGM, \( pdgfb \) mRNA: 5.1±1.7, \( il6 \) MO: 3.5±1.7, \( pdgfb \) mRNA+\( il6 \) MO: 3.6±1.9, \( n \geq 15/\text{condition, } *p \leq 0.05, **p \leq 0.005 \)) (Figure 2.7 D,E). In contrast, overexpression of \( il6 \) mRNA increased \( runx1 \) expression in the AGM at 36hpf (Figure 2.8 E,F) and the percentage of Flk1\(^+\);cMyb\(^+\) HSPCs at 48hpf as assessed by flow cytometry (1.49-fold vs. control, \( p \leq 0.02 \)) (Figure 2.7 F). Furthermore, \( il6 \) mRNA significantly increased the number of phospho-histone H3 (pH3)-positive cells in the AGM region, similar to that seen with \( pdgfb \) injection (Uninjected: 5.1±2.5 cells/AGM, \( pdgfb \) mRNA: 7.9±2.5, \( p \leq 0.014; \) \( il6 \) mRNA: 7.4±2.2, \( p \leq 0.002, \) \( n \geq 10/\text{condition} \)) (Figure 2.7 G), suggesting that the PDGF-B/IL-6 signaling axis increases HSPCs via proliferative expansion. Altogether these data indicate that IL-6 functions downstream of PDGF-B/PDGFR\( \beta \) to increase AGM HSC production.
Figure 2.8

(A) qPCR analysis of inflammatory genes in pdgfb mrna injected embryos showed increased expression of il1b, mmp2, and mmp9 (*p≤0.05, **p≤0.01, two-tailed t-test, n≥3).

(B) qPCR analysis using FACS-sorted populations from Tg(flk1:dsRed;cmyb:egfp)p embryos at 48hpf showed that il6 and its receptors are expressed in both the vasculature (Flk1:dsred+ ;cMyb:gfp-) and HSCs (Flk1 :dsred+ ;cMyb:gfp+) (normalized to tbp).

(C) Exposure to the gp130 inhibitor SC-144 (1μM) blocked the ability of pdgfb mRNA to increase runx1;cmyb expression at 36hpf.

(D) Qualitative phenotypic distribution of embryos from panel 2.8C (n ≥20/condition x 3 replicate clutches).

(E) il6 overexpression strongly increased runx1 expression in the AGM at 36hpf.

(F) Qualitative phenotypic distribution of embryos from panel 2.8E (n ≥20/condition x 3 replicate clutches).
Figure 2.8 (Continued)

A. qPCR at 36hpf

<table>
<thead>
<tr>
<th>Gene</th>
<th>Control</th>
<th>pdgfβ mRNA</th>
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<td><img src="chart2.png" alt="" /></td>
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<tr>
<td>mmp2</td>
<td><img src="chart3.png" alt="" /></td>
<td><img src="chart4.png" alt="" /></td>
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<tr>
<td>mmp9</td>
<td><img src="chart5.png" alt="" /></td>
<td><img src="chart6.png" alt="" /></td>
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B. WT FACS sorted cells at 36hpf

<table>
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<tr>
<th>Gene</th>
<th>Control</th>
<th>Fk1&lt;sup&gt;−&lt;/sup&gt; cMyb&lt;sup&gt;−&lt;/sup&gt; (endothelial)</th>
<th>Fk1&lt;sup&gt;+&lt;/sup&gt; cMyb&lt;sup&gt;+&lt;/sup&gt; (hemogenic)</th>
</tr>
</thead>
<tbody>
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<td><img src="chart8.png" alt="" /></td>
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<tr>
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<td>gp130</td>
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<td><img src="chart14.png" alt="" /></td>
<td><img src="chart15.png" alt="" /></td>
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</tbody>
</table>

C. Control | pdgfβ mRNA

D. runx1;cmyb expression

E. Control | il6 mRNA

F. runx1 expression

runx1; cmyb

runx1
**IL-6 signaling acts downstream of Hif1α to increase AGM HSPC production**

Our previous studies indicated that elevated glucose metabolism caused ROS-mediated Hif1α stabilization allowing increased developmental HSPC specification and proliferative expansion (Harris et al., 2013). To further strengthen the regulatory network connecting Hif1α and PDGFRβ signaling to HSC regulation via IL-6, we directly examined the role of IL-6 signaling downstream of Hif1α induction on embryonic HSPC formation. Hif1α stabilization with CoCl₂ during the period of HSC specification (12-36hpf) significantly up-regulated the expression of *il6* (*p*<0.01) and its receptor *il6r* (*p*<0.05) by RT-qPCR (**Figure 2.9** A); furthermore analysis of sorted HSPC fractions showed the predominant responses were found in the Flk1⁺;cMyb⁻ endothelial and Flk1⁺;cMyb⁺ HSC populations (**Figure 2.10** A), similar to that seen for *pdgfb* and *pdgfrb*. Modified epistasis experiments indicated that blocking IL-6 signaling using either the *il6* MO or SC-144 suppressed the effect of CoCl₂ on *runx1;cmyb* expression in the AGM, suggesting that IL-6 signaling acts to further mediate the effect of Hif1α on AGM HSPCs (**Figure 2.9** B,C; **Figure 2.10** B,C). To determine whether IL-6 signaling is sufficient to increase HSPC induction in the absence of Hif1α activity, similarly to that observed for PDGF-B, embryos were co-injected with dominant-negative *hif1a* (*dnhif1a*) (Elks et al., 2011) and *il6* mRNA. Inhibition of Hif1α function decreased *runx1;cmyb* expression in the AGM, consistent with prior observations (Harris et al., 2013); however, overexpression of *il6* mRNA was able to restore *runx1;cmyb* expressing HSPCs to that seen in controls (**Figure 2.9** D,E), indicating that IL-6 signaling functions downstream of Hif1α to influence HSPC production. Collectively, our data reveal a PDGF-associated signaling network connecting Hif1α-stabilization to IL-6
Figure 2.9

(A) RT-qPCR analysis showed *il6* and its receptor *il6R* are increased by CoCl$_2$ exposure (*p*<0.05, **p**<0.01, one-tailed t-test, n≥3).

(B) Morpholino knockdown of *il6* blocked the ability of CoCl$_2$ to increase *runx1; cmyb* expression in the AGM as determined by WISH analysis at 36hpf.

(C) Qualitative phenotypic distribution of embryos from panel 2.9B (n values as in 2.1B).

(D) Injection of *dnhif1a* mRNA decreased *runx1; cmyb* expression in the AGM at 36hpf, which could be partially ameliorated by overexpression of *il6*.

(E) Qualitative phenotypic distribution of embryos from panel 2.9D.
**Figure 2.10**

(A) qPCR analysis of FACS sorted populations showed that *il6* and its receptor *il6r* were upregulated by CoCl$_2$ in both the endothelium (Flk1:dsRed+;cMyb;GFP-) and HSCs (Flk1:dsRed+;cMyb;GFP+).

(B) gp130 inhibitor SC-144 (1μM) blocked the ability of CoCl$_2$ to increase runx1;cmyb expression in the AGM.

(C) Qualitative phenotypic distribution of embryos from panel 2.10B (n ≥20/condition x 3 replicate clutches).
signaling that provides a method to regulate HSPC production through inflammatory cascades during development in response to environmental stimuli.

**Discussion**

The generation and production of HSCs from hemogenic endothelium is an intricate and multifaceted process involving multiple signaling mechanisms and molecular inputs that are spatially as well as temporally regulated (Clements and Traver, 2013; Kim et al., 2014b). It is becoming increasingly clear that environmental and external factors can act upon and feed into the normal process of HSC generation, given that the developing embryo is exposed to fluctuating levels of oxygen, nutrients and energy supply (Harris et al., 2013; North et al., 2009). The importance of the hypoxic sensor Hif1α is now well documented in the adult BM niche, where local hypoxia and resultant Hif1α levels serve a key physiological mechanism to regulate HSC number and intracellular damage by maintaining low metabolic rate and HSC cell cycle quiescence (Takubo et al., 2010). Interestingly, we previously showed that Hif1α can also regulate embryonic HSC numbers and function, in this case by mediating the metabolic response to glucose metabolism to control induction of HSCs from the hemogenic endothelium and their subsequent proliferative expansion in the embryo (Harris et al., 2013); a role for Hif1α as an inductive rather than quiescent factor was similarly demonstrated in mammalian embryos (Imanirad et al., 2014). While Hif1α has been long established to act as a physiological sensor to regulate downstream targets such as Erythropoietin (Epo) and Vascular Endothelial Growth Factor (VEGF) to maintain erythroid homeostasis and vascular remodeling in response to hypoxia (Semenza, 2012), the functional targets of Hif1α that could act to stimulate HSPC production in an expansive niche remain to be identified. In this paper, we show a novel
mechanism of action mediated by Hif1α, which through regulation of PDGFRβ signaling ensures proper production of HSCs through regulation of proproliferative inflammatory signaling during periods of metabolic stimulation during embryonic development.

Expression of PDGF-B and its receptor is induced as a result of increased metabolic activity as result of glucose exposure (Harris et al., 2013; North et al., 2009) as well as direct CoCl2-mediated Hif1α stabilization. PDGF-B has previously been shown to promote the ability of primitive hematopoietic precursor cells to form multilineage colonies in culture, as well as the ex vivo expansion of CD34+ human cord blood cells, although it has been unclear whether the expansion is a result of direct stimulation or secondary to autologous effects on stromal supporting cells (Yan 1993, Su 2002). Our results show that PDGF-B signaling is necessary for the Hif1α-mediated induction of HSCs, and that overexpression of PDGF-B is furthermore sufficient to stimulate increased HSPC production during hematopoietic development in vivo. Recently, PDGF-B activity in trophoblasts was found to be a vital component of the placental niche where it functions to protect HSPCs from premature differentiation into red blood cells by suppressing the production of EPO (Chhabra et al., 2012); this data together with our own presented here suggests that PDGF-B may be playing a role in regulating the balance between HSPC maintenance and differentiation during development. In our studies, we further identify IL-6 signaling downstream of PDGFRβ activity in mediating the effect of metabolic induction of HSCs, as the effect of PDGF-B overexpression on HSPCs number can be attenuated by morpholino knockdown of IL-6. Inflammatory cytokines have been well characterized to activate the mobilization, proliferation, and differentiation of HSCs to ensure an adequate response to infection or injury during demand-driven hematopoiesis (Schuettpelz and Link, 2013; Takizawa et al., 2012), and have also recently been shown to play an important role in
embryonic HSC development (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014). We have identified IL-6 as an additional inflammatory factor that can influence magnitude of developmental HSC production under the control of the Hif1α-PDGFRβ signaling axis. Interestingly, hypoxia has been shown previously to induce the expression of cytokines and proinflammatory mediators, including IL-6 (Tamm et al., 1998; Yan et al., 1995). As there exist many regulatory inputs that converge to influence hematopoiesis, IL-6 may serve as a regulatory node where key environmental stimulus signal into to ensure right number of HSC production and balance of hematopoiesis. In our studies, pdgfb expression was found to be increased specifically in the endothelial cells in response to CoCl₂ mediated Hif1α stabilization, while pdgfrb was found upregulated in both the endothelial as well as hemogenic endothelial/HSC population. IL-6 and IL-6R were similarly upregulated in both the endothelium and HSPCs in response to CoCl₂. While significant further investigation involving cell-type specific studies is warranted, we propose a possible mechanism of action whereby PDGF-B, acting through both an autocrine and paracrine mechanism, can influence IL-6/IL-6R signaling to thereby regulate the overall production of HSPCs.

While mechanistic investigations into how growth factor signaling involving PDGF-B can lead to the activation of IL-6 were beyond the scope of this study, there are many other reports that provide insight into the myriad of possible routes of regulation. In osteoblastic cells, Protein Kinase C (PKC) activation of members of the activator protein-1 (AP-1) complex was determined as having a direct role in the PDGF-B induction of IL-6 (Franchimont et al., 1999). In contrast in glioma-initiating cells, a PDGF-driven signaling axis involving NO-dependent inhibitor of differentiation 4 (ID4) has been shown to promote JAGGED1–NOTCH activity (Jeon et al., 2014). Related, both direct and non-canonical regulation of IL-6 expression by
Notch has been previously reported (Jin et al., 2013; Wongchana and Palaga, 2012). As it is well established that generation of HSCs requires multiple Notch signaling inputs (Butko et al., 2016), it will be interesting to investigate whether Notch signaling may be a part of the Hif1α-PDGFRβ signaling axis in regard to the ability of IL-6 to enhance production of HSPCs. Furthermore, while we report increased proliferation as assessed by PH3+ cell counts following pdgfb and il6 overexpression, whether we are affecting hemogenic endothelial competence for HSC generation or the proliferation of specified HSCs is complicated by the fact that both functions are contemporaneous. Future studies involving live cell imaging using transgenic reporters labeling endothelial and hematopoietic populations may help clarify the exact process in HSPC development that is altered by the Hif1α-PDGFRβ-IL-6 signaling axis.

In summary, we have shown that during development, PDGFRβ can function downstream of Hif1α signaling to regulate the scale of definitive HSPC formation via IL-6 mediated inflammatory regulation. We anticipate that further understanding of exactly how environmental and physiological inputs can induce cell signaling events, in this case metabolic induction of PDGFRβ signaling, to affect the normal course of HSPC production in vivo will provide new insights into mechanisms to improve in vitro generation or expansion of HSCs for research and therapeutic purposes.

Methods
Zebrafish husbandry
Zebrafish were maintained according to IACUC-approved protocols. Tg(-6.0itga2b(CD41):eGFP (Lin et al., 2005), Tg(kdrl(flk1):dsRed), and Tg(cmyb:eGFP), Tg(gata1:dsred) lines (North et al., 2010) were described previously.
Chemical treatments and evaluation

Zebrafish embryos were exposed to compound-modifiers in E3 water in multi-well plates for durations noted. Compounds utilized were: DMPQ (10µM, Cayman), cobalt (II) chloride (500µM, R&D Systems) DMOG (75µM, Cayman), AG1295 (10µM, Calbiochem), AG1296 (2.5µM, Cayman), SC-144 (1µM, EMD Millipore). Whole-mount in situ hybridization (WISH) was performed as previously described (Bertrand et al. 2010). Qualitative phenotypes for individual embryos (n≥20 embryos/condition, ≥2 replicate clutches) were scored as relatively high/medium/low in expression compared to sibling controls and graphically depicted as the percentage falling into each of the 3 phenotypic expression bins; “medium” expression was set as the most representative phenotype in the normal bell-curve distribution of each cohort of control embryos per experiment.

Fluorescence Activated Cell Sorting

FACS analysis was performed using double transgenic Tg(flk1:dsRed;cmyb:GFP) HSPC reporter embryos as previously described (Bertrand et al., 2010). Embryos (pools of 3-5 embryos per sample, ≥5 replicates) were dissociated, resuspended in 1xPBS, and analyzed on a BD FACSCanto II (BD Biosciences, San Jose, CA) in the presence of SYTOX Red Dead Cell Stain (5nM, Life Technologies, Waltham, MA). Data was analyzed using FlowJo X software (TreeStar, Ashland, OR). For isolation of endothelial (Flk1:dsRed⁺cMyb:GFP⁺) and HSPC (Flk1:dsRed⁺cMyb:GFP⁺⁺) fractions, pooled embryos (n>1000) were sorted using FACSARia (BD Biosciences, San Jose, CA). After cell collection, RNA was extracted, treated with DNaseI (RNAqueous-Micro Total RNA isolation Kit, Life Technologies, Rockville, MD), and amplified with Ovation RNA Amplification System V2 (NuGEN).
**Morpholino and mRNA Injection**

vhl and hif1a MOs (GeneTools, Philomath, OR) were injected as described previously (Harris et al., 2013; North et al., 2007). pdgfrb MO (5’ ACA GGA ACT GAA GTC ACT GAC CTT C 3’). was microinjected at 0.2mM in 1-cell stage embryos and allowed to develop to the timepoint of interest before processing with matched sibling controls for evaluation. For mRNA generation, pdgfb and il6 Coding Data Sequences were amplified by PCR (see primers pairs below) from IMAGE clones 6330609 and 40130735 respectively and cloned into pCS2+ (EcoRI/XbaI). mRNA was in vitro transcribed from NotI linearized constructs using the SP6 mMESSAGE mMACHINE kit (Life Technologies) and injected at the 1-cell stage. Mouse Pdgfb mRNA was injected at 25ng/μl, mouse il6 mRNA was injected at 200ng/μl, and dnhif1 mRNA (Elks et al., 2011) was injected at 200ng/μl.

**pdgfb**

F: 5’ GATGGAATTCCATGAATCGCTGCTGGGCG  
R: 5’ GATGTTCTAGACTAGGCTCCGAGGGTCTCC

**il6**

F: 5’ GATGGAATTCCATGAAGTTCTCTGCAAG  
R: 5’ GATGTTCTAGACTAGGTTTGCCGAGTAGATC

**Generation of hsp70:ca-pdgfrb expression construct and heat-shock induction**

Mouse pdgfrb Coding Data Sequence was amplified from the IMAGE clone 30060666 and cloned into the pENTR-D-TOPO vector (Invitrogen) using the following primers:

F 5’ CACCATGGGGCTTCCAGGAGTGATACCAG  
R 5’ CTACAGGAAGCTCTCTGCTGCTTCAGCC
The D849V amino acid substitution used to generate the constitutively activating mutation (ca-pdgfrb), as described in murine Pdgfrb (Magnusson et al 2007), was created by site-directed mutagenesis. The hsp70:ca-pdgfrb construct was generated using the multigateway LR Clonase (Invitrogen) Gateway reaction. The resulting plasmid was microinjected with tol2-transposase RNA into 1-cell-stage embryos (Kawakami et al., 2004). Tg(hsp70:ca-pdgfrb) and WT sibling control embryos were heat-shocked at 27hpf by incubation in a 37°C water bath for 1 hour. Embryos were fixed at 48 hpf for analysis and processed as above.

**Microscopy**

Fluorescent embryos were treated as above and imaged by fluorescence microscopy using a Zeiss Discovery V8/Axio Cam MRC and Axiovision LE software (Carl Zeiss) as previously described (North et al., 2007). Cell counts were quantified using ImageJ (NIH). Two-tailed Student’s t-tests were performed: data are presented as mean±SEM, and p-values less than 0.05 were considered significant.

**Quantitative RT-PCR**

qPCR was performed on cDNA isolated from pooled embryos at timepoints indicated (n=25 embryos/variable) using ABI PRISM 7900HT (Invitrogen). Primer pairs utilized are listed in the table below (Methods Table 2.1). Samples were run in technical triplicate with ≥3 biological replicates/condition. Ct values were determined using PCR Miner (Zhao and Fernald, 2005) and fold-change calculated by the ddCt or R0 method with tbp or B-actin as the reference gene. Two-tailed Student’s t-tests were performed, unless otherwise indicated.
### Methods Table 2.1

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Chapter 3:
The Impact of Chronic Stimulation of Glucose Metabolism on Embryonic Hematopoietic Stem Cell Production and Function

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Attributions

SEL, VE, JMH and GF performed embryo exposures and WISH. SEL and VE did FACS, and qPCR analysis. MC and SEL conducted FACS sorting for microarray analysis. SEL performed Western Blotting. SEL, VE, WG, and TEN designed experiments and evaluated results. We thank: B. Paw, A. Wagers, and D. Langenau for suggestions and reagents.
Introduction

There are several cases in the literature that suggest high glucose exposure in utero alters hematopoietic homeostasis. Meta-analyses have revealed that children born to mothers with gestational diabetes have an increased risk of developing acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Hjalgrim et al., 2003). There is also a strong correlation between early onset leukemic risk and Type I diabetes (Hemminki et al., 2012). Further, umbilical cord blood from neonates born to mothers with gestational diabetes showed decreased platelet function and impaired neutrophil mobility (Mehta and Petrova, 2005). These data suggest that sustained exposure to high blood glucose levels during development may have profound and long term consequences on the hematopoietic program.

Hematopoietic stem cells (HSCs) can both self-renew and differentiate into all mature blood cell lineages and thereby reconstitute the entire blood system (Orkin and Zon, 2008). In vertebrates, hematopoiesis occurs in two distinct waves and is accompanied by shifting sites of production and colonization (Cumano and Godin, 2007). The first wave, termed primitive, begins at E7.5 in the yolk sac in mammalian embryos. The primitive wave produces mostly erythroid cells, and is thought to be a transient mechanism to provide the embryo with oxygen as passive diffusion becomes inadequate. The definitive wave, beginning at E10.5, produces the long-term repopulating hematopoietic cells that can give rise to erythroid, myeloid, and lymphoid cells. The definitive wave occurs in the hemogenic endothelium of the dorsal aorta in the intraembryonic site called the AGM (aorta-gonadal-mesonephros) region in vertebrates (Dzierzak and Speck, 2008). Runx1 (AML1/Cbfa2), frequently the target of genetic alterations in human leukemia (Kalev-Zylinska et al., 2002), is a transcription factor required for definitive hematopoiesis (North et al., 1999; Wang et al., 1996); Runx1 is necessary for HSC induction from arterial hemogenic endothelium (Chen et al., 2009; North et al., 2002). The HSCs and
progenitors that arise from the AGM colonize subsequent sites of definitive hematopoiesis including, the fetal liver, thymus, spleen, and bone marrow, where they undergo further maturation and expansion (Orkin and Zon, 2008).

Zebrafish have emerged as an advantageous model system for the study of hematopoiesis due to their embryonic transparency, high fecundity, and strong evolutionary conservation with mammals for hematopoietic genes. In zebrafish, primitive hematopoiesis occurs in ventral mesoderm derived tissues in the intraembryonic regions called the intermediate cell mass (ICM) and anterior lateral mesoderm (ALM) (Detrich et al., 1995). Definitive hematopoiesis, marked by runx1 and cmyb expression, appears robustly by 36hpf in the AGM (Murayama et al., 2006). HSCs then colonize the caudal hematopoietic tissue (CHT), an intermediate site of hematopoiesis thought to be equivalent to the mammalian fetal liver. HSCs subsequently migrate to the thymus by 3 dpf to initiate lymphopoiesis. By 4 dpf, HSCs can be found in the kidney marrow, the zebrafish equivalent of the mammalian bone marrow (Orkin and Zon, 2008).

In vertebrates, Hypoxia Inducible Factor (HIF) functions as the master regulator of the adaptive response to low oxygen levels, or hypoxia (Hickey and Simon, 2006). HIF1 is a transcription factor composed of the oxygen-responsive HIF-1α subunit, and the constitutively expressed HIF1β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT). Under normoxia, HIF1α is hydroxylated and marked for degradation by the von Hippel-Lindau tumour suppressor protein (VHL). Hypoxia inhibits HIF1α degradation, allowing it to interact with HIF-1β to activate transcription of hypoxic responsive genes, which affect a wide range of cellular processes including: enhanced glucose metabolism, erythopoiesis, and angiogenesis (Simon and Keith, 2008). Additional gene targets of hypoxia/HIF1α are
growth factors, including VEGF (Vascular Endothelial Growth Factor) and PDGF-B (Platelet-Derived Growth Factor-B) (Semenza, 2001). While the role of HIF on cellular metabolism and proliferation in cancer has been well studied, the specific function for HIF1α in hematopoiesis during vertebrate embryogenesis remains unknown (Semenza, 2003).

Hypoxia has recently been identified as playing a role in adult HSC quiescence in the murine bone marrow (Suda et al., 2011). Serial transplantation of Hif1α−/− HSCs resulted in loss of long term reconstitution (Takubo et al., 2010). Hif1β has also been implicated in hematopoiesis; Hif1b deletion resulted in embryos that lacked blood-filled vitelline vessels, suggesting a deficiency in hematopoiesis in addition to previously seen vascular defects (Adelman et al., 1999; Maltepe et al., 1997). Hematopoietic colony formation assays using Arnt−/- E9.5 yolk sac also yielded a significant decrease in the number of colony-forming-unit progenitors including CFU-E and CFU-GM compared to their sibling controls (Adelman et al., 1999). Explants from Arnt−/- embryos also exhibited non-cell autonomous defects in vasculo- and hematopoietic colony formation in vitro, which could be rescued by addition of VEGF (Ramirez-Bergeron et al., 2006). However, as homozygous deletion of Arnt leads to lethality by E10.5, and as the vascular niche affects HSC formation, the requirement for HIF and the transcriptional response to hypoxia directly on HSCs has not been studied in vivo in mammals (Ramirez-Bergeron et al., 2006).

Recently, our lab has uncovered a novel role of transiently elevated glucose levels in expanding HSCs during embryonic blood development through reactive oxygen species (ROS) mediated induction of the Hif1α hypoxic response (Harris et al., 2013). However, the impact of chronically elevated glucose levels on the developing hematopoietic system has not been well characterized. Interestingly, children of mothers with gestational diabetes are at a greater risk of
developing leukemia, suggesting that blood glucose levels can affect the formation and differentiation potential of HSCs long-term. Here, we find that in contrast to the relatively beneficial response of HSCs to transient flux in metabolic rate, chronic blood glucose elevation during embryogenesis leads to lineage skewing during larval stages via combined action of Hif1α and Insulin/FOXO signaling, which is maintained from embryonic to adult stages.

**Results**

**Chronic glucose elevation increases developmental HSPC production**

Our earlier studies indicated that transient (acute) exposure to heightened glucose concentrations lead to increased production of HSCs in the zebrafish embryo, which was mediated by metabolism induced ROS production and subsequent HIF1a activity (Harris et al., 2013). In order to begin to assess the impact of chronic glucose exposure on HSC production and function in the vertebrate embryo, we exposed zebrafish embryos to 1% glucose in the fish water (equivalent to a 2-fold increase in internal glucose concentration (Harris et al., 2013) from 24hpf to 5dpf (120hpf), the time point when HSCs robustly begin to populate the kidney marrow. Chronic glucose exposure (24-120hpf) during the onset and progression of definitive hematopoietic increased cmyb expression by whole mount in situ hybridization (WISH) analysis (Figure 3.1 A, B); this effect was confirmed by RT-qPCR on embryos exposed to glucose from 12-120hpf (p<0.001) (Figure 3.1 C). To investigate impact at the cellular level, fluorescence microscopy and FACS analysis of the Tg(-6.0itga2b(CD41):eGFP transgenic reporter embryos was employed: FACS analysis indicated that chronic glucose exposure led to increased numbers of CD41:GFP+ HSCs (Figure 3.1 D), consistent with prior observations for acute treatment
Figure 3.1

(A) Embryos exposed to chronic glucose (1%) during HSC production (24-120hpf) exhibited increased *cmyb* expression, particularly in the developing kidney marrow (n≥18/condition).

(B) Qualitative phenotypic distribution of embryos from panel 3.1A scored with low, medium or high *cmyb* expression in the KM.

(C) qPCR analysis showed *cmyb* was significantly upregulated in chronic glucose treated embryos (tx 12-120hpf) compared to untreated controls (p<0.001).

(D) FACS analysis of *Tg(CD41:eGFP)* embryos showed that chronic glucose treated embryos (tx 24-120hpf) exhibited a statistically significant increase in the CD41^{hi} thrombocyte population (p<0.05), but not in the CD41^{lo} HSC population at 120hpf.
(p<0.01) (Harris et al., 2013). Interestingly, preliminary assays using further segregation of the CD41 population by FACS analysis revealed that while the CD41\(^{hi}\) thrombocyte population had increased, there no longer appeared to be a significant change in the CD41\(^{lo}\) HSC population with chronic treatment at 120hpf (Figure 3.1 D), suggestive of potential alterations in lineage commitment, including myeloid expansion, with chronic glucose treatment. As segregation into CD41\(^{hi}\) vs CD41\(^{lo}\) populations may be influenced by maturation status of the thrombocyte population and gating strategies, we will perform analysis of CD41:GFP\(^{+}\) HSCs in embryos crossed to the Tg(gata1:dsred) line to more definitely characterize the impact of chronic glucose elevation.

**HSPC function is altered by chronic developmental exposure to heighten glucose levels**

As definitive HSPCs contribute to hematopoietic lineage production by 120hpf, we assessed the effect of chronic glucose elevation on the erythroid, myeloid and lymphoid lineages. Chronic glucose (24hpf-120hpf) exposure led to the appearance of increased myeloid cell numbers in the kidney marrow, and throughout the embryo, as assessed by whole mount in situ hybridization (WISH) for myeloperoxidase (mpo) (Figure 3.2 A,C); this finding was confirmed by RT-qPCR analysis (p<0.01) at 5dpf (Figure 3.2 D). Importantly, this phenotype did not appear to be due to sickness or infection related to treatment (data not shown); furthermore, gata1 expression was likewise elevated by qPCR analysis (Figure 3.2 D), consistent with the established role for Hif1\(\alpha\) activity in erythroid development (Haase, 2013), implying specific effects of chronic glucose exposure on hematopoietic regulation. To confirm whether the increase in mpo expression was indicative of elevated myeloid cell numbers, fluorescence microscopy and FACS analysis using lineage reporters was employed: total Mpo\(^{+}\) cells were
Figure 3.2

(A) Embryos exposed to chronic glucose elevation during HSC production (24-120hpf) exhibited increased myeloid expression as assessed by mpo WISH (n≥20/condition).

(B) Embryos exposed to chronic glucose (24-120hpf) exhibited increased lymphoid expression as assessed by rag1 WISH (n≥20/condition).

(C) Qualitative phenotypic distribution of embryos from panels 3.2A (mpo, top) and 3.2B (rag1, bottom).

(D) qPCR analysis showed mpo was significantly upregulated in chronic glucose treated embryos (tx 12-120hpf) compared to untreated controls (p<0.001); cd41 and gata1 expression were also enhanced in chronic glucose treated embryos.

(E) FACS analysis of myeloid specific lines at 120hpf showed increased Mpo+ (p<0.05), Lyz+ (p<0.05), and Mpeg+ (p=0.06) cells in chronic glucose treated embryos.

(F) FACS analysis of lymphoid specific lines at 120hpf showed moderate increase in Rag+ and Lck+ cells in chronic glucose treated embryos.
significantly increased (p<0.001) in Tg(mpo;GFP) embryos by FACS at 120hpf following chronic glucose exposure (Figure 3.2 E). Analysis of other myeloid specific lines: Tg(lyz;dsred) and Tg(mpeg:gfp) using FACS revealed similar increases in these specific myeloid lineage subpopulations (27% for Lyz:dsRed (p<0.05); 30% increase for Mpeg;GFP (p=0.06) (Figure 3.2 E). A moderate increase in lymphoid cells, as assessed by rag1 WISH was also observed for prolonged glucose exposure (Figure 3.2 B,C), and confirmed RT-qPCR for rag1 and lck (Figure 3.2 F), together indicative of a total increase in hematopoietic content.

To further dissect the effect of increase in overall HSC number from that of an effect on lineage differentiation, we treated embryos at later time intervals from 72-120hpf, after the emergence of HSCs in the AGM. While an increase was still seen in the myeloid population with this later treatment window by mpo WISH and RT-qPCR (Figure 3.3 A, E), we now saw a decrease in the lymphoid population, as assessed by rag1 and lck WISH and RT-qPCR analysis (p<0.05) (Figure 3.3 B-E). The increase in Mpo\(^+\) cells was confirmed by FACS (Figure 3.3 F) at 120hpf. We further quantified the decrease in lymphoid cells on embryos treated from 72-132hpf using the Tg(rag2;gfp) and Tg(lck:gfp) transgenic lines and saw a 0.86 (p<0.05) and 0.89-fold reduction (p<0.005), respectively (Figure 3.3 G). Interestingly, by RT-qPCR (Figure 3.3 E), we also observed an increase in expression of the early lymphoid progenitor marker ikaros, but decrease in foxp3, a marker of mature T-cells, suggestive of a potential differentiation arrest, reminiscent of the lymphoid differentiation block and susceptibility to ALL seen in children born to mothers with gestational diabetes. Together, this data indicates that chronic exposure to glucose has sustained effects on HSC number, which can impact hematopoietic lineage production, however, those cells maybe susceptible to differentiation blocks or bias.
Figure 3.3

(A) Embryos exposed to elevated glucose levels during the period of HSC maturation (72-120hpf) exhibited increased mpo expression in the KM and throughout the embryo.

(B) Embryos exposed to elevated glucose levels from 72-120hpf exhibited decreased rag1 expression in the thymus at 120hpf.

(C) Embryos exposed to elevated glucose from 72-120hpf exhibited decreased lck expression in the thymus at 120hpf.

(D) Qualitative phenotypic distribution of embryos from panel 3.3B

(E) qPCR analysis showed embryos exposed to elevated glucose from 72-120hpf exhibited an increase in the myeloid genes mpo (p<0.001) and lyz. These embryos exhibited a concomitant decrease in expression of lymphoid genes such as rag2 (p<0.001) and lck (p<0.05); foxp3 expression was similarly decreased (p<0.05), while ikaros expression was increased (p<0.001).

(F) FACS analysis showed an increase in Mpo⁺ cells in embryos treated with glucose from 72-120hpf (*p<0.05).

(G) FACS analysis of lymphoid reporter lines confirmed a decrease in Rag2⁺ and Lck⁺ cells in embryos exposed to elevated glucose during the period of HSC maturation (72-132hpf) (*p<0.05, ***p<0.001).
Chronic elevations in developmental glucose levels has long-term hematopoietic consequences

We had previously demonstrated that short-term embryonic glucose stimulation can increase the number of HSCs with adult kidney marrow repopulating potential (Harris et al., 2013); further, we demonstrated that transient glucose modulation in the adult could similarly enhance hematopoietic stem and progenitor recovery after irradiation injury (Harris et al., 2013). To begin to interrogate long-term consequences of chronic glucose exposure during embryonic development (24-120hpf) on adult HSC function, we assessed isolated KM for impact on the HSC precursor population and differentiated blood lineages based on forward scatter (cell size) and side scatter (granularity) properties (Traver et al., 2003) at 1 and 3 months post-treatment. Chronic glucose exposure during embryonic development led to subtle increases in the lymphoid and precursor populations at 6 weeks (Figure 3.4 A). Similar analysis was conducted using the Tg(rag2:GFP) line at 3 months; interestingly, in female fish at 3 months post glucose treatment, we found increased (p<0.05) numbers of cells in the “lymphoid gate” (Figure 3.4 B), which includes both lymphoid cells and HSCs. As our earlier embryonic analysis suggested negative impact on lymphogenesis, to better determine which cells in the lymphoid pool were expanded, we further looked at the Rag2+ population within the lymphoid gate: this assay showed an increased number of Rag2+ cells in the lymphoid gate (p<0.05; n=10) with embryonic glucose exposure (Figure 3.4 B), indicating that the increased cell fraction in our SSC/FSC analysis was attributable, at least in part, to an increased number lymphoid cells. To further decipher this effect, a similar experiment was conducted using Tg(rag2:dsred;cd41:gfp) double transgenic fish. In preliminary studies at just over 1 month post treatment, a statistically significant increase in the precursor population (p<0.01) was observed in males (Figure 3.4 C), but not females exposed to chronically elevated glucose. Interestingly, females, showed a slight
Figure 3.4

(A) KM from 6 week adults that had been exposed to chronic glucose elevation during embryonic development from 24-120hpf was analyzed by FACS. KM from adults exposed as embryos to elevated glucose levels showed slightly increased numbers of cells in the lymphoid and precursor gates then matched sibling controls.

(B) Tg(rag2:GFP) adults that had been exposed to chronically elevated glucose during embryonic development from 24-120 hpf showed an increased number of Rag2⁺ cells in the lymphoid gate (p<0.05; n=10).

(C) Male, but not female, Tg(rag2:dsred;cd41:gfp) double transgenic fish at 7 weeks post treatment with excess glucose (24-120hpf) showed a statistically significant increase in the precursor population (p<0.01).
Figure 3.4 (Continued)

A  
Control  
1% Glucose  

FSC/SSC KM FACS  
lymphoid 12.3%  8.7%  
precursor 20.7%  21.9%  
myeloid  14.4%  10.3%  

WT Adults 6 weeks post Glucose Exposure  

B  
Control  
1% Glucose  

FSC/SSC KM FACS  
lymphoid 14.5%  12.6%  
precursor 52.4%  53.6%  
myeloid  17.1%  11.5%  

Rag2:dsRed Adults 3 months post Glucose Exposure  

Rag2:dsRed Adults 3 months post Glucose Exposure  

C  
Males  
Females  

Control  1% Glucose  Control  1% Glucose  
Rag2:dsRed;CD41:GFP Adults 7 weeks post Glucose Exposure  

**p < 0.01
increase in Rag\(^+\) cells in the lymphoid gate (*data not shown*), while the cellular source of the effect on males was less clear, indicating that the phenotype present at 3 months in the prior study may be related to aging, and/or differentially influenced by gender. While in contrast to our evaluation of T-cell development at 120hpf (5-day), the finding of an enhanced pool in the lymphoid gate is reminiscent of clinical observations connecting gestational diabetes to lymphoid leukemias. Importantly, an impact on B-cell development, which occurs at approximately 3 weeks post fertilization (Page et al., 2013), was not previously evaluated in our embryonic studies. Together this data suggests that chronic exposure to glucose in the embryo has little effect on HSC numbers in the adult, but may impact their differentiation capacity (B vs T cells), and/or stimulate possible compensatory mechanism allowing for the production of adequate or excess lymphoid cells later in the adult.

**Hif1α is partially responsible for the effects of chronic excess glucose on HSPCs**

As our lab (Harris et al., 2013), and others (Takubo et al., 2010), previously showed that Hif1α can mediate the effects of metabolic/hypoxic regulation on HSPCs during embryonic development, we examined whether the increase in HSPC number and/or impact on lineage differentiation with chronic glucose dysregulation was due to Hif1α activity. As seen with acute treatment (North et al., 2009), chronic glucose exposure from 12-120hpf led to significant impact on the expression of a number of hemato-vascular associated genes by qPCR; Hif1α targets, such as *pdgf* and *vegf* increased, as did HSC-relevant factors including *nos2* (**Figure 3.5 A**). Chemical inhibition of Hif1α function with YC1 (Segawa et al., 2006) (tx 24-120hpf) was found to block the myeloid increase seen with chronic glucose exposure, by *mpo* WISH (*data not
Figure 3.5

(A) qPCR analysis showed chronic glucose exposure (tx 12-120hpf) led to increased expression of Hif1α-target and insulin-related genes (*p<0.05, **p<0.01, ***p<0.001).

(B) Hif1α-target genes observed to be downregulated in chronic glucose exposed embryos (*p<0.05, ***p<0.001) by qPCR.

(C) Chemical inhibition of Hif1α with YC-1 (2.5µM) blocked the increase in myeloid cell number with chronic glucose exposure (tx 24-120hpf) of Tg(mpo:gfp) by fluorescence microscopy.

(D) Qualitative phenotypic distribution of embryos from panel 3.5D
Figure 3.5 (Continued)

A. qPCR 12hpf - 5dpf upregulated genes

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B. qPCR 12hpf - 5dpf downregulated genes

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C. Control vs 1% Glucose

- DMSO
- YC1 (2.5 μM)
- Mpo:GFP at 120hpf (tx 24-120hpf)

D. Mpo:GFP 120hpf

- Untreated
- glucose
- YC1
- + YC-1

% Embryos
shown) and fluorescence microscopy for Mpo\(^+\) cells (Figure 3.5 C, D). In addition to sustained activation, some genes previously shown to be positively stimulated by Hif1\(\alpha\) with acute glucose exposure, were actually found to be downregulated by chronic glucose stimulation, including \textit{igf2} and \textit{nos1} (Figure 3.5 B). This finding indicates that direct or indirect alterations in these factors could be contributing to the differential effects on HSC maintenance and differentiation potential found with short versus long-term elevation in glucose metabolism and Hif1\(\alpha\) function.

To begin to determine whether Hif1\(\alpha\) stabilization is sufficient to induce lineage bias, zebrafish embryos were exposed to either CoCl\(_2\) or DMOG during hematopoietic development (24-120hpf). WISH analysis revealed that similar to glucose, long-term exposure to CoCl\(_2\) increased the appearance of \textit{mpo}\(^+\) cells in the zebrafish embryo (Figure 3.6 A,C); this effect was confirmed by FACS evaluation for Mpo\(^+\) cells using the Tg(\textit{mpo}:GFP) line (Figure 3.6 B). Long term exposure to CoCl\(_2\) and DMOG also led to decreased \textit{rag} expression by WISH analysis (Figure 3.6 A,C). To further dissect the role of Hif1\(\alpha\) in regard to the lineage phenotypes associated with chronic glucose exposure, we created a \textit{hsp70:dnhif-mcherry} transgenic line using a published dominant negative hif (\textit{dnhif}) construct (Elks et al., 2011). The heat shock line has been functionally validated as active (data not shown); ongoing studies to optimize the heat shock conditions will allow us to determine if blocking Hif1\(\alpha\) function genetically will attenuate lineage dysregulation in the setting of metabolic stimulation, as well as provide a tool to interrogate potentially causative versus passenger genetic alteration further.
Figure 3.6
(A) Embryos exposed to Hif1α stabilizers CoCl₂ or DMOG during HSC production (24-120hpf) exhibit skewed lineage potential, favoring myeloid over lymphoid production, as assessed by mpo and rag1 WISH.
(B) FACS analysis showed embryos exposed to CoCl₂ from 24-120hpf exhibit increased Mpo⁺ cells (p<0.05)
(C) Qualitative phenotypic distribution of embryos from panel 3.6A for mpo (left) and rag1 (right).
**Chronic glucose elevation initiates an insulin response that can impact HSPCs**

In our previous analysis, we determined that acute glucose exposure and metabolic stimulation of HSPC production during early development had no impact on nor elicited a classical insulin response in the embryo (Harris et al., 2013), likely due to the fact that this organ is immature during this early window of development (pre-36hpf). However, as pancreatic regulation of glucose metabolism via insulin production is thought to begin around 48hpf (Jurczyk et al., 2011), we examined whether an insulin response was initiated by chronic glucose exposure. Consistent with feedback regulation in the adult, insulin expression was induced following sustained glucose exposure from 24hpf-120hpf (see Figure 3.5 A). To begin to understand if insulin regulation had an impact on either HSPC production or lineage commitment, we made use of a previously published nitroreductase cell ablation line (Pisharath et al., 2007). Ablation of islet cell-mediated insulin production via metronidazole exposure (72-120hpf) of Tg(ins:nfsB-mCherry) embryos antagonized myeloid lineage dysregulation caused by chronic glucose exposure (Figure 3.7 A, B); importantly, ablation had very little effect on mpo expression in the absence of glucose treatment. The lymphoid lineage was also reduced slightly as marked by rag1 WISH in the thymus (Figure 3.7 C, D); however, metronidazole exposure alone decreased lymphoid expression, precluding a definitive examination by this methodology. Together, these findings suggest that insulin production is stimulated by chronic glucose exposure in the 72-120hpf embryo, and it may contribute either directly or indirectly to the observed hematopoietic phenotypes.

One possible mechanism of insulin action is the activation of phosphatidylinositol 3-kinase/Akt signaling, which regulates downstream targets such as TSC/mTOR, GSK3b, and
Figure 3.7
(A) Ablation of islet cell-mediated insulin production via metronidazole exposure (72-120hpf) of Tg(ins:nfsB-mCherry) embryos blocked the increase in myeloid cells caused by chronic glucose exposure as assessed by mpo WISH.
(B) Qualitative phenotypic distribution of embryos from panel 3.7B.
(C) Ablation of insulin production via metronidazole exposure (72-120hpf) led to a reduction in the lymphoid lineage as marked by rag1 WISH in the thymus; metronidazole exposure alone also decreased lymphoid expression.
(D) Qualitative phenotypic distribution of embryos from panel 3.7C.
(E) Western blot analysis of embryos exposed to chronically elevated glucose (24-120hpf) showed increased phosphorylation of FOXO1, 3a, and 4 proteins.
(F) qPCR analysis of embryos exposed to glucose from 60-120hpf showed increased expression of FOXO target genes including catalase and cdkn1a (*p<0.05, ***p<0.001).
Figure 3.7 (Continued)

A  
\[\text{mpo in situ (tx 72-120 hpf)}\]

B  
Phenotypic distribution
ins:nfsB
mpo expression

C  
\[\text{rag in situ (tx 72-120 hpf)}\]

D  
Phenotypic distribution
ins:nfsB
rag expression

E  

F  
gPCR Analysis

\[\text{tx 60-120 hpf}\]
FOXOs, involved in cell survival, protein synthesis, cell cycle status, and the response to physiologic oxidative stress (Manning and Cantley, 2007; Tothova et al., 2007). Knockout mice of several of these pathway components have been documented to have an impact on hematopoietic stem cell functions (Rossi et al., 2012), with FOXO-1, -3 and -4 knockout mice in particular exhibiting increased cycling, increased myeloid population, and decreased lymphoid cells (Tothova et al., 2007) in adults, reminiscent of the chronic glucose-associated embryonic phenotype. Significantly, phosphorylation of FOXOs 1/3/4, downstream targets of insulin signaling, was increased in embryos treated with glucose from 60-120hpf (Figure 3.7 E); FOXO target genes, including catalase and cdkn1a, were likewise upregulated (Figure 3.7 F) by chronic glucose exposure. Intriguingly, treatment with the FOXO1 inhibitor AS1842856 (100nM) alone from 60-120hpf increased myeloid differentiation by WISH at 120hpf (Figure 3.8 A, B), indicating a potential role for this regulatory cascade in mediating our phenotype. Furthermore, the effect of AS1842856 treatment 72-120hpf increased mpo expression by RT-qPCR (p<0.05) (Figure 3.8 C), with a concomitant decrease in lck expression (p<0.001) similar to that seen for chronic glucose stimulation. To connect alterations in FOXO phosphorylation to insulin signaling, kinase activity downstream of receptor activation was examined: preliminary analysis indicated that glucose exposure from 72-120 led to increased p-AKT levels (data not shown). Importantly, treatment with the PI3K inhibitor, LY294002 (10µM) antagonized the myeloid lineage dysregulation caused by chronic glucose treatment as indicated by mpo WISH (Figure 3.8 D, E); however, LY294002 exposure alone also decreased the appearance lymphoid cells as marked by rag1, (data not shown) indicating that targeting PI3K may not be specific to all aspects of the effect of chronic glucose exposure, or causes global inhibition of HSPCs and all resultant downstream lineages.
Figure 3.8

(A) Chemical inhibition of FOXO1 using AS182856 produces an increase in myeloid cells and decrease in lymphoid cells as marked by mpo and lck.

(B) Qualitative phenotypic distribution of lck WISH embryos from panel 3.8C.

(C) qPCR analysis of embryos exposed to AS182856 (tx 72-120hpf) confirmed inhibition of FOXO1 increased mpo and decreased lck expression (*p<0.05, ***p<0.001).

(D) Exposing embryos to the PI3K inhibitor LY294002 (tx 72-120hpf) partially blocks the increase in myeloid cells elicited by chronic glucose exposure.

(E) Qualitative phenotypic distribution of embryos from panel 3.8A.

(F) Embryos exposed to the mTOR inhibitor Torin exhibited a reduction in myeloid and lymphoid cells marked by mpo and lck (n≥17).
Figure 3.8 (Continued)

**A**

DMSO

AS1842856

mpo in situ  lck in situ

(tx 60-120 hpf)

N = 32

↑ 100%

**B**

Phenotypic distribution

lck (120hpf)

% Embryos

DMSO  AS1842856

**C**

gPCR Analysis

mpo (tx 72-120 hpf)

Relative Expression

mpo  lck

DMSO  AS1842856

E

Phenotypic distribution

mpo (120hpf)

% Embryos

DMSO  glucose  LY294002  glucose + LY294002

High  Medium  Low

**D**

Control  1% Glucose

DMSO  LY294002

N ≥ 14

mpo in situ (tx 72-120 hpf)

**F**

DMSO  Torin

N = 20

N = 17

mpo in situ  lck in situ

(tx 72-120 hpf)

↓ 100%

↓ 100%
In adult hematopoietic regulation, mTOR activation is known to increase proliferation of committed progenitors at the expense of HSC maintenance, indicating a role of nutrient availability in regulating adult hematopoietic quiescence (Huang et al., 2012). To assess the potential effect of mTOR signaling downstream of insulin receptor activation, the chemical inhibitor Torin was utilized: Torin treatment (1µM) from 72-120hpf decreased both myeloid cells as accessed by mpo and lymphoid cells as accessed by lck WISH (Figure 3.8 F), indicating that mTOR may also elicit global inhibition of HSPCs production and/or function, including impact on the downstream differentiated lineages. To further elucidate the role of insulin-associated Akt and TSC/mTOR function, protein levels and phosphorylation status will be assessed in embryos treated with glucose from 24-120hpf prior to initiation of epistasis studies.

**Informatics analysis reveals potential mediators of HSPC dysregulation from excess glucose**

In an effort to try to further elucidate the pathways that may be dysregulated by exposure to chronically elevated levels of glucose, which may be mediating our various lineage phenotypes, microarray analysis was performed. 50,000 CD41+ HSCs were FACS sorted from 120hpf Tg(CD41:gfp) control embryos and their matched siblings that had been exposed to glucose from 24-120hpf. Ingenuity pathway analysis (IPA) (Figure 3.9 A) revealed that among the top networks affected were those associated with: Metabolism, Cellular assembly and Organization; Humoral Immune Response/Inflammatory Response, and Cell Cycle/Energy Production consistent with our prior analyses for acute glucose stimulation (Harris et al., 2013). IPA also indicated that among the top canonical pathways affected were Oxidative Phosphorylation and Mitochondrial Dysfunction (Figure 3.9 B), clearly highlighting the use of
Figure 3.9

(A) Microarray analysis was performed on RNA isolated from sorted CD41+ cells from embryos exposed to chronically elevated glucose from 24-120hpf compared to untreated sibling controls (n≥120). Ingenuity Pathway Analysis (APA) indicates Top [5] Networks affected.

(B) IPA Top Canonical Pathways affected by chronic glucose elevation.

(C) GSEA Analysis of the upregulated gene set following chronic glucose exposure.

(D) GSEA Analysis of downregulated gene set with chronic glucose treatment.
### A

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<tr>
<th>ID</th>
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<td>1</td>
<td>Cellular Assembly and Organization, Cell-To-Cell Signaling and Interaction, Reproductive System Development and Function</td>
<td>50</td>
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<tr>
<td>2</td>
<td>Cellular Function and Maintenance, Small Molecule Biochemistry, Developmental Disorder</td>
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<td>3</td>
<td>Humoral Immune Response, Inflammatory Response, Infectious Disease</td>
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<td>4</td>
<td>Cell Cycle, DNA Replication, Recombination, and Repair, Energy Production</td>
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<td>5</td>
<td>Tissue Morphology, Inflammatory Disease, Respiratory Disease</td>
<td>28</td>
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### B

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<th>Ratio</th>
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<tr>
<td>Oxidative Phosphorylation</td>
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<td>1.33E-01</td>
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<td>Mitochondrial Dysfunction</td>
<td>4.13E-02</td>
<td>7.44E-02</td>
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<tr>
<td>Regulation of Actin-based Motility by Rho</td>
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<td>Mechanisms of Viral Exit from Host Cells</td>
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<td>1.56E-01</td>
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<td>Glutrogenesis</td>
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<td>RhoGDI Signaling</td>
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<td>6.44E-02</td>
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<tr>
<td>Phosphatidyl C Kinase signaling</td>
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<td>6.06E-02</td>
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<th>Description</th>
<th># Genes in Overlap (K)</th>
<th>K/K</th>
<th>P-value</th>
<th>q-value</th>
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<td>DIAZ_CHRONIC_MEYLOGENOUS_LEUKEMIA_UP [182]</td>
<td>76</td>
<td>Genes up-regulated in CD34+ [GeneID=947] cells isolated from bone marrow of CML (chronic myelogenous leukemia) patients, compared to those from normal donors.</td>
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<td>1.1</td>
<td>e^{1.73}</td>
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<td>ROME_INSULIN_TARGETS_IN_MUSCLE_UP [442]</td>
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<td>Genes up-regulated by 3 h of hyperglycemic hyperinsulinemic clamp in the vastus lateralis muscle of healthy lean subjects.</td>
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<td>1.64</td>
<td>e^{3.87}</td>
<td>e^{3.87}</td>
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<td>PDGF_UP_V1_UP [146]</td>
<td>11</td>
<td>Genes up-regulated in S1/SYSY cells (neuroblastoma) in response to PDGF [Gene ID=] stimulation.</td>
<td>11</td>
<td>1.88</td>
<td>e^{9.98}</td>
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### D

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<tr>
<th>Gene Set Name</th>
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<th>Description</th>
<th># Genes in Overlap (K)</th>
<th>K/K</th>
<th>P-value</th>
<th>FDR q-value</th>
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<td>PTEN_DN.V1_DN [187]</td>
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<td>Genes down-regulated upon knockdown of PTEN [Gene ID=5728] by RNAi.</td>
<td></td>
<td>1.32</td>
<td>e^{2.3}</td>
<td>2.86 e^{3}</td>
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excess glucose as metabolic fuel in embryonic HSCs. Gene Set Enrichment Analysis (GSEA) documented that the upregulated genes from our microarray dataset overlapped with published gene sets upregulated in “Chronic Myelogeneous Leukemia”, “Insulin Targets in Muscle”, and “Genes up-regulated (in SH-SY5Y cells) in response to PDGF Stimulation” (Antipova et al., 2008; Diaz-Blanco et al., 2007; Rome et al., 2003) (Figure 3.9 C). Interestingly, GSEA analysis of the downregulated genes from our microarray dataset revealed overlap with “Genes downregulated upon knockdown of PTEN,” a negative regulator of AKT signaling (Vivanco et al., 2007) (Figure 3.9 D). In contrast, expression of FOXO3a was upregulated 1.2-fold in FACS-sorted glucose exposed HSCs by microarray analysis (data not shown). Together, this analysis supports our findings that both Hif1α- and Insulin-mediated responses are activated by chronic glucose exposure and can influence HSC production and function.

Validation of the role of downstream targets of glucose stimulation in hematologic dysregulation

Consistent with alterations in myeloid production seen throughout our phenotypic analysis, exposure to chronically elevated glucose was associated with a strong inflammatory signature by microarray and subsequent pathway analysis. Among the inflammatory genes upregulated were il8 (1.8-fold), il1b (1.9-fold), and tnfα (2-fold) (Figure 3.10 A); mpeg1.2, a gene associated with macrophages, recently shown by our lab and others to be necessary for embryonic HSC production (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014) was upregulated by 2.4-fold. Confirmation of potential regulatory targets of chronic glucose stimulation by qPCR analysis revealed induction of several
Figure 3.10

(A) Select upregulated and downregulated genes, potentially relevant to the observed hematopoietic phenotypes, and their fold changes by microarray analysis of CD41+ HSCs isolated from chronically elevated glucose exposed embryos vs. controls. (*p<0.05, **p<0.01, ****p<0.0001)

(B) qPCR analysis of embryos exposed to chronically elevated glucose levels (tx 24-120hpf) showed increased expression of inflammatory cytokines and their receptors.

(C) Treatment with the Notch inhibitor DAPT (20µM) phenocopied the decrease in lymphoid cells marked by lck in chronic glucose exposed embryos.

(D) Qualitative phenotypic distribution of embryos from panel 3.10C.

(E) qPCR analysis of embryos treated with the FOXO1 inhibitor AS1842856 showed a decrease in notch1a and notch1b expression (p<0.05).
Figure 3.10 (Continued)

<table>
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<th>T value</th>
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<td>mpeg1.2</td>
<td>2.37978</td>
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<td>il3</td>
<td>2.166128</td>
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<td>tnfa</td>
<td>2.041332</td>
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<td>il1b</td>
<td>1.93368</td>
<td>0.991</td>
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<td>il8</td>
<td>1.844284</td>
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<td>hdac3</td>
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<td>mil4b</td>
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<td>notch1a</td>
<td>-5.31479</td>
<td>0.991</td>
<td>6.2346</td>
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qPCR Analysis
tx 24-120 hpf

Interferon signaling

Interleukin signaling

Control
Glucose

Phenotypic distribution

\( lck \) (120hpf)

<table>
<thead>
<tr>
<th>% embryos</th>
<th>DMSO</th>
<th>glucose</th>
<th>DAPT</th>
<th>glucose + DAPT</th>
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Relative Expression

notch1a
notch1b

DMSO
20uM DAPT

20uM DAPT

\( lck \) in situ tx 72-120
interleukins, including \textit{il1, il6} (see PDGF chapter for additional details on the role of IL-6 in HSPC regulation), and \textit{il4} (\textbf{Figure 3.10 B}). This data is in line with clinical observations showing an altered inflammatory profile in diabetic patients (Grossmann et al., 2015). As inflammatory dysregulation is associated with enhanced cell proliferation, including HSCs, and the potential for stem and progenitor cells exhaustion, it will be important to determine if this cellular pressure contributes to the propensity for leukemic transformation in children born to mothers with gestational diabetes. We have recently created an inducible \textit{il1b} line to interrogate the role of that inflammatory cytokine, previously associated with AGM HSC production in the mouse (Orelio et al., 2008) and zebrafish embryo (Esain et al., 2015), in glucose-mediated alterations in HSC number and/or function.

In addition to metabolic and inflammatory regulation, Notch signaling appears to be impacted by chronic glucose exposure. Our prior study indicated that Notch activity was not significantly modified by acute glucose treatment (Harris et al., 2013). In contrast, \textit{notch1a} was significantly downregulated (5-fold) in HSCs exposed to chronic glucose elevation (\textbf{Figure 3.10 A}). \textit{Notch1a} is known to play a role in the differentiation and maturation of T cells (Radtke et al., 2010), suggesting that it could be playing a role in the early lymphoid differentiation arrest seen with chronic glucose exposure. Intriguingly, treatment with DAPT, an inhibitor of Notch signaling, phenocopies the decrease in lymphoid cells we see with chronic glucose exposure by \textit{in situ} (\textbf{Figure 3.10 C, D}). In ongoing studies we will determine if re-activation of Notch signaling using the previously established \textit{hs:Gal4; UAS:NICD} system can partially rescue the decreased appearance of lymphoid cells due to chronic glucose exposure. Interestingly, qPCR analysis of FOXO inhibitor treated embryos also showed decreased expression of both \textit{notch1a} and \textit{notch1b}, further supporting its role as a relevant downstream target (\textbf{Figure 3.10 E}).
Together these preliminary findings imply Notch may be acting alone or in concert with additional factors to impact HSC differentiation and/or maturation to the lymphoid lineage downstream of metabolic dysregulation caused by chronic glucose elevation.

In contrast to the negative effect on Notch signaling, *insulin growth factor 2 binding protein 1 (igf2bp1)*, was found to be upregulated 1.8 fold in glucose treated HSCs (Figure 3.10 A). Prior studies have identified significant correlations between *IGF2BP1* overexpression and B-cell Acute Lymphocytic Leukemic (B-ALL) (Stoskus et al., 2011), which has strong associations with gestational diabetes. *Igf2bp1* is known to bind to Insulin Growth Factor 2 (IGF2) and regulate its translation (Bell et al., 2013). IGF2 was identified as a Hif1α regulated gene that, interestingly, shows differential responses to acute (Harris et al., 2013) versus chronic glucose exposure (see Figure 3.5 B). *IGF2BP1* is predominantly expressed in embryonic tissues, but can be reactivated and overexpressed in various human neoplasias (Ioannidis et al., 2005). While FACS analysis using available lines does not distinguish between T and B cells in the lymphoid gate or with the Rag2+ reporter, the stalled maturation of embryonic T-cell development together with Notch downregulation, and the increased lymphoid pool seen in adults following embryonic exposure to chronically elevated glucose implies that B-cells may be preferentially expanded via alterations in IGF expression and function.

Finally, the microarray results also indicated several epigenetic modifiers were upregulated in glucose treated HSCs, including *histone deactylase 3 (HDAC3)* and *mixed lymphoid leukemia (MLL)* (Figure 3.10 A) This finding is intriguing given that our analysis of 3 month old adults, exposed to chronically elevated glucose during embryogenesis, appear to show sustained dysregulation of blood cell homeostasis (see Figure 3.4). HDAC3 is known to associate with oncoproteins that drive leukemia and lymphoma (Summers et al., 2013).
Similarly MLL, as the name implies, is a frequent target of mutation in leukemia; MLL is a histone methyltransferase that when mutated in murine embryos leads to significant impact on in vitro HSPC production, including both smaller (growth retarded) and fewer colony forming units-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-macrophage (CFU-M), and burst-forming unit-erythroid (BFU-E) (Hess et al., 1997; Yagi et al., 1998). It is interesting to speculate that changes in the action of these epigenetic modifiers by chronic glucose elevation may target genes found to be over or under-expressed by 120hpf, causing sustained alterations in expression that impact adult HSC number and function as seen in our ongoing evaluations.

**Discussion**

Our ongoing investigations have prospectively elucidated the impact of chronic heightened glucose levels and subsequent metabolic activity on hematopoiesis during gestation. Interestingly, our work not only identified immediate impact downstream of combinatorial regulation mediated by Hif1a and Insulin/FOXO activity, but revealed long-term consequences to metabolic dysregulation for the hematopoietic system. Importantly, these findings almost directly parallel clinical literature, suggesting that further investigation in this area may speak to the correlations between gestational diabetes and hematopoietic dysfunction, including leukemogenesis, and may also provide a panel of rational targets to control and/or correct particular aspects of the effects of chronically elevated glucose on HSC production and function.

The prevalence of metabolic disorders with high blood glucose levels, including gestational diabetes, has significantly increased in developed countries in recent decades (Wang et al., 2012). Excess nutrients, provided by diabetic mothers, and resulting biological responses,
such as insulin production, can have far-reaching consequences on the developing vertebrate (Luo et al., 2012; Sermer et al., 1995): higher birth weight is the most obvious outcome of continuously elevated glucose, insulin and IGF levels in the placental circulation, while impacts on neural and cardiovascular development have also been described (Vambergue and Fajardy, 2011). Despite significant advances in the detection of maternal diabetes and regulation of glycemic control, both acute and chronic exposure to hyperglycemia and/or hyperinsulinemia affect 3 to 10% of all pregnancies (Nold and Georgieff, 2004) and is anticipated to rise over the following decades; furthermore, even under good maternal glycemic control, a significant fraction of infants are still born macrosomic, suggesting mild chronic elevations in physiological glucose levels and/or hyperglycemia prior to midgestation when the embryonic pancreas is sufficiently developed to mount an insulin response can have significant effects on embryonic and fetal development (Nold and Georgieff, 2004). Hyperglycemic memory of issues with maternal glycemic control are associated with defects in pancreatic function such as diabetes, metabolic disturbances including a propensity toward obesity in offspring, as well as risk of hypertension and cardiovascular disease (Vambergue and Fajardy, 2011); recent studies have indicated this memory may be mediated by epigenetic changes, and in the case of vascular endothelial cells, appears to be due to a persistent proinflammatory or stress response (Brasacchio et al., 2009) to glucose. Meta-analyses of birth records world-wide have revealed significantly enhanced risks for acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) with increasing birth weight, particularly in children under the age of 2 (Feltbower et al., 2004; Hjalgrim et al., 2004; Hjalgrim et al., 2003). ALL is also more frequently found in children and adults with diabetes, highlighting the sustained regulatory impact of glucose metabolism and response on HSC fate (Shu et al., 2010; Zendehdel et al.,
Further, cord blood from neonates born to mothers with gestational diabetes showed decreased platelet function (Strauss et al., 2010) and neutrophil mobility (Mehta and Petrova, 2005), suggesting glucose levels can modify hematopoiesis in the absence of mutation. Together, these data imply fluctuations in nutrient supply exert direct hematopoietic influence, which may have relevance to human HSC function, including disease onset and progression; the studies described here will help to prospectively illustrate how and why physiological modulations of glucose levels may lead to hematopoietic overproduction or dysregulation.

Understanding the factors that regulate the hematopoietic niche has significant therapeutic value; the phenomenon of shifting sites of hematopoietic induction during development has long been described, however the physiological rationale and cues that initiate these changes have not been identified. Recent studies reveal the importance of the local environment, the HSC niche, in this process; we have shown that HSC induction from the vascular niche (AGM region) coincides with the onset of the heartbeat and subsequent blood flow (North et al., 2009) and requires active participation of the primitive myeloid population (Li et al., 2014). Previously, we examined whether embryonic hematopoietic niches localize to sites of active nutrient and oxygen exchange (Harris et al., 2013), including whether physiological fluctuations in metabolic activity correlate with Hif1α status and transcriptional regulation of hematopoietic targets. Significant developmental delay and embryonic lethality had prevented definitive conclusions concerning the requirement for Hif1α function in HSC formation in mammalian models (Semenza, 1994); however, recent work using and inducible model confirmed our findings in zebrafish, demonstrating that metabolic regulation via Hif1α can significantly impact HSC development (Imanirad et al., 2014). However, the consequences of
prolonged or repeated exposure to hyperglycemia and/or resultant insulin responses mimicking disease states such as gestational diabetes were unknown.

Here, we prospectively demonstrated a potent impact of long-term (chronic) exposure to elevated glucose levels on HSCs, and further showed that this effect was likely mediated only in part via Hif1α. Interestingly, we also observed that a subset of factors positively regulated by acute glucose exposure, were negatively impacted by chronic dysregulation. In future studies, the implications of this differential regulation of Hif1α targets and known HSC regulatory factors will need to be directly investigated in more detail using inducible regulatory constructs allowing precise spatio- and/or temporal- regulation. In particular, as the IGF family and its receptors are not only well established as a hematopoietic regulators, but can co-ordinately act with the insulin signaling cascade, it will be important to determine how that interplay may influence downstream signaling and HSC fate. Furthermore, we clearly identified a role for metabolism-induced insulin stimulation and subsequent signaling through the FOXO family as mediating effects on lineage differentiation that paralleled clinical observations. While a precise understanding of which FOXO members are acting to mediate this phenotype and the targets contributing to alterations in HSC function remain to be determined, this work illustrates that complex alterations in both autonomous and non-cell autonomous signaling that could lead to the phenotypes seen in patients born to mothers with gestational diabetes. Finally, and perhaps most intriguingly, we demonstrated that metabolic dysregulation during embryonic development can have long-term consequences for the hematopoietic system, which may be relevant to the associations between metabolic dysregulation and leukemia. While not evaluated here, coupled with the reduced T-cell formation seen in the embryo, the increase in lymphoid gate in the adult analysis suggests potential expansion of the B-cell population; significantly, this unanticipated
observation mirrors that observed in ALL patients. In addition to the anticipated alterations in hematopoietic associated gene targets, our microarray analysis illustrated significant changes in the expression of established epigenetic regulators known to be active in blood cells. It will be interesting to determine if these factors, or related candidates provide the “memory” of the developmental microenvironment that could explain long-term consequences of altered embryonic glucose levels that influences HSC numbers and function.

In summary, we recently showed that glucose metabolism controls the onset and magnitude of HSC induction in vivo. In zebrafish, transient glucose elevation elicited dose-dependent effects on HSCs mediated by elevated metabolic activity and subsequent ROS-mediated induction of Hif1α. Here we show that chronic exposure to excess glucose also significantly impacts hematopoiesis; in contrast to the relatively beneficial response of HSCs to moderate flux in metabolic rate, chronic blood glucose elevation during embryogenesis leads to lineage skewing during larval stages resulting in elevated erythrocyte and myeloid cell numbers, with decreased lymphoid production. These results are due to combinatorial interplay of Hif1α-mediated transcriptional regulation and insulin homeostasis-induced FOXO activity and resulted in long-term consequences for HSC numbers and function. Together, these studies indicate that both acute and chronic alterations in metabolic state affect HSCs and may further explain immunological phenotypes associated with metabolic syndromes and cancer.

**Methods**

**Zebrafish husbandry**

Zebrafish were maintained according to BIDMC IACUC approved protocols. Tg(-6.0itga2b(CD41):eGFP) (North et al., 2009), Tg(cmyb:eGFP) (North et al., 2009),
Tg(mpeg:eGFP) (Ellett et al., 2011), Tg(lyz:dsred) (Stachura et al., 2013), Tg(mpo:Egfp) (Hall et al., 2007), Tg(rag2:gfp) (Esain et al., 2015) and Tg(lck:gfp) (Langenau et al., 2004) lines were described previously.

**Chemical treatments and evaluation**

Zebrafish embryos were exposed to compounds in E3 (fish) water in multi-well plates from 5-somites (12hpf) until 120hpf, unless otherwise noted; glucose concentration was 1%, unless indicated. Compounds and doses utilized were as follows: YC-1 (2.5µM, Cayman), Torin (1µM, Cayman), AS1842856 (100 nM, Calbiochem), LY294002 (10µM, Cayman), CoCl₂ (500µM, R&D Systems), DMOG (Cayman), DAPT (20µM, Tocris Bioscience). *In situ* hybridization was performed using published protocols ([http://zfin.org/ZFIN/Methods/ThisseProtocol.html](http://zfin.org/ZFIN/Methods/ThisseProtocol.html)) and probes. Phenotype distribution is summarized as: #-altered/ #-scored per treatment (tx); ≥2 independent experiments were conducted per analysis.

**Fluorescent microscopy and FACS analysis**

Fluorescent reporter embryos were exposed to compounds as indicated above. Embryos were imaged by fluorescence microscopy as previously described (Harris et al., 2013); images were acquired using a Zeiss Axio Imager A1 or Zeiss Discovery V8/Axio Cam MRC and Axiovision LE software (Carl Zeiss, Oberkochen, Germany). Fluorescence Activated Cell Sorting (FACS) analysis of transgenic reporters (pools of ≥3 embryos x >5 biological replicates) was performed as previously described (Harris et al., 2013).
**Quantitative RT-PCR and microarray analysis**

qPCR was performed on cDNA isolated from pooled embryos (n=25/variable) using a iQ5 RTPCR Detection System (BioRad) as previously described (North et al., 2007). Primer pairs utilized are listed in the table below (Methods Table 3.1). Microarray (Affymetrix) experiments were performed in duplicate using embryos at 120hpf and analyzed using Ingenuity Pathway software as described (Harris et al., 2013).

**Methods Table 3.1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>gata1</td>
<td>TGAATGTGTGAATGTTGTTG</td>
<td>ATTCGCTTCCCATAGTGTGTT</td>
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<tr>
<td>lyz</td>
<td>GTGAAAATGGACGGGTGCAAA</td>
<td>CTTTTGTTTGGCTGCTCACA</td>
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<tr>
<td>mpo</td>
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<td>GAGCTGTGTTTCTTGGTTGTTGT</td>
</tr>
<tr>
<td>rag2</td>
<td>ACGCTCATGCTCAACTGGGATA</td>
<td>CTCTGCTGCTCTACGCTCAAACATGTA</td>
</tr>
<tr>
<td>lck</td>
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<td>il7r</td>
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### Methods Table 3.1 (Continued)

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<td>notch1a</td>
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</tr>
<tr>
<td>notch1b</td>
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<tr>
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<td>CACTTGCGAACGCTCCTGTTG</td>
</tr>
<tr>
<td>FOXO1a (2)</td>
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<td>ACTCCACCTGCCCATCAC</td>
</tr>
<tr>
<td>FOXO3b</td>
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<td>CTGTGAGAGACCGAGCAAT</td>
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<td>c-myc</td>
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<td>GCTGCTGGATCTGCTGGAT</td>
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</table>

**Western Blot Analysis**

Western blot analysis was conducted on pooled embryo lysates as described (n=30 embryos/condition) and repeated in duplicate (Li et al., 2014).
Chapter 4:

Discussion
Conclusions and Discussion

HSCs comprise the base of the entire hematopoietic system and alone are capable of both self-renewal and differentiation into all the mature blood lineages, thereby maintaining immune function, tissue perfusion and hematopoietic homeostasis. HSCs are therapeutically valuable for the treatment of hematological malignances, immunodeficiencies and bone marrow (BM) failure. Understanding the factors that contribute to HSC formation in the embryo is essential for its application to regenerative medicine, as it can aid the ex vivo generation and/or expansion of functional and transplantable HSCs for the treatment of the blood diseases and disorders. As elucidated in this thesis, HSC generation during development requires the complex interplay between both cell-extrinsic and cell-intrinsic cues and regulatory pathways, which can affect multiple stages of HSC development from establishment of the hemogenic niche to the induction and production of HSPCs from the hemogenic endothelium and their eventual expansion and migration to secondary sites of hematopoiesis. Beyond its role in the regulation of HSC homeostasis in the bone marrow niche, we, and others, previously demonstrated the importance of the hypoxic sensor Hif1α in controlling the scale of HSC development in vertebrate embryos. However, prior to the investigations detailed here, the downstream mechanism of action of Hif1α-mediated HSC regulation during embryogenesis had remained elusive.

Role of PDGF in hematopoietic production

We previously demonstrated that heightened glucose metabolism increased HSC formation in the Aorta-Gonad-Mesonephros (AGM) region of zebrafish embryos through elevated ROS levels and Hif1α stabilization (Harris et al., 2013). Exposure of zebrafish embryos to excess glucose during HSC formation enhanced pdgfb expression as assessed by quantitative
RT-PCR (qPCR). Using zebrafish, we characterized the role of PDGF–B signaling as an inducer of expanded developmental HSC production downstream of metabolic stimulation. Platelet-Derived Growth Factors (PDGFs) are a family of growth factors regulating cell proliferation, survival, migration and differentiation. A long line of research has established PDGFs as having an essential function in vascular remodeling and angiogenesis. PDGF-B signaling has also been reported to regulate a variety of embryonic and adult hematopoietic functions including the migration, proliferation or differentiation of platelets, macrophages and erythrocytes. However, a role for PDGF-B in developmental blood stem cell formation and function had not been elucidated. In the present study, we reveal that PDGF-B signaling through PDGFRβ is required downstream of Hif1α to increase HSC production. Overexpression of pdgfb enhanced HSC formation, even in the presence of Hif1α inhibition, suggesting PDGF-B signaling is sufficient to induce HSC numbers. The finding that PDGF-B signaling acts to promote HSC production in response to changes in the microenvironment is a key finding that can be translated to promote the expansion of HSCs for therapeutic purposes.

Recently, PDGF-B signaling in the placenta was identified as necessary for restricting the differentiation of HSPCs into erythrocytes through inhibition of EPO, through a non-cell autonomous manner (Chhabra et al., 2012). Chhabra et al. had discovered that loss of PDGF-B, which is mainly expressed in the endothelium, led to upregulation of EPO in the PDGFRβ expressing sinusoidal trophoblast giant cells, a population known for its role in secreting essential factors in fetal circulation (Chhabra et al., 2012). This suggests paracrine signaling between the endothelium and trophoblasts acting to direct cell fate. Similarly, in our studies, PDGF-B upregulation via Hif1α stimulation was evoked specifically in the endothelial cell population; given the present literature, a cell-non autonomous mechanism of signal propagation
may be likely, but will require promoter specific modulation of PDGFRβ to confirm. The studies by Chhabra et al. indicated that the upregulation of EPO due to PDGF-B loss in the placenta was mediated through a hypoxia independent mechanism (Chhabra et al., 2012). Hif1α is well established to induce EPO expression and erythropoiesis as a response to hypoxic conditions (Semenza, 2007); as such, it is interesting to speculate whether during periods of stimulation by Hif1α, which is known to increase erythropoiesis, a certain degree of PDGF-B signaling may necessary to simultaneously restrict differentiation to ensure that proper numbers of HSCs are maintained.

**HSCs and the concept of demand-driven hematopoiesis**

The hematopoietic system has evolved mechanisms to dynamically respond to stress, such as inflammatory signals produced during injury or infection, by communicating stress-induced changes in the peripheral blood system back to the bone marrow where cellular output is increased to meet the needs of the host. This adaptive regulation of hematopoiesis has been termed demand driven hematopoiesis (Hall et al., 2016; Takizawa et al., 2012). It has become increasingly recognized that hematopoietic progenitors can respond directly to such injury or infection induced inflammatory signals through expression and downstream activation of cytokine receptors. Recent studies have indicated that HSPCs can respond directly to inflammatory cytokines including interferon (IFN)-α/β, IFNγ, and tumor necrosis factor (TNFα), to become activated and increase proliferation (Baldridge et al., 2011; King and Goodell, 2011). These signals would then allow for demand driven output of hematopoietic cells, leading to increased proliferation and skewed differentiation toward immune cell lineages equipped to fight microbicidal infiltration (Takizawa et al., 2012). There is evidence that during stress-
induced hematopoiesis, HSCs can directly upregulate cytokines that are critical in mediating inflammatory responses, including proliferation, migration and/or differentiation (Zhao et al., 2014), highlighting the importance of cytokine signals in the regulation of stress hematopoiesis.

A Role for inflammatory signaling during embryonic hematopoiesis

In addition to the role of inflammatory signals in maintaining adult hematopoietic homeostasis, prior studies in mice have indicated that cytokines, including IL-1 and IL-3 are involved in the embryonic development of HSPCs in the AGM. IL-1 signaling has been reported to act within the mouse AGM to suppress differentiation of HSCs along the myeloid lineage (Orelio et al., 2008). While an overt hematopoietic phenotype had not been reported in IL3 knockout mice, AGM explants from IL3 knockout mice were unable to repopulate the bone marrow upon transplantation, while addition of IL-3 could rescue the decrease in AGM HSC numbers in AGM explants due to Runx1 haploinsufficiency (Robin et al., 2006). As a possible mechanism of action, IL-3 was found to promote the proliferation and/or survival of embryonic HSCs (Robin et al., 2006). PGE2 (North et al., 2007) and NO (North et al., 2009), discovered to be required for HSC development, also fall under the category of inflammatory molecules.

In this study, we found using a candidate approach for genes regulated by PDGF-B that the expression of il6 and its receptors is increased upon pdgfb overexpression or Hif1α stabilization. Modified epistasis experiments confirmed IL-6 is required downstream of the Hif1α-PDGF-B axis. IL-6 is a pleiotropic cytokine involved in immune regulation, hematopoiesis, inflammation, and oncogenesis (Kishimoto, 2010). While IL-6 is well appreciated as an important regulator of myeloid differentiation (Zhao et al., 2014), previous research also provides supporting evidence for a role of IL-6 in regulating HSCs. Impaired
hematopoiesis has been reported in knockout mice for gp130, the co-receptor in IL-6 signaling (Yoshida et al., 1996). Furthermore, the combination of IL-6, soluble IL-6 receptor chain (sIL-6Ra), and SCF was found to promote in vitro expansion of human cord blood CD34+ hematopoietic progenitors (Sui et al., 1999); an increase of hematopoietic progenitors in IL-6/sIL-6Ra double transgenic mice has also been reported (Peters et al., 1997). To this literature, our study now adds that IL-6 can act to promote hematopoietic production during embryogenesis in response to increased demand or stress via induction by Hif1α.

More recently, work by our lab and others have identified that many pro-inflammatory cytokines are both necessary and sufficient for vertebrate HSCs emergence (Espin-Palazon et al., 2014; He et al., 2015; Li et al., 2014; Sawamiphak et al., 2014). In the absence of IFNγ signaling, zebrafish embryos exhibited fewer HSCs and downstream lymphoid progenitors; similar reductions in HSPCs were observed in mouse embryos lacking IFNγ or IFNα signaling (Li et al., 2014). IFNγ was also found by another group to contribute to HSC emergence, increasing EHT rather than altered endothelial/HSC proliferation or survival (Sawamiphak et al., 2014). TNFα/Tnfr2 signaling, established as a fundamental driver of inflammation, was found to act within endothelial cells upstream of NF-kb activation to affect the emergence of HSCs (Espin-Palazon et al., 2014). He et al. also showed that TLR4-MyD88 and Gcsfr are required for HSC emergence through NF-kb signaling (He et al., 2015). Interestingly, many of these studies found that these inflammatory signals are regulated by or act upstream of Notch signaling, which has been established by multiple studies to be fundamental in HSC development. The effect of TNFα/Tnfr was found to be driven by the action of Notch, mediated by the Jag1a ligand and Notch1a receptor, within emergence HSCs (Espin-Palazon et al., 2014). Infγ was found to be regulated by Notch signaling during HSC development (Sawamiphak et al., 2014). The Tlr4bb-
MyD888-NFkB signaling axis was found to act upstream of Notch signaling (He et al., 2015). Interestingly, IL-6 is a known target of NF-Kb, now understood to act via Notch to induce HSCs. As Notch is a fundamental player in the emergence of HSCs, it will be interesting to explore a connection of the Hif1α-PDGF-IL6 signaling axis with Notch signaling. Such studies will extend our understanding of how stress and inflammatory signaling pathways can converge during early development to collectively help orchestrate HSC production during development.

The emergence and growth of HSCs in the midgestation mouse embryo is a complex process requiring HSC formation to maturation and influenced by the specific microenvironments of the various hematopoietic sites (Robin et al., 2006). A study evaluating the transcriptomes of definitive HSC ontogeny has described that relative to the FL and BM, the AGM is enriched in IL-3 and IL-6 signaling pathways (Kim et al., 2016; McKinney-Freeman et al., 2012). Nascent AGM HSCs have been shown to exhibit molecular signatures reminiscent of their endothelial origin (Kim et al., 2016; McKinney-Freeman et al., 2012). Further investigation will be needed to understand the developmental timing of action of the Hif1α-PDGF-IL6 signaling on HSC production as well as the cellular mechanism involved. Our cell sorting experiments have suggested an endothelial source of PDGF-B upon Hif1α stimulation, whereas IL-6 was upregulated in both the endothelial and HSC population. Further studies expressing the dominant negative/constitutively active receptors of PDGF or IL-6 under tissue specific promoters combined with confocal imaging of hemogenic endothelial reporter lines will help to further elucidate the spatio-temporal activity and cellular mechanism involved. Interestingly, PGE2 and PKA-CREB signaling have also been found to activate IL-6 expression in vascular smooth muscle cell migration and fibroblasts (Chava et al., 2009; Raychaudhuri et al., 2010). As the AGM is enriched for shear-stress mediated PGE2, which has been shown to act through
PKA/CREB signaling in HSC formation (Kim et al., 2016), IL-6 may be a relevant expansion factor downstream of many signaling factors for the enhancement of HSCs. Indeed in our studies, it appears as IL-6 serves as a common node upon which multiple signaling can converge onto to boost production of HSCs during times of stress in a demand driven manner. Finally, additional work is needed to determine the regulatory network downstream of IL-6 that impacts HSPC expansion. Stat3 has been identified as one of the prime Stat/Jak targets downstream of IL-6 signaling (Kishimoto, 2010). Accordingly, Stat3 knockout mice have significant reduction in the numbers of KSL CD34- cells, which represent a pool of LT-HSCs (Mantel et al., 2012). These follow up studies will further our discovery that the Hif1α-PDGF-B axis promotes HSC production in the hemogenic endothelial niche via upregulation of pro-inflammatory cytokines such as IL-6.

Long term consequences of Hif1α and inflammatory activity

While we and others have revealed that inflammatory cytokines, macrophages, and glucose metabolism can have a positive outcome on the production of HSCs, it is clear that these factors contribute also to negative outcomes and even drive tissue dysfunction in the context of chronic inflammation and autoimmune diseases (Medzhitov, 2008). Clinical observations indicate significant correlation between dysregulation of these pathways and negative outcome in the transplant setting. As an example, IL-1β, which is known as a key emergency cytokine signal that rapidly activates host defense and repair in many tissues, including the blood system, is secreted by BM stromal cells upon irradiation, IL-1β (Bigildeev et al., 2013); injection of IL-1β can drastically increase the survival of lethally irradiated mice, presumably by stimulating expression of downstream cytokines and growth factors such as IL-3, IL-6, G-CSF and GM-CSF.
Yet many of the inflammatory disease conditions associated with chronic IL-1 production such as rheumatoid arthritis, obesity, and type-2 diabetes also feature severe hematological complications, including overproduction of tissue-damaging myeloid cells, loss of lymphoid cell production, and chronic anemia (Cain et al., 2009; Pietras et al., 2016; Smith et al., 1992). Work by Pietras et al. recently demonstrate that IL-1 acts as a double-edged sword for HSC function: during acute need, IL-1 can promote myeloid regeneration without functional cost to HSCs, but following chronic exposure, IL-1 significantly impairs HSC self-renewal and lineage output (Pietras et al., 2016). Concordantly, in our studies into the long term consequences of pathway modulation described in this thesis, we showed that chronic exposure to glucose, invoking hyperglycemic conditions, led to a myeloid bias, creating increased myeloid cells at the expense of lymphoid cells. Understanding how HSC production and homeostasis is affected by a chronic inflammatory state, such as in the setting of therapy-induced hyperglycemia, is of critical interest for developing strategies to control hematopoietic outcome and alleviate negative effects on the patient in the setting of hematopoietic stem cell transplant therapy. It is clear that there is an optimal therapeutic window whereby glucose metabolism and/or inflammatory factors can act to modulate HSCs in a positive manner; more research must be done to identify this optimal window of time. The ultimate goal would be to utilize specific factors to promote HSC production in vitro and/or in vivo but bypass the negative effects of chronic exposure that might impact differentiation into myeloid cells and HSC exhaustion.

**Closing Summary**

The work presented in this thesis has delineated several relevant factors acting downstream of metabolic stimulation and Hif1α signaling during vertebrate hematopoietic
development in vivo. A role for PDGF in hematopoietic formation in vivo has not been appreciated previously. Specifically, we have identified PDGF as functioning downstream of Hif1α stimulation in hematopoietic stem cell production during early development. Secondly, we have identified IL-6 is one of the regulatory targets of PDGF responsible for the effects on HSC production. This work demonstrates that IL-6 is a critical player downstream of the Hif1α-PDGF-B axis that acts to ensure adequate HSC production in the changing embryonic microenvironment. In addition to the Hif1α-PDGF-B-IL6 regulatory cascade, we have also investigated the impact of Insulin/FOXO-mediated feedback regulation in mediating long-term consequences for metabolic stimulation in the embryo, impacting lineage homeostasis in the embryo and adult. Our hope is that work from this thesis, and future studies clarifying the impact of long-term exposure to glucose metabolism and inflammatory factors on hematopoietic development, can be translated for use in regenerative therapies and/or form the basis for rational approaches to minimize the hematopoietic impacts of metabolic disease, such as diabetes.
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display a hypoxic response and recapitulate key aspects of Chuvash polycythemia. Blood 113, 6449-6460.


