Hemogenic Endothelium within the Zebrafish Caudal Hematopoietic Tissue illustrates the Common Ties of the Vascular and Hematopoietic Systems

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ABSTRACT

MELISSA JO WOOLLS. Hemogenic Endothelium within the Zebrafish Caudal Hematopoietic Tissue illustrates the Common Ties of the Vascular and Hematopoietic Systems.
(Under the direction of Suk-Won Jin)

Hemogenic endothelium involves the specification of a hematopoietic stem cell (HSC) from an existing endothelial cell. It, along with common developmental origins, co-regulation, and shared niches are examples of the close ties the hematopoietic and endothelial lineages share in development. As a significant portion of total HSCs are generated via a hemogenic endothelium intermediate, modulation of this pathway is expected to impact both hematopoietic and endothelial development. Currently, our understanding of how endothelial cells transition to the HSC lineage is still limited. We found that non-aortic endothelial cells, specifically venous endothelial cells, can undergo the transition to an HSC lineage, suggesting that hemogenic capacity is a more general characteristic of endothelial cells then previously appreciated. Upon further analysis, we found that they were positive for venous specific markers and hematopoietic transcription factors. We identified a potential homolog of Platelet Endothelial Cell Adhesion Molecule 1 (PECAM1) in zebrafish, characterized its expression during development. It is expressed in a manner consistent with PECAM1 and is involved in flow-dependent process. Finally, we find that loss of this PECAM1-like molecule is capable of modulating hematopoiesis, suggesting that the vascular and hematopoietic share common machinery responsible for the observed response to blood flow.

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CHAPTER 1

Hematopoietic and Vascular Systems Overlap to Coordinate Function

The endothelial and hematopoetic lineages share a close developmental relationship, characterized by a common progenitor, close development, shared signaling cues, and even the ability of endothelial cells to transdifferentiate into hematopoietic progenitors[1-6]. The interdependence of the hematopoietic and vascular systems was first demonstrated by detailed studies of the vascular mutant *cloche* which lacks all endothelial and hematopoietic cells[7]. Though these mutants lack all cells of both lineages, the defect in endothelial cells is cell-autonomous, while the defect in hematopoietic cells is non-cell autonomous[7]. This suggests that in *cloche* mutants, the hematopoietic system is suffering from the lack of an intact vascular system. Understanding the interconnectedness of these two systems has significance, as dysfunction in either system has the ability to perturb human health. The cardiovascular system is responsible for the delivery of blood, nutrients, and oxygen to all tissues and organs of the body. Dysfunction of the vascular endothelium has been tied to diabetes, atherosclerosis, and tumor angiogenesis[8-11]. The blood carried by the cardiovascular system includes erythrocytes and immune cells produced by the hematopoietic system. Specific misregulation of the hematopoietic system can result in leukemias and auto-immune disease as well as generally perturb nutrition and health[12, 13]. Given the necessity of endothelial cells in regulation of the hematopoietic system, it is likely that better understanding of the interaction between these two lineages will aid our current knowledge of the

role of endothelial cells in regulating hematopoietic cells, and add significantly to our knowledge about organism health.

Vascular Development of Zebrafish

The vascular system is characterized by a lumenized vessel lined with endothelial cells carrying blood to the tissues of an organism. The endothelial cells lining the vessels have a stereotypic morphology, polarized and aligned in the direction of blood flow. They are identified by expression of specific vascular genes, including Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1), Vascular Endothelial Growth Factor Receptor-2 (VEGFR2, or *kdrl* in zebrafish), and Vascular Endothelial Cadherin (VE-Cadherin). In zebrafish, expression of vascular markers, such as *kdrl* and *ve-cadherin*, is detectable as early as 12 hours post fertizlization (hpf)[14]. However, the predisposition of certain cells towards endothelial (and hematopoietic) lineages begins much earlier than that, starting during gastrulation of the embryo[1]. During gastrulation, endothelial cells are specified from the ventral mesoderm. These cells migrate to the lateral plate mesoderm at 12hpf, where they coalesce to form a vascular cord. By 18hpf, these endothelial cells have formed a single vessel throughout the length of most of the organism[15]. As the endothelial cells coalesce, an additional subset of endothelial migration occurs[15]. These two waves of endothelial specification and migration are followed by the lumenization of the vascular cord and the determination of arterial or venous identity. Within the vascular cord, individual endothelial cells begin to express either Ephrin Type-B Receptor 4 (EphB4) in veins or Ephrin-B2 in arteries[16]. Once arterial/venous

identity is determined, venous endothelial cells are separated from their arterial neighbors and migrate to form the cardinal vein[16]. By 24hpf, the initial wave of vascular patterning is complete. Embryos have a dorsal aorta spanning the length of the embryo, carrying blood flow away from the heart, a cardinal vein, also spanning the length of the embryo, carrying blood back to the heart, and intersomitic vessels (ISVs) have sprouted dorsally from the aorta and are beginning to anastomoze to form the dorsal longitudinal anastomotic vessel (DLAV) (Figure 1.1)[15, 17, 18].

At 24hpf, the 2-chambered zebrafish heart has formed and begins contracting which pushes blood throughout the body of the embryo. Though, at this early stage, the flow is primarily of plasma, it allows a flow-dependent remodeling of the venous vascular plexus. In the caudal-most region of the vascular system, the cardinal vein sprouts ventrally to form a second venous vessel[19, 20]. This second vessel, termed the ventral vein, is mostly intact by 36hpf, and by 48hpf, it carries an equal share of the venous blood flow. As blood flow is transferred to the ventral vein, the dorsal vein constricts and carries less blood. By 72hpf, the dorsal vein is no longer a significant carrier of blood flow and has become predominantly a site of hematopoietic development (Figure 1.1).

As remodeling of the caudal plexus is occurring, differentiation of endothelial cell identity is stabilizing, such that markers of venous and arterial identity are detectable in specific vessels. EphB4, Disabled homolog 2 (Dab2), and Fms-related tyrosine kinase 4 (Flt4) are all expressed in venous endothelial cells, whereas Notch, T-box 20 (Tbx20), and EphrinB2 are expressed in arterial cells. An additional third

class of molecules is expressed heterogeneously throughout all endothelial cells, such as SRY-related HMG-box transcription factor SOX17 (Sox17), and even within arteries, Delta like ligand 4 (Dll4) and Flt-4 levels vary depending on tip or stalk cell identity. Thus, endothelial cells are a diverse set of cells as they are specified at varied timepoints, have different identities, and different subsets of gene expression within those identities. Though it seems that endothelial cells are a heterogeneous population, for these studies, a uniform cell identity of *kdrl* expression was used to define endothelial cells (Figure 1.1).

Hematopoietic Development in Zebrafish

The hematopoietic system is also a diverse set of cells. Erythrocytes, macrophages, myeloid cells, thrombocytes, and lymphocytes (both B and T-cells) are all derived from the same hematopoietic stem cells (HSCs)[21, 22]. Thus the development and specification of HSCs is of incredible interest to the fields of immunity, nutrition, and health. Multiple studies have sought to characterized the HSCs, and have identified multiple markers of hematopoietic potential, including *c-myb, runt-related transcription factor 1 (runx1), CD41, c-kit* and VE-cadherin. Unfortunately, many of these markers have limitations as to their usefulness for scientific investigation. For instance, CD41 is expressed on lymphocytes, and VE-Cadherin is a marker of endothelial cells[21, 23-25]. Runx1 expression is more variable, due to being expressed more transiently during hematopoietic specification[26, 27]. Though *c-myb* is expressed in some neuronal tissues, it

remains as the most reliable single marker of hematopoietic stem cell fate for experiments where more than one marker cannot be utilized.

During development, hematopoietic cells have heterogeneous in origin. Most notably, hematopoiesis is initiated two different forms[28-30]. The first wave of hematopoiesis, termed primitive hematopoiesis, initializes in the yolk sac by 16hpf. It occurs primarily in the ventral mesoderm and intermediate cell mass (ICM) of zebrafish, similar to the ventral mesoderm and the yolk sac blood islands in mice. This form of hematopoiesis does not self-renew and only produces erythrocytes and myeloid cells[21, 30, 31]. In zebrafish, it is replaced starting at about 24hpf, by definitive hematopoiesis. By 36hpf, definitive hematopoiesis is the primary form of hematopoiesis occurring in the embryo and will continue throughout the life of the organism. Definitive hematopoiesis is distinguished from primitive hematopoiesis in that it: 1) produces all blood lineages including erythrocytes, lymphocytes, macrophages, and myeloid cells, and 2) includes HSCs capable of self-renewal, which allows them to be maintained throughout the life of the organism (Figure 1.1)[21, 32].

This wave of definitive hematopoiesis will continue past development and into adulthood, in specialized niches. During development, these niches are transient and colonized successively. In zebrafish, definitive hematopoiesis is initiated in the Aorta-gonad-mesonephros (AGM), however, it progresses to the caudal hematopoietic tissue (CHT), and finally to the kidney (Figure 1.1)[30]. This is very similar to the situation in mammals, where the onset of definitive hematopoiesis is also in the Aorta-Gonad-Mesonephros (AGM) region, and later it can be detected in

the fetal liver, and then finally in the bone marrow[21]. Post-development, hematopoietic stem cells settle into a final specialized vascular niche, and hematopoiesis continues. For mammals, this adult niche is the bone marrow, and for zebrafish it is the kidney marrow[21]. This may be due to the lack of bone marrow in zebrafish to provide the microenvironment for HSCs. Interestingly, it is not clear if these niches are capable of producing hematopoietic progenitors *de novo* from the surrounding tissue and/or endothelium, or if the HSCs arising from the AGM colonize each successive niche. This is especially pertinent as reports have documented the *de novo* development of definitive hematopoiesis in tissues outside of the AGM, such as the placenta.

Association between the endothelial and hematopoietic lineages during development

Though it is interesting to note the many of instances of heterogeneity occurring within endothelial and hematopoietic lineages, it is also noteworthy that while these two lineages are quite diverse within their cell type, they also have many ties common to both lineages. They can arise from a common progenitor, termed a hemangiobast. They are specified in close spatial and temporal proximity, and they have multiple common signaling pathways, including Notch, Bone Morphogenic Proteins (BMPs), and hemodynamic force. Moreover, specific subtypes of endothelial cells can trans-differentiatiate as hematopoietic progenitors (Figure 1.2).

The hemangioblast is a progenitor cell capable of giving rise to both hematopoietic and endothelial lineages. It was originally hypothesized to exist due

to the close spatial and temporal development of blood and endothelial cells, as is seen in blood islands. Lineage tracing studies and single cell derived colony forming assays have since demonstrated that such progenitors do exist[1, 33]. As endothelial cells are being specified during gastrulation, a small subset of those cells specified will give rise to both endothelial and hematopoietic cells[1, 15]. However, this cell type is rare, and most specified cells will produce either hematopoietic or endothelial cells, but not both[1]. The significance of this population is not yet clear (Figure 1.2A).

One potential regulator of the hemangioblast 'switch' is Notch signaling, which has been shown to modulate the number of endothelial or hematopoietic cells at the expense of the other cell type[2]. Thus, Notch can drive the endothelial-hematopoietic balance. Notch signaling has emerged as a key regulator of multiple facets of hematovascular biology. Within endothelial cells, Notch signaling promotes arterial fate[34]. Increased notch activity promotes tip cell identity and loss of the inhibitory ligand, dll4, results in increased sprouting and ectopic angiogenesis[35, 36]. Within hematopoietic cells, Notch activity is required for cell identity. Notch mutants, *mindbomb*, fail to specify HSCs[2, 29, 37-39]. Interestingly, these roles for Notch appear to be distinct in that arterial specification is not required for hematopoietic development (Figure 1.2B).

Other than Notch signaling, multiple other signaling cues are shared between the hematopoietic and vascular systems. BMPs, wnts, and Fibroblast Growth Factors (FGFs) have all been shown to elicit responses on both the hematopoietic and vascular fields[40-44]. In each of these cases, specificity appears to be

achieved by modulation of the specific ligand/receptor complex. For example, BMP2 signaling acts specifically on veins to promote angiogenesis of existing vessels, while BMP4, which is structurally similar to BMP2, has been shown to drive activation of hematopoietic cells from the hematopoietic niche (Figure 1.2B)[19, 20, 45, 46].

Hemodynamic Force

In addition to diverse signaling pathways which influence both lineages, environmental factors also substantially affect the development of both endothelial and hematopoietic lineages. For instance, blood flow appears to modulate both hematopoietic and endothelial cells[47-49]. The flow of blood through a vessel, whether arterial or vein, has been shown to elicit responses from the endothelium. It is thought that the movement of the blood over the endothelial cells exposes the endothelium to a shear stress, resulting in a pulling on the endothelium via PECAM and Integrin dependent mechanisms[50]. This stress promotes the polarization of endothelial cells and their alignment in the direction of blood flow. The effects of shear stress can be clearly observed in the area where the endothelial cells are exposed to disturbed flow. In areas of disturbed or turbulent flow, endothelial cells fail to align and are prone to atherosclerotic lesions[51]. Molecularly, this is characterized by changes in endothelial Nitric Oxide Synthase (eNOS) activity, integrin activation, and alignment of stress fibers[52, 53]. Though the biological mechanism of this response remains elusive, the significance of it has been demonstrated in its involvement in atherosclerosis and inflammation (Figure 1.2B).

Multiple recent reports have documented the role of blood flow in regulation of the hematopoietic system. Loss of blood flow in zebrafish impairs hematopoietic development, resulting in decreased *cmyb* and *runx1* expression. In experiments where blood flow was blocked via morpholino knockdown of the *cardiac Troponin Type 2 (tnnt2)* gene, decreased *cmyb* expression was observed in all hematopoietic tissue observed[47]. This approach is limited in that it cannot distinguish between effects on hematopoietic specification and hematopoietic maintenance. If HSCs were correctly specified but quickly recruited to downstream *cmyb*$^-$ compartments, these experiments would yield the same result. However, from these studies, it is clear that blood flow is providing some biological regulation of the HSC compartment. Additionally, it has been shown that exposure of FACS sorted HSCs increases HSC marker expression, such as *cmyb* and *runx1[54].* The mechanism by which this regulation occurs is almost completely unknown, though it is presumed that it would be similar to the mechanisms observed already in endothelial cells.

Hemogenic Endothelium

In 1926, Florence Sabin and Charles Doan noted the appearance of apparent endothelial cells derivatives in the blood[3]. These cells were seen nearly constant and could be considered a normal constituent of the blood. Though they could not know the significance of these cells, they hypothesized that they were upregulated during pathogenic conditions. The possibility that endothelial cells could produce hematopoietic cells has persisted. In 2008, Iruela-Arispe's group undertook extensive lineage tracing experiments using VE-Cadherin:Cre mice and observed

that endothelial cells in the dorsal aorta of mice appeared to be ‘budding’ into the lumen of the vessel[55]. This was direct evidence that endothelial cells were capable of producing non-endothelial, blood components.

Since those studies, multiple reports have furthered our understanding of the hemogenic endothelium. Hemogenic endothelial cells have been shown to express *c-myb, runx1,* and *c-kit[56]. Runx1* has been shown to be required transiently for the actual budding, but not to be required for the maintenance of the HSC population once it is specified[26, 57]. Recently, direct imaging experiments have shown budding of *c-myb* positive progenitors from the dorsal aorta of both zebrafish and mice[6]. It is now a broadly accepted phenomenon that endothelial cells are capable of producing HSCs and that these HSCs will contribute to hematopoiesis for the life-span of the organism. Currently, one question that remains in the field is the extent to which all endothelial cells are hemogenic. Most studies have focused on the aorta as the site of hemogenic endothelium, but given that definitive hematopoiesis is observed in non-embryonic tissues, it is possible that other vascular populations retain hemogenic ability[58, 59] (Figure 1.2C).

The Caudal Hematopoeitic Niche

The common ties between hematopoietic and endothelial cells are especially interesting when considering the hematovascular niche, which consist of hematopoietic stem cells surrounded by a vascular bed. HSCs must migrate through the endothelial layer during the process of differentiation to their downstream lineages[21]. The signaling cues which regulate this process, such as

blood flow, would be received from the lumen of the vessel. Thus, it seems that direct communication of the endothelial and hematopoietic cells would be necessary to coordinate HSC recruitment. Given the common signaling pathways between endothelial and hematopoietic lineages, it would be likely that the signaling cues controlling this process in the HSCs also affect the endothelium, and that the responses are coordinated and synergistic. Dysfunction in the niche can result in skewed populations or dysfunctional components (T-cells that don't fight infection).

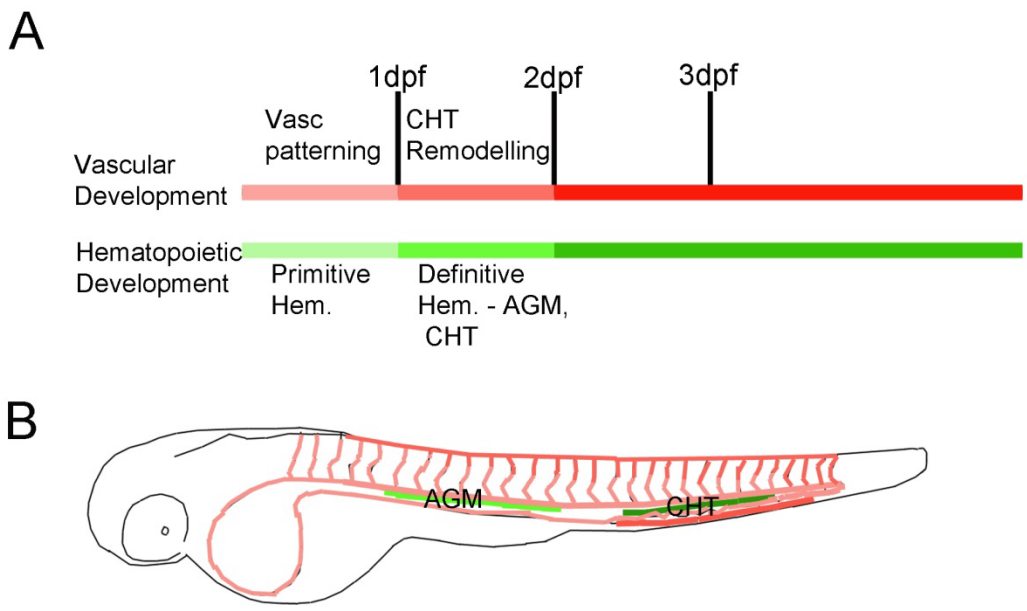
Within the series of vascular niches colonized by HSCs in the zebrafish, the caudal hematopoietic niche is of particular interest (Figure 1.3). By 24hpf, the zebrafish has a contracting heart, driving blood flow through the vascular system[15, 17]. In the caudal tissue of the embryo, an aorta carries blood away from the heart and a single vein returns blood flow to the heart. However, starting about 24hpf, the vein of the caudal tissue begins BMP-driven sprouting angiogenesis[20]. Remodeling of the vein in this tissue drives the formation of a second vein and the initial vein, now termed the dorsal vein, constricts. At the same time this caudal vein plexus (CVP) is remodeling, HSCs are colonizing the tissue previously occupied by the dorsal vein. By 72hpf, the dorsal vein is almost completely constricted, permitting little blood flow, the ventral vein is the primary conduit of blood flow, and HSCs have robustly populated the tissue. Though the colonization by HSCs occurs concurrently with the venous remodeling, little is known about the relationship between the two processes and whether hematopoietic colonization could occur in the absence of the venous remodeling. Interestingly, blood flow has been shown to regulate both the hematopoietic development of the CHT and vascular

remodeling[47]. In the absence of blood flow, *cmyb* fails to be expressed in the CHT, and while the sprouting from the vein occurs, the dorsal vein fails to constrict, and both veins dilate to such an extent that it appears as one large vessel occupying the CHT[47] (unpublished data). The failure of the dorsal vein to constrict and the concurrent venous dilation has confounded attempts to understand the role of blood flow in regulation of this remodeling process. What is clear is that in the absence of blood flow, vascular remodeling is aberrant and HSC colonization does not occur, further strengthening the ties between these two processes.

In this thesis, I present evidence that non-aortic endothelial cells can also serve as hemogenic sources for HSCs. Specifically, I show that venous endothelial cells within the zebrafish CHT can be hemogenic. This suggests that hemogenic ability may be a more general property of endothelial cells than previously appreciated. Furthermore, I show that HSCs are responsive to blood flow, even after the development of the niche. This indicates that blood flow is a regulator, not merely of hematopoietic development, but of the hematopoietic niche. Finally, I identify a PECAM1-like molecule in zebrafish as a potential mediator of this response. Together, these findings increase our understanding of the endothelial identity, and the potential role of PECAM1 in hematopoietic regulation. The need for careful term definition in vascular biology occurs because both blood and endothelial lineages can be diverse in nature, markers, and function. For the purposes of this report, hematopoietic cells will refer to hematopoietic stem cells capable of self-renewel and producing the major blood lineages, while endothelial cells will be vascular cells lining the lumen of blood-carrying vessels.

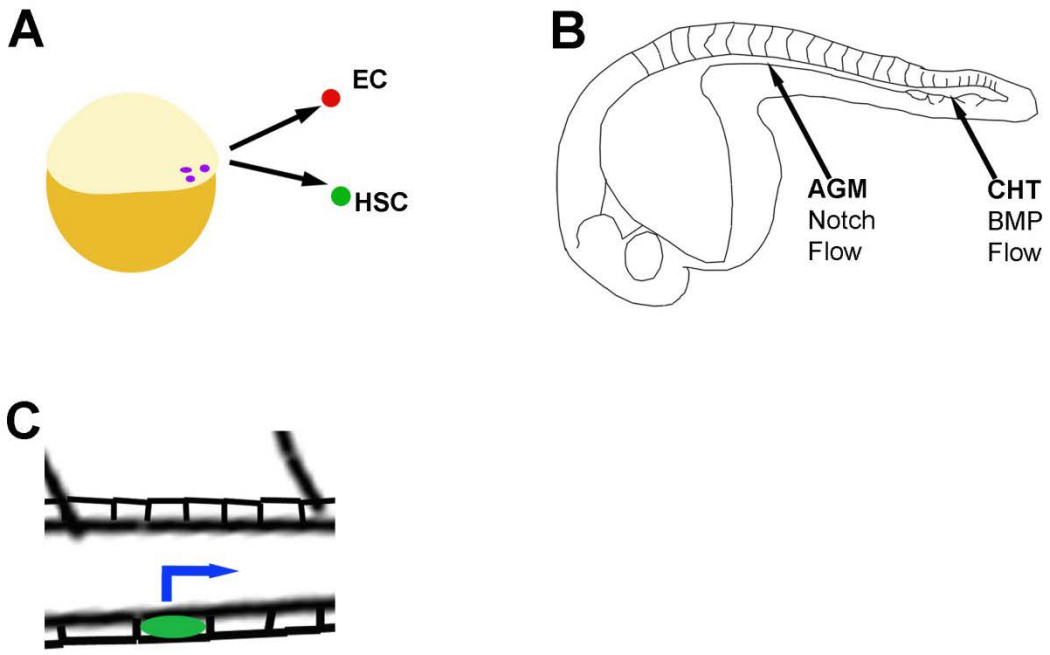
FIGURES

Figure 1.1: Concurrent development of the hematopoietic and vascular systems.



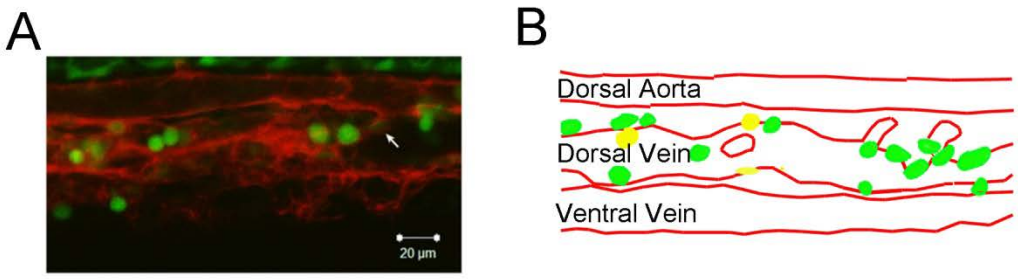
The hematopoietic and vascular systems develop alongside each other, both concurrently (A) and spatially (B). Prior to one day post fertilization, vascular (vasc) patterning is occurring during primitive hematopoiesis (hem) development. After one day post fertilization, the Caudal Hematopoietic Tissue (CHT) is remodeling, while definitive hematopoiesis is occurring the the Aorta-Gonad-Mesonephros (AGM) and CHT.

Figure 1.2: Common hematovascular ties.



Endothelial and hematopoietic cells have many common ties. They can be specified by a common progenitor, termed the hemangioblast (purple cells in A). They share many of the same niches and signaling cues, such as Notch and blood flow in the Aorta-Gonad-Mesonephros (AGM) and BMPs and blood flow in the Caudal Hematopoietic Tissue (CHT), (B). Certain endothelial cells, hemogenic endothelium (green cell in C) can produce hematpoietic stem cells.

Figure 1.3: The caudal hematopoietic niche.



The caudal hematopoietic niche is both a site of hematopoietic development and vascular remodeling. A confocal micrograph of a *Tg(cmyb:GFP;kdrl:mCherry)* embryo is shown (A) with a budding *c-myb*$^{+}$ progenitor (arrow). A schematic representation of this specialized niche is shown (B). *Cmyb*$^{+}$*/kdrl*$^{+}$ cells are shown as yellow, while *cmyb*$^{+}$*/kdrl*$^{-}$ cells are shown as green.

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CHAPTER 2

Hematopoietic Potential of Venous Endothelial Cells[1]

OVERVIEW

Since 1926, the existence of blood-producing endothelial cells has been suggested by scientific investigation[1]. These cells have been termed hemogenic endothelial cells--endothelial cells capable of producing the hematopoietic stem cells responsible for all hematopoietic lineages. Current evidence suggests that this ability is limited to a specialized subset of endothelial cells, residing in particular niches[1-5]. In this study, we identify the zebrafish Caudal Hematopoietic Tissue (CHT) as a vascular bed capable of producing hematopoietic stem cells (HSCs). We examined the co-expression of hematopoietic and venous markers within the zebrafish cardinal vein during development, using in situ, immunofluorescence, and time-lapse imaging. We found that venous endothelial cells in the cardinal vein co-expressed hematopoietic markers, that these cells were capable of budding to produce hematopoietic progenitors, and though most previously identified hemogenic vascular beds have been arterial, we find that hemogenic venous endothelial cells also exist and are present throughout development. We also find that blood flow exerts not only a developmental effect, but that it regulates these cells beyond specification of the cell type.

[1] Assistance was provided with Dab2 immunofluorescence by Jun-Dae Kim. Caroline Burns, Yong Zhou, and Neal Chi provided key reagents.

INTRODUCTION

The hematopoietic and vascular systems share several extensive ties throughout development, such that they can be collectively referred to as the hematovascular system. The relationship between these two systems is complex and intricate, involving common progenitors, similar signaling pathways, and multiple molecular switches between the two lineages. Additionally, transitions between the two systems appears to be possible in the existence of hemogenic endothelium—specialized endothelial cells which retain the ability to produce hematopoietic stem and progenitor cells (HSCs/HPCs)[2, 4-15]. Hemogenic endothelial cells reside precisely at the intersection of the hematopoietic and vascular systems. The possible existence of hemogenic endothelium was first documented in 1926 by Florence Sabin and Charles Doan, who noticed that endothelial cells occasionally appeared in the blood of certain individuals[1]. Further studies since then have elucidated molecular markers of these endothelial cells, such as *c-myb*, *runt-related transcription factor 1 (runx1)*, Vascular Endothelial Cadherin (VE-Cadherin), and more recently, *c-kit[16].* It has also become clear, that *in vivo,* not all endothelial cells are hemogenic at the same time. Multiple lines of evidence have indicated that the dorsal aorta is a site of definitive hematopoiesis, and given the importance of this region, many of investigations into hemogenic endothelium have focused on the aorta-gonad-mesonephros (AGM) as an early site of definitive hematopoiesis and hemogenic endothelium. Others have identified other sites of hematopoiesis, such as the placenta, the yolk sac, and the vitelline and umbilical arteries[17-23]. Though, these investigations have revealed that the AGM is not the only source of

hemogenic endothelium, whenever examined, the hemogenic endothelium was arterial in nature. As hematopoiesis appears to be a migratory process, colonizing several distinct niches temporarily before finally settling in the mammalian bone marrow or the teleost kidney, we sought to determine if these different niches were capable of producing their own hemogenic endothelium, or if each niche was sequentially colonized by a migrating population, all arising from the same initial niche. The implications that defects in the hematopoietic system could be regulated, impacted, or resolved by modulation of endothelial signaling has huge clinical relevance in the treatment of hematopoietic disorders, transplant rejections, etc.

Though the ability of endothelial cells to produce hematopoietic progenitors has been well documented across multiple species, the nature of this process remains unclear, and multiple studies have focuses on determining the sites of hemogenic endothelial cells. Here, we expand upon existing knowledge about hemogenic endothelium and show for the first time, that a non-arterial vascular bed retains the ability to become hemogenic. Further, we elucidate the regulation of these venous hemogenic endothelial cells by blood flow, a common regulator of both hematopoietic and vascular cells.

EXPERIMENTAL PROCEDURES

Zebrafish husbandry, microinjection, and immunohistochemistry

Zebrafish were maintained according to IACCUC-approved protocols. The following transgenic lines were used: *Tg(kdrl:ras-mCherry)*^{s896}, *Tg(cmyb:GFP)*^{zf169}, *Tg(ubb:LoxP-AmCyan-LoxP-ZsYellow)*^{fb5}, and *Tg(ubb:loxP-EGFP-loxP-mCherry)*^{cz1701}.

Embryos were injected into the yolk sac at the one-cell stage as previously described[24]. To block heart beat, cardiac Troponin Type 2 (*tnnt2)* MO was used (5- CATGTTTGCTCTGATCTGACACGCA-3')[25]. Immunohistochemistry were performed as previously described[26], using mouse anti-Disabled homolog 2 (Dab2) antibody (Abcam, 1:500).

FACS Analysis and qRT-PCR

28hpf *Tg(kdrl:ras-mCherry)*^{s896}*;Tg(cmyb:GFP)*^{zf169} embryos were dissociated using liberase at 37°C. Subsequently, suspension of dissociated cells were sorted on a Fluorescence-activated cell sorting (FACS) Aria. Four distnict populations of cells (*GFP*$^{-}$*/mCherry*$^{-}$, *GFP*$^{-}$*/mCherry*$^{+}$, *GFP*$^{+}$*/mCherry*$^{-}$ and *GFP*$^{+}$*/mCherry*$^{+}$) were collected and RNA was extracted to perform qRT-PCR to determine their molecular identities.

For qRT-PCR, the following primers were used:
cmyb-f, TCCCGGCTCCATCCCTAGAGC; *cmyb*-r TTGTTGGCCCAGACTGATGGGG; *c-kit*-f, ACAAGTGCGCTGTGTGGCCG; *c-kit*-r, GGCTCTCCACTGCGCCTTTCC; *runx1*-f, CGCTGAGCTCCGCAACGCTA; *runx1-r*,

ATCCGGCTTCTGTCGGTGGC; *gata1a*-f, CGAGATGGGACAGGCCACTACCT; *gata1a*-r, GCAGTTGGCGCACTGCGTTC; *18s*-f, cacttgtccctctaagaagttgca; *18s*-r, ggttgattccgataacgaacga; *b-actin*-f, AAGCTGTGACCCACCTCACGC; *b-actin*-r, TCAACGACCAGGGCAGCGATT.

Image acquisition, quantification and statistical analyses

For time lapse imaging, wild-type embryos were anesthetized in 640μM of tricaine methanesulfonate (Tricane) and embedded on glass-bottom dishes in 1% low-melt agarose dissolved in Tricaine and embryo medium (1mM NaCl). Subsequently, embryos were imaged on a Zeiss 710 two-photon microscope for 12 hours at 4 minute intervals starting at 24hpf. For lineage tracing, spectral imaging using Zeiss 510 with Meta-detection was performed. The acquired images were analyzed using Volocity.

To quantify *cmyb*$^{+}$ cells contained within the CVP, embryos were imaged at 40x magnifications through the caudal vascular plexus (CVP) starting at the end of the yolk extension (approximately correlating to somites 16-20). Individual z-slices were exported to Photoshop, and GFP expression (labeled by *cmyb:GFP* transgene) which persisted for at least three z-slices (approx. 30μm) was defined as a *cmyb*$^{+}$ cell. Individual *cmyb*$^{+}$ cells were numbered and then assessed for mCherry expression (labeled by *kdrl:mCherry* transgene). At least ten embryos per condition were examined to provide statistical analyses.

To assess the distribution of *cmyb*$^{+}$ cells within the CVP, a cartograph was generated by tracing all *cmyb*$^{+}$*/kdrl*$^{-}$ and *cmyb*$^{+}$*/kdrl*$^{+}$ cells within the CVP of each

24hpf or 72hpf embryo. These individual cartographs were overlayed onto a single composite, which was divided into five segments, corresponding to somites.
To determine the statistical significance of differences between percentages of *cmyb*$^{+}$*/kdrl*$^{+}$ cells in control and *tnnt2* MO injected embryos, one-tailed, student t-tests were performed.

Chemical Treatment

(2,3)-Butanediomemonoxime (BDM) and epinephrine(EPI) were obtained from Sigma Aldrich. Starting at either 67hpf or 69.5hpf, zebrafish embryos were treated for either 5 hours or 2.5 hours, respectively, with BDM or epinephrine. For BDM treatments, embryos were pulsed with 30uM BDM to cause cessation of the heartbeat and then maintained in 25uM BDM for the duration of the experiment. For epinephrine treatments, embryos were maintained in 10ug/mL epinephrine for the duration of the experiment. Both drugs were dissolved directly in egg water to permit the use of egg water as the control treatment.

Plasmid construction and transient lineage tracing

To perform transient lineage tracing, *kdrl:Cre-ER*^{t2} construct was generated by subcloning Cre-ER^{t2} under the regulation of the *kdrl* promoter in a Tol2 based vector[26-28]. To generate somatic mosaicism, *Tg(cmyb:EGFP)*^{zf169}*;Tg(kdrl:mCherry)*^{s896} double transgenic individuals were crossed with *Tg(ubb:LoxP-AmCyan-LoxP-ZsYellow)*^{fb5} transgenic individual. The resulting embryos were co-injected at the one-cell stage with transposase mRNA and

kdrl:Cre-ER^{t2}. Embryos were reared at 28°C and treated with 4μM tamoxifen from 18 to 24hpf as previously described, and analyzed at 36 and 48hpf.[29] Similarly, embryos were treated 4μM tamoxifen from 66hpf to 72hpf and imaged at 84hpf and 96hpf.

RESULTS

Venous endothelial cells express hematopoietic markers

Using transgenic zebrafish expressing GFP under control of the hematopoietic stem cell marker, *cmyb,* and mCherry under control of the *kdrl* promoter, we first sought to determine whether non aortic endothelial cells could express hematopoietic markers during development. The Caudal Vein Plexus (CVP) of *Tg(cmyb:EGFP)*^{zf169}*;Tg(kdrl:mCherry)*^{s896} zebrafish embryos was examined by confocal microscopy at 24hpf (Figure 2.1A). HSCs were detected of two fluorescent profiles, *cmyb*$^{+}$*/kdrl*$^{+}$ and *cmyb*$^{+}$*/kdr*$^{-}$ cells (GPF+/mCherry+ and GFP+/mCherry-, respectively). As nascent hematopoietic progenitors quickly lose the expression of vascular genes after delamination, the *cmyb*$^{+}$*/kdrl*$^{+}$ population likely represents a population originating from endothelial cells within the CVP (Figure 1.1A).[5, 30] Given that the caudal hematopoietic tissue (CHT) is a later developing niche, we hypothesized that, if it had hemogenic activity, there would be nascent hematopoietic progenitors present later in development. To examine this possibility, the cardinal vein of zebrafish was imaged at different points in development. Double transgenic zebrafish Tg(*kdrl:mCherry*);Tg(*cmyb:GFP*) embryos were imaged at 24hpf and 72hpf. At all timepoints examined, *cmyb* expression could be detected within venous cells of the zebrafish caudal hematopoietic tissue (Figure 2.1A, B). At 24hpf, an average of six cells within the wall of the caudal vein were *cmyb*$^{+}$*/kdrl*$^{+}$ (n=10, *s*=4) (Figure 2.1C). The number of *cmyb*$^{+}$*/kdrl*$^{+}$ cells significantly increased to an average of 12 cells (n=10, *s*=7) by

72hpf (Figure 2.1C). This indicates that this population of cells is present through the development and patterning of this endothelial niche.

To further substantiate that *cmyb* expression was within the endothelial cells population, embryos were dissociated and cell populations were assessed via Fluorescence Activated Cell Sorting (FACS) analysis. Tg(*kdrl:mCherry*);Tg(*cmyb:GFP*) embryos at 28hpf and 72hpf were dissociated and analyzed by sorting on a FACS Diva. In the total embryo at 28hpf, $cmyb^{+}$ endothelial cells were 20 percent of the endothelial population. Consistent with confocal results, this population was maintained as a percentage of total endothelial cells (Figure 2.1E). At 72hpf, $cmyb^{+}$ endothelial cells were 7% of the total endothelial population (Figure 2.1E). However, when only the CHT was used for dissociation, $cmyb^{+}$ endothelial cells were 19 and 21 percent of the $kdrl^{+}$ population at 24hpf and 72hpf, respectively, confirming that a $kdrl^{+}/cmyb^{+}$ population of endothelial cells is present within the zebrafish CHT cells throughout development (Figure 2.1E). Together, this data shows that *cmyb* is expressed in a subset of endothelial cells within venous endothelial cells of the zebrafish CHT and that this population of cells persists later into development than previously reported. FACS analysis corroborated our finding that CVP contains $cmyb^{+}/kdrl^{+}$ cells both before and after the proposed colonization of the AGM-derived hematopoietic progenitors (Figure 2.1E). Taken together, the data support that the $cmyb^{+}/kdrl^{+}$ population persists at 72hpf after the dissolution of the AGM.

Given that these HSCs were located outside of the typical AGM hematopoietic niche, we sought to determine if they were in fact not arterial, but

instead venous in nature. To test this, we examined co-expression of the venous marker, Disabled homolog 2 (Dab2), at 36hpf. To confirm that these endothelial cells were venous in nature, embryos were fixed and stained for the venous marker, Dab2. Dab2 expression is restricted to the vein by 36hpf in the developing zebrafish. Transgenic embryos, Tg(*cmyb:GFP*) were fixed at 36hpf and co-stained for GFP and Dab2. Co-expression of GFP and Dab2 was observed, consistent with the live-imaging results and further demonstrating that venous endothelial cells are capable of expressing hematopoietic markers (Figure 2.1F). The *cmyb*$^{+}$*/kdrl*$^{+}$ cells in the CVP strongly expressed venous specific marker, *dab2*, indicating their venous identity (Figure 2.1F).

To rule out the possibility that the *cmyb*$^{+}$*/kdrl*$^{+}$ cells within the CVP are the previously described *GATA-binding factor 1* (*gata1)*$^{+}$*/pu.1*$^{+}$ erythromyeloid progenitors (EMP), we examined the expression of *gata1* from the isolated *cmyb*$^{+}$*/kdrl*$^{+}$ cells. [31] While the expression of *cmyb and c-kit* were strongly detected, the expression of *gata1* was not detected in this population, suggesting that *cmyb*$^{+}$*/kdrl*$^{+}$ cells within the CVP are not likely to be the EMPs, but are more likely to be HSCS capable of producing all hematopoietic lineages (Figure 2.2).

cmyb^{+} cells within the zebrafish CHT are located in the wall of the vessel

To confirm that *cmyb*$^{+}$*/kdrl*$^{+}$ cells are within the vessel wall, Z axes of each confocal image were analyzed (Figure 2.1A, B). Three-dimensional reconstructions of the vein confirmed that the observed cmyb expression was within the wall of the

vessel, suggesting the ability of venous endothelial cells to become hemogenic (Figure 2.3A-D).

cmyb^{+} progenitors arise de novo in the CHT

It is known that hematopoietic progenitors bud from the hematopoietic tissue of the AGM and enter circulation to colonize other tissues. To rule out the possibility that the *cmyb*$^{+}$*/kdrl*$^{+}$ cells detected in the CHT were the result of cells budding from the AGM, we blocked circulation using a morpholino targeting *cardiac Troponin Type 2* (*tnnt2*) which would prevent colonization by HSCs from other tissues, such as the aorta-gonad-mesonephros (AGM). We examined whether both cell types could be detected in the CHT in the absence of blood flow. The morpholino targeting *tnnt2* was injected into the one-cell embryos, and embryos were screened at 24hpf. We found that *cmyb*$^{+}$*/kdrl*$^{+}$ cells could be detected in the *tnnt2*-MO injected embryos (Figure 2.4A-B). Furthermore, *cmyb*$^{+}$*/kdlr*$^{-}$ cells, which, as previously reported, are rapidly depleted in the absence of blood flow, represent a lower percentage of the overall population. *cmyb*$^{+}$*/kdrl*$^{+}$ cells were 50% and 75% of the total *cmyb*$^{+}$ population in control and *tnnt2*-MO injected embryos respectively (Figure 2.4C). The total number of *cmyb*$^{+}$*/kdrl*$^{+}$ cells actually increased from 4 cells in control injected to 41 cells in *tnnt2*-MO injected embryos (Figure 2.4D). The reason for this transient increase is not yet clear. As previously reported, the majority of *cmyb*$^{+}$*/kdr*$^{-}$ cells are lost in the absence of flow. It remains a possibility that blood flow required for the controlled regulation of these cell subtypes. We found that in the absence of circulation, as previously reported, cells from the AGM do not colonize the CHT.

However, *cmyb* expression could still be detected within the endothelial cells of the CHT. Due to the lack of additional progenitors from the AGM, most of the *cmyb*$^{+}$ cells observed were positive *for kdrl*, suggesting that they are new progenitors, just being derived from the endothelial cells in the CHT.

cmyb^{+}*/kdrl*$^{+}$ progenitors preferentially localize in the CHT

We next investigated whether *cmyb*$^{+}$*/kdrl*$^{+}$ cells in the CHT had a preferentially localization in this niche. To examine the spatiotemporal pattern of *cmyb*$^{+}$*/kdrl*$^{-}$ and *cmyb*$^{+}$*/kdrl*$^{+}$ within the CVP, we generated cartographs at 24 and 72hpf and placed the localization of *cmyb*$^{+}$*/kdrl*$^{+}$ and *cmyb*$^{+}$*/kdrl*$^{-}$ cells onto each cartograph at 24hpf or 3dpf as appropriate. We found that at 24hpf, the *cmyb*$^{+}$*/kdrl*$^{+}$ cells were preferentially located in the anterior regions of the CHT (Figure 2.5A, C). While the *cmyb*$^{+}$*/kdrl*$^{-}$ and *cmyb*$^{+}$*/kdrl*$^{+}$ cells are clustered anteriorly at 24hpf (Figure 2.5A, C), they become more dispersed within the CVP (n=10) at 72hpf (Figure 2.5B, D).

The distribution of HSCs within the different vessels of the CVP was also examined. Interestingly, the dorsal wall of the cardinal vein and the dorsal wall of the dorsal vein were the most prominent sites to detect *cmyb*$^{+}$*/kdrl*$^{+}$ cells—41% of *cmyb*$^{+}$*/kdrl*$^{+}$ cells resided in association with these surfaces at either 24hpf or 72hpf (Figure 2.5E, F). That this percentage was constant at 24hpf and 3dpf suggests a stable characteristic of the dorsal vein.

***cmyb*$^{+}$ cells within the CHT bud from the endothelium.**

To confirm that hematopoietic progenitors arise directly from venous endothelial cells of the CHT, we performed time-lapse imaging. Fish were immobilized in agarose and imaged for 12 hours beginning at 24hpf. *cmyb*$^{+}$*/kdrl*$^{+}$ cells could be observed to bud from the dorsal vein (Figure 2.7, 2.8). This budding was abluminal in nature and was sometimes followed by the nascent progenitor entering circulation. Interestingly, this is at the same time which this tissue is remodeling and the dorsal vein of the cardinal tissue is constricting, suggesting a possible fate for the endothelial cells of the dorsal vein. To exclude the possibility that the *cmyb*$^{+}$ cell migrates and is incorporated into the vessel wall within the area of observation, we analyzed the z-slices 20μm above and below the forming *cmyb*$^{+}$*/kdrl*$^{+}$ cell (Figure 2.8A). We found that *kdrl*$^{+}$ cells within the wall of vein initiate the expression of *cmyb*, and a subset of these cells subsequently delaminate from the vessel wall and enter into the circulation (Figure 2.7, 2.8), suggesting that *cmyb*$^{+}$*/kdrl*$^{+}$ cells in the CVP undergo similar transition as those in the dorsal aorta.

To further delineate the ontogeny of the *cmyb*$^{+}$*/kdrl*$^{+}$ cells in the CVP, we examined performed short-term lineage tracing (Figure 2.9). *cmyb*$^{+}$*/kdrl*$^{+}$ cells were observed to lose *kdrl* expression and produce *cmyb*$^{+}$*/kdrl*$^{-}$ cells. Thus, single cell lineage tracing supports that these venous endothelial cells can differentiate as hemogenic endothelium and eventually contribute to the hematopoietic progenitor population, suggesting that hemogenic potential is not restricted to only arterial endothelial cells (Figure 2.9).

cmyb^{+} cells within the CHT display unique behaviors

We performed time-lapse imaging of the tissue to observe the behavior of these *cmyb*$^{+}$*/kdrl*$^{+}$ cells. While the majority of the *cmyb*$^{+}$*/kdrl*$^{+}$ cells within the CVP maintain stereotypic endothelial morphology, time-lapse imaging revealed dynamic behavior of these cells. HSCs within the vessel wall were capable of proliferating (Figure 2.10A), and a subset of cells occasionally protruded toward the luminal side of the vein and buds off from the vein wall (Figure 2.10B), as observed in the AGM region. We found that *cmyb*$^{+}$*/kdrl*$^{+}$ cells within the dorsal vein were capable of proliferating within the wall of the vessel with a cell division perpendicular to the vessel lumen (Figure 2.10A). Furthermore, these cells were capable of protruding into the vessel lumen, and a diverse range of migratory behaviors, including crawling along the vessel, or rolling in circulation (Figure 2.10B). Together, this suggests that the behavior of these cells is dynamic and exposes them to a wide variety of environments within the niche.

cmyb^{+}*/kdrl*$^{+}$ cells respond distinctly to the loss of blood flow

Previous reports have shown that blood flow acts as a regulator for both endothelial and hematopoietic cells. Interestingly, no reports have specifically addressed the effect of blood flow on hemogenic endothelium. We investigated by confocal microscopy the ability of blood flow to regulate the hemogenic endothelium within the zebrafish CHT. To eliminate possible effects on development of the hematopoietic tissue, embryos were raised to 68hpf. Definitive hematopoiesis begins at 24hpf, with highest detectable activity at 36-48hpf. By waiting until 68hpf,

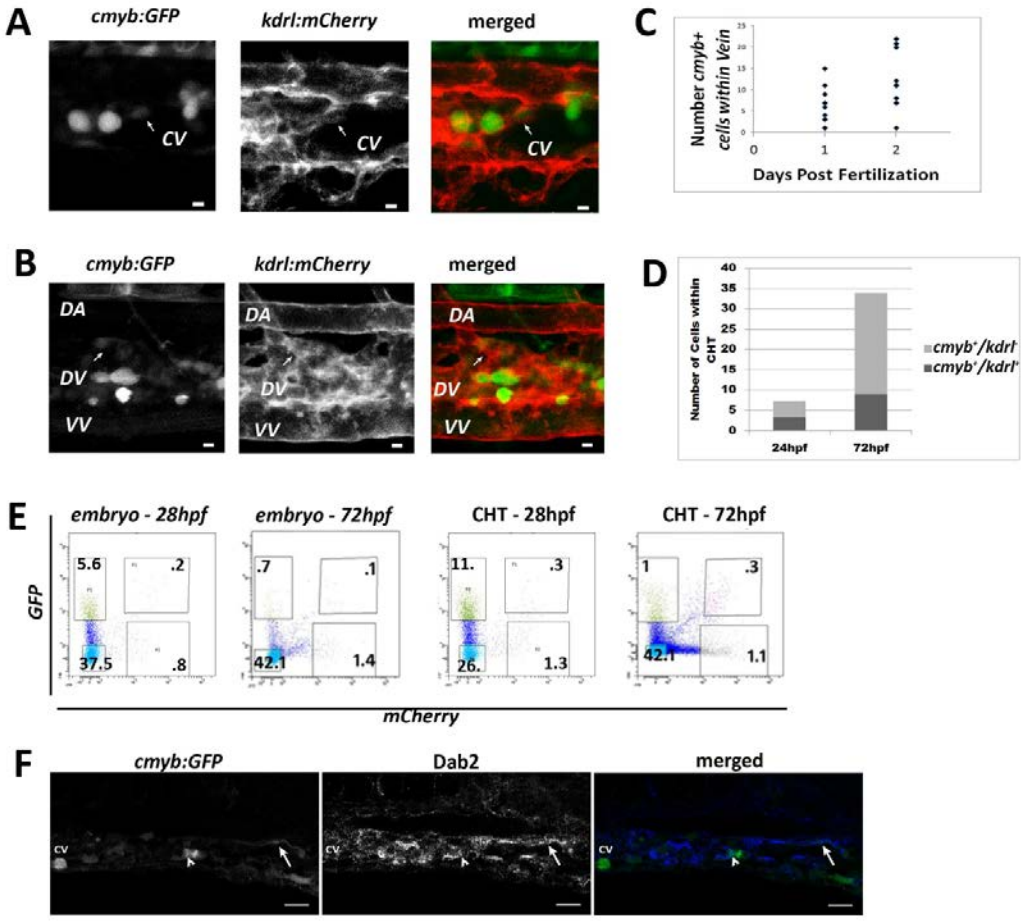
we hoped to only observe effects on the hemogenic endothelium of the CHT. At 68hpf, embryos were incubated in either BDM or epinephrine, to either block or increase blood flow, respectively. We found that blood flow tightly regulated *cmyb* expression within the CHT. We found that BDM treatment resulted in the rapid depletion of *cmyb* expression within the CHT. By 5hr of BDM treatment, both the number of *cmyb*$^{+}$ cells and the expression levels had decreased. By contrast, stimulation of blood flow by epinephrine had the opposite effect, and increased *cmyb* expression could be detected (Figure 2.11). Loss of blood flow resulted in loss of the *cmyb* expression, while increased blood flow, as caused in the epinephrine treatment, caused increased *cmyb* expression (Figure 2.11).

DISCUSSION

To our knowledge, this is the first detailed study of a non-aortic bed of hemogenic endothelium. We demonstrate that endothelial cells outside of the AGM are capable of producing *cmyb*$^{+}$ progenitors, that these cells can be venous in nature, and that they respond distinctly to changes in blood flow. Previous reports had focused on the dorsal aorta as a site of hemogenic endothelium. Because of this, these studies have not been able to elucidate the ability of all endothelium to become hemogenic. Though our study falls short of demonstrating that hematopoietic potential is an intrinsic property of most endothelial cells, we broaden the scope of hemogenicity in endothelial cells by examining that property in venous endothelial cells. Furthermore, we demonstrate the ability of blood flow to regulate hematopoietic potential of endothelial cells. Though flow has been implicated in the development of hematopoietic niches and specification of hematopoietic stem cells, this is the first report that demonstrates that blood flow is a regulator of the HSCs post-development.

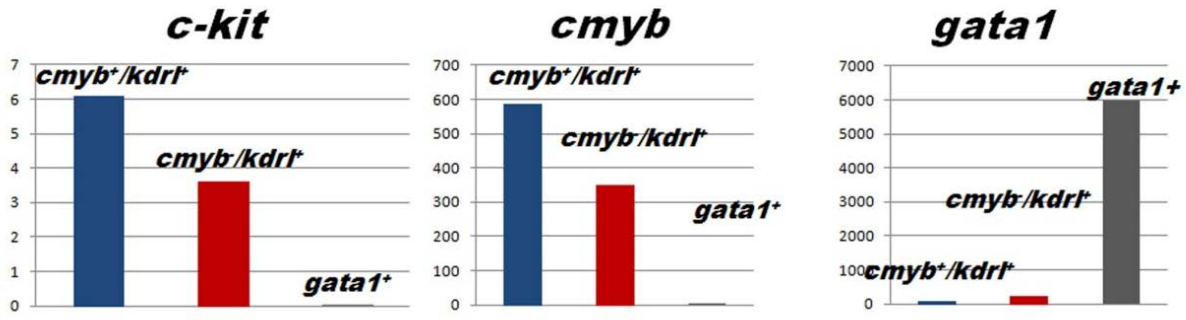
FIGURES

Figure 2.1: Detection of *cmyb* in venous endothelial cells throughout development.



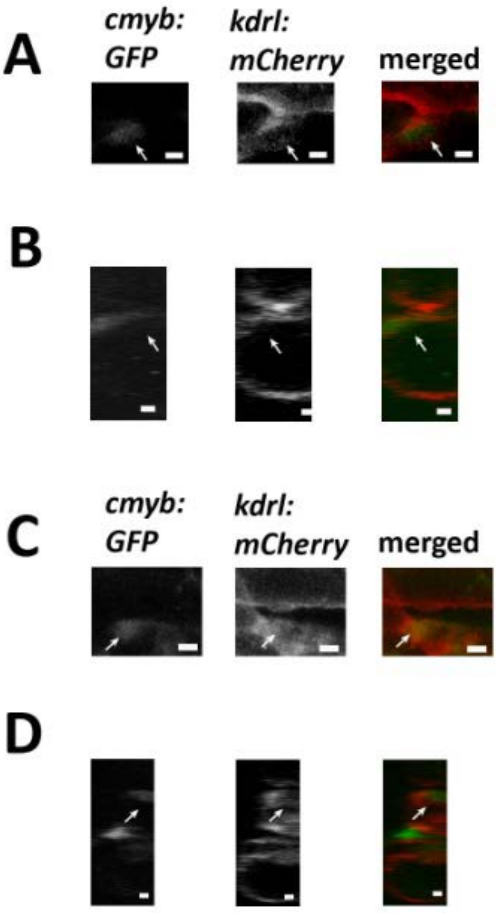
Tg(*cmyb:GFP;kdrl:mCherry*) embryos were imaged by confocal microscopy for the existence of co-postive cells within the vessel wall at 24hpf (A) and 72hpf (B). The number of *cmyb*$^{+}$ cells within the vessel wall is quantified (C) as is the total number of *cmyb*$^{+}$ progenitors within the CHT (D). FACS analysis was done on embryos or CHTs of 28hpf and 72hpf embryos to determine if co-positive progenitors were detected (G). Tg(*cmyb:GFP*) embryos were fixed at 36hpf and stained for the venous marker, Dab2 (F).

Figure 2.2 *cmyb*$^{+}$/*kdrl*$^{+}$ cells express hematopoietic markers.



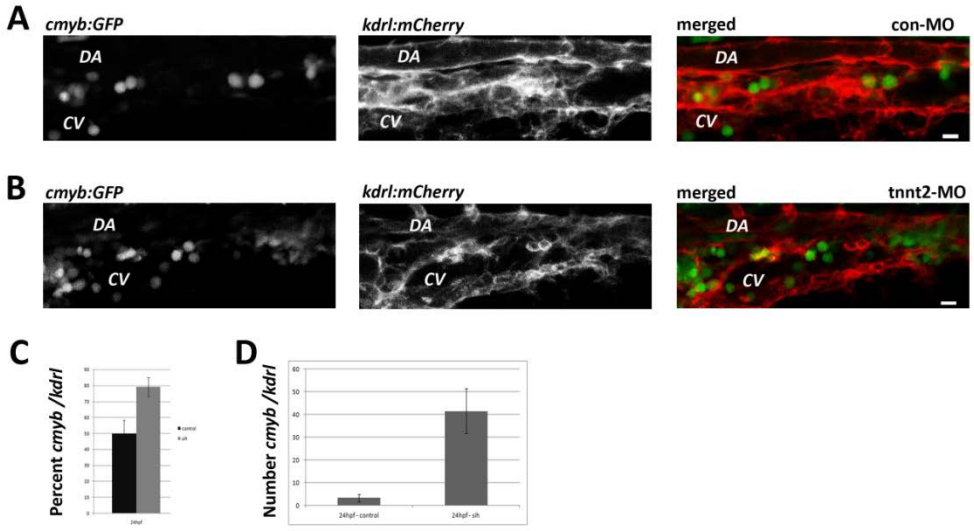
Endothelial cells were sorted for Tg(*cmyb:GFP*) expression. FACS sorted cells were subjected to QPCR for *c-kit*, *cmyb*, and *gata1*.

Figure 2.3 Detection of *cmyb* within the venous vessel wall.



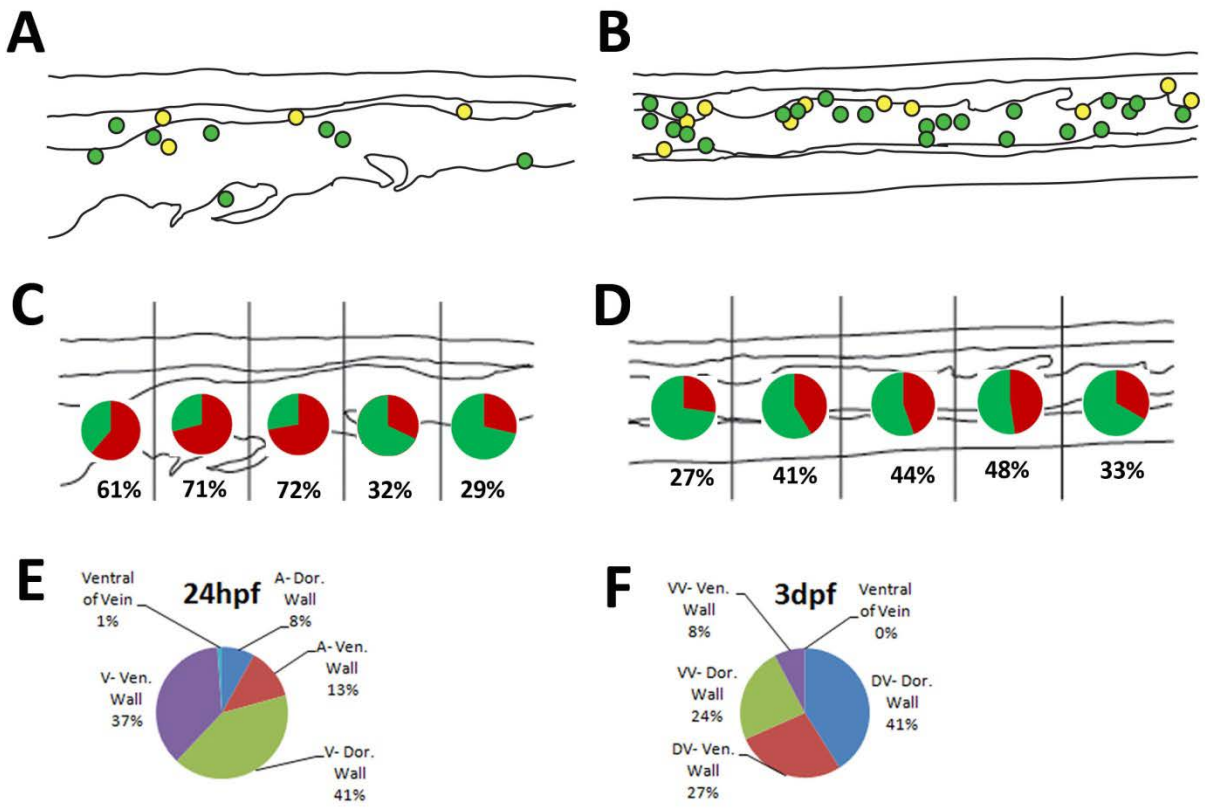
Three-dimensional reconstructions were created of *cmyb:GFP*$^{+}$ cells within the vessel wall of 24hpf (A-B) and 72hpf (C-D) embryos.

Figure 2.4 *cmyb*$^{+}$ endothelial cells arise de novo from the cardinal vein.



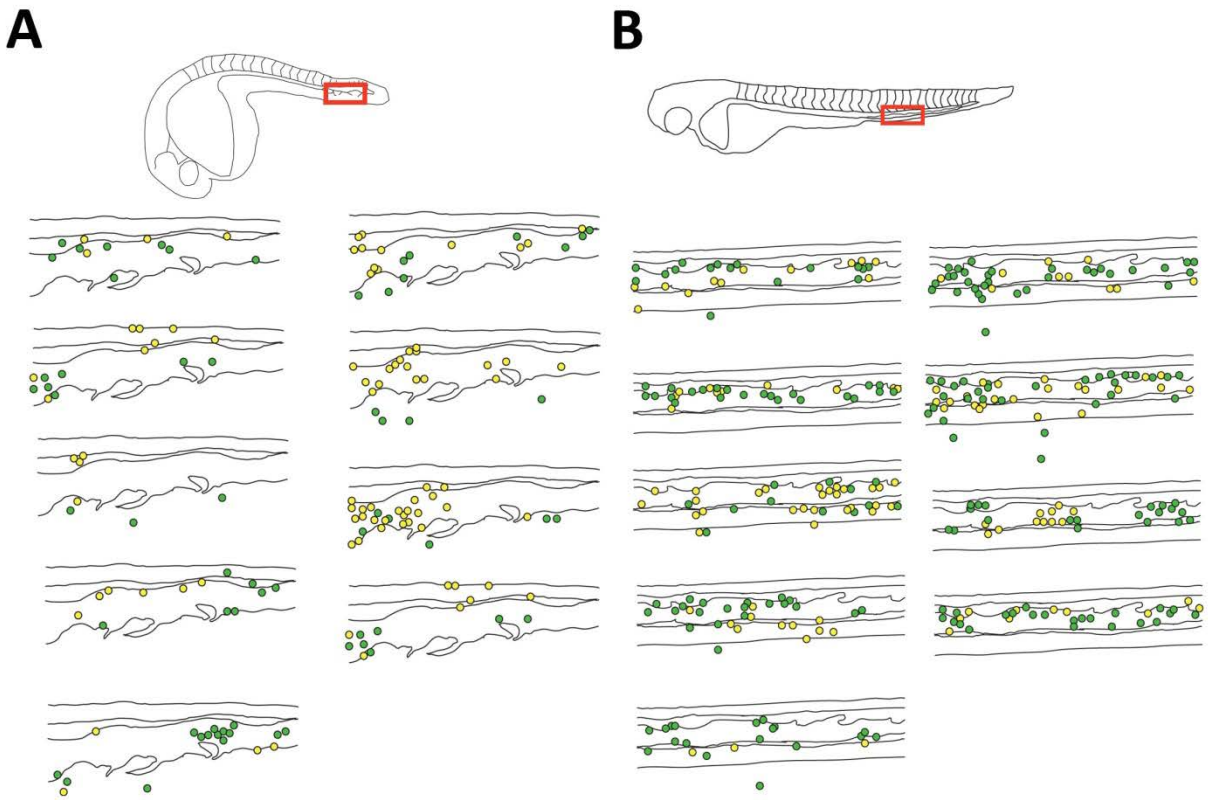
Tg(*cmyb:GFP;kdrl:mCherry*) double transgenics were examined by confocal microscopy. Control embryos (A) and tnnt2-MO injected embryos (B) both showed *cmyb*$^{+}$*/kdrl*$^{+}$ double positive cells at 24hpf. The results are quantified by number of co-postive cells (D) and the percentage of total *cmyb* cells expressing *kdrl* (C).

Figure 2.5: Preferential distribution of *cmyb*$^{+}$ endothelial cells.



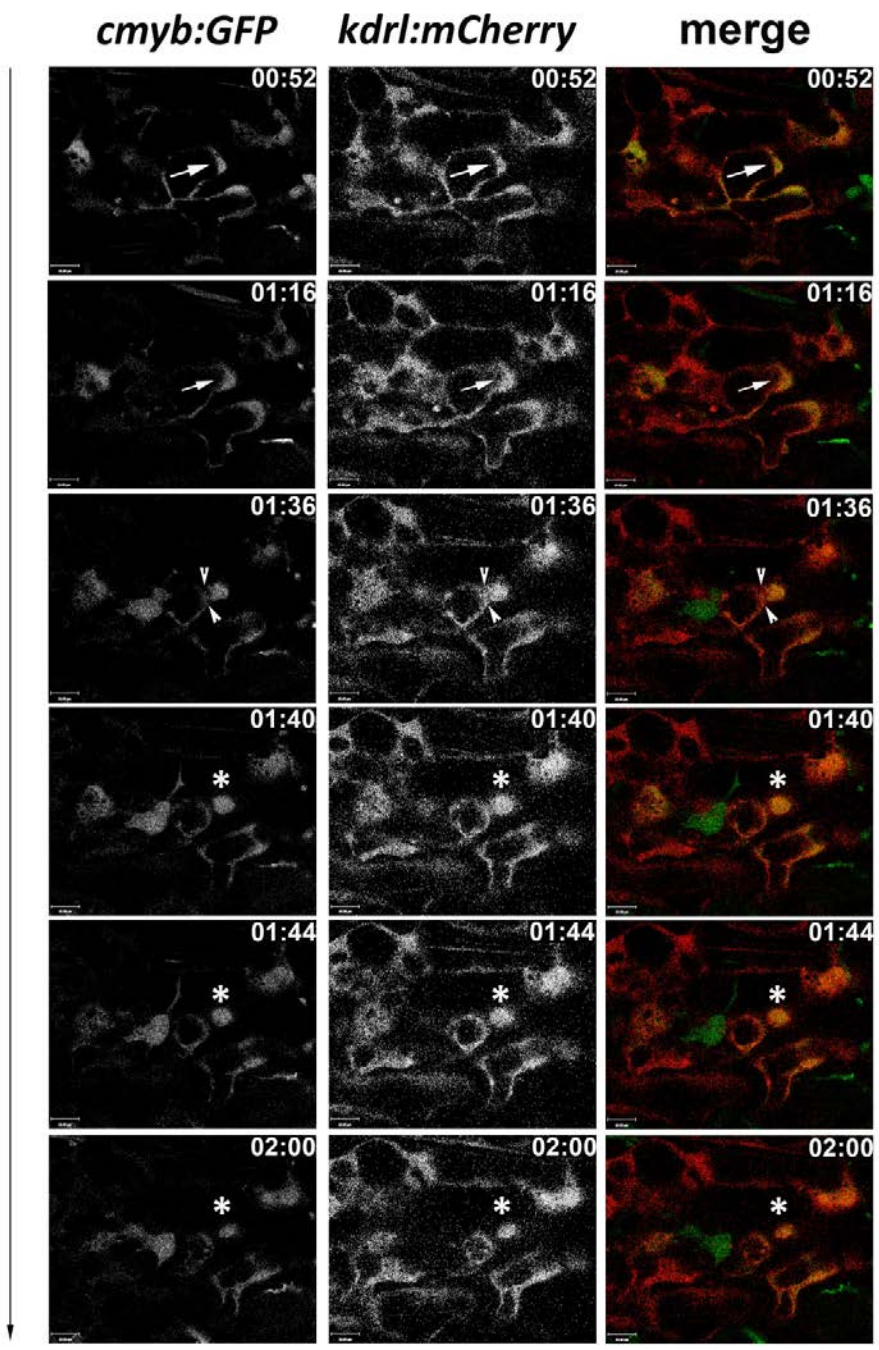
The location of *cmyb*$^{+}$*/kdrl*$^{+}$ and *cmyb*$^{+}$*/kdrl*$^{-}$ cells was plotted on detailed cartographs. Representative cartographs are shown for 24hpf (A) and 72hpf (B). The distribution anterior to posterior is quantified for 24hpf (C) and 72hpf (D). The distribution of arterial versus venous associations is quantified for 24hpf (E) and 72hpf (F).

Figure 2.6 Cartographs reveal preferential localization of *cmyb*$^{+}$ endothelial cells.



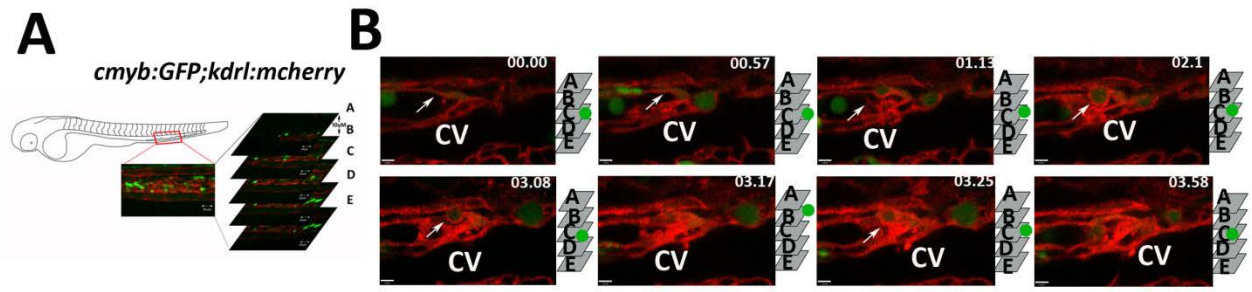
The location of *cmyb*$^{+}$*/kdrl*$^{+}$ and *cmyb*$^{+}$*/kdrl*$^{-}$ cells was plotted on detailed cartographs. Nine cartographs are shown for 24hpf (A) and 72hpf (B).

Figure 2.7 *cmyb*$^{+}$ endothelial cells bud from the vascular wall.



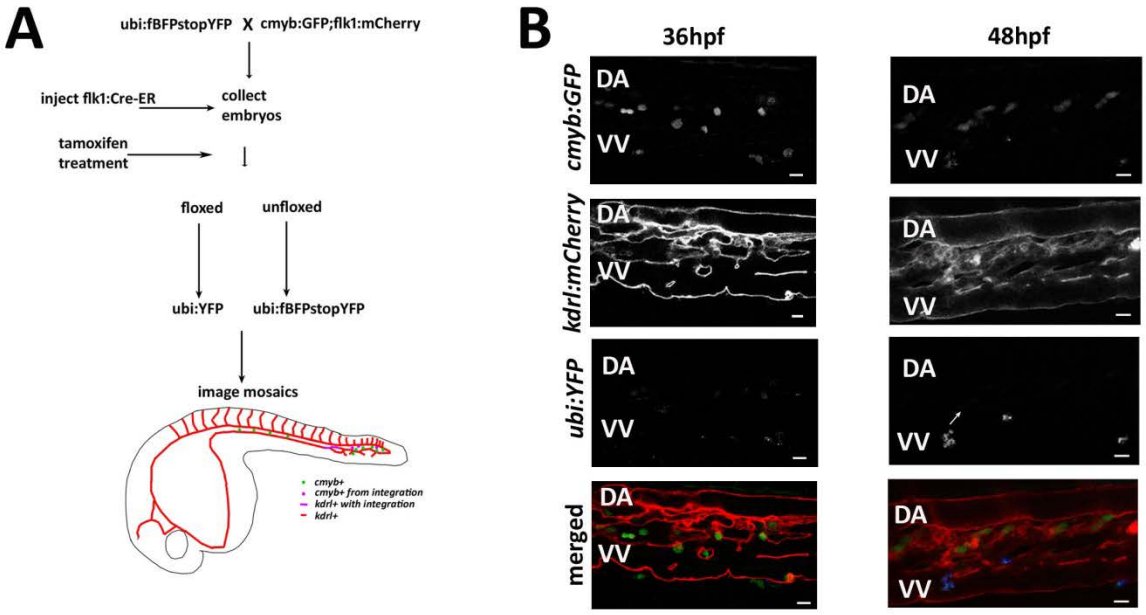
Tg(*cmyb:GFP,kdrl:mCherry*) double transgenic embryos were examined by time-lapse confocal microscopy. Cells were observed to bud (arrow) and exit the vessel (asterisk).

Figure 2.8: *cmyb*$^{+}$ endothelial cells bud from the vascular wall and enter circulation.



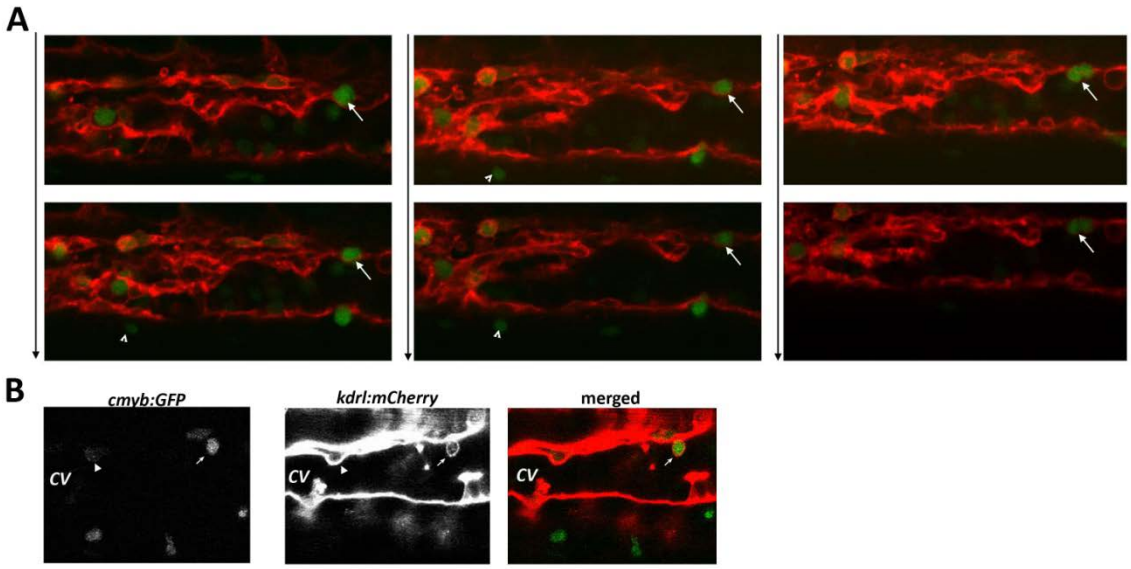
Tg(*cmyb:GFP,kdrl:mCherry*) double transgenic embryos were examined by time-lapse confocal microscopy. A schematic of the z-slices is shown (A). Cells were observed to bud from the vessel (arrow, B) and enter circulation.

Figure 2.9: *cmyb*$^{+}$/*kdrl*$^{+}$ cells give rise to *cmyb*$^{+}$/*kdrl*$^{-}$ cells.



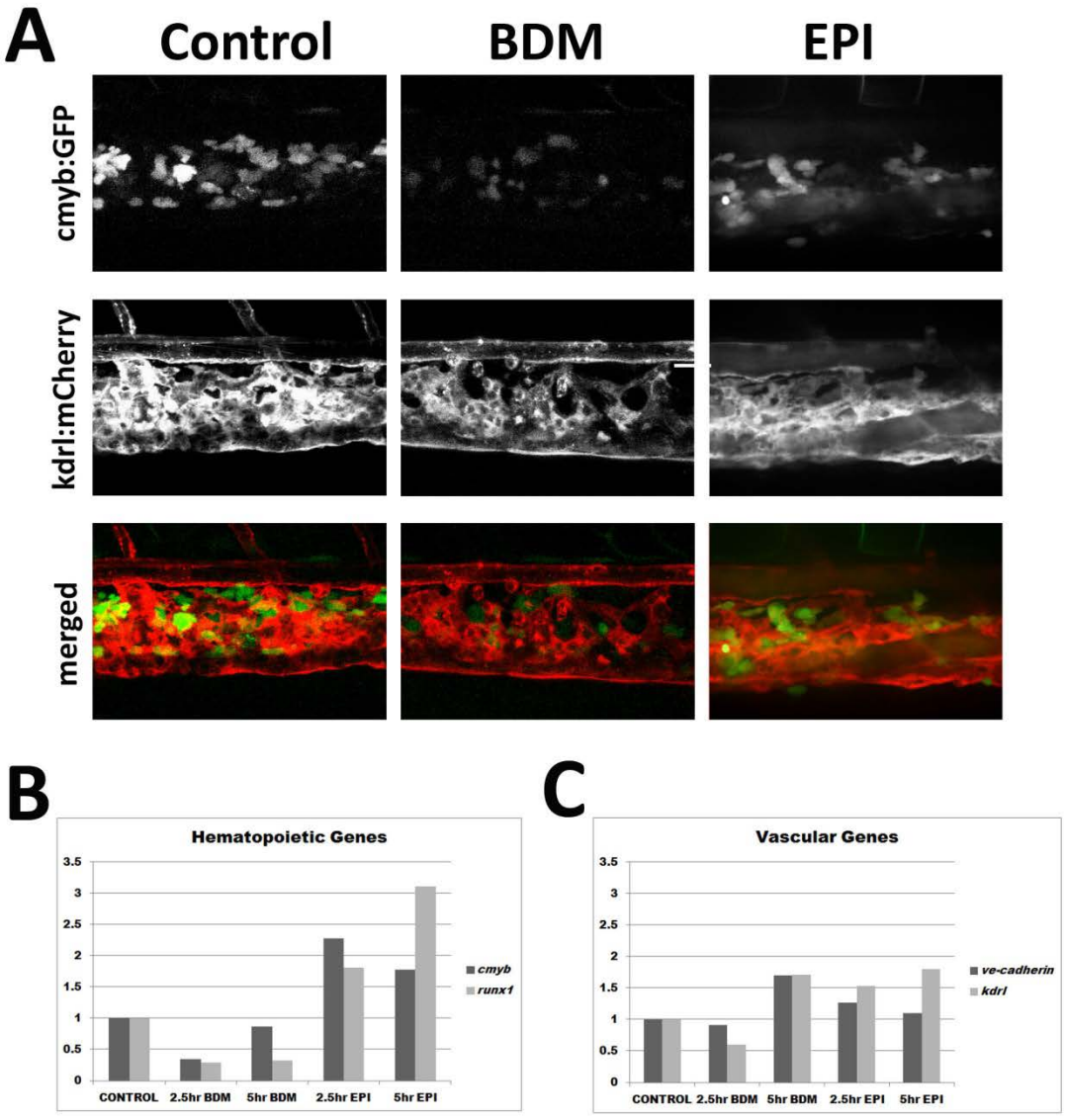
Tg(*ubb:LoxP-AmCyan-LoxP-ZsYellow*);Tg(*kdrl:mcherry*);Tg(*cmyb:GFP*) embryos were injected with *kdrl:CreER*^{t2} plasmid. The experimental design is diagrammed (A). The injected embryos were treated with tamoxifen for six hours and imaged by confocal microscopy (B).

Figure 2.10: *cmyb*$^{+}$ endothelial cells proliferate and migrate in the CHT.



Tg(*cmyb:GFP,kdrl:mCherry*) double transgenic embryos were examined by time-lapse confocal microscopy. Cells were observed to undergo mitosis (A), budding (arrow, B).

Figure 2.11: Blood flow regulates venous hemogenic endothelium.



Tg(cmyb:GFP;kdrl:mCherry) double transgenic embryos were treated for 5 hours with vehicle, (2,3)-BDM, or epinephrine at 67 hpf. Embryos were examined at 72hpf by confocal microscopy (A). RNA was extracted on used to perform QPCR of hematopoietic (B) or vascular (C) genes.

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CHAPTER 3

PECAM1-mediated Regulation of Hematopoietic Potential within Endothelial Cells

OVERVIEW

Both the hematopoietic and vascular systems have flow-sensitive mechanisms of development. In endothelial cells, multiple aspects of the response to blood flow have been shown to be regulated by the association of including Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1) with Vascular Endothelial Cadherin (VE-Cadherin). This association has been shown to be induced in response to blood flow and triggers activation of endothelial Nitric Oxide Synthase (eNOS) and an alignment of endothelial cells in the direction of blood flow. In this report, we sought to identify a zebrafish homolog of PECAM1 and to investigate a potential role of PECAM1 in hematopoietic development. We find that the zebrafish gene XP_697859.3 has homology with mammalian PECAM1, has a vascular expression profile, and that loss of XP_697859.3 has hematopoietic and va remodeling defects, consistent with a role for PECAM in hematopoietic and vascular function.

INTRODUCTION

The response of endothelial cells to blood flow is an area of intense investigation. It has been shown to include alignment of endothelial cells in the direction of blood flow, increased endothelial Nitric Oxide Synthase (eNOS) activity, and Krueppel-like factor 2 (klf2) regulation[1-6]. Within the organisms, there are two

types of blood flow, depending on the nature of the vessel. Elongated, straight vessels experience a constant, uniform blood flow, termed laminar flow. Endothelial cells in areas of the vessel where bifurcations or turns occur experience a less uniform flow, where blood can be pushed in different directions as the vessel turns. This is termed turbulent or disturbed flow.

The importance of this response is shown in susceptibility to atherosclerosis. Areas of the endothelium under sustained laminar flow, align, polarize, and are less prone to develop atherscolerotic lesions. Alternatively, areas of the endothelium under disturbed or turbulent blood flow do not align and are much more susceptible to develop atherosclerotic plaques[7, 8]. Though the initial sensor of blood flow remains unidentified and the subject of intense research, one of the mediators of this response has been shown to be including Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1), which within endothelial cells exposed to blood flow, becomes associated with Vascular Endothelial Cadherin (VE-Cadherin) and Vascular Endothelial Growth Factor Receptor-2 (VEGFR2, or *kdrl* in zebrafish) in a 'mechanosensory complex.[1]' Endothelial cells lacking PECAM1 fail to align in the direction of blood flow and fail to increase eNos production in response to blood flow[1, 3, 4]. Additionally, PECAM1 loss promotes the development of atherosclerotic lesions in areas of laminar flow—areas normally protected from the development of atherosclerosis[1, 9-11].

Interesting, in mammalian systems where PECAM expression is known, it is not restricted to endothelial cells, but is also expressed in hematopoietic stem cells (HSCs), as is VE-Cadherin[12, 13]. This is given more significance by the discovery

that HSCs are also regulated by blood flow. Exposure of HSCs to blood flow was shown to increase expression of hematopoietic stem cell markers[14]. Concurrently, it was shown that loss of blood flow in the organism results in loss of HSC development, either by lack of specification of lack of adequate maintenance[15].

Given that both endothelial cells and hematopoietic cells respond to blood flow, we hypothesized that PECAM1 or PECAM1-like signaling would mediate this flow response in hematopoietic, as well as endothelial, cells in the zebrafish. We investigated this possibility by first identifying a zebrafish homolog of PECAM1, characterizing its expression in development, and then examining its role in the response of HSCs to blood flow.

EXPERIMENTAL PROCEDURES

Zebrafish husbandry

Zebrafish were maintained according to IACCUC-approved protocols in an Aquatic Habitats (AHAB) facility. The following transgenic lines were used: *Tg(kdrl:ras-mCherry)*^{s896}, *Tg(cmyb:GFP)*^{zf169}. Embyros were raised at 28degC until the specified timepoints.

Identification of XP_697859.3

An amino acid blast was performed of mmPECAM1 against all putative open reading frames (ORFs) of the zebrafish genome, www.zfin.org . Reciprocal best hits were assessed using the identified best zebrafish hit as a query in the ensemble blast against mammalian, both human and mouse, genomes.

Syntenic and Phologenic Analysis

Syntenic analysis was performed using Cinteny, the server for synteny identification and analysis of genome rearrangement, http://cinteny.cchmc.org/ . Human chromosome 17, containing PECAM1, and danio rerio chromosome 3, containing XP_697859.3, were compared. A minimum syntenic length of 70kb was used to define a syntenic block. Phylogenic analysis was performed using http://www.phylogeny.fr/.

In situ hybridization

In situ analysis was performed as previously described[16]. Briefly, embryos at the appropriate timepoint were fixed overnight at 4degC in 4% paraformaldehyde. Embryos were dehydrated in methanol overnight at -20degC. The full-length transcript of XP_697859.3 was cloned into pCS2+ and anti-sense, dig-labeled probe was synthesized using a Dig-labeling kit, Ambion Cat#1234.

Morpholino Injection

Embryos were injected into the yolk sac at the one-cell stage as previously described[17]. To block heart beat, *tnnt2* MO was used (5-CATGTTTGCTCTGATCTGACACGCA-3'). Two morpholinos were used targeting XP_697859.3: MO1 and MO2. MO1 targeted the start codon. The sequence of MO1 is: (5'-CCGCTCCCATCCTCACAGGTGAA-3'). MO2 targeted the splice site from exon3 to intron3. The sequence of MO2 is: (5'AACGCAGTGTTTCTGCTCACCTGTG-3'). A standard 1x concentration of .4pmol morpholino per embryo was injected.

mRNA Injections

The coding sequence of XP_697859.3 was cloned into pCS2+. Capped mRNA was synthesized using a SP6 polymerase kit from Ambion. For rescue, .1ng coding mRNA was injected at the one cell stage. For over-expression analysis, either .1ng or .2ng coding mRNA was injected at the one cell stage.

RESULTS

By an amino acid blast of mammalian PECAM1 against all putative open-reading-frames (ORFs) in the zebrafish genome, we isolated XP_697859.3. XP_697859.3 has 35% identity and 40%similarity with mammalian PECAM1 (Figure 3.1A). When blasted against the mammalian genome, XP-697859.3 showed the highest similarity to PECAM1. To further investigate the evolutionary relationship between these two genes, the chromosomes containing each of them was compared using syntenic alignment (Figure 3.1B). Though there is a break in synteny right at the XP_697859.3 locus, the chromosome including XP_697859.3 is syntenic to the mammalian chromosome including PECAM1 at multiple other loci, spaced throughout the chromosomes. When phylogenic analysis was performed using the mouse, frog, and zebrafish potential homologs, the zebrafish gene was the most diverged from the mouse gene (Figure 3.1C). Taken together, this suggests that though the amino acid sequences show some homology, and they are likely descendents from the same ancestral gene, the DNA sequence in teleosts has diverged significantly from that in mammalian systems, and demonstration of their relationship will require functional conservation.

To further determine if XP_697859.3 is a functional homolog of PECAM1, we characterized its expression by RT-PCR and *in situ* hybridization. RNA was extracted from embryos at 3 hours post fertilization (hpf), 18hpf, 24hpf, 3 days post fertilization (dpf), and one week. XP_697859.3 was detectable by RT-PCR starting at 18hpf. Expression increased until 72hpf and was still present at one week (Figure 3.2E). This expression profile is consistent with a vascular gene, as vascular

patterning begins just prior to 18hpf. VE-Cadherin and VegfR2 have similar expression profiles[18, 19]. RT-PCR of XP_697859.3 in *cloche*, a mutant lacking both endothelial and hematopoietic cells, showed loss of XP_697859.3 expression in the *cloche* background (Figure 3.2F). In wild-type embryos, i*n situ* hybridization of XP_697859.3 showed a vascular pattern at 24hpf and 48hpf (Figure 3.2A-D). Interesting at 48hpf, by *in situ,* XP_697859.3 expression appeared to be increased within the caudal vein plexus (CVP)—a vascular and hematopoietic niche, undergoing extensive remodeling at that timepoint (Figure 3.2 B, D). To further determine the endothelial expression of XP_697859.3, QPCR of sorted endothelial cells was performed. Briefly, Tg(cmyb:GFP);Tg(kdrl:mCherry) embryos were dissociated and sorted for GFP or mCherry expression. A 15-fold induction was seen in the endothelial (mCherry+/GFP-) specific population only (Figure 3.2 G). This is similar to the expected fold change for known endothelial markers (kdrl). *In situ* analysis of XP_697859.3 expression in the avascular mutants, *cloche* and *groom of cloche,* both of which lack endothelial cells, though *groom of cloche* retains hematopoietic cells, was not as informative. Though XP_697859.3 expression appeared reduced in *cloche, groom of cloche* appeared to express higher levels of XP_697859.3, either due to the presence of hematopoietic cells, the remnants of some endothelial cells, or increased background expression (data not shown).

To further determine the function of XP_697859.3, we designed two morpholinos. Morpholino 1 (MO1) targeted the start codon of XP_697859.3 while morpholino 2 (MO2) targeted the splice site of exon 2-intron 2, potentially resulting in incorporation of intron 2, and 9 termination codons. As MO1 blocks the ATG start

site of the transcript, it is anticipated that it should block all translation of the XP_697859.3 gene, resulting in loss of most, if not all protein, from this transcript. To determine if XP_697859.3 was involved in a flow response, it was injected alongside a morpholinos targeting *cardiac troponin, tnnt2*. *Tnnt2* is expressed specifically in cardiac muscle, and when silenced using a morpholinos, results in specific lack of a beating heart. Development is unperturbed and vascular patterning is retained, but the heart fails to contract and the endothelial cells are never exposed to blood flow. This has become a useful tool to understanding the effects of blood flow on the vascular system. As previously reported, loss of *tnnt2* resulted in dilation of the cardinal vein, and failure of hematopoietic cells to colonize the tissue. To test the role of XP_697859.3 in these same processes, MO1 was injected into Tg(*cmyb:GFP);*Tg*(kdrl:mCherry*) embryos, and the embryos were screened at 36hpf. When injected at the 1x concentration, MO1 recapitulates the *tnnt*-morpholino injections. The cardinal vein is dilated, indicated a failure of the tissue to remodel, and decreased *cmyb* expression is detected in the CHT (Figure 3.3A). This phenotype could be rescued by co-injection of wild-type mRNA, indicating that it is the result of specific targeting of the XP_697859.3 transcript (Figure 3.3A).

Interestingly, injection of the MO2 morpholino, which targets the splice site of exon 1 to intron 1, did not have the same result. Injection of MO2 at the 1x concentration consistently resulted in no remodeling defect of the cardinal vein and furthermore, consistently resulted in the upregulation of *c-myb* expression within the CHT (Figure 3.3B). To determine the effect of MO2 on XP_697859.3 transcript

levels, mRNA was extracted at 24hpf, and QPCR was performed of the XP_697859.3 transcript. We found that MO2 resulted in a slight upregulation of the XP_697859.3 transcript (Figure 3.3E). Thus, it is likely that MO2 causes either increased stability of the mRNA, or otherwise results in the altered splicing of the transcript such that it is expressed at higher levels. To confirm that increased expression of XP_697859.3 could result in the observed phenotype, mRNA was injected at .1ng and at .2ng per embryo, similar to the concentrations used to ectopically expressing other genes, and the embryos were screened at 36hpf. When mRNA was injected to overexpress XP_697859.3, there was no defect of vascular remodeling in the CHT and increased c-myb expression was observed (Figure 3.3B). Thus, injection of MO2 is consistent with an overexpression phenotype of XP_697859.3. Concurrent with this possibility is that co-injection of the wild-type XP_697859.3 transcript does not restore the normal phenotype.

To further investigate these somewhat contradictory phenotypes, MO1 was coinjected with MO2. We found that coinjection of the two morpholinos resulted in the rescue of the vein dilation and restoration of the *c-myb* expression within the CHT (Figure 3.3C). Thus, it is likely that both morpholinos are targeting the same gene, but with different effects on protein levels. Interesting, co-injection of MO1 with a morpholinos targeting p53 also rescued the MO1, loss of flow phenotype (Figure 3.3C). This is possibly due to a known role of PECAM1 in apoptotic signaling. In mammalian systems, PECAM1 has been shown to protect from apoptosis by acting through p53. It is possible that loss of XP_697859.3 activates a p53-dependent pathway and that cell death is partially responsible for the observed

phenotype. Finally, to test how these two morpholinos were interacting, QPCR was performed of vascular and hematopoietic genes on mRNA from embryos injected with one or both of the morpholinos (Figure 3.3D). Injection of MO1 resulted in upregulation of both hematopoietic and vascular markers, while injection of MO2 only resulted in increased *cmyb* transcript. Again, co-injection of either the p53 morpholino or MO2 with MO1 suppressed the MO1 phenotype, suggesting that the two morpholinos cause opposing phenotypes and that MO1 acts through p53.

DISCUSSION

Though Pecam1 is often used as a stereotypic marker for endothelial cells, its expression profile includes multiple hematopoietic cell types including platelets, lymphocytes, and hematopoietic stem cells[12, 13]. Its role in many of these tissues remains unclear, and though the knock-out mouse is viable, Pecam1 has been shown to have important roles in angiogenesis, and the response of endothelial cells to blood flow[1, 20]. As both endothelial and hematopoietic stem cells respond to blood flow and both express Pecam1, we sought to determine if Pecam1 could be a mediator in the hematopoietic response to blood flow. To do this, we first identified a potential homolog of Pecam1 in zebrafish and then characterized its role in hematopoietic development. We found that danio rerio XP_697859.3 displayed homology to PECAM1, was located on a chromosome syntenic to the human chromosome containing PECAM1, and the furthermore, modulation of XP_697859.3 affected known flow sensitive pathways. Interestingly, modulation of XP_697859.3 was sufficient to also alter hematopoietic development, suggesting that indeed, PECAM1 could be involved in the response of HSCs to blood flow. Though the mechanism by which this occurs remains uninvestigated, it is interesting to speculate that it is similar to the pathway in endothelial cells.

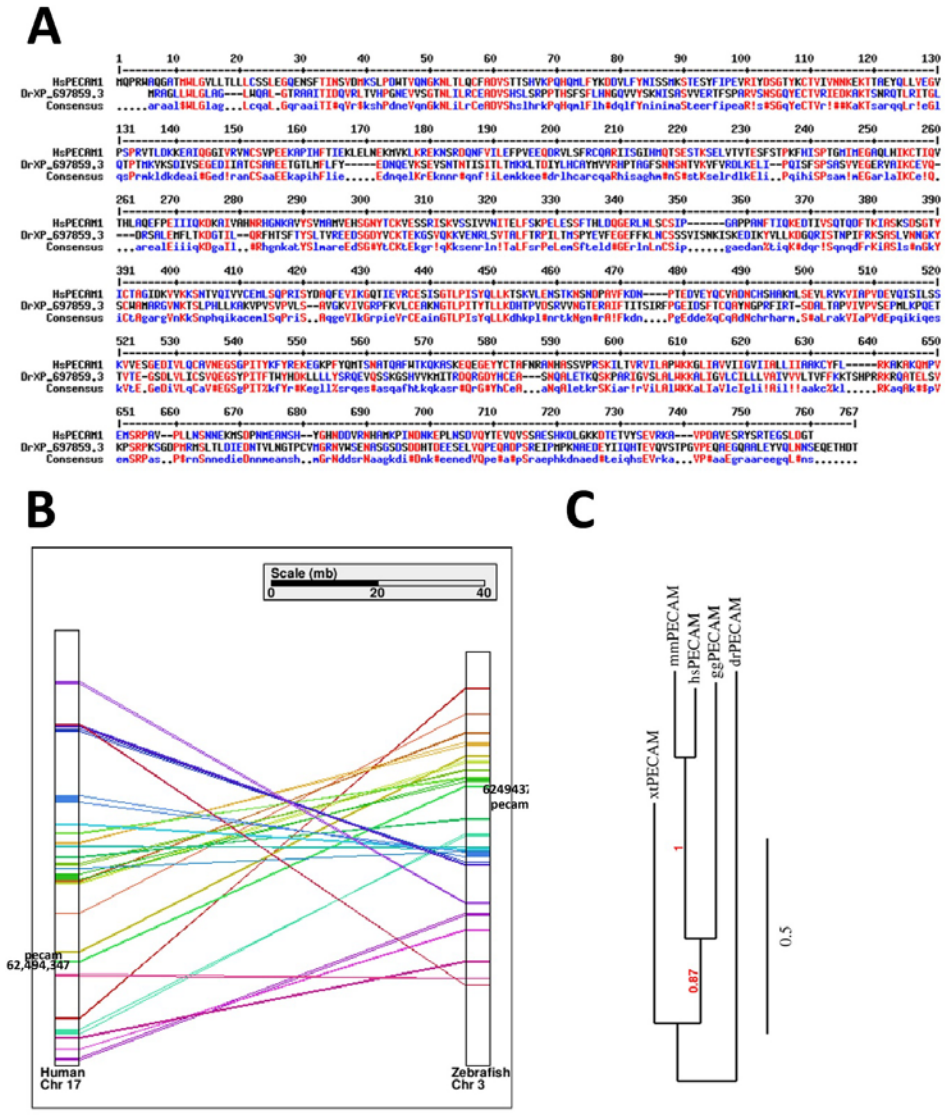
In endothelial cells, Pecam1 becomes associated with the mechanosensory complex in response to hemodynamic force[1]. Interestingly, the mechanosensory complex within endothelial cells consists of PECAM1, VE-Cadherin, and VegfR2. Given that both PECAM1 and VE-Cadherin are expressed in hematopoietic stem cells which also respond to changes in blood flow, there is the possibility of a second

mechanosensory complex which exists in hematopoietic stem and progenitor cells. In this scenario, it would be interesting to consider that either the mechanosensory complex in HSCs responds differently than in endothelial cells, either due to the absence of VegfR2 or because of the inclusion of a different receptor tyronsine kinase.

Recently, an additional gene has been identified in zebrafish that has homology to PECAM1. This newly isolated gene, still identified as si:dkey-2370.8 has 27% identity and 44% similarity to murine PECAM1. Additionally, at least one of the ITIM phoshorylation sites appears to be conserved in this homolog. It is possible that closer inspection of these genes will yield interesting divergent roles for PECAM1-like molecules in zebrafish. Clearly, it is necessary to further investigate these genes, as well as the possible overlap and divergence of their functions before making any claims as to their effects on hematopoiesis in zebrafish.

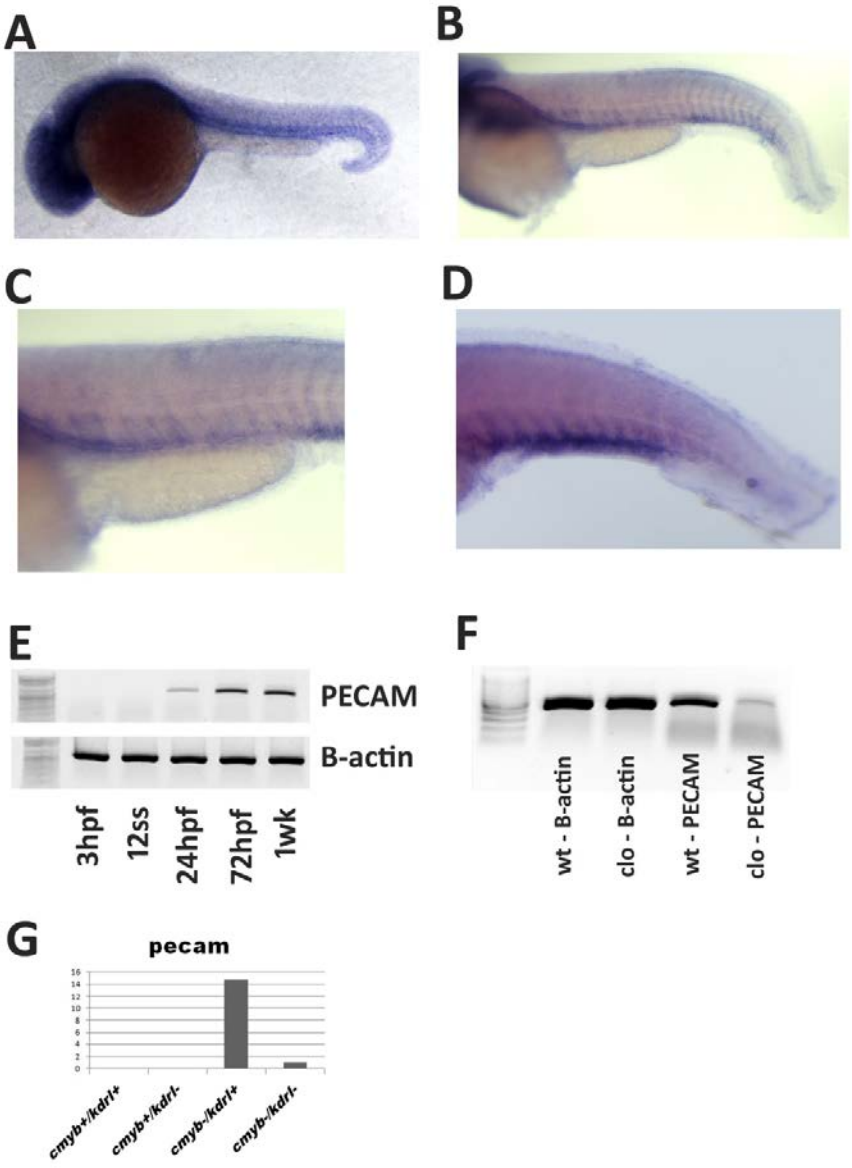
FIGURES

Figure 3.1: Identification XP-697859.3 as a potential homolog to PECAM1.



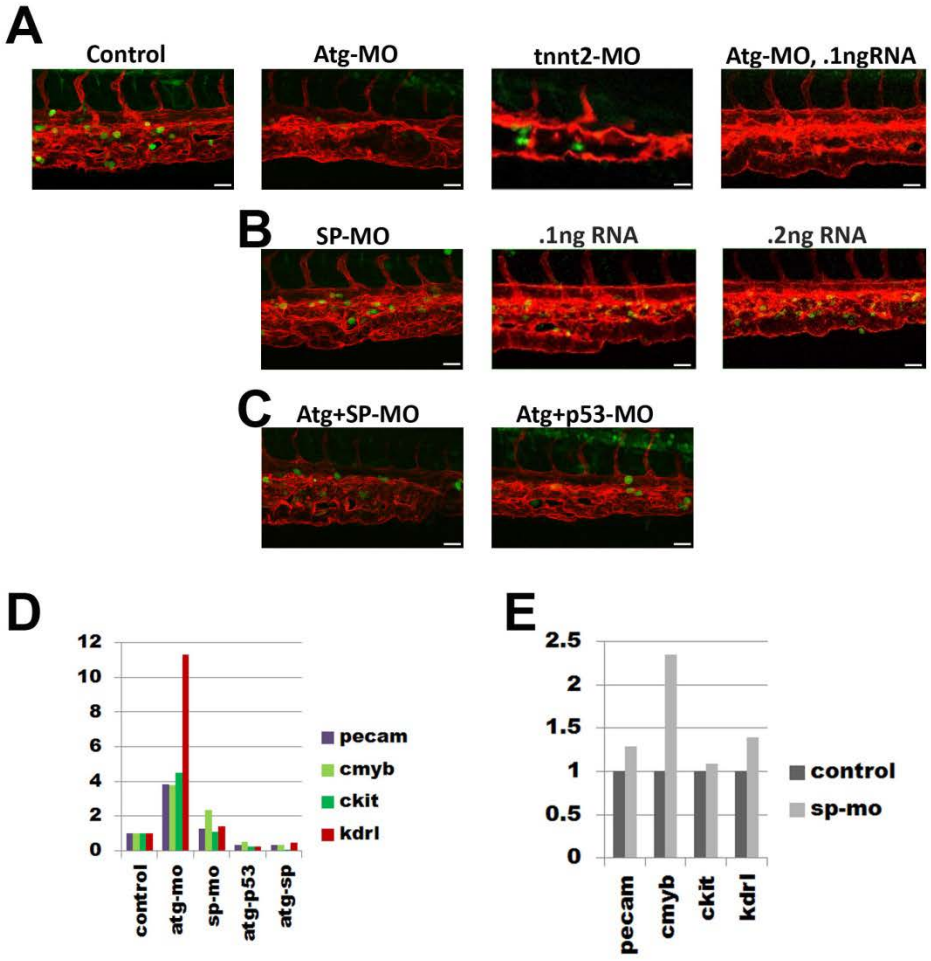
HsPECAM1 was aligned against danio rerio XP_697859.3 using *multalign* (A). The synteny of the chromosomes containing human PECAM1 and danio rerio XP_697859.3 (chromosomes 17 and 3 respectively) were examined using cinteny (B). The phylogenic analysis of the putative PECAM homologs is shown (C).

Figure 3.2: Characterization of XP_697859.3.



The full-length transcript of XP_697859.3 was used to probe for expression at 24hpf (A) and 48hpf (B). Higher magnifcations of the 48hpf expression profiles are shown of the AGM (C) and the CHT (D). RT-PCR was performed at multiple time-points to determine when XP_697859.3 was expressed (E). RT-PCR was performed from wild-type and *cloche* mutants (F). QPCR was performed on sorted endothelial populations (G).

Figure 3.3: Characterization of XP_ 697859.3 morpholinos.



Morpholinos were injected against control, *tnnt2*, or XP_697859.3. The phenotype of MO1 or MO1 + wild-type transcript was compared with either control or *tnnt2*-MO injections (A). The phenotype of MO2 was compared with mRNA injections (B). MO1 was co-injected with either MO2 or p53-MO (C). QPCR was performed on morpholino-injected embryos (D, E).

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CHAPTER 4

Conclusions and Perspectives

The first observation of potential hematopoietic cells descending from the endothelium, made by Florence Sabin in the 1920s, was a powerful discovery for multiple reasons[1]. It suggested that endothelial cells were not completely a terminally differentiated cell, an idea that may have seemed contrary to conventional thinking but has since found more acceptance. The idea that a differentiated endothelial cell could become undifferentiated and become something different, especially a hematopoietic stem cell (HSC) capable of self-renewal and differentiation into multiple other lineages, must have seemed an interesting concept at first glance. What was this cell doing? Why would an endothelial cell do that?

As studies have progressed, scientists have been able to add substantial knowledge to the regulation of this process. We now have multiple markers of hemogenic endothelium, we know that it is conserved across species, and we know that it occurs in more than one vascular bed[2-4]. However, there are still many questions unanswered. It is still not clear why some endothelial cells are hemogenic and others are not. The pathways that promote hemogenic behavior are being elucidated, but slowly, mostly due to complications in distinguishing changes in overall hematopoiesis from changes in specific descendents of hemogenic endothelium[5]. The biological significance of this phenomenon remains almost completely untested, due to the near impossibility of eliminating HSCs descending from endothelial cells while leaving other HSCs intact. One interesting question is

whether all endothelial cells possess the ability to become hemogenic, and to what extent these endothelial cells are hemogenic.

My work has helped to clarify that non-aortic endothelial cells also possess the ability to produce HSCs. We have provided evidence that venous endothelium can provide HSCs. Though previously described hemogenic endothelium has been arterial in nature, studies have suggested that arterial differentiation was not a necessary component of hemogenic activity[3, 6-11]. The ability of venous endothelial cells to be hemogenic confirms that the arterial nature is not an absolute requirement of hemogenic endothelium. This is interesting, because it presents the possibility that hematopoietic activity may be a more general property of endothelial cells than previously appreciated. Also, it suggests that the pathways promoting hemogenic activity occur alongside of, but independent from, those promoting endothelial differentiation. This second idea is supported by reports on *stem cell leukemia* (*scl)* activity in endothelial and hematopoietic tissues. Loss of *scl* was shown to cause lack of hematopoietic specification, while not affecting angioblast development until a later angiogenic stage[11, 12].

As hematopoietic activity does occur specifically in endothelial cell—as opposed to randomly occurring in any stromal cell available, the implication is that there is a common denominator between the endothelial and hematopoietic pathways or that endothelial cells are more capable of becoming hemogenic than another cell type. The questions that arise are what are the common denominators and at what point do hemogenic endothelial cells diverge from the endothelial program? Current reports suggest, though they do not conclusively demonstrate,

that while it uses some of the same mediators, it is mechanistically independent of the arterial/venous specification, though occurring concordantly temporally.

The role of blood flow in regulation of this pathway is also still unclear[5, 13]. We identify blood flow as a regulator of hematopoietic maintenance. This suggests that previous reports, detailing the effects of blood flow on hematopoietic development may be over-simplified, confounded with changes of hematopoietic maintenance. While our results support that at least some hemogenic endothelium may be specified in the absence of blood flow, there is no expansion of this population, it may not bud from the vessel wall, and instead the HSCs are rapidly depleted. Furthermore, our data support the hypothesis that Platelet Endothelial Cell Adhesion Molecule-1 (PECAM1), a mediator of the flow response in endothelial cells, is also involved in the response within hematopoietic cells. There are several reasons this could be the case. It could be an instance of HSCs sensing and responding directly to the presence of blood flow. As HSCs retain a close association with the endothelium, it is formally possible that they remain in some contact with the vessel lumen and can sense and respond directly to blood flow. Alternatively, the flow signal could be relayed through the endothelial cells, and in the absence of that signal from the endothelial cells, the HSCs become depleted. Given the uniform depletion of *cmyb*$^{+}$ cells we observed, and that not all *cmyb*$^{+}$ cells were in direct contact with the vessel, this is more likely to be the case. In either case, the role of blood flow in regulation of HSC maintenance is instructive as to the cues directing HSC recruitment to the downstream lineages. Resolving this quandary will require sophisticated cell-autonomy experiments and/or conditional

knock-outs. Though it is possible, and likely, that PECAM1 is involved in the transendothelial migration of hematopoietic cells, the role this plays in our observed phenotype is unclear.

Future directions

Our experiments demonstrate the existence of hemogenic endothelium outside of the aorta-gonad-mesonephros (AGM), suggesting that more endothelial cells may possess hemogenic ability than previously appreciated. However, these studies were subject to several limitations. First, due to the limited availability of hemogenic markers, we relied substantially on the Tg(*cmyb:GFP)* line. Exhaustive studies would require the generation of multiple hemogenic markers. Currently, candidates such as *runt-related transcription factor 1 (runx1),* provide the most promising alternatives, and we are actively working to generate transgenic lines utilizing the hematopoietic specific enhancers of the *runx1* gene. However, evidence has suggested that event his approach will have limitations, as the requirement of *runx1* in hematopoiesis is unclear. Multiple studies have suggested that loss of *runx1* does not have irreparable deleterious effects on hematopoietic development[14, 15]. It is likely that there are better markers that have yet to be characterized. Identification of these markers would be aided by array experiments on isolated hemogenic endothelium.

Further work, that would be instrumental in moving the field forward, would address the biological consequences of loss of hemogenic endothelium. As reports have shown that HSCs can have endothelial or non-endothelial origins, it is possible

that loss of either of these populations would have no effect on overall organism health, or alternatively, both populations could be absolutely required. To address this issue, a combination of lineage tracing with cell-ablation technology could be employed[16, 17]. This would allow for the specific ablation of endothelium-derived HSCs, and then the questions of biological significance could be addressed.

Once the characterization of pathways directly involved in production of hemogenic endothelium have been clearly elucidated and distinguished from other populations of HSCs, the benefits to human health could be significant. In cases where hematopoiesis has failed, or has become dysfunctional at the expense of the individuals health, endothelial cells could be the reservoir from which normal hematopoiesis could be restored.

In our experiments where blood flow was modulated using chemical treatments, we showed that transendothelial migration of HSCs across the endothelium was not required to occur before *cmyb*$^{+}$ cells could become depleted. As the XP-697859.3 morpholino injected embryos displayed a similar hematopoietic defect, it is suggested that PECAM1's involvement in this process may be more as a flow sensor/responder or signaling molecule, and less as a mediator of transendothelial migration. There are several experiments necessary to validate these findings. We would need to first determine if the zebrafish homolog of PECAM1 acts in a similar manner as mammalian PECAM1 has been shown to act. We would need to test whether it was involved in integrin activation, changes in b-catenin association, and phosphorylation of Vascular Endothelial Growth Factor Receptor-2 (VEGFR2, or *kdrl* in zebrafish). Furthermore, the demonstration that XP-

697859.3 is a functional homolog of PECAM1 has remained elusive. A clear demonstration that mammalian PECAM1 can rescue the changes we see in the XP-697859.3 morpholino injected embryos would clarify the functional relationship between these two genes.

Taken together, the data all suggest that these two systems, hematopoietic and vascular, are intrinsically linked: they develop concordantly, they are co-regulated, and they cross-talk. The idea of transdifferentiation of specified cell types is still unusual in many systems, though now we know, that in the correct conditions, many cell types can be undifferentiated and driven towards alternative cell fates[18, 19]. What makes hemogenic endothelium still unique, is that it occurs normally in the organism, be it zebrafish or mice.

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