PLATELET DERIVED GROWTH FACTOR RECEPTOR B (PDGFRB) EXPRESSING CELLS DURING ZEBRAFISH CORONARY VESSEL DEVELOPMENT

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A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Juancarlos Fierros
June 2017
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Approved by:

Nicole Bournias-Vardiabasis, Ph.D, Committee Chair, Biology

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ABSTRACT

Coronary heart disease is a prevalent issue in developed countries throughout the world. It can have crippling effects on the quality of life and even lead to mortality, in the case of myocardial infarction. Part of the problem is the lack of a robust regenerative response in mammals after injury. Zebrafish have an amazing ability to regenerate after injury, and studies have demonstrated that the regenerative response recapitulates embryonic development. Our lab previously reported the first analysis of coronary vessel development in zebrafish and demonstrated that coronary endothelial cells undergo angiogenesis to form a vascular network. The roles of perivascular cells in this process have not been examined in zebrafish. Using a transgenic reporter line marking pdgfrß expression, I found that pdgfrß is first observed in epicardium at the AV canal. At later stages of coronary vessel development, pdgfrß positive cells become localized to the perivascular region of mature vessels. I also observe that early in development, Tcf21 and pdgfrß co-express, which suggests a close relationship between the epicardium and pdgfrß+ cells. Previous findings from our lab revealed that cxcl12b+ cells localize to large coronary vessels during development. My findings reveal that pdgfrß+ marks perivascular cells of both capillaries and large coronary vessels. Lineage tracing analysis revealed that a subset of pdgfrß+ perivascular cells derive from tcf21 labeled epicardial cells. To see if disruption of Pdgfrß signaling impacts coronary development, I examined pdgfrß mutant hearts. In the Pdgfrß mutant, a mature coronary vessel network
fails to form, and instead we observe isolated endothelial cell islands. Lastly, I characterized a transgenic line that expresses a dominant negative form of Pdgfrβ (dnpdgfrβ) and can be potentially used for later developmental and/or regenerative studies. My findings indicate strong dnpdgfrβ induction can be achieved at adult stages. My studies will greatly enhance our current understanding of coronary vessel development, and can be used as the basis for studying perivascular cells and their interactions with endothelial cells after cardiac injury in regeneration
ACKNOWLEDGEMENTS

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CHAPTER ONE
INTRODUCTION

Background

Coronary Heart Disease

Coronary heart disease is a major concern for industrialized nations all over the world. Cardiovascular disease has deep implications on both human health and economics. According to the American Heart Association (AHA), in 2011-2012 the sum of both direct and indirect costs of cardiovascular disease were 316.6 billion dollars. In 2013, coronary heart disease was responsible for approximately 1 in 7 deaths in the United States. Each year, an estimated 660,000 Americans suffer a new coronary attack (defined as myocardial infarction) (Mozaffarian et al., 2015). Myocardial Infarction (MI), also known as a heart attack, is a common end result for people suffering from coronary heart disease. Myocardial Infarction results when cholesterols and other plaques accumulate over time and reduce or block blood flow in the coronary arteries. When blood flow is disrupted, proper oxygen and nutrient delivery to the remaining cardiac tissue is affected. When the tissues downstream of the blockage are deprived of oxygen, cardiac tissue will begin to be lost through the processes of apoptosis and necrosis (American Heart Association). Therefore, revascularization is one major therapeutic approach after myocardial infarction resulted from coronary heart disease. In addition to adult coronary disease,
congenital coronary artery anomalies can also be life threatening without surgical intervention. Despite the importance in both children and adults, the development of coronary vessels and revascularization after injury remain poorly understood.

**Zebrafish as a Model to Study Cardiovascular Development and Regeneration**

Zebrafish use as a model system was first established in the 1970’s by Streisinger and colleagues to study vertebrate development. Recently, the use of Zebrafish as a research model has seen a sharp increase due to the many advantages compared to other model organisms, such as mice. Zebrafish can be used to study human cardiac development disorder, since the genetic networks that regulate cardiac development are highly conserved (Fahed, Gelb, Seidman, & Seidman, 2013; Moorman & Christoffels, 2003). Due to the small size of zebrafish embryos, until the coronary vasculature forms, zebrafish can obtain their oxygen requirements through diffusion. This allows for the study of severe cardiovascular defects that might not be observable in other animal models, since the absence of a cardiovascular system leads to death (Bakkers, 2011; J. N. Chen et al., 1996; Sehnert et al., 2002; Stainier et al., 1996; Strecker, Seiler, Hollert, & Braunbeck, 2011). Zebrafish also have relatively large clutch sizes, which allows for greater statistical certainty. It is not uncommon for zebrafish pairs to lay hundreds of embryos per mating (Brown, Samsa, Qian, & Liu, 2016). Another advantage is the external development of the embryos which allows for easier embryo manipulation, as opposed to embryos in utero. Zebrafish embryos are optically transparent, which is a useful property in studying development.
Embryo clarity is useful to observe gene expression when researchers use whole-mount in situ hybridization and transgenic reporter lines for analyses. Zebrafish phenotyping and screening can be done using transgenic reporter lines, just by observation of the embryo live under fluorescence (Huang, Tu, Hsiao, Hsieh, & Tsai, 2003; Jin, Beis, Mitchell, Chen, & Stainier, 2005; Long et al., 1997; Perner, Englert, & Bollig, 2007). Lastly, zebrafish are amenable to other forward and reverse genetic approaches, which increase their usefulness in the lab.

**Zebrafish Heart**

Similar to all other vertebrates, the heart is the first organ to form during zebrafish development (Bakkers, 2011). The result of zebrafish cardiac development is a two-chambered heart, which is composed of one ventricle and one atrium. The atrium consists of a thin layer of muscle, whereas the ventricular myocardium consists of a thick outer compact layer and inner trabeculae layer (Hu, Yost, & Clark, 2001; Menke, Spitsbergen, Wolterbeek, & Woutersen, 2011; Poss, Wilson, & Keating, 2002). Incoming oxygenated blood flows from the atrium to the ventricle via the atrial-ventricular valve. At the base of the ventricle is a structure known as the bulbus arteriosus, which acts as the outflow tract so that the blood can reach the gill arches. Similar to mammals, the zebrafish heart is also composed of three tissue layers: epicardium, myocardium (compact and trabecular), and endocardium (Bakkers, 2011; Lien, Harrison, Tuan, & Starnes, 2012).
Mammalian Coronary Vessel Development

Although coronary vessel development is a crucial step in mammalian development, for decades the origin of these coronary vessels was unknown. Throughout the years, different theories were proposed to explain the origin of the coronary vasculature. Early anatomical studies proposed that the coronary arteries arose by budding from the aorta. Chick-quail chimaera studies proposed that proepicardial cells, which are progenitors of the epicardium, undergo epithelial-to-mesenchymal transition (EMT) to form the endothelial tubes, which will become the mature vessels (Hutchins, Kessler-Hanna, & Moore, 1988; Majesky, 2004). These widely held views were challenged when recent lineage tracing studies revealed that the proepicardium gave rise to few, if any coronary endothelial cells (Cai et al., 2008; Merki et al., 2005; Wilm, Ipenberg, Hastie, Burch, & Bader, 2005; Zhou et al., 2008). Recent work with mice, has revealed that the coronary vasculature is derived from existing endothelial cells that reside in the sinus venosus (SV). When examining the nascent plexus originating in the sinus venosus, vessels at this location expressed angiogenic sprout markers: Vegfr3, Dll4, and Pdgfrβ (Hellstrom et al., 2007; Kidoya et al., 2008; Suchting et al., 2007; Tammela et al., 2008). More importantly, consistent with previous lineage racing results, the group did not observe proepicardial cells becoming endothelial cells (K. Red-Horse, H. Ueno, I. L. Weissman, & M. Krasnow, 2010). To test their model that the sinus venosus was required for coronary vessel development, they turned to an In Vitro culture system for help. In culture, hearts
with sinus venosus intact were compared to hearts with sinus venosus removed. Intact hearts developed coronary vessels, whereas dissected hearts displayed no coronary vessels. (K. Red-Horse, H. Ueno, I. L. Weissman, & M. A. Krasnow, 2010). Clonal analysis demonstrated that most coronary arterial cells are clonally related to cells that reside in the sinus venosus, and that a single cell originating from the sinus venosus can give rise to the three coronary cell types: arteries, veins, and capillaries through reprogramming (K. Red-Horse et al., 2010). Follow-up studies supported the model that the sinus venosus contributes to coronary vasculature and also revealed that the endocardium can also give rise to regions of the coronary vasculature (Tian et al., 2013; Wu et al., 2012). Interestingly, the proepicardium was also shown to give rise to a uniform portion of vasculature around the heart. It was also observed that the dorsal coronary vasculature originates from the sinus venosus and is dependent on epicardial secreted VEGFC, which has been shown to activate endothelial cell migration. The importance of VEGFC to sinus venosus derived coronary vessels was demonstrated through VEGFC knockout mice. VEGFC knockout coronary vessels were wider and flatter, and contained fewer branch points, compared to wild type. The ventral coronary vasculature was shown to be derived from the endocardium and acts in a VEGFC independent manner (H. I. Chen et al., 2014). Ultimately, their model suggests that these two populations (Dorsal and Ventral), along with a minor contribution from the proepicardium, intermix to give rise to an
interconnected vasculature network during embryonic development (H. I. Chen et al., 2014)

Zebrafish Coronary Vessel Development

Although studies in mice elucidated the source of coronary vessels in mammals, the origin of the coronary vasculature in zebrafish remained unclear. Our lab demonstrated that CXC chemokines play a major role in zebrafish coronary vessel development (Harrison et al., 2015). This was not surprising, since cxc chemokines have been shown to regulate cell adhesion and cell migration during development. CXCR4 and CXCL12 knockouts in mice display defects in cell migration (Raz & Mahabaleshwar, 2009). Zebrafish contain two cxcl12 genes, cxcl12a and cxcl12b, along with two receptors, cxcr4a and cxcr4b (Boldajipour et al., 2011). With the aid of transgenic lines that fluorescently tag endothelial cells, fli1a:EGFP, our lab discovered that the coronary vasculature does not form until 1-2 months post fertilization, a time point where other groups have demonstrated that the ventricular myocardium undergoes significant expansion. (Gupta & Poss, 2012; Harrison et al., 2015; Hu, Sedmera, Yost, & Clark, 2000). At 1-2 months post fertilization, endothelial cells emerge from the atrial-ventricular canal, a region between the atrium and ventricle that has direct connection to the endocardium. Using lineage tracing and clonal analysis, our lab showed that the endocardium at the AV canal is in fact the source of the beginning angiogenic sprouts. After these endothelial cells emerge from the AV canal, they first migrate to the bulbus arteriosus and then the angiogenic sprouts
continue cover the rest of ventricle as the heart expands. It has been shown by other groups that during formation of the lateral aorta and brain capillaries, endothelial cells expressing Cxcr4a migrate towards Cxcl12b expressed by the endoderm (Bussmann, Wolfe, & Siekmann, 2011; Siekmann, Standley, Fogarty, Wolfe, & Lawson, 2009). During our studies we observed strong cxcl12b expression in the myocardium before endothelial cells emerge at the AV canal. This strong expression in the myocardium is maintained until the vasculature network forms, which at that time cxcl12b expression downregulates in the myocardium, but upregulates in a cell population located on large coronary vessels, which were presumed to be perivascular cells, based on location (Harrison et al., 2015). Cxr4a was only expressed after endothelial cells emerge. Strong expression of cxr4a was observed on the migratory endothelial cells, but was downregulated as the vasculature network formed. In cxcr4a mutant zebrafish, the coronary vasculature fails to form and endothelial cells that do migrate to the surface appear abnormal and lack coordinated migration. Live imaging studies have shown that endothelial cells from cxcr4a mutants lack coordinated migration and do not form interconnections with other endothelial cell sprouts (Harrison et al., 2015). In addition, cxcl12a/b double mutants did not survive past larval stages and did not form vasculature. Cxcr4a-Cxcl12b signaling also has a role in regeneration. cxcr4a mutants did not regenerate after heart amputation and instead a collagenous scar remained after injury (Harrison et al., 2015; Poss et al., 2002). More work remains to determine the exact role of
Cxcr4a-Cxcl12 signaling during regeneration. These works suggest a model where myocardial expressed Cxcl12b provides migratory cues for endothelial cells expressing Cxcr4a, and disruption of this signaling results in lack of coronary vasculature during development and reduced regenerative capacity after injury.

Pericytes

Pericytes were first described over 100 years ago, by Charles-Marie Benjamin Rouget, who originally described them as a population of contractile cells that surround blood vessel endothelial cells (Armulik 2011). It is now clear that there is a variety of different cell types in close proximity to endothelial cells, but correct identification can still be problematic (Armulik, Genove, & Betsholtz, 2011; Krueger & Bechmann, 2010). Contributing to this problem is the fact that several molecular markers, complied by different reviews, have also been shown to label pericytes and other cell types such as smooth muscle cells. Unfortunately, there is not one “universal marker” that can label all pericytes and expression of cell markers can be dynamic (Armulik, Abramsson, & Betsholtz, 2005; Armulik et al., 2011; Diaz-Flores et al., 2009; Krueger & Bechmann, 2010). However, several groups have reported Pdgfrβ to be a reliable pericyte marker in brain micro vessels (Lindahl, Johansson, Leveen, & Betsholtz, 1997; Winkler, Bell, & Zlokovic, 2010). Much of what we currently know about pericyte morphology was achieved through electron microscopy. Electron microscopy has also given us our current accepted definition of a mature pericyte, which is that of
a cell that is embedded within the vascular basement membrane (Sims, 1986). Pericytes also possess a cell body with a prominent nucleus, and several branching processes (Bergers & Song, 2005). Pericytes extend processes that run along the surface of the endothelial cells, some of which can span several cells. Secondary processes, which branch off primary processes, also extend to encircle the endothelial cells (Armulik et al., 2011). As mentioned previously, pericytes surround endothelial cells, but how this occurred was unknown. We now know that adhesion plaques allow pericytes to attach to endothelial cells, while peg-and-socket contacts allow pericytes to penetrate spaces in the endothelial cells and achieve a tighter connection between the two cell types (Rucker, Wynder, & Thomas, 2000).

The location of pericytes can vary depending on vessel type. Pericytes irregularly cover veins and capillaries, whereas arteries are covered with thick layers of smooth muscle cells (Cleaver & Melton, 2003). Pericytes have been shown to express smooth-muscle actin, which adds to the confusion on how to label cells that are in close proximity to endothelial cells. Current mice models suggest during development, cells from the epicardium undergo epithelial-to-mesenchymal transition (EMT) and migrate deep into the myocardium to form epicardium derived cells (EPDC’s), such as fibroblasts, pericytes, and smooth muscle cells (Mellgren et al., 2008; Smith, Baek, Sung, & Tallquist, 2011). Since pericytes share many characteristics between smooth muscle cells and possibly share the same lineage, this had led many people to refer to pericytes as smooth
muscle cells, which will explain the interchangeable use in the literature (Bergers & Song, 2005).

**Pericyte Function**

Pericytes play a major role in coronary vessel development, and have a wide array of functions. Some known roles for pericytes include regulation of the vascular diameter, through the act of vasoconstriction or vasodilation (Bergers & Song, 2005; Rucker et al., 2000). Pericytes are able to carry out this function, due to the fact that they express smooth muscle-like properties. Pericytes also play a role in vessel stability and integrity. Mice severely deficient in pericytes exhibit vessel hyperdilation and edema formation, both of which contribute to embryonic lethality (Lindahl et al., 1997). Part of this response, has to do with disruption of a key signaling mechanisms for pericytes, which is Pdgfb/Pdgfrβ signaling. During vessel development, endothelial cells will secrete factors such as PDGF-B, which then recruits pericytes to the newly formed vessels (Betsholtz, 2004). Once the pericytes attach to the endothelial cells, they can influence endothelial differentiation and endothelial cell mitotic rate, by arresting endothelial cell growth (Gerhardt & Betsholtz, 2003; K. K. Hirschi, Rohovsky, & D'Amore, 1998; Sims, 2000). Researchers have further determined that pericytes function to recruit endothelial cells to developing and regenerating vasculature (Armulik et al., 2005; Gaengel, Genove, Armulik, & Betsholtz, 2009). Recent work suggests that pericytes in the human heart express pdgfrβ and mesenchymal stem cell markers. These pericytes are able to form capillary
networks in culture and more extensive vascular networks when co-cultured with endothelial cells (C. W. Chen et al., 2013). In contrast to pericytes sampled from skeletal muscle, human heart pericytes increase the angiogenic response during hypoxic conditions (C. W. Chen et al., 2013). This data suggests that pericyte location can influence pericyte function.

A recent mouse study suggested that pericytes can also act as progenitors for coronary artery smooth muscle cells (Volz et al., 2015). Using Tbx18-Cre lineage tracing and the MADM reporter system, it was revealed that pericytes near capillaries are clonally related to coronary artery smooth muscle cells (caSMC’s), but retain the function of pericytes (Cai et al., 2008; Volz et al., 2015; Zong, Espinosa, Su, Muzumdar, & Luo, 2005). Using the NG2-Cre and Notch3-Cre lines, pericytes were labeled at a time point where caSMC’s were absent (Fre et al., 2011; Zhu et al., 2011). At later time points where caSMC’s should be present, they observed caSMC’s were labeled. These two lineage tracing experiments strongly suggest that pericyte bridge the gap from epicardium to smooth muscle cells, by acting as the intermediate progenitor to caSMC’s (Volz et al., 2015). The transition from pericytes to caSMC’s required Notch3 signaling, and in Notch3 null mice, pericytes fail to differentiate into caSMC’s (Volz et al., 2015).

**PDGF Signaling**

Platelet-derived growth factor (PDGF) was first identified in platelets as a mitogenic factor for smooth muscle cells, fibroblasts, and glial cells. Subsequent
studies have shown this factor to play a role in responses such as survival, migration, and remodeling factors (Bergers & Song, 2005; Betsholtz, Karlsson, & Lindahl, 2001; Hoch & Soriano, 2003; Ross, Glomset, Kariya, & Harker, 1974). In mice and humans, PDGF’s consist of four ligands (PDGF A-D), and two receptors (Pdgfra and Pdgfrβ) (Hoch & Soriano, 2003). PDGFRs are receptor tyrosine kinases that dimerize upon ligand binding. After ligand binding the tyrosine kinase domains activate, which then autophosphorylate several tyrosine residues in the receptor cytoplasmic domains (Hoch & Soriano, 2003). The four PDGF ligands can bind to either of the two receptors, but PDGF-B preferentially binds to Pdgfrβ (Bergers & Song, 2005). Our lab has established that zebrafish Pdgf-A and Pdgf-B are evolutionary distinct, while phylogenetic studies revealed that Pdgf and Pdgfrβ proteins were closely related to respective vertebrate homologs (Wiens et al., 2010). During mammalian angiogenesis, PDGF-B is expressed by sprouting endothelial cells, while Pdgfrβ is expressed by pericytes (Enge et al., 2002; Hellstrom, Kalen, Lindahl, Abramsson, & Betsholtz, 1999; Karen K. Hirschi, Rohovsky, Beck, Smith, & D’Amore, 1999; Lindahl et al., 1997; Lindblom et al., 2003; Wiens et al., 2010)

**PDGF/PDGFRB Function in Development and Regeneration**

Our lab has demonstrated disruption of Pdgf signaling has a significant effect on zebrafish intersegmental vessel growth (Kim et al., 2010). Chemical inhibition of Pdgfr resulted in a significant reduction of complete intersegmental vessels at 48 hpf, compared to controls. Using a heat-shock inducible dominant
negative Pdgfrβ transgenic zebrafish line, our lab observed defective intersegmental vessel formation after heat shock (Wiens et al., 2010). These results imply PGDF signaling is crucial in vessel development in vivo. A key step in the current model of pericyte formation in the heart involves epithelial-to-mesenchymal transition (EMT) to form epicardial derived cells, some of which become pericytes. In zebrafish heart explant cultures, treatment of epicardial cells with PDGF-BB induced the epicardial cells to adopt mesenchymal-like morphology and reorganization of subcortical actin into stress fibers (Kim et al., 2010). These are two important hallmarks of EMT in mice models. PDGF signaling also plays a role in epicardial cell migration. Mice epicardial knockouts of PDGFR exhibited a loss of EPDC’s in the underlying myocardium. Mesenchymal marker vimentin and undifferentiated EPDC marker WT1 were severely reduced in the epicardial knockouts in the myocardium (Smith et al., 2011). To support this, other studies have shown that although vimentin is severely reduced in the myocardium, it is still expressed in the epicardium at E15.5. This suggests that epicardial cells might initiate EMT, but subsequent migration is impaired leading to reduced or absent mural cell populations (Mellgren et al., 2008).

In addition, PDGF signaling also impacts epicardial proliferation in vivo during regeneration. Treatment of regenerating zebrafish with PDGFR inhibitor resulted in only 14% of epicardial cells proliferating compared to 45% in DMSO controls (Kim et al., 2010). These results demonstrate the importance of PDGF
signaling in epicardial proliferation. Our lab has also demonstrated the effect of disruption of PDGF signaling during heart regeneration. During heart regeneration, a key step is the revascularization of the injured area. Our lab has demonstrated, that disruption of PDGF signaling results in impaired regeneration after injury. Treatment with PDGFR inhibitor resulted in lack of blood vessels in the regenerating myocardium, compared to DMSO controls which contained blood vessels in the regenerating area (Kim et al., 2010). Mice studies also revealed impaired coronary artery development in Pdgfrβ mutants. At E14.5, Pdgfrβ mutant mice possessed abnormal clusters of endothelial cells and no dominant vessels formed on the ventral surface. Several studies have established the importance of PDGF-B/PDGFRB signaling during vascular development. Pdgfrβ knockout mice die perinatally and exhibit extensive hemorrhaging, and consistent with other studies, vessels lacked or sparsely covered by pericytes (Leveen et al., 1994; Soriano, 1994). Also, endothelial sprouts are unstable and vulnerable to degradation. (Enge et al., 2002; Hellström et al., 2001; Lindahl et al., 1997).

**Pericytes During Zebrafish Cardiac Development**

Much of what we know from pericyte and coronary vessel development comes from murine studies. Whether these findings translate to zebrafish remains unclear. Until recently, the origin of zebrafish coronary vessels was unknown. In addition, it was not until 2016, that a reliable marker for zebrafish pericytes was discovered, although the group did not address if this marker was
could be utilized during zebrafish cardiac development. My interest was to
determine if Pdgfrβ can be used as a reliable marker for pericytes during cardiac
development. In addition, if Pdgfrβ labels pericytes, where do these pericytes
originate from? Another aspect that I wanted to look into was the effect on
coronary vasculature in the absence of Pdgfrβ, by utilizing a previously published
$pdgfr\beta$ mutant line. Lastly, could a dominant negative form of Pdgfrβ ($dnpdgfr\beta$)
be induced in adult zebrafish, to temporally control Pdgfrβ signaling? Confocal
imaging should allow us to visualize the pericytes during development and by
taking advantage of the Cre-Lox system, we should be able to do lineage tracing
studies to determine the origin of cardiac pericytes. Previous research has shown
that $dnpdgfr\beta$ induction can be achieved in embryos, so with some possible
modification it should be possible to achieve induction in adults.
CHAPTER TWO
MATERIALS AND METHODS

Fish Strain, Care and Treatment

Zebrafish Strains

All fish care, maintenance and experimental procedures have been approved by CHLA IACUC animal care protocols.

$\text{Tg}(\text{fli1a}:\text{EGFP})^{Y1}$, $\text{Tg}(\text{fli1a.ep}:\text{DsRedEX})^{\text{um}13}$ also known as $\text{fli1a}:\text{DsRed}$, $\text{TgBAC(cryaa:EGFP,tf21:Cre-ERT2)}^{\text{pd}42}$ also known as $\text{tcf21}:\text{CreERT2}$, $\text{TgBAC(tf21:DsRed2)}^{\text{pd}37}$, $\text{Tg(cxcl12b:YFP)}$, $\text{TgBAC(pdgfr}^b\text{:EGFP)}$, $\text{TgBAC(pdgfr}^b\text{:mCitrine)}$, $\text{Tg(-3.5ubi:loxP-EGFP-loxP-mCherry)}$ also known as $\text{ubi:Switch}$, $\text{Tg(gata5:loxP-mCherry-STOP-loxP-nucEGFP)}^{\text{pd}40}$ also known as $\text{gata5:RnG}$, $\text{pdgfr}^b_{\text{um}148}$ (mutant strain), $\text{cxcr4a}_{\text{um}20}$ (mutant strain), $\text{Tg(-6.5kdrl:mCherry)}^{\text{ci}5}$, $\text{hsp70:Gal4}$, $\text{UAS:dpdgfr}^b\text{-yfp}$

4-Hydroxytamoxifen (4-OHT) Labeling

Embryos were treated daily with 4-hydroxytamoxifen (Sigma) from 0-5 days post fertilization (dpf) in E3 media with a concentration of 10µM, from a stock solution of 10mM. Residual 4-OHT was rinsed off the embryos with system water before being placed back on the system.

Heat Shock

Zebrafish were placed in 1L tanks containing 250ml water and heat shocked at 38°C for 45 minutes, followed by a 15-minute recovery at room
temperature. Following this recovery, the zebrafish were placed back on the system to allow for recovery.

Histology

Paraffin Embedding

Tissue was collected from terminally anesthetized fish and fix overnight in 4% paraformaldehyde (PFA) at 4°C. For subsequent steps, the samples were placed on a rocker. DEPC-PBS incubation for 15 minutes, followed by: 30% EtOH for 15 minutes, 50% EtOH for 15 minutes, and 70% EtOH for 15 minutes. This is followed by 95% EtOH twice for 60 minutes each time, 100% EtOH twice for 60 minutes each time, and 100% toluene twice for 60 minutes each time. For the subsequent steps, the samples were handled in the core embedding station. Samples were placed in 1:1 mix of 100% toluene and 100% paraffin overnight at 60-62°C. Samples were then incubated in 100% paraffin twice for 60 minutes each time. To embed each heart, the heart was placed at the bottom of a steel embedding tray containing liquid paraffin. The paraffin block was allowed to solidify on ice overnight. Paraffin blocks were stored at 4°C

Paraffin Sections

Heart tissue paraffin blocks were sectioned at a 7µm thickness using a Leica RM2235 rotary microtome. Sections were placed in DEPC-treated water and mounted on glass slides, and dried overnight at 37°C and stored at room temperature in a slide box
**In Situ Hybridization**

This 3-day procedure was performed on zebrafish heart section slides.

Day 1: 100% toluene for 5 minutes, 100% toluene for 10 minutes, 100% EtOH for 5 minutes, 80% EtOH for 10 minutes, DEPC-PBST for 5 minutes, 3% H$_2$O$_2$ in methanol for 10 minutes, DEPC-PBST for 5 minutes, proteinase K in DEPC-PBST for 10 minutes, DEPC-PBST for 5 minutes, 4% DEPC-PBS PFA with 0.2% glutaraldehyde for 20 minutes, and DEPC-PBST for 5 minutes. Following this, a hydrophobic ink pen was used to draw a circle around the tissue. Incubate slides in pre-warmed hybridization buffer for 1 hour at 68°C. After incubation, mix probe with new hybridization buffer (2 µl for every 500 ml hybridization buffer) and add 250 ml of this mix to the slides and place a coverslip on each slide. Slides were incubated overnight at 70°C.

Day 2: Remove coverslip and rinse in washing solution for 15 minutes at 65°C, 30 minutes at 65°C, and 30 minutes at 65°C. Rinse three times in TBSTL for five minutes each time. Next, incubate slides for 60 minutes in the embryo blocking solution. Dilute anti-DIG antibody 1:1000 in antibody blocking solution. Mix the diluted anti-DIG antibody 1:1 in TBSTL containing 1% FBS. Incubate the slides for 1 hour in this mixed and diluted anti-DIG antibody solution at room temperature. For the color reaction: wash slides in TBSTL for five minutes three times, NMTL for five minutes twice, and incubate slides in pre-warmed staining solution at 37°C overnight in the dark.
Day 3: To stop the color reaction, rinse the slides in tap water for 15 seconds. Incubate in nuclear fast red counterstain for five minutes, followed by a rinse in water until red color disappears from slides. Incubate slides in tap water for five minutes. Slides were then incubated in 95% EtOH two times for 1 minute each time, and 100% EtOH two times for 1 minute each time. Incubate in Citrisolv two times for 1 minute each time. Finally, add 2-3 drops of Cytoseal and place coverslip on slide. Slides can be stored at room temperature.

Imaging

Whole Mount Confocal Imaging of Heart Tissue
Zebrafish were anesthetized in tricane for 2 minutes. Hearts were removed from terminally anesthetized fish and rinsed for 1 minute in PBS, followed by 1 minute in 4% PFA. Hearts were mounted in 1% low melting point agarose on a glass-bottom dish. Z-stacks and maximum intensity projections were acquired using Zeiss LSM710 and LSM700 confocal microscopes.

Live Imaging
Hearts were collected from terminally anesthetized pdgfrβ:EGFP;kdrl:mCherry zebrafish. Hearts were then placed in 5ml of Ringers solution containing 100µl/ml Primocin and 150U/ml heparin. The steps above can be done outside of a culture hood, but the following steps need to be done inside a culture hood. L-15 media (supplemented with 10% FCS, 100µg/ml Primocin, 1.25mM CaCl2, and 800mg/l glucose) was added to a glass-bottom dish and
hearts were placed at the bottom of the dish towards the middle of the dish. Pre-
cut filter paper was placed on top of the hearts to prevent movement. Z-stack
images were obtained over the course of 2 days, using a Leica DM IRE2
microscope. The images were used to form a time-lapse movie.

Genotyping

CreERT2 genotyping

Portion of the caudal fin was amputated and placed in a PCR tube
containing 50µl of a 50mM NaOH solution. After amputation, fish were kept in
separate tanks to avoid intermixing. Extraction of DNA was carried out by a
thermal cycler (Applied Biosystems verity 96 well) by heating sample for 20
minutes at 95°C. Master mix preparation for 1 sample is as follows, multiplied by
the number of samples needed: 5µl 5X buffer, 2µl of 25mM MgCl₂, .5µl 10mM
dNTP’s, .5µl of 20µm Cre S3 forward primer, .5µl of 20µm Cre AS3 reverse
primer, .2µl GoTaq flexi DNA polymerase (Promega), and 13.3µl distilled water.
For the PCR reaction each PCR tube contained 23µl of master mix and 2µl DNA.
PCR program is as follows: 95°C for 5 minutes, 95°C for 30 seconds, 55°C
(annealing temperature) for 1 minute, 72°C for 1 minute, and 72°C for 7 minutes.
Reactions were run out on a 1% TAE agarose gel.

Pdgfrβ +/+,-/- Genotyping

Pdgfrβ +/+,-/- genotyping protocol the same as the CreERT2
genotyping protocol, except for the following changes:
• Master mix preparation for 1 sample is as follows, multiplied by the number of samples needed:
  o 5µl 5X buffer
  o 2µl of 25mM MgCl₂
  o .5µl 10mM dNTP’s
  o .1µl of 100µm pdgfrβ tetra WT FWD primer
  o .1µl of 100µm pdgfrβ mut REV primer
  o .1µl of 100µm tetra outer REV primer
  o .1µl of 100µm tetra outer FWD primer
  o .2µl GoTaq flexi DNA polymerase (Promega)
  o 14.9µl distilled water

• PCR program was based on the touchdown genotyping method and contained 13 stages.
  o Stage 1: 95°C for 1 minute
  o Stage 2: 95°C for 15 seconds followed by 75°C for 1 minute
  o Stage 3: 95°C for 15 seconds followed by 74°C for 1 minute
  o Stage 4: 95°C for 15 seconds followed by 73°C for 1 minute
  o Stage 5: 95°C for 15 seconds followed by 72°C for 1 minute
  o Stage 6: 95°C for 15 seconds followed by 71°C for 30 seconds and 72°C for 30 seconds
Stage 7: 95°C for 15 seconds followed by 70°C for 30 seconds and 72°C for 30 seconds

Stage 8: 95°C for 15 seconds followed by 69°C for 30 seconds and 72°C for 30 seconds

Stage 9: 95°C for 15 seconds followed by 68°C for 30 seconds and 72°C for 30 seconds

Stage 10: 95°C for 15 seconds followed by 67°C for 30 seconds and 72°C for 30 seconds

Stage 11: 95°C for 15 seconds followed by 66°C for 30 seconds and 72°C for 30 seconds

Stage 12: 95°C for 15 seconds followed by 65°C for 30 seconds and 72°C for 30 seconds

Stage 13: 72°C for 5 minutes. Reactions were run out on a 2% TAE agarose gel.

**Cxcr4a +/+,+-,-/- Genotyping**

**Cxcr4a +/+,+-,-/-** genotyping protocol the same as the CreERT2 genotyping protocol, except for the following changes: for the master mix preparation, replace Cre forward and reverse primers with Cxcr4a mFWD and Cxcr4a mREV primers. Also reactions were run overnight on a 3% TBE gel, to allow for optimal band separation.
**Pdgfrβ;mCitrine Genotyping**

mCitrine genotyping protocol the same as the CreERT2 genotyping protocol, except for the following changes: for the master mix, replace forward and reverse Cre primers with pdgfrβ genotyping FWD and mC/mT/EG FP 5' REV primers. The PCR program is: 95°C for 1 minute, 95°C for 15 seconds, 51°C (annealing temperature) for 15 seconds, 72°C for 15 seconds, and 72°C for 7 minutes.

**Gal4 Genotyping**

Gal4 genotyping protocol the same as the CreERT2 genotyping protocol, except for the following changes: for the master mix, replace forward and reverse Cre primers with Hsp:gal4 FWD and HSP:gal4 REV. The PCR program is the same as CreERT2 genotyping.
Table 1. Primer Sequences Used for PCR Genotyping

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>pdgfrβ genotyping FWD</td>
<td>TGT TTC CTT TGG CTT TGA GG</td>
</tr>
<tr>
<td>mC/mT/EG FP 5’ REV</td>
<td>GAA CTT CAG GGT CAG CTT GC</td>
</tr>
<tr>
<td>pdgfrβ tetra mut REV</td>
<td>GGA GGA CGA GTT GAT GGA CAG GAT CGT</td>
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<tr>
<td>pdgfrβ tetra WT FWD</td>
<td>GAG CTC AGT CCC AGC GCT CCA CAG AT</td>
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<tr>
<td>pdgfrβ tetra outer FWD</td>
<td>ATA AGT GCA GCT GGA AGG GCG TCC TCT G</td>
</tr>
<tr>
<td>pdgfrβ tetra outer REV</td>
<td>CAC ATA AAC CCC GGT GTG TCT CCA GGT C</td>
</tr>
<tr>
<td>cxcr4a mFWD</td>
<td>CCA ACT TTG AGG TCC CGT GTG</td>
</tr>
<tr>
<td>cxcr4a mREV</td>
<td>TCC AAA CTG ATG AAG GCG AGG</td>
</tr>
<tr>
<td>Cre S3</td>
<td>CGT TTT CTG AGC ATA CCT GGA</td>
</tr>
<tr>
<td>Cre AS3</td>
<td>ATT CTC CCA CCG TCA GTA CG</td>
</tr>
<tr>
<td>HSP: gal4 FWD</td>
<td>CGC TAC TCT CCC AAA ACC AAA AGG</td>
</tr>
<tr>
<td>HSP: gal4 REV</td>
<td>TCT CTT CCG ATG ATG ATG TCG CAC</td>
</tr>
</tbody>
</table>

Stereotyping

Tcf21:DsRed Stereotyping

All stereotyping was done using a fluorescent stereoscope. At 4 dpf, red fluorescence can be observed as a layer surrounding the ventricle.

Tcf21:Cre Stereotyping

At 3-4 dpf, GFP fluorescence can be observed in the lens.
**Pdgfrb:mCitrine Stereotyping**

At 5-6 dpf, YFP expression can be found in the jaw region and aortic arches. Expression levels can be variable from strong to weak.

**Fli1a:EGFP Stereotyping**

Beginning at 1 dpf, strong GFP expression can be visualized in the vessels. Intersegmental vessels running down the trunk are clearly marked. Vessel can be seen in head region as well.

**Gata5:RnG Stereotyping**

At 3-4 dpf, strong RFP expression can be observed throughout the entire heart.

**Ubi:Switch Stereotyping**

Beginning at 1 dpf, the complete embryo strongly expresses GFP.

**Pdgfrb:EGFP Stereotyping**

From 3-6 dpf, strong GFP expression can be observed in the jaw region, aortic arches, and intersegmental vessels.

**Cxcl12b:YFP Stereotyping**

Observable only at early 1 dpf. YFP expression can be observed at the tip of the tail, but expression is faint.

**Fli1a:DsRed Stereotyping**

At 4-6 dpf, weak diffused RFP expression can be observed near the aortic arches.
UAS:dnpdgrfbyfp Stereotyping

at 4-6 dpf, strong RFP expression can be observed throughout the whole heart.

Statistical Analysis

Data was reported as mean ± SEM. Graphs were generated using Graphpad Prism software. P values < 0.05 were significant.
CHAPTER THREE

RESULTS

Pdgfrβ Labels Pericytes During Zebrafish Coronary Development

Platelet-derived growth factor (PDGF)-B/PDGF receptor β (PDGFRβ) signaling has been shown to be essential in the development of mural cells such as vascular smooth muscle cells (vSMC’s) and pericytes, which line and stabilize nascent vascular endothelial sprouts (Lindahl 1997; Hellstrom 2001; Leveen 1994; Karlsson 2000). In addition to the importance of PDGF-B/PDGFR β signaling, previous work has also identified Pdgfrβ as a reliable marker for mural cells (Lindahl 1997; Winkler 2010). Although previous studies have shown the origin and fate of pericytes in zebrafish cranial and intersegmental vessel development, these studies did not address the development of pericytes in coronary vasculature development (Mochizuki, 2016) (Wiens et al., 2010). To investigate the role of pericytes in zebrafish coronary vasculature, I crossed Tg(flia1:DsRed), a transgenic line which labels endothelial cells, with Tg(pdgfrβ:mCitrine), which has been previously characterized to label cranial vessel pericytes (Vanhollebeke et al 2015). I observed pericyte development from early embryonic stages through late-juvenile stages. At the earliest time point of observation (Figure 1A), pdgfrβ expression is weakly expressed near the atrio-ventricular (AV) junction (Figure 1A’), which is a region where endoderm-derived endothelial cells originate during zebrafish coronary angiogenesis.
(Harrison et al., 2015). $\text{pdgfr}\beta$ expression at this time point exhibits no obvious cellular morphology, but is diffused and non-specific. At 31 dpf, $\text{pdgfr}\beta$ is strongly expressed at the AV junction (Figure 1B, insert), but expression can also be found at the base of the ventricle and the bulbus arteriosus. As the vasculature develops and endothelial cells migrate to cover the ventricle, $\text{pdgfr}\beta$ expression exhibits a close and extensive association to the developing vessels (Figure 1C). $\text{pdgfr}\beta$ expression starts to adopt a cellular morphology (Figure 1C’, arrow), unlike earlier time points. During late-juvenile development, extensive ventricular vessel coverage was observed, and $\text{pdgfr}\beta^+$ pericytes are closely associated with the vessels (Figure 1D’, arrow). Interestingly, $\text{pdgfr}\beta$ expression during late-juvenile stage is localized to specific cellular structures on the vessels, and no obvious localization of $\text{pdgfr}\beta$ expression can be observed in the atrio-ventricular junction. Previous research has noted that pericytes extend processes that can span several endothelial cells and even bridge capillaries that are in close proximity. Consistent with this, we observed pericytes that are near vessel branch points and extend processes that seem to connect neighboring vessels.

**Tcsf21, and Pdgfrβ Co-express During Early Coronary Vessel Development**

During cardiac development in mice, it has been show that the epicardium can undergo EMT and contribute to the pericyte population. Additionally, Pdgfrβ has been shown to have a close association to the epicardium, but this had not been examined in zebrafish previously. To address the epicardial-$\text{pdgfr}\beta$
relationship early in zebrafish coronary development, I utilized the following transgenic zebrafish lines which demark the epicardium (tcf21:Dsred) and pericytes (Pdgfrβ:mCitrine). Consistent with earlier zebrafish findings, the epicardium surrounds the ventricle and the atrium (Figure 2A’). Full coverage was not achieved, but this can possibly be attributed to differences in expression between different transgenic lines. Interestingly, pdgfrβ expression seemed to closely track the epicardial expression, except for the B.A (Figure 2A’’). Both transgenes seem to, at least partially, cover the ventricle and the atrium. On the surface of the ventricle and the AV region we can see epicardial cells, which also express the pericyte marker Pdgfrβ. This seems to support earlier mice findings that the epicardium can express pdgfrβ during development. My findings seem to suggest that early in coronary vessel development, the epicardium expresses pdgfrβ. One thing I took into consideration is the possible color bleed which could affect our results. When comparing the RFP and GFP channel, we can see that the bright green expression in the B.A is not mirrored in the B.A of the RFP channel. This suggests that our channels are not bleeding into each other and validates our imaging. These findings, that the epicardium expresses pdgfrβ early in cardiac development (30 dpf), give me a good foundation to explore the epicardial contribution to the pericyte population observed at coronary vessel maturity. Lineage tracing will enable us to better answer these questions in our future studies.
Recently, our lab has demonstrated the origin of coronary vessels in zebrafish development (Harrison et al, 2015). In contrast to mice, where coronary vessels originate from the sinus venosus (Red-Horse et al., 2010), zebrafish endothelial cells migrate to the surface from the endocardium near the AV canal. This process is directed by Cxcr4a-Cxcl12b signaling, which has been shown to have a role in cell migration during development (Boldajipour et al, 2011). The angiogenic sprouts that arise from the endocardium express cxcr4a, and are then guided to their respective positions by the myocardial secreted ligand Cxcl12b. Our lab demonstrated that cxcl12b expression downregulates in cardiomyocytes after formation of the vasculature, but is upregulated in perivascular cells that line the coronary arteries (Harrison et al, 2015). Unfortunately, we did not have the tools at the time to definitively identify these cells as pericytes. To aid in my studies, we used a zebrafish line, Tg(pdgfrβ:EGFP), that labels pericytes to determine if the cxcl12+ perivascular cells are indeed pericytes (Mochizuki, 2016). I found that cxcl12b-expressing cell coverage was not uniform, but a consistent pattern emerged. Along some of the larger coronary vessels, cxcl12b-expressing cells are in close association with the coronary vasculature (Figure 3A-B, arrows). Co-localization of cxcl12b and pdgfrβ is mostly seen along the major vessels, with very few cells outside of the major vessels. In addition, some hearts expressed cxcl12b in the myocardium (Figure 3B, asterisk). Although, cxcl12b downregulates in cardiomyocytes after the vasculature forms, it would
not be surprising to observe some expression remaining in the myocardium, due to the variable nature of coronary vessel formation. Using the line that labels pericytes, Tg(pdgfrβ:EGFP), I was able to determine that the cxcl12b expressing cells previously observed, are indeed pericytes. I was not able to observe complete overlap of the two markers, which could indicate that not all cxcl12b expressing cells are pericytes, instead probably a subset.

**Lineage Tracing Reveals Epicardial Contribution to the Pericyte Population**

Although I established that pericytes reside in close proximity to mature coronary vessels, the origin of these pericytes remained unclear. Evidence from mouse studies suggests that pericytes are derived from the epicardium, through a process of endothelial-to-mesenchymal transition (EMT). I was interested in seeing if this was the case for coronary vessel pericytes as well. For our lineage tracing experiment, I crossed our pdgfrβ:mCitrine line to the tcf21:cre;ubi:switch line. Tcf21:Cre is expressed by the epicardium, and ubi:switch is a line that near-ubiquitously labels all cell types in the zebrafish. Together this combination should allow tracking of the descendants of the epicardium though development. To ensure maximum labeling efficiency, I treated our embryos from 0-5 dpf with 10uM 4-OHT (4-Hydroxytamoxifen). My results reveal that some of the pdgfrβ:mCitrine expressing pericytes co-localize with mcherry (Figure 4B, arrows), which indicates the epicardium contributes to cardiac pericytes. Interestingly, I also observe pdgfrβ:mCitrine expressing pericytes that do not co-
localize with mcherry (Figure 4B, asterisk). One explanation for this is that 4-OHT labeling efficiency was not 100 percent, and it is possible that these pericytes are derived from the epicardium, but not labeled due to this lack of 100 percent labeling. Another possibility is that these pericytes are not derived from the epicardium at all, but rather another cell population, as some recent findings have suggested. In addition, we observe mcherry positive cells that do not overlap with pdgfrβ:mCitrine (Figure 4B, arrowhead). It is possible that these descendants of the epicardium are not pericytes, but another cell type such as fibroblasts or vascular smooth muscle cells, which would explain why they do not express pdgfrβ. To support this, mice studies have shown that in addition to pericytes, the epicardium can give rise to fibroblasts or vascular smooth muscle cells, which could explain our findings. To see if our results were due to 4-OHT recombination and not due to “leaky” Cre activity, we looked at our non 4-OHT controls. Our controls exhibited no mcherry expression (Figure 4A’”), which means that recombination did not occur in the absence of 4-OHT.

**Coronary Vessel Defects Present in Pdgfrβ Mutants**

Pericytes are crucial to vessel stability, and studies have shown that vessels are vulnerable to degradation in the absence of pericytes, and hemorrhaging often results. To investigate any coronary vessel defects in the absence of pdgfrβ, I used the pdgfrβ mutant line with the endothelial cell reporter fli1a:EGFP, to track vessel growth. Previous studies in mice have shown that
endothelial cells, especially tip cells, secrete Pdgf-B, which then acts as a paracrine signal that recruits pdgfrβ+ pericytes to the vessels. This pdgfrβ mutant line has been shown to exhibit extensive hemorrhaging in the head region, but no effects were described in heterozygotes. At our early time points (98 dpf), heterozygote and mutant hearts displayed similar vessel coverage (Figure 5 A-B'). Inserts for both groups reveal vessels that have a typical appearance of immature vessels (Figure 5 A-B, inserts). What this could suggest is that early in development, mutants can form vessels in the absence of pdgfrβ, similar to that of the heterozygotes. I next asked how vessel development at a later time point, where a vessel network should be established, would be affected between the two groups? Although it was reported that no effects are exhibited in the heterozygotes, coronary vessel development was not addressed. Is it possible that heterozygotes and mutants still exhibit abnormal vessel phenotypes at later stages, if any? At our last time point (167 dpf), heterozygotes exhibit robust coronary vessel coverage compared to sparse coverage of the mutants (Figure 5 C-D). Vessels on the heterozygote hearts have a normal tube-like structure and sprouts interconnect (Figure 5C, insert), whereas the mutant endothelial sprouts are very thin in appearance and do not interconnect (Figure 5D, insert). Although mutant hearts do not form a coronary vasculature network, I did observe a number of endothelial cell "islands" that do not form interconnections (Figure 5D'). To compare coverage between the two groups at different time points, I quantified vessel coverage. When comparing vessel coverage between the
heterozygotes and mutants at 98 dpf, I concluded that there was no significant difference between them (Figure 5E). When comparing the two groups at 167 dpf, I found that there was a very significant difference in vessel coverage (Figure 5F). In mice, studies have shown that disruption in Pdgfr/pdgfrβ signaling leads to unstable vessels and increased susceptibility to degeneration. To support my findings, previous work has revealed significant reduction in pericytes leads to functionally and structurally abnormal vessels. These findings, along with my results, suggest the following as a possible mechanism: pdgfrβ mutants might form some sort of vessel network early in development, but pdgf/pdgfrβ disruption between endothelial cells and pericytes, leads to reduced pericyte coverage. If the vessels lack pericycle coverage, they can degenerate and lose the interconnections, which could explain the “islands” we observed.

Characterization of Dnpdgfrβ Transgenic Zebrafish Line

To further analyze the question, what happens to the pericycle population after Pdgfrβ signaling disruption in development and regeneration, I characterized a heat-shock inducible dominant negative Pdgfrβ (dnpdgfrβ) adult transgenic zebrafish line. This line, Tg(UAS:dnpdgfrβ-yfp;hsp70:Gal4;fli1a:EGFP), replaces the intracellular tyrosine kinase domain of pdgfrβ, with YFP. This inactive fusion protein binds to endogenous Pdgfrβ and prevents auto-phosphorylation (Wiens et al., 2010). The advantages to using this dnpdgfrβ line is that it gives us the ability to temporally control disruption of Pdgfrβ signaling as opposed to pdgfrβ mutants, which could display cumulative
developmental defects. Previously, our lab has demonstrated that after 20 minutes in 40°C water, strong YFP expression could be observed in embryos, and disruption of pdgfrβ signaling resulted in impaired intersegmental vessel growth. Since my interest is with the pdgfrβ+ expressing pericytes, I crossed Tg(UAS:dnpdgfrβ-yfp;hsp70:Gal4) to Tg(Pdgfrβ:EGFP), which labels pericytes (Mochizuki, 2016). This will allow us to observe the effects of Pdgfrβ signaling disruption on the pericyte population. Although our lab utilized this line to analyze intersegmental defects in embryos, this line has not been characterized in adult zebrafish hearts. To analyze induction in adult hearts, I separated our Tg(UAS:dnpdgfrβ-yfp;hsp70:Gal4;Pdgfrβ:EGFP) zebrafish line into 2 groups, non-heat shock and heat shock, which was subjected to 38°C for an hour, for 14 days. Confocal imaging revealed that the non-heat shocked induced fish displayed pericytes but no YFP expression could be observed in the ventricle (Figure 6 A-A”). When looking at the heat shocked induced group, I observed pericyte coverage similar to the non-heat shocked group (Figure 6B’). Interestingly, I was able to observe strong YFP induction in the heat-shocked group, as opposed to the non-heat shocked group (Figure 6 B-B”). This is the first time that dnpdgfrβ-YFP induction has been shown in adults using this line. This transgenic line will be useful as an alternative to the Pdgfrβ mutant line, as it will allow us to temporally control Pdgfrβ expression.
Conclusion

A crucial aspect of zebrafish heart regeneration is the invasion of blood vessels into the damaged area, but the role of pericytes during this process remains unclear. By understanding the role of Pdgfrβ during development, we will better understand the role of pericytes during development. My findings have shown that Pdgfrβ originates at the AV region of the ventricle and is expressed in pericytes near mature vessels. I also demonstrated a strong association of Pdgfrβ with Tcf21, which suggests a relationship between Pdgfrβ and the epicardium. With the aid of a Pdgfrβ reporter line, I was also able to confirm that previously described cxcl12b expressing perivascular cells are a subset population of pericytes. Lineage tracing experiments revealed the potential origin of the pdgfrβ expressing population. My findings revealed that the epicardium contributes to the pericyte population at maturity. Further lineage tracing studies will help to determine the full extent of the epicardium contribution to populations other than pericytes, as shown by my results. Using mutant zebrafish deficient in Pdgfrβ, I observed that at late stages of development (167 dpf), mutants fail to form a proper coronary vasculature network, compared to heterozygote controls. The addition of a Pdgfrβ transgenic reporter to the mutant line will help to elucidate what occurs to the pericyte population in the pdgfrβ mutant background. Lastly to aid our future regeneration studies, I characterized dnpdgfrβ, via heat shock induction in adult zebrafish. This line will allow us temporal control of the dnpdgfrβ domain. Pdgfrβ and the pericyte population have
been described previously in other organs in zebrafish, but not during coronary vessel development. I hope my findings will contribute to more research on this topic to help elucidate the mechanism of pericyte contribution in development and regeneration.
Figure 1. *Pdgfrβ* expression in the developing zebrafish heart. At 24 dpf (days post fertilization), no endothelial cells are present at the atrio-ventricular (AV) canal, but *pdgfrβ* expression can be observed in this region (A-A’). *Pdgfrβ* expression at this stage has no obvious cell morphology, but rather diffused in appearance. In addition, *pdgfrβ* is strongly expressed in the bulbus arteriosus at 24-31 dpf, and downregulated at later stages. At 31 dpf, *pdgfrβ* expression adopts a more tube like appearance along the AV canal (B-B’), but endothelial cells are still not present on the ventricle. At 43 dpf, endothelial cells were observed on the surface of the myocardium forming coronary vessel sprouts. At this stage *pdgfrβ* expression adopts a cell-like shape and exhibits close association with the developing vessels (C-C’, arrow). At my last time point (74 dpf), the endothelial cells have formed interconnections to form a vast vasculature network, while *pdgfrβ* expressing pericytes strongly associate with the vessel walls (D-D’, arrow). (V=ventricle, A=atrium, B.A= Bulbus Arteriosus). (n=5 for each time point)
Figure 2. Overlapping expression of Tcf21 and Pdgfrβ during early (30dpf) zebrafish coronary development. Merged image of Tcf21:DsRed and Pdgfrb:mCitrine expression (A). Epicardial expression, as shown by DsRed expression, surrounds the ventricle and the atrium, but exhibits reduced coverage at the Bulbus Arteriosus (A'). mCitrine expression closely matches DsRed expression, except the B.A where mCitrine exhibits intense expression not seen with Tcf21 (A''). (n=4)
**Figure 3. Cxcl12b positive cells co-express perivascular marker pdgfrβ.**

Strong cxcl12b expression is localized to the vessel walls of larger coronary vessels (A, arrow). Cxcl12b expressing cells co-localize with the pericyte marker pdgfrβ (B, arrows). In addition to being expressed by a sub-section of pericytes, Cxcl12b expression could also be found in the myocardium surrounding the coronary vessels (B, asterisk). No noticeable expression of cxcl12b was observed in the pericytes of smaller coronary vessels. (n=5)
Figure 4. Tcf21 lineage tracing reveals epicardial contribution to pericytes during coronary vessel development. Non 4-OHT group merged images, followed by split channel images (A-A'''). Pericytes could be observed in the BFP (mCitrine) channel, but no observable RFP (mcherry) recombination could be observed (A''-A''''). 4-OHT induced group merged images, followed by split channel images (B-B'''). mcherry expression could be observed in the 4-OHT treated group, indicating successful recombination (B''). Interestingly, mCitrine expression co-localizes with a subset of mcherry expression, which suggests that the epicardium, at least partially, contributes to the pericyte population (B, arrows). In addition, I also found mcherry expression that did not co-localizes with mCitrine, which could suggest that the epicardium also contributes to other populations besides pericytes (B, arrowhead). (n=5 for 4-OHT, n=4 for Non 4-OHT)
Figure 5. pdgfrβ mutants fail to form a coronary vessel network. At our earliest time point (98 dpf), pdgfrβ heterozygote nascent coronary vessels are expanding to cover the ventricle. At this time point, a complete coronary vessel network was not observed (A-A’). At this same time point, pdgfrβ mutants also contain nascent coronary vessels, although vessels appear thinner and flatter in shape, not the typical tube structure that has been observed previously (B-B’). At our last time point (167 dpf), pdgfrβ heterozygotes express a robust coronary vasculature that covers the entire ventricle. Small and larger coronary vessels can be observed, and the vessels express typical tube like structure (C-C’). At the same time point, pdgfrβ mutants lack a distinct coronary vasculature. Interestingly, isolated endothelial cells are observed on the ventricle, with no interconnection between them (D-D’). Quantification of vessel coverage at 98 dpf revealed no statistical difference between pdgfrβ heterozygotes and mutants (n=6 for heterozygotes, n=8 for mutants) (E). Quantification at 167 dpf revealed a very significant statistical difference in coronary vessel coverage (n=4 for heterozygotes, n=4 for mutants) (F).
Figure 6. Heat-Shock induction of dnpdgfrβ-YFP. Non-Heat-Shock induced fish double transgenic fish (A). GFP channel shows pericytes covering the ventricle (A'). YFP signal absent in non-heat shock induced fish (A''). Heat-Shock induced fish double transgenic fish (B). GFP channel shows pericyte coverage similar to non-heat shock induced fish (B'). Strong YFP expression can be observed in the zebrafish ventricle, compared to non-heat shocked induced fish (B''). H.S= heat shock. (n=5 for Non-H.S, n=6 for H.S)
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