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Cheng Jiao April 7, 2020

Healing Hearts: Co-dependent FGF-VEGF mediation of coronary vasculature

by

Cheng Jiao

Jinhu Wang
Adviser

Department of Biology

Jinhu Wang

Adviser

Andreas Fritz

Committee Member

Nawazish Naqvi

Committee Member

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Abstract

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Unlike most vertebrates, following a myocardial infarction, zebrafish have the remarkable ability to regenerate their hearts instead of forming permanent scar tissue. Previous studies have indicated that to support this replenishment of lost tissue, there needs to be subsequent rapid revascularization of the damaged area. Here, I show that zebrafish coronary vasculature is modulated by fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) co-dependent pathways. By observing juvenile *delta;*EGFP zebrafish, I was able to demonstrate that growth factor pathways other than VEGF have a role in vessel development and rapid coronary vascularization. Analysis of FGF and VEGF inhibitory assays indicate that both angiogenic pathways are necessary for the upkeep of the coronary vasculature during adulthood. Investigations in the past have previously shown that FGF signaling can promote VEGF enhancer activity and upregulate VEGFR expression. Likewise, my results demonstrate a temporal disparity between FGF and VEGF inhibition that is indicative of some relationality between the two pathways. When FGF was blocked, it potentially had less initial effect upon the endothelial cells. But due to FGF-VEGF crosstalk, the FGF inhibition could also downregulate VEGF expression, leading to the observed increased effect upon coronary vessels seen later on. Importantly, the combinational inhibition of VEGFR and its ligand suggests that there is a possible impedance of FGF-directed rescue of VEGF expression because *vegfa* pulldown makes the additional VEGFR receptors meaningless. Additionally, the implications of this FGF-VEGF signal transduction can directly impact heart regeneration because VEGF was reaffirmed to be an essential factor in vascular development after cardiac injury. Altogether, my findings about the interaction of angiogenic growth factor systems provide for a more holistic approach toward investigating the interplay between revascularization and subsequent cardiac regeneration.

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I. Introduction and Literature Review

A. Pathophysiology of human myocardial infarction

Throughout a person's lifespan, the human heart pumps relentlessly to provide blood for the entire body. As such a critical component of survival, the heart demands a continuous supply of oxygen and nutrients to invigorate the heart muscle cells, cardiomyocytes (CMs). The coronary vasculature is made up of a network of arteries, veins, and capillaries that meet those demands and also provides a route for immune cells and other regulatory cells to consistently maintain proper function (Kapuria et al., 2018). Thus, it is not surprising that coronary artery disease is one of the leading causes of mortality in the United States. Previous epidemiological studies estimate that every 90 secs someone dies due to myocardial infarctions (MI), also known as "heart attacks," and the costs associated with heart disease, including MI, exceed any other diagnostic group (Mozaffarian et al., 2015).

In adults, most MIs result from a thrombus forming over an atheromatous plaque (Gonzalez-Rosa et al., 2017) (Fig. 1). When the coronary vessels become occluded, the heart undergoes prolonged ischemia, leading to immediate CMs death. Following the infarction, the injury to the heart triggers the release of pro-inflammatory cytokines and the infiltration of splenic neutrophils and monocytes (Swirski et al., 2009). Percutaneous coronary intervention can open the blocked coronary artery and improved the myocardium salvage, but this effect is temporary as the oxidative stress increases CMs death. Furthermore, complications with microvascular obstruction (MVO) caused by the endothelial damage persist in up to 50% of patients after reperfusion (Niccoli et al., 2009). Over the following days, the infarct expands as inflammation drives further damage to the border zones. Subsequently, the fibroblasts and immune response activate myofibroblasts

that deposit collagen matrix and replace the necrotic muscle with noncontractile fibrotic scar tissue (Gonzalez-Rosa et al., 2017).

The irreversible loss of heart muscle cells and pathological remodeling compromises ventricular wall integrity and pump function. Due to the reduced cardiac output, the body tries to maintain blood pressure and proper circulation by triggering the release of angiotensin II and aldosterone, driving fluid retention, while also promoting the activation of vasoconstriction (Cahill et al., 2017). Ultimately, the culmination of these compensatory mechanisms is pathological: driving fluid overload, myocardial hypertrophy, continuous CMs death, and, eventually, congestive heart failure (Jessup and Brozena, 2003; Kehat and Molkentin, 2010).

Despite medical advances, heart failure currently remains incurable without a heart transplant, and standard treatment remains to be primarily palliative (Augoustides and Riha, 2009). As a result, MI patients experience adverse quality of life and often die prematurely. Patients diagnosed with heart failure carry a worse prognosis than most cancer patients, with a survival rate of only 50% in 5 years (Stewart et al., 2001). Therefore, therapies that stimulate heart regeneration have the capability to significantly reduce the morbidity and mortality for millions of people annually.

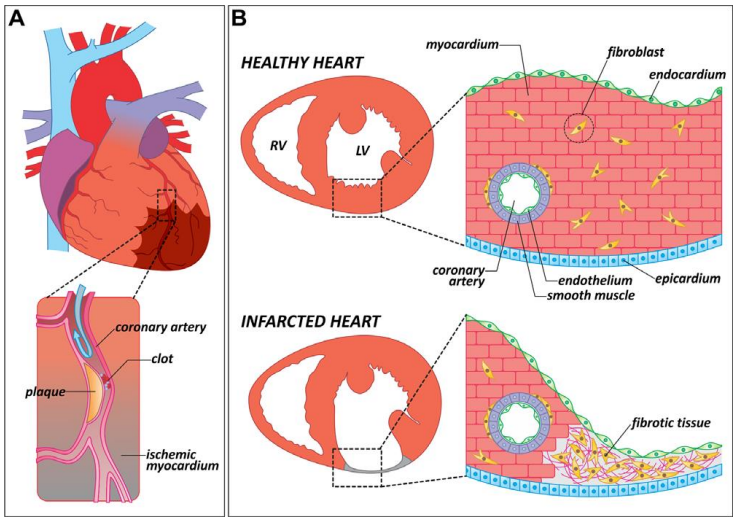

Figure 1. Cause and results of MI in humans. (A) Schematic of coronary clot preventing blood flow into region of MI (brown) leading to CM death. (B) Comparison of healthy and infarcted heart, showing CM replacement with fibrotic scar. (Gonzalez-Rosa et al., 2017)

B. Heart regeneration in vertebrates

In recent decades, most clinical trials for heart failure and acute myocardial infarction have relied on cell-based therapies, including mesenchymal stem cells, bone-marrow-derived cells, and presumed cardiac progenitor cells. Unfortunately, few of these studies have significantly improved ventricular function, promoting the search for novel approaches (Cahill et al., 2017). An area of high interest has been towards endogenous regeneration. In the past, a longstanding dogma was that mammalian heart could never regenerate and that myocardium was terminally differentiated. However, in 2011, Porrello and colleagues reported transient cardiogenesis in neonatal mice and showed nearly complete regeneration of myocardial tissue after resection of the cardiac apex or surgically induced MI (Porrello et al., 2011). Furthermore, several interesting case reports have observed children making a full functional recovery after MI or after cardiac surgery, providing evidence for some regenerative capacity in humans (Fratz et al., 2011; Haubner et al., 2016). Although this response seems to be age-dependent, recent data using isotopic tracing during DNA replication indicate that there is continuous cardiomyocyte generation and turnover throughout the

human lifespan – limited to only <4% annually in adults (Bergmann et al., 2015). Although the rate of cardiomyocyte renewal is insufficient to recover the infarcted tissue following MI, this revelation shows the potential for bolstering endogenous human regeneration to promote myocardial healing.

To date, the seminal study by Poss and Keating described the most robust cardiac regeneration in a vertebrate. Remarkably, after 20% ventricular amputation, zebrafish (*Danio rerio)* hearts quickly form a fibrin clot, but they do not undergo intense collagen deposition and permanent scarring, as seen in adult mammalian hearts. Instead, they show massive cell proliferation, replacing lost CMs and recovering normal contractile function by 60 days post-injury (Poss et al., 2002; Wang et al., 2015).

Even though zebrafish adult hearts are smaller (1-2mm^3) and simpler (two-chambers) than mammalian, they have similar histological composition to other vertebrates (Uygur and Lee, 2016). Furthermore, the extensive availability of genetic and molecular tools makes the zebrafish one of the best model systems for heart regeneration. The insights gathered from the zebrafish can further understanding of the innate mechanism necessary for organ repair.

C. Zebrafish cardiac injury response

Zebrafish studies have shown that the regenerating CMs do not originate from stem cell sources. Instead, Cre-based genetic fate mapping has shown that pre-existing CMs exhibit partial sarcomere disassembly and entrance into a de-differentiated phenotype, which is characterized by the downregulation of sarcomeric myosin and re-expression of embryonic proteins (Jopling et al., 2010; Kikuchi et al., 2010). When the zebrafish heart is initially injured, an early inflammatory response is immediately triggered. This immune activation results in the secretion of cytokines

that stimulate the Jak1/Stat3 pathway within CMs, upregulating *relaxin*3a, and stimulating proliferation (Fang et al., 2013).

In concomitant, non-muscular cells become robustly proliferative and migrate to cover the wound area, forming a "regenerative scaffold" (Gonzalez-Rosa et al., 2017). Both the endocardium and the epicardium become activated organ-wide and re-express embryonic genes. Shortly after the injury, epicardial tissue migrates directionally toward the wound, from the base of the bulbous arteriosus to the apex of the ventricle, to provide support and guidance during myocardial regeneration (Wang et al., 2015). Furthermore, a subpopulation of epicardial cells gives rise to epicardial-derived cells (EPDC), through a process of epithelial to mesenchymal transition (EMT) (Lepilina et al., 2006). Of particular importance is the *fgfb* secreted by the cardiomyocytes that induce the epicardial EMT and the mobilization of the derived cells. Inhibition of the FGF pathways results in the failure to recruit epicardial cells into the cardiac wound, leading to arrested development of new coronary vasculature and incomplete heart regeneration (Lepilina et al., 2006). In line with this understanding, recent studies have shown that the epicardium and EPDC strongly express extra-cellular matric proteins such as fibronectin, which guide cardiomyocytes into the wound area and are essential to correct CM integration (Wang et al., 2013). Furthermore, the epicardium plays an essential role in CM survival and proliferation as a source for paracrine signaling, a supply of perivascular cells, and a mediator of inflammation (Huang et al., 2012; Song et al., 2012; Wang et al., 2015). Altogether, FGF is a key modular of the epicardium and EPDCs, which provide the necessary cellular signals for the proliferation of wound-edge cardiomyocytes and overall tissue recovery.

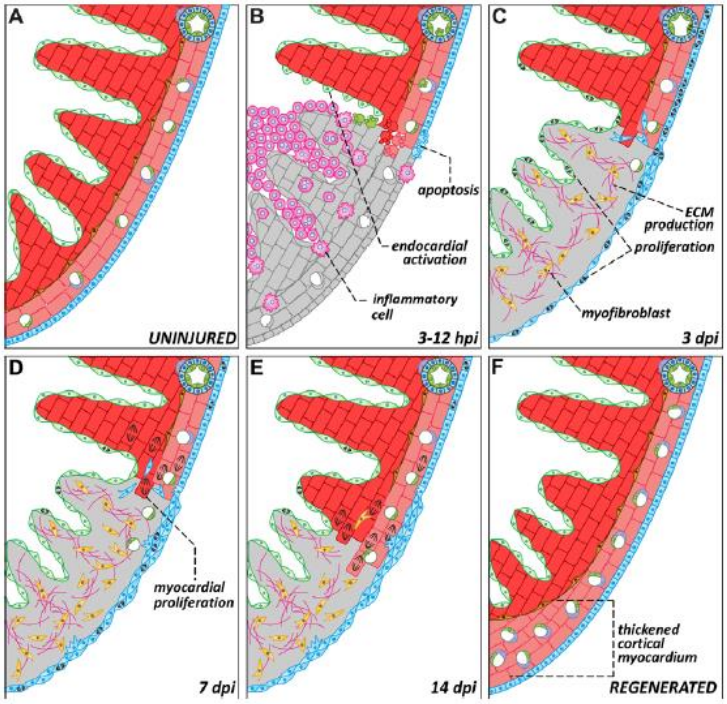

Figure 2. Mechanism for zebrafish heart regeneration (A) Representative regions in the uninjured heart (B) Immediate after injury. Necrosis (gray) of cardiac tissue. Cell death trigger inflammation and endocardial activation. (C) Days after the injury, FGF signaling promote the mesenchymal transition of epicardium. Epicardial and endocardial cells quickly proliferate and migrate to cover the wound. Epicardium produce signaling factors and ECM, which later guide CMs. (D-E) Weeks after injury. Wound-edge cardiomyocytes repopulate the injury site causing fibrotic tissue to progressively disappear. (F) Completed restoration of zebrafish myocardium. (Gonzalez-Rosa et al., 2017)

D. Importance of coronary revascularization in regeneration

Recent attempts toward therapeutic CM repair have resulted in little success partially due to early apoptosis, revealing the urgent need to understand better the systems supporting CMs (Nadal-Ginard et al., 2018). In previous cardiac regeneration studies, cardiomyocyte growth often overshadowed angiogenesis. Nonetheless, the formation of a proper vascular support system is crucial to the repopulation of healthy tissue, and these two processes are not wholly independent (Kapuria et al., 2018). CM hyperplasia and thickening of the ventricular wall is tightly coupled to coronary angiogenesis (Karra et al., 2018). In a regenerative context, Marin-Juez et al. showed that

15 hours post cryo-injury early vascular sprouting would enter into the wound region (Marin-Juez et al., 2016). Similarly, in neonatal mammalian hearts, coronary capillaries began to invade the injury site within two days, and by five days, they had matured into full arteries (Ingason et al., 2018). Further inhibition studies using an overexpression dominant-negative form of *vegfaa* showed reduced vascular density at the injury area by ~75%, and this led to an almost 60% decrease in CM proliferation at seven days post-injury. Additionally, the *vegfaa*$^{-/-}$ fish exhibited more collagen deposition in the damaged area, which eventually caused permanent fibrotic scaring and incomplete healing (Marin-Juez et al., 2016). Adult *cxcr4a*$^{-/-}$ animals indicated similar results as they were unable to establish vascular networks and likewise could not regenerate fully (Harrison et al., 2015). In summary, the revascularization of the wound site precedes cardiomyocyte migration and is vital to the new CM population.

Several cellular signaling mechanisms that regulate coronary revascularization also co-express within the epicardium. Vascular endothelial growth factor (VEGF) signaling regulates the rapid angiogenesis necessary for further cardiac wound repair. In *TgBAC(vegfaa;*EGFP) fish, *vegfaa* expression is significantly upregulated as early as one-day post cryoinjury. Specifically, around the wound area, the epicardium has an increase in *vegfaa* expression. Under a global genetic cardiomyocyte ablation model, endocardial *vegfaa* expression permeated throughout the heart (Karra et al., 2018). Taken together, these study results suggest that *vegfaa* expression in the epicardium and endocardium are adjacent to regions of regenerating muscle. In the ventricular amputation model, the CMs and many other cells in the regenerated myocardial wall upregulate the expression of the fibroblast growth factor (FGF) ligand, *fgf17b*, starting from as early as seven days post-injury and maintaining it until day 30. At the same time, epicardium surrounding the injury site has increased *fgfr2* and *fgfr4* expression. Together, FGF activity promotes EMT of the

EPDC and the integration of epicardial cells into the regenerating tissue. Inhibition of the Fgf signaling severely impairs coronary neovascularization and the resulting cardiac regeneration, leaving scar tissue 30 days post-injury (Lepilina et al., 2006).

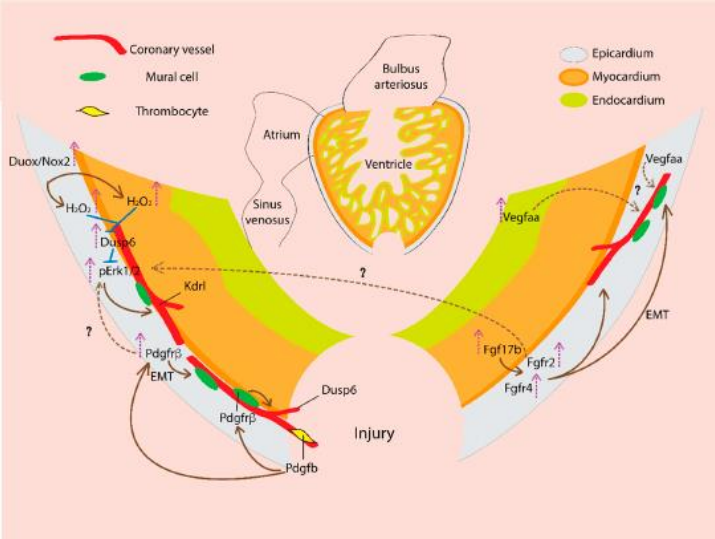

Figure 3. Signaling pathways regulating coronary revascularization during zebrafish cardiac regeneration. VEGF expression is required for angiogenesis. Vegfaa expression in the epicardium and endocardium is upregulated surrounding the injury sites. FGF ligands are upregulated in CMs, and there is increased FGF receptor expression in epicardium. FGF signaling promote EMT of epicardial cells, which benefits neovascularization and resulting CM proliferation. (Kapuria et al., 2018)

E. VEGF and FGF communication and potential combinational impact

Even with these compelling studies, as of now, little is known about the molecular mechanisms that regulate the coordination between rapid revascularization and subsequent CM regeneration. A potential candidate behind this inter-relationality is FGF. As previously discussed, FGF induces epicardial migration and CM proliferation (Lepilina et al., 2006; Yu et al., 2016). Fgf also has a role in de novo neovascularization, but its primary function is limited to endothelial maintenance. However, FGF still has the potential to moderate the early sprouting

from pre-existing blood vessels (angiogenesis) found post-cardiac injury. FGF-FGFR binding allows for crosstalk with the VEGF system through intracellular transduction. When FGF binding activates Erk1/2, it translocates to the nucleus and promotes the binding of *Vegfr2* enhancer, resulting in increased VEGFR2 transcription and expression (Murakami et al., 2011). VEGF is one of the most prominent angiogenic growth factor families and is integral to vessel maintenance, akin to FGF, development, and repair due to its ability to pass through interstitial space and bind receptors stimulating endothelial sprouting (Ritenour and Dickie, 2017). Previous cardiac regeneration studies have identified VEGF and FGF as two separate important determinants of heart regrowth; yet, to date, there have not been combinational studies of angiogenic and CM growth factors.

Here, I used small molecule inhibitors to examine the coordination between FGF and VEGF response, which underlies vascular network development, maintenance, and cardiac regeneration. During organogenesis, coronary vessel formation was sensitive to not only VEGF inhibitors, but also more broad tyrosine blockers, suggesting that multiple signaling factors are involved and not just VEGF. In adult zebrafish, FGF and VEGF signaling pathways have a role in vascular maintenance. Using a functional assay, I was able to provide evidence that FGF blockage induced additive inhibition that potentially proceeds VEGF and utilizes the same pathway. More importantly, combinational inhibition shows that FGF receptor activation could counteract VEGF inhibition, but this effect was lost when VEGF ligand was sequestered making the additional FGF-induced VEGFR expression useless. Together, these results provide evidence for FGF and VEGF coordinated angiogenesis, which is a vital component to rapid revascularization and subsequent CM regeneration and overall heart recovery.

II. Materials and Methods

Zebrafish husbandry and line:

delta:EGFP transgenic zebrafish line marked vascular endothelium and were used to visualize the coronary vessel network. The zebrafish were raised and maintained on 10-h dark/14-h light cycle at 26.5°C (Kenneth D. Poss, 2002). Before experimental operations, zebrafish were anesthetized in 0.02% 1M tricaine (Sigma). The zebrafish facility and procedures were approved by the Emory Institutional Animal Care and Use Committee.

Ex vivo heart explant culture:

Before examination of the heart vascularization, *delta*:EGFP fish were killed by a lethal dose of tricaine. After using micro forceps to make small chest incision, the heart was exposed. The bulbus arteriosus and ventricle was isolated and placed in Dulbecco Modified Eagle Medium (DMEM). To protect against degradation, the media also contains antibiotic and 2-Mercaptoethanol. Within the hour, samples were mounted using an 1% agarose DMEM-FBS solution. The solution was created by gradually heating DMEM until the agarose was homogenously dissolved. After cooling back down to room temperature, fetal bovine serum (FBS) was added, and the entire solution was stored in a warm water bath to prevent early congealing. The samples were incubated at 28°C and 5% carbon dioxide for up to 3 days post-extraction.

Inhibitor treatment:

Hearts were exposed to the pharmacological inhibitors by culturing samples with DMEM solution infused with the drug. In a similar fashion to before, the culturing media would still be removed during imaging and replaced with fresh media afterwards.

Orantibib (SU6668) was used to target PDGFRβ, VEGFR2, and FGFR1 inhibition, (Darren W. Davis, 2005). Inhibition of all VEGF-mediated angiogenesis was done through Vatalanib (PTK787) (Ritenour and Dickie, 2017). Drugs were mixed into DMEM-based culture media to form final concentration 3 uM, which was previously shown to be significantly effective and had no gross signs of toxicity (referenced above). For blockage of the FGFR family of receptors, Erdafitinib (JNJ-42756) was administered into culture media at a concentration of 1uM, 3 uM, 5uM and 10 uM (Perera et al., 2017). Based on dose-response experiments, 3uM was the minimal dosage concentration for the small molecule inhibitors for statistically significant effects to vessel coverage. To maintain consistency throughout the groups, all other inhibitors were also tested at 3 uM. Previous literature supports that this 3uM concentration for the following drugs did not express toxicity, and all were well below the EC50 concentration for the half-maximal response (refer to each drugs' citations). The anilinoquiazoline ZM323881 was used to specifically inhibit the kinase activity of VEGFR2 with no off-targeting to VEGFR1, EGF, or FGF (Whittles et al., 2002; Zhang et al., 2017). Ponatinib (AP24534) was utilized as a pan receptor tyrosine inhibitor that has been shown to be very active across a broad range of growth factors, especially toward angiogenic signals. (Gozgit et al., 2012; Singh et al., 2019).

To sequester VEGFA ligand, tinzaparin sodium was used. This low weight heparan sulfate does not affect the tyrosine kinase receptor. Instead, it competitively inhibits growth factor signaling by pulling down the ligand (Norrby, 2006). Prior studies have shown that heparin fractions with MWs of 4.8-5.4 kDa block the binding of VEGFA to its endothelial cell receptor. Furthermore, tinzaparin with an MW of 5.0 kDa was shown to systematically inhibit VEGF-A-mediated angiogenesis within adult rats (Norrby, 2000). However, additional studies have found

evidence that tinzaparin attenuates FGF-related vascularization (Mousa and Mohamed, 2004). Taken together, tinzaparin has some degree of off targeting depending on its molecular weight, but a majority of studies, to date, have confirmed tinzaparin's specific efficacy on VEGF-mediated angiogenesis.

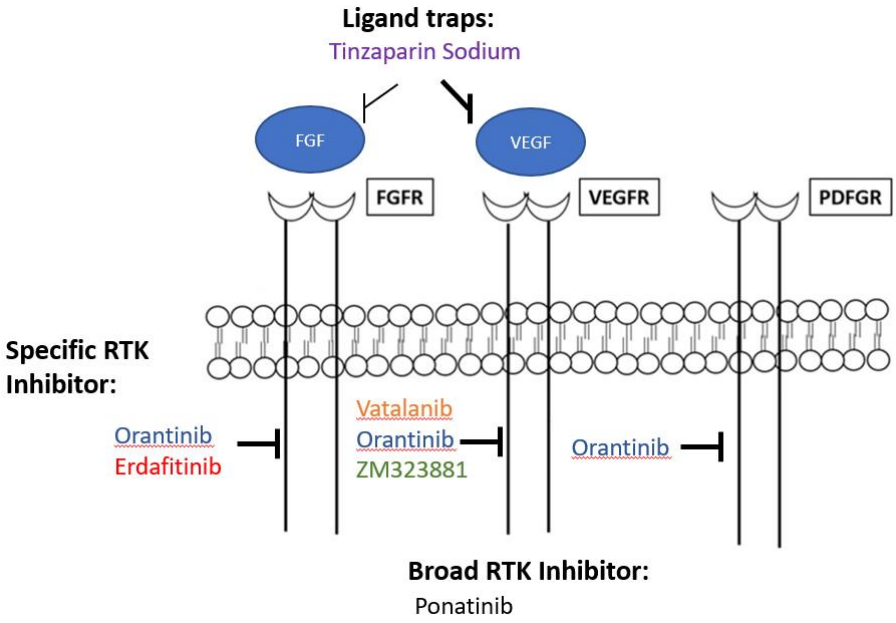

Figure 4. Target inhibition pathways. Vatalanib (PTK787) is specific toward the VEGFR family. ZM323881 has even higher VEGF specificity, only blocking VEGFR2. Erdafitinib (JNJ-42756) is effective particular toward the FGFR family. Orantibib (SU6668) is effective against FGFR, VEGFR, and PDGFR. Tinzaparin sodium somewhat indiscriminate amongst FGF and VEGF, but studies have mainly shown to be effectiveness toward VEGF ligand.

Apical resection surgery:

Anesthetized adult zebrafish sustained cardiac injury. Using micro-forceps, a ventral incision was created to expose the heart. Curved micro-scissors were then used to remove 10-20% of the ventricular apex quickly. After ensuring that the heart tissue was completely cut away, the heart was slipped back inside the chest. Once the bleeding has clotted, the fish was returned to the fresh fish water tank.

Coronary vessel network quantification:

When capturing daily *ex vivo* images, the DMEM FBS liquid media was removed. Then the ventricle was observed using Lecia M165 FC fluorescent stereomicroscope. Afterward, the media was replaced with fresh solution. After the three-day *ex vivo* culture, the images were collected and quantified using Angiotool (Zudaire et al., 2011). In this process, only strong fluorescent signals that had blood vessel-like structures were skeletonized into vectors preserving the extent and connectivity of the vessels; weaker fluorescent signals that were similar to background were ignored. Further spatial and morphological analysis of the vascular network was then conducted to identify critical structural motifs such as percent vessel coverage, junction density, and average vessel length. Using these quantitative metrics, a comparison was made in a spatiotemporal manner across all the sample groups.

III. Results

A. Organogenesis promote the growth of new vessels from pre-existing

Similar to the mammalian heart, the zebrafish heart receives nutrients and oxygen through luminal blood vessels. To meet the high metabolic demands of the cardiomyocytes, zebrafish need to form early coronary vasculature. During the juvenile stage (<3 months old), the heart will gradually increase myocardial layers. In correlation to the timing of myocardial growth, is the emergence of the coronary circulatory system. This well-regulated process is dependent on a combination of factors, including de nova neovascularization, arterial maturation, and angiogenesis (Kapuria et al., 2018). In this state of rapid development, the coronary circulatory system will quickly expand until adulthood. Similar to the process of regeneration, during organogenesis, the heart is undergoing a process of myocardial expansion and the rapid growth of coronary vasculature.

To define the spatiotemporal pattern of vascular growth seen in development, I am using a transgenic line with the specific endothelium-expressed regulatory sequence, *delta*, directing the expression of a fluorescent reported, EGFP. Using juvenile fish, I was able to visualize the process of coronary development ex vivo over five days. As reported in previous literature, the coronary network emerged from a plexus around the aorta and grew outward toward the apex of the ventricle, until it entirely covered the myocardium (Kapuria et al., 2018). During this period of expansion, there was little evidence of de novo formation of vessels. Instead, a majority of vascular development involved pre-existing vessels undergoing angiogenesis as they continued to grow forward and extend. Additionally, there were new vasculature branch points, where the previous vessel would bifurcate. Often, these new branch vessels would start growing in the same direction as another sprouting vessel. As these two new vessels linked together, they formed a more

interconnected network of circulation. Overall, the early angiogenic process combines these two processes of branching and elongation to achieve more overall coronary vessel coverage (Fig 5a).

Quantification of the coronary vasculature using Angiotool (Zudaire et al., 2011) indicated that within the five days, there was exponential development of the vessel network. There was a significant increase in junction density and average vessel length at all time points following the second day (Fig. 5c and d). The vessel coverage significantly changed by the third day, and at the end of the observation period, there was, on average, 11.2% additional vascular area (Fig. 5b). Taken together, the developmental results indicate that during this early growth period, coronary vasculature is rapidly expanding as new vessels are continually forming from pre-existing ones through a process of exponential elongation and bifurcation.

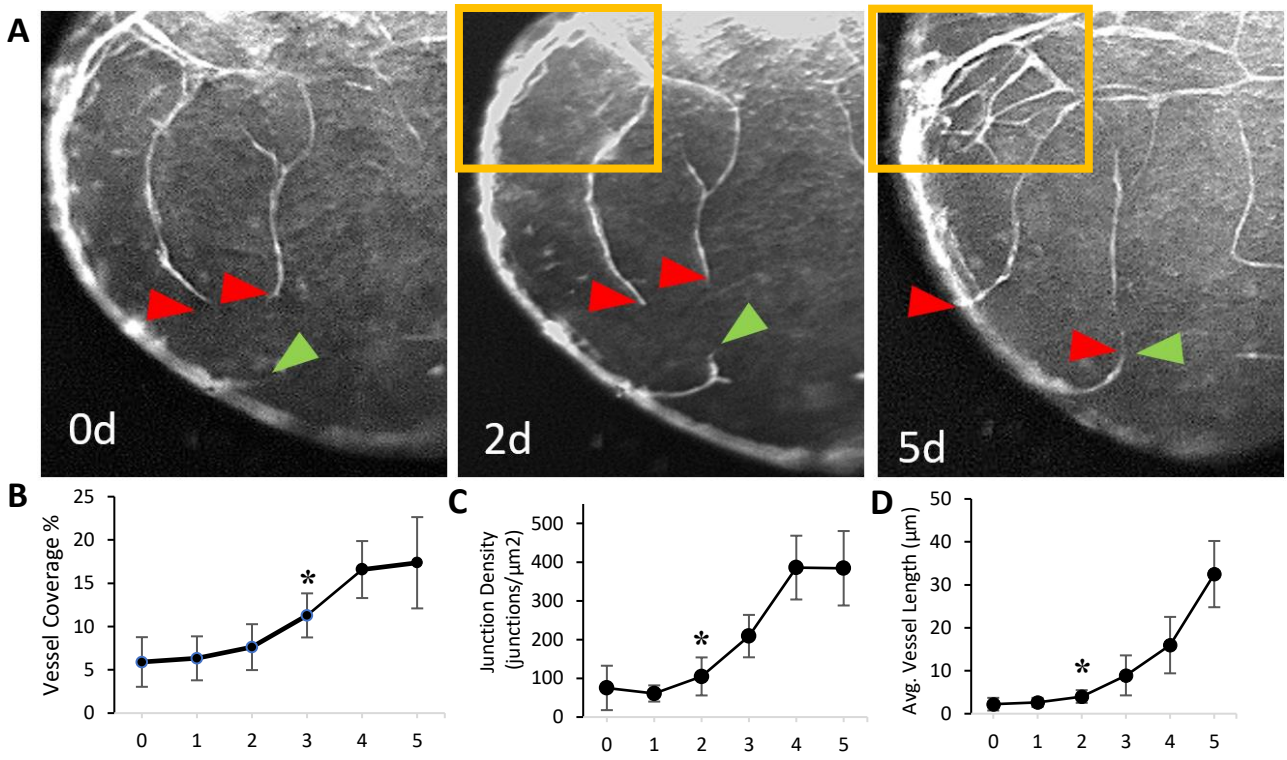

Figure 5. Development of coronary vasculature in 5 day ex vivo culture of *delta;*EGFP (n=8). (A) Red arrows show vessels of interest. Green arrow indicate site of new branching and elongation. By day 5, new vessels have connected with each other expanding the circulatory network. Yellow square highlights region that form new coronary plexus (B-D) Quantification of rapid vessel development. Exponential increase in vessel coverage, junction density and average vessel length. *, indicate first significant change from day 0. ($p < 0.05$). Aligning with observed image, day 2 has significant increase in junctions and branches.

B. Non-VEGF growth factors can modulate developmental angiogenesis

To further understanding of the development angiogenesis process, juvenile hearts were exposed to antiangiogenic small molecules. For testing the effects of PDGFRβ, VEGFR2, and FGFR1 inhibition, I administered orantibib (SU6668) in DMEM culture media (Darren W. Davis, 2005). Furthermore, inhibition of all VEGF-mediated angiogenesis was observed by preparing a separate DMEM solution of vatalanib (PTK787) (Ritenour and Dickie, 2017). Both drugs were dilated from 10mM stock to a final concentration of 3 uM. The concentration used was shown to be significantly effective and has no gross signs of toxicity (Fig. 6a), further evidenced by previous dose-response studies (referenced in the above citations).

Previous experimental data indicated that by the third day, there would be a significant increase in vessel coverage. By day 3, the control samples displayed the same rapid vascularization seen before. However, in the drug-treated samples, there was not only a lack of angiogenesis but also a regression in the vasculature (Fig. 6a). Looking at the percent change from day 0, both forms of growth factor inhibition significantly decreased the vascular coverage, junction density, and the average vessel length compared to the control. Between the VEGF-specific and more comprehensive inhibitor, there was no significant difference. Nonetheless, samples treated with orantibib, on average, displayed more severe deterioration and had a lower overall p-value, showing more deviance from the control. This more robust inhibition of developmental angiogenesis by the broad tyrosine blocker suggests that multiple signaling factors are involved and not merely VEGF.

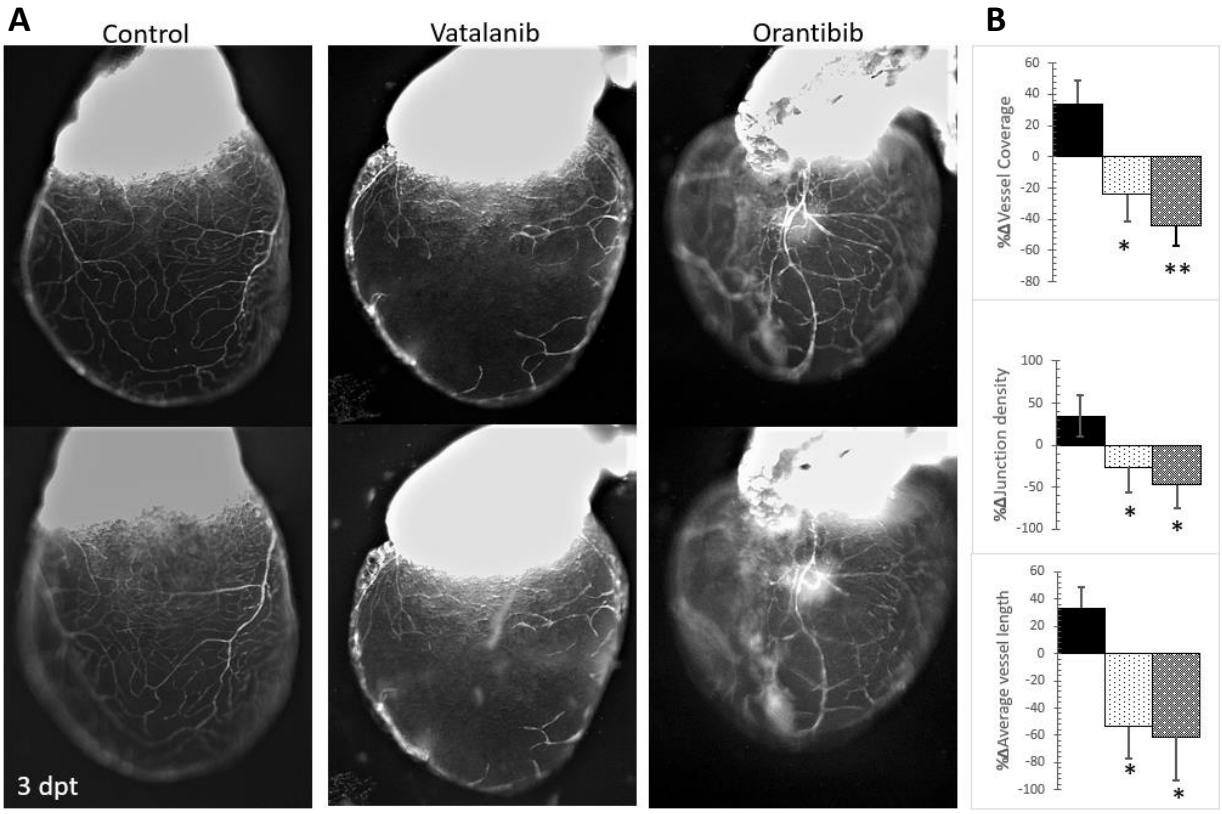

Figure 6. Comparison of juvenile *delta;*EGFP (n=6) hearts with anti-angiogenesis inhibitors at 3 um in 3-day ex vivo culture. Vatalanib is VEGFR specific blocker and Orantibib inhibits PDGFR, FGFR and VEGFR. (A) Representative images demonstrating how growth factor inhibition prevents the normal growth of coronary network. (B) Instead of absolute measurement, resulting vascular changes are presented as percent difference from 0-day. This helps account for potential individual variance amongst the hearts. Broad inhibition affects angiogenesis more than VEGF-specific. *, p-value < 0.05; **, p-value<0.02

C. Growth factor inhibition has differential spatiotemporal effects on adult heart

To determine if VEGF and FGF pathways are involved with the maintenance of adult coronary vasculature, I conducted an ex vivo functional assay using FGF and VEGF-specific antagonist that will either bind growth factor molecules or directly block tyrosine activity on the receptor. To specifically inhibit the kinase activity of VEGFR2, I used ZM32388, and for blockage of the FGFR family of receptors, erdafitinib (JNJ-42756) was administered into culture media at a concentration of 1uM, 3 uM, 5uM and 10 uM. Based on dose-response experiments, 3uM was the minimal dosage concentration for statistically significant effects (Fig 6a). To

maintain consistency and a standard of comparison, all other treatments groups were also administered at 3uM concentration. The wide spectrum RTK inhibitor was ponatinib (AP24534) used to examine the full extent growth factor blockage on coronary vessels. To sequester VEGF ligand, I utilized tinzaparin sodium. As a low weight heparan sulfates, this inhibitor does have some off-targeting to FGF, but due to tinzaparin specific MW, it is most effective towards VEGF-mediated angiogenesis (Norrby, 2006).

After imaging the adult *delta;*EGFP (n=4) hearts every 24 hours for three days, I found that all the treated samples showed significant loss of coronary vessels by the third day (Fig. 6b-c). In some controls, the vasculature was lessened; however, this was not to a significant degree. Hearts treated with erdafitinib, the FGFR inhibitor, had one of the most significant decreases in vessel coverage, indicating the FGF pathway is a vital modulator of vessel integrity. This result is consistent with the previous reports demonstrating that FGF affects the maintenance of adult blood vessels (Kapuria et al., 2018). Only, the potent broad RTK inhibitor ponatinib had a slightly higher effect upon vessel coverage because it, as expected, can impede an extensive array of growth factor pathways. The samples treat with tinzaparin demonstrated similar levels of vessel degradation as the ZM, which makes sense because they are both acting to suppress VEGF expression. Intriguingly, on the first day, erdafitinib had less impact on vasculature than ZM, the VEGFR2 specific inhibitor. This temporal disparity between FGF and VEGF inhibition may indicate that there is some relationality between the two pathways. The inhibition of FGF would initially have less of an effect on endothelial cells, but because FGF has a role in VEGFR expression, its drug would have the potential for combinational inhibition by downregulating both pathways.

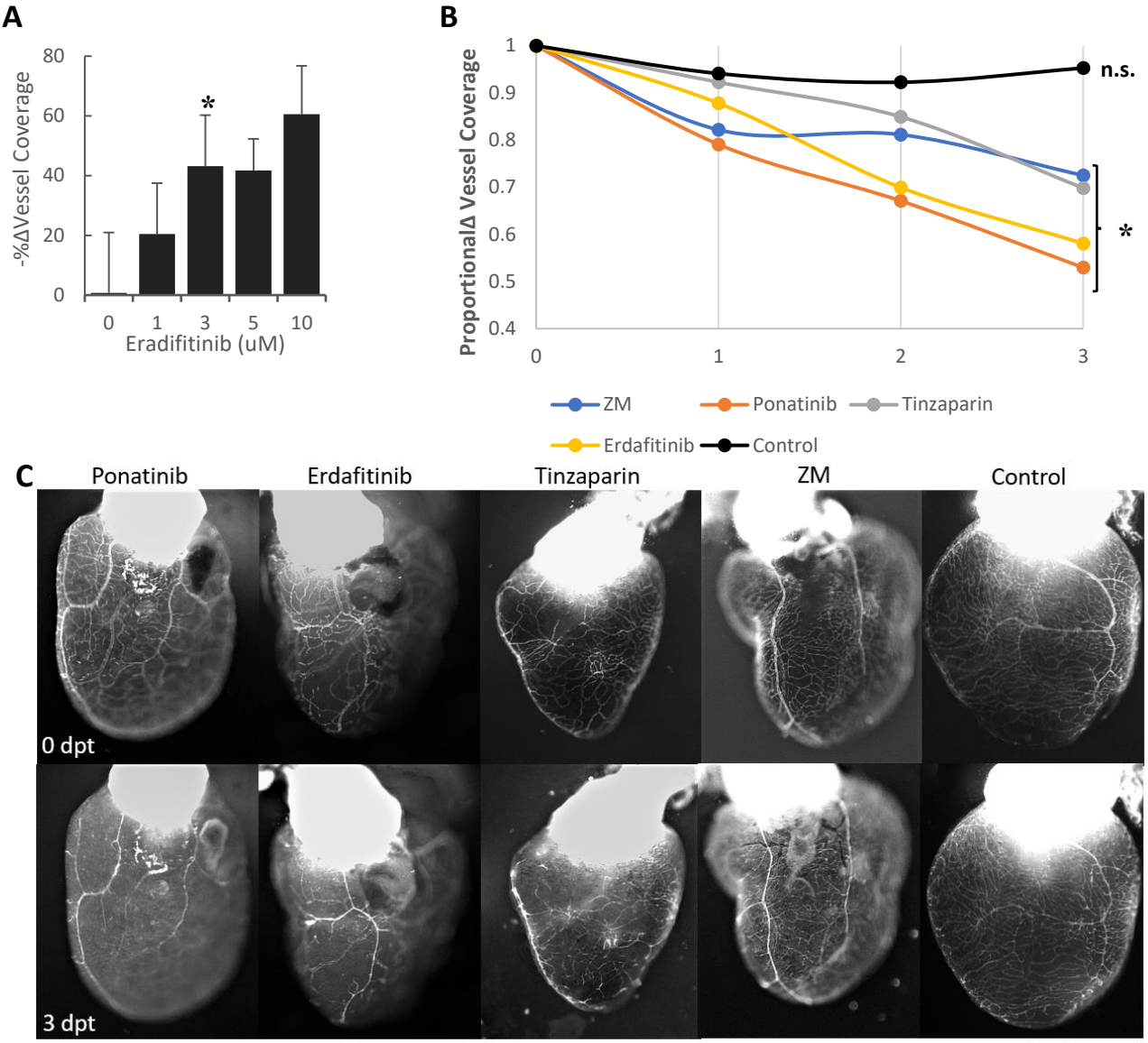

Figure 7. Comparison of anti-angiogenesis inhibitors using adult *delta;*EGFP (n=4) ex vivo. Erdafitinib is FGFR specific blocker, ZM323881 (ZM) specifically inhibits VEGFR2, tinzaparin pulls down primarily VEGF ligand, ponatinib is a potent wide-spectrum RTK inhibitor. (A) Dose-response of erdafitinib based on proportional decrease compared to original vessel coverage. The minimum effective dose is 3uM. (B) Differing rate of coronary vessel degradation based on inhibition target. Day 0 vessel coverage is 1.0, and then following data points track the proportional loss of original vasculature. All inhibitors showed significant change ($p<0.05$) in coronary vessel coverage by third day. The control showed some degradation, but this was nonsignificant. (C) Representative images of hearts from each sample group.

D. Maintenance of adult vasculature dependent on the FGF-VEGF coordination

To test the hypothesis that FGF signaling regulates VEGF function and controls adult vascular network formation, I used a combinational assay that targets FGF and VEGF pathways concurrently. The drug pairs were all at a final concentration of 3uM with 1.5 uM of each inhibitor.

Ponatinib with tinzaparin produced the most drastic results as it was able to reduce a broad range of receptors, while also pulling down the growth factor ligands (Fig. 8a-b). Furthermore, samples that had inhibited FGF family of receptors and VEGF2 receptors displayed similar levels of coronary disruption as the ponatinib, suggesting that the knockdown of these two pathways accounted for most of the effect seen in the broad RTK inhibitor. Furthermore, this combination of erdafitinib and ZM produced similar levels of vessel degradation as with the tinzaparin and erdafitinib.

Interestingly, blockage of VEGFR and FGFR produce a much stronger loss of vasculature than with either VEGF ligand pulldown or VEGFR blockage alone. When it was just tinzaparin treatment targeting VEGF ligand, FGF signaling could still engage transcriptional enhancers to upregulate VEGFR2 expression and potentially overcome the receptor inhibition, However, with both ZM and erdafitinib, rescue was impossible because the erdafitinib was preventing FGF receptor activity. Further evidence is shown by ZM and tinzaparin. Samples that have only VEGF inhibition through inhibition of VEGF receptor and its ligand show less lost coronary vasculature as compared to the simultaneous inhibition of FGFR and VEGFR.

Additionally, when VEGFR and VEGF ligand are combinational inhibited, it produces a greater effect upon the vascular coverage as compared to just VEGFR inhibition. This observation suggests that even when FGF receptors are still responsive, the lack of VEGF ligand makes the additionally VEGFR2 receptors useless because the ligand is being sequestered away by

tinzaparin. Taken together, my functional assays indicate that there is a degree of interaction between FGF and VEGF signaling that produce differential effects upon adult coronary vessel maintenance.

A

Target	Drug	Day 1 (%)	Day 2 (%)	Day 3 (%)	Day 3 SD	Day 3 P-value
VEGF ligand, All RTK	Tinzaparin-Ponatinib	-19.49	-33.81	-51.65	20.80	3.23E-08
All RTK	Ponatinib	-20.93	-32.92	-47.03	14.69	3.03E-03
VEGFR, FGFR	ZM-Erdafitinib	-19.56	-34.30	-44.91	14.39	2.81E-05
VEGF ligand,FGFR	Tinzaparin-Erdafitinib	-15.92	-31.95	-45.15	8.08	5.69E-04
FGFR	Erdafitinib	-12.20	-30.08	-41.93	6.91	1.11E-04
VEGFR, VEGF ligand	ZM-Tinzaparin	-18.11	-32.42	-36.87	12.81	1.08E-03
VEGF ligand	Tinzaparin	-7.75	-15.09	-30.17	8.27	4.56E-02
VEGFR	ZM	-17.84	-18.86	-27.51	16.36	4.22E-02
None	Control	-5.90	-7.75	-4.74	19.86	6.43E-01

B

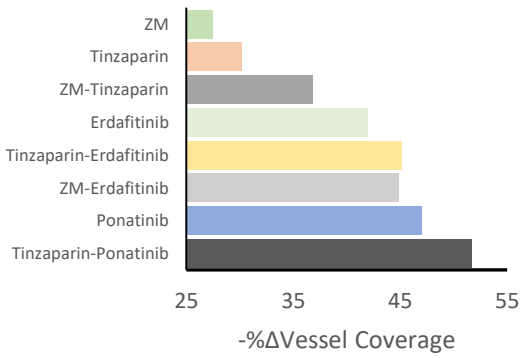

Figure 8. Analysis of combined anti-angiogenesis inhibitors using adult *delta;*EGFP (n=4) ex vivo. Table showing the drug treatment, inhibition targets, and the daily percent decrease compared to the original vascular coverage (day 0). P-value calculated based on if there was a significant variation of vessel area during the 3-day culture. (B) Graph of the percent decrease in vessel coverage for each of the treatment groups.

E. VEGF mediates rapid revascularization following heart injury

Previous studies have shown that zebrafish can completely regrow their lost cardiac tissue following amputation of up to 20% of their ventricle (Karra et al., 2018; Poss et al., 2002; Rosenblatt-Velin et al., 2005). For years, this tissue removal surgery has been used as an injury model to examine the process of heart regeneration.

I conducted an ex vivo observation of adult *delta;*EGFP hearts two days post-injury. In supporting of the literature, the hearts display fast revascularization of the damaged area (Fig. 9a) (Marin-Juez et al., 2016). As early as three days post-injury (1-day ex vivo observation), the first early vessel sprouts were entering into the ablation area. These new vessels were originating from preexisting ones, suggesting that the endothelial cells are undergoing angiogenesis. There is the

possibility that there are some instances of de nova neovascularization, but this was not observed. By the second day, a coronary plexus had formed along the injury border, and the large vessels were seen to have branched into an increasingly dense capillary system. Revascularization was seen to have started proximal to the injury site and gradually working toward the apex, most likely following the same directional regeneration as epicardium and later CMs (Wang et al., 2015).

To assess if revascularization involves VEGF signaling, I conducted a dosed inhibitory study, using 3.0uM and 1.5uM of ZM. Within the three-day observational period, the untreated samples were able to more than double their original vascular coverage, and generally, able to entirely cover the injury site with new coronary vessels (Fig. 9b). With VEGFR2-specific blockage, there was still a significant level of revascularization, but it was less than the control. Further evidence that this effect is dependent on VEGF is the dose-dependent manner of the inhibition. Samples cultured with 3.0uM exhibited less vascular regeneration than the hearts exposed to 1.5uM and much less than the untreated heart. Combining these result with the previous experiment, it would be reasonable to assume that FGF could also have role in this rapid revascularization. During the previous combinational drug analysis, FGF signaling was shown to coordinate with VEGF and effect coronary vasculature. Therefore, FGF could be an indirect modulator of angiogenesis. However, due to time limitations within this study, this hypothesis was unable to be fully assessed.

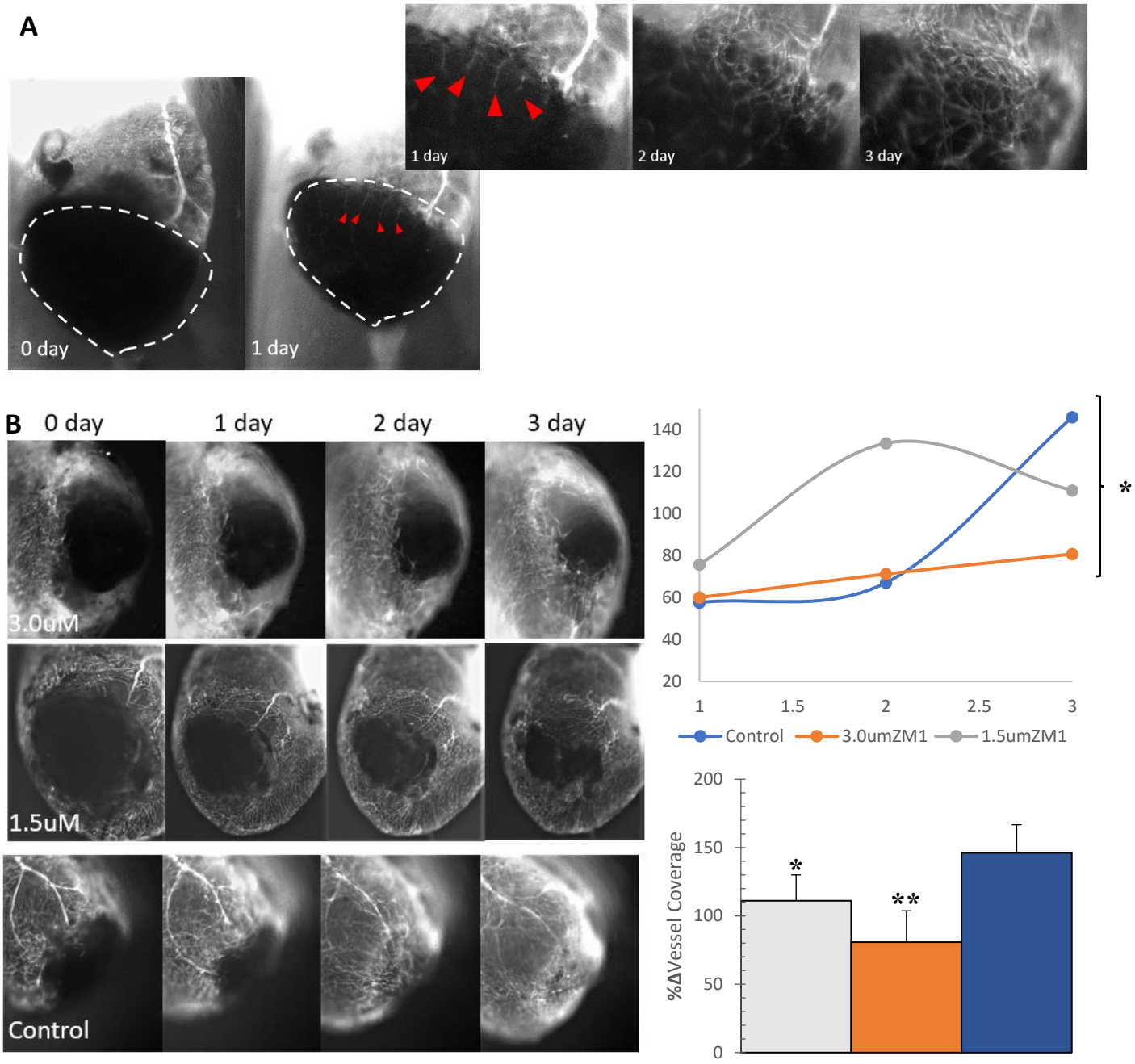

Figure 9. 3 day ex vivo observation of *delta;*EGFP (n=4) 2 days after apical resection (Day 1 observation = 3 days post injury) (A) Example of early revascularization. Dotted white line show initial injury region. Red arrow heads point to sprouting vessel that quickly form dense vascular network that will be critical for supporting subsequent cardiac regeneration. (B) Dose response ZM at 3.0uM and 1.5uM. Less revascularization after treatment with VEGFR2 specific inhibitor. Line graph tracking daily increase of vessel coverage in proportion (%) to the initial (Day 0). Bar graphs give comparison of total level of growth. *, $p<0.05$. All groups showed significant revascularization.

IV. Discussion

Recent, therapeutic attempts toward cardiomyocyte repair have produced little success partially due to early apoptosis, revealing the urgent need to better understand the systems supporting CMs (Nadal-Ginard et al., 2018). In previous cardiac regeneration studies, cardiomyocyte growth often overshadowed angiogenesis. However, many recent studies has shown that *vegfa* expression is upregulated early after heart injury, inducing rapid angiogenesis and that this increased expression can influence cardiac growth (Karra et al., 2018; Marin-Juez et al., 2016). Even with these compelling results, as of now, little is known about the molecular mechanisms that regulate the dialogue between the cardiac tissue and vascular endothelium. In zebrafish, one potential coordinator of innate heart regeneration and angiogenesis is FGF signaling. After injury, the myocardium secretes *fgfb*, which gives rise to epicardial motility. The epicardium and mesenchymal EPDC cells proliferate to cover the injury and form the ECM foundation for later CM integration and proliferation (Wang et al., 2013). Additionally, FGF activation is able to stimulate Erk1/2, which promotes VEGFR2 transcription and expression (Murakami et al., 2011). As one of the most prominent angiogenic growth factor families, VEGF is able to induce endothelial sprouting and potential activate the early revascularization required for subsequent CM recovery.

Here, I show that zebrafish coronary vasculature is modulated by co-dependent FGF and VEGF pathways. During juvenile development, there is a process of rapid revascularization that is analogous to the rapid angiogenesis that Marin-Juez et al documents as a crucial precursor to cardiac regeneration. Previous studies focused on *vegfaa* as a key modulator of this process, but my data shows that other growth factor pathways also have a role in this coronary expansion. Using an inhibitory assay, I show that both FGF and VEGF are necessary to for upkeep of the coronary

vasculature during adulthood. The temporal disparity between FGF and VEGF inhibition indicates some relationality between the two pathways. The initial impact of FGF endothelial cells is less than direct VEGFR blockage, but as time goes by, FGF blockage potentially can downregulate both angiogenic pathways because FGF is a modulator of VEGFR expression (Murakami et al., 2011). Furthermore, combinational inhibition of VEGFR and its ligand prevents FGF signaling benefits because growth factor pull down makes additional VEGF receptors meaningless. Importantly, the implications of this FGF-VEGF signal transduction has the potential to affect overall cardiac regeneration because my results reaffirm the importance of VEGF as a critical factor in vascular development after cardiac injury. In the future, conducting the inhibitory assay in the injury model can help better understand if the coordination of FGF and VEGF persists during states of vascular development.

Taken together, my results show that FGF signaling has broad functional effects upon VEGF expression and coronary vasculature. Here, I devised an ex vivo inhibitory assay that specifically blocks the FGF family of receptors, VEGF2 receptors, and VEGF ligand. Using this system, I showed that downregulation of VEGF could be mitigated as long as its ligand was present, in conjunction with FGF signal, to rescue VEGF expression. Thus, my study has paved the way for future zebrafish research that helps understanding the coordinated effect of FGF and VEGF on coronary vasculature. By furthering investigation of the interconnection amongst endogenous heart repair mechanisms, therapies can be more holistic on their approach and better match the signaling dynamic necessary for integrating angiogenesis and myocardial proliferation.

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