TRPM5's Role in Vascular Development

Megan Goeckel

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WASHINGTON UNIVERSITY IN ST. LOUIS

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Department of Cell Biology and Physiology

TRPM5’s Role in Vascular Development

by

Megan Goeckel

A thesis presented to
Washington University in St. Louis
in partial fulfillment of the
requirements for the degree
of Master of Arts in Biology

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Megan Goeckel

Washington University in St. Louis

May 2023
Dedicated to my fiancé.
ABSTRACT OF THE THESIS

TRPM5’s Role in Vascular Development

by

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Master of Arts in Biology

Washington University in St. Louis, 2023

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Blood vessels are composed of two main cell types, endothelial cells (ECs) and mural cells (MCs). Endothelial cells comprise of the inner-most layer of the vessel and sense changes in blood flow to recruit mural cells. The mechanism for how endothelial cells sense these hemodynamic changes is not well understood and deciphering this mechanism is the focus of this thesis. We hypothesize that transient receptor potential melastatin 5 (TRPM5), a transmembrane channel protein, is involved in this process. Here, using cultured human endothelial cells and the zebrafish model, we show that inhibition of TRPM5 decreases vessel formation and increases EC-MC association during vascular development.
Chapter 1: Introduction to Blood Vessel Structure and Development

This chapter provides an introduction to the development of vascular biology.
1.1 Blood Vessel Structure

The vascular system is a complex circuit of vessels that provide nutrients to and carry waste away from cells located in all corners of the body. In a human, the vascular system is so extensive that if stretched out in a single line it would cover 60,000 miles	extsuperscript{1}. The vasculature is composed of arteries—vessels transporting blood away from the heart—which flow to a smaller network of vessels known as capillaries followed by veins—vessels which carry blood back to the heart	extsuperscript{2-9}. The heart is responsible for the movement of the vessel contents through the system as it serves as a pump	extsuperscript{2-9}. Due to the heart’s pump-like activity and the large distance that the blood travels through the body, the type of blood flow that leaves the heart is vastly different than the type of blood flow that returns	extsuperscript{10-12}. Blood flow leaving the heart—through the arteries—appear to have a ‘go-stop-go’ flow type called pulsatile flow. Blood flow that is returning to the heart—through the veins—appear to have a more consistent flow type called laminar flow	extsuperscript{13, 14} (Fig 1.1).

Each of the vessel types exhibit distinct structural phenotypes to compensate for these differences in blood flow	extsuperscript{15-17}. All blood vessels, though, are comprised of two distinct cell layers, an inner layer of endothelial cells which interact with the blood, and an outer layer of vascular smooth muscle cells or pericytes—collectively called mural cells—which serve as supporting cells	extsuperscript{15-17}. Due to the higher amount of pressure exerted on the walls of these arteries from the pumping of the heart, multiple layers of mural cells surround these vessels. Capillaries, due to their small diameter, are only two cells thick, one endothelial cell and one pericyte cell. Veins have a distinct endothelial cell layer but lack a continuous mural cell layer	extsuperscript{15-17} (Fig 1.2).
1.2 An Overview of Vascular Development

Since arteries and veins have distinctly different muscle layers, it is thought that differences in flow type mediate the recruitment of mural cells through flow recognition by endothelial cells\textsuperscript{18,19}. During development, endothelial cells produce platelet derived growth factors (PDGFs) which bind to receptors on mural cells and recruit these supporting cells to the vascular endothelium\textsuperscript{20-22}.

Krupple-like factors (KLFs) are zinc finger proteins capable of binding to DNA and regulating transcription of the genome\textsuperscript{23}. KLF2 has been shown to inversely regulate PDGFb production\textsuperscript{24}. Increased KLF2 activity results in a decrease in \textit{pdgfb} transcription and decreases the production of PDGFb resulting in less mural cells recruited to vascular endothelial cells\textsuperscript{24}; the same is true of the inverse (Fig 1.3). KLF2 expression is also blood flow regulated; its activity increases when the endothelial cell experiences laminar flow and decreases with pulsatile flow\textsuperscript{25-28} (Fig 1.4). To put this together, arteries experience pulsatile flow which results in decreased KLF2 activity allowing for an increase in PDGFb production and recruitment of mural cells. Veins experience the opposite with laminar flow allowing for an increase in KLF2 activity which inhibits the production of PDGFb and reduces the amount of mural cell recruitment. Although this pathway seems complete, the means for how endothelial cells recognize these differences in blood flow types to alter vessel structure throughout development is an area requiring further investigation. The goal of the Stratman Lab is to identify mediators of blood flow recognition in endothelial cells throughout the course of vascular development.
1.3 Figures

Figure 1.1. Diagram representing the cardiovascular system, including the types of vessels and the types of flow. This schematic represents the cardiovascular system with arteries carrying blood with pulsatile flow away from the heart and blood with laminar flow towards the heart. Capillaries are connecting the arteries and veins. Figure was designed using BioRender.
Figure 1.2. Diagram showing the layers of the different blood vessels. This schematic shows the layer of endothelial cells lining the vessels. On the arteries and smaller vessels, there are mural cells. Figure was designed using BioRender.
Figure 1.3. KLFs inversely transcribe PDGFb, the signaling molecule recruiting vSMCs to blood vessels. This schematic represents the relationship between KLFs and PDGFs. Figure was designed using BioRender.
Figure 1.4. Diagram representing the relationship between the blood vessels, KLF activity, PDGF production, and vSMC recruitment to blood vessels. This schematic shows that KLF activity is decreased in arteries, allowing for more PDGF production. PDGF production recruits vSMCs to blood vessels. Veins have an increase in KLF activity. This decreases PDGF production, resulting in fewer vSMCs localizing to blood vessels. Figure was designed using BioRender.

1.4 References


Chapter 2: Determining Mediators of Vascular Development Using Zebrafish

This chapter describes the zebrafish model as a tool for studying vascular development and changes in hemodynamic forces.
2.1 The Benefits of the Zebrafish Model for Studying Vascular Biology

The Stratman Lab utilizes the zebrafish model for studying vascular development. Zebrafish provide a great model for this area of study for a range of reasons. First, a single pair of zebrafish parents can produce 100s of embryos in a single setting. These embryos are externally fertilized and mature ex-utero. This allows for the embryos to be easily genetically modified via CRISPR/Cas9 injections of genetic material at the one-cell stage\(^1\)\(^-\)\(^5\) (Fig 2.1). Of these genetic modifications, the insertion of transgenic tags to fluorescently visualize different areas of the fish is often utilized. Zebrafish embryos are optically transparent, making visualization of the transgenic markers in living embryos attainable (Fig 2.1).

Another benefit of the zebrafish model that is of particular interest for investigating the impact of changes in blood flow is the fact that zebrafish embryos can live up to five days post fertilization (dpf) without blood flow. These embryos can survive due to their thin layer of skin. Necessary cellular nutrients and the removal of waste can be fulfilled through diffusion across the skin, mitigating the need for the vascular system at early stages of development. The thin skin layer is also beneficial when performing pharmaceutical assays; small molecule drugs can be delivered to deep tissue via diffusion without the requirement of blood flow\(^6\) (Fig 2.1). This reduces the need for injection of the drug and eliminates the risk of injury and other complications to the fish.

Lastly, and most importantly, are the similarities in vascular development between humans and zebrafish. Zebrafish also have a heart which pumps blood through the arteries to the capillaries and to the veins with the same blood flow types previously mentioned\(^7\). The
recruitment of mural cells to vascular endothelium follows the same patterns noted in the fish\textsuperscript{7}. Furthermore, nearly 80\% of the zebrafish genome overlaps the human genome; proving more than just some pathway similarities between the fish and the human\textsuperscript{8}!

2.2 Determination of Genes Regulated by Changes in Hemodynamic Forces

Since zebrafish embryos are capable of surviving nearly a week without blood flow, the pharmacologic inhibitor BDM at 20uM was used to stop the heartbeat at 22hpf\textsuperscript{9}. Blood flow was stopped for 12 hours. HA tagged ribosomes in endothelial cells and mural cells were then collected and the mRNA bound to them were isolated and purified for sequencing. RNAseq was then performed on the collected RNA sequences. Of the sequences collected, \textit{trpm5} showed an increase in transcript values in endothelial cells and a decrease in mural cells compared to samples collected from fish that did not have their heartbeats stopped (Fig 2.3). Further investigation into how differential regulation of \textit{trpm5} transcript in response to flow by each cell type is the focus of this thesis.
2.3 Figures

Figure 2.1. Diagram representing a range of the benefits of the zebrafish model. Top left) Male and female zebrafish in a small mating tank laying eggs. Top right) An injection rig assembled to inject genetic materials into a fertilized zebrafish embryo at the single cell stage. Bottom right) A zebrafish embryo in a petri dish with water. A drug canister and pipette showing the addition of the pharmaceutical to the water. Bottom left) A zebrafish embryo with a green, fluorescent tag marking the cells of the vasculature. Center) An adult female zebrafish with a 5dpf zebrafish embryo (not to scale). Figure was designed using BioRender.
Figure 2.2. Diagram representing the collection of mRNA sequences via RNAseq. This schematic represents the TRAP assay performed by AN Stratman, unpublished data. Blood flow in zebrafish embryos was stopped and actively translating mRNA was purified from tagged ribosomes in either endothelial or mural cell populations. Figure was designed using BioRender.

Figure 2.3. Differential expression of TRPM5 transcript values upon the stopping of blood flow. qPCR values were normalized to GAPDH and numerically represented as ‘1’ as indicated by the dashed line. n=2, 6,000 individuals
2.4 References


Chapter 3: Transient Receptor Potential

Melastatin 5

This chapter describes the structure and function of TRPM5.
3.1 Structure of TRPM5

TRPM5 is one of eight members of the melastatin subfamily within the transient receptor potential superfamily\(^1\). Transient receptor potential melastatin 5 is a transmembrane channel\(^2\). Most TRP channels are permeable to calcium, but TRPM5 is one of only two TRP channels that are not and instead are permeable to monovalent cations; TRPM4 is the other channel\(^3-6\).

Calcium, though impermeable to the channel, still plays a critical role in TRPM5 signaling. TRPM5 is activated by intracellular calcium through its binding to two binding sites, one in the intracellular domain and the other on the transmembrane domain\(^3\). Binding of calcium to both sites cause conformational changes which allow for the opening of the channel and the influx of monovalent cations\(^3\).

3.2 Function of TRPM5

Other than endothelial cells and mural cells, TRPM5 can also be found on tuft cells\(^3,7\) and pancreatic beta cells\(^8,9\), but it is most characterized on type two taste cells for its role in taste transduction. When sweet, umami, or bitter tastants bind to a G-protein coupled taste receptor on a type two taste cell, this results in IP\(_3\) release and binding to its receptor on an intracellular calcium compartment. The binding of IP\(_3\) to its receptor releases calcium into the cytoplasm for it to then bind and activate TRPM5. TRPM5 then undergoes a conformational change to allow for an influx of monovalent cations into the cell. This causes depolarization of the cell leading to the production of an action potential and the release of the neurotransmitter ATP into the synapse where neurons deliver the message of taste recognition to the brain for comprehension\(^2,3,10,11\).
Though TRPM5’s role in taste transduction is well defined, its role in vascular development is yet to be described.

### 3.3 References


Chapter 4: Determination of TRPM5’s Role in Vascular Development

This chapter illustrates the role of TRPM5 in vascular development within the context of our system.
4.1 Introduction

Sequencing data collected from zebrafish embryos with altered blood flow has shown changes in \textit{trpm5} transcript values in both endothelial cells and mural cells—cell types necessary for proper vascular assembly (Stratman, \textit{et al.}, unpublished data). Next is answering the pertinent question of ‘why?’ We now look to our in vivo model, the zebrafish, as well as an in vitro model, human umbilical vascular endothelial cells (HUVECs) to determine the role of TRPM5 in vascular development. We hypothesize that TRPM5 mediates vascular development via two independent pathways, one affecting endothelial cells and one affecting the vascular smooth muscle cells.

4.2 Results

4.2.1 \textit{In vitro} analysis of cultured endothelial cells with inhibited TRPM5

Though zebrafish provide a great model for vascular analysis, whole organisms introduce a level of complexity. Thus, we first started our studies of TRPM5 using cultured human umbilical vascular endothelial cells (HUVECs); this removes the complex variables of blood flow and other physiological conditions such as interactions with other types of cells from the model, and allows us to address the effects of TRPM5 on endothelial cells in a minimal model.

To our cultured cells, we added a TRPM5 small molecule inhibitor, Ketoconazole\textsuperscript{1} (Fig 4.1A). This allows us to ask the question ‘how do endothelial cells—and endothelial cells only—respond to the inhibition of TRPM5’.
Under normal conditions, HUVECs are motile and form tube-like structures in our three dimensional in vitro model, but when Ketoconazole was added to the cultures, the treated cells did not form the normal tube structures (Fig 4.1B,D). In fact, they did not form tubes at all (Fig 4.1C).

4.2.2 Endothelial cells in vivo: Cranial vessels

We then tested this inhibitor in our zebrafish model to see if a similar phenotype, i.e. lack of endothelial cell motility, occurred in vivo. Endothelial cell migration and expansion of the cranial vasculature in the midbrain of the zebrafish occurs starting around 32hpf². Ketoconazole was added at 25uM to the water with zebrafish embryos at 30hpf, and cranial vessels were imaged at 78hpf, allowing for inhibition of TRPM5 throughout the key time for cell movement and vessel expansion.

To analyze vessel expansion and the endothelial cell motility required for this event, the number of branches to the vascular network were counted in the midbrain of the zebrafish. The vessels between the middle cerebral vein (MCeV) and the mesencephalic vein (MsV)³ behind the eye provided boundaries for analysis (Fig 4.2A). Vessel expansion was significantly impaired in the ketoconazole treated embryos compared to control siblings (Fig 4.2C). Quantification of vessel branches, or the amount of new vessel directions after an intersection, between the MCeV and the MsV showed that fish treated with ketoconazole had a dramatic decrease—approaching significance—in the number of branches compared to control fish. Addition of additional animals to the analysis will likely bring this data to significance.
4.2.3** Mural cell-Endothelial cell association increases with Ketoconazole**

So far, we have only considered one of the cell types required for vascular development and long-term stabilization—the endothelial cell. The recruitment of and stabilization provided by mural cells, a perivascular cell population that works like bricks and mortar around the ‘hallways’ of the blood vessels, are also necessary for proper vessel assembly. At 36hpf, endothelial cells begin to produce PDGFb, a signaling molecule used to recruit mural cells to the newly formed vessels. Our goal is to test whether embryos deficient in TRPM5 activity have normal mural cell recruitment and organization around the vasculature.

Zebrafish embryos were treated with 12.5uM of Ketoconazole at 24hpf and RNA was collected at either 48hpf or 102hpf for qPCR analysis (Fig 4.3A,C), with the chemical inhibitor exchanged every 24 hours. *pdgfb* expression in zebrafish treated with the TRPM5 inhibitor increased at the 102hpf time point, consistent with the time range for regulation of mural cell recruitment. This indicates that endothelial cells increase mural cell recruitment molecules under inhibited TRPM5 conditions.

To check for phenotypic consequences of the increased PDGFb expression, changes in mural cell-endothelial cell association on the dorsal aorta and cardinal veins of the zebrafish were analyzed (Fig 4.4A). Zebrafish embryos were treated with 12.5uM of ketoconazole starting at 30hpf until they were imaged at 102hpf. Embryos treated with the TRPM5 inhibitor showed a significant increase in mural cells associated with the dorsal aorta (Fig 4.4B,C), while mural cells associated with the cardinal vein did not show any significance (Fig 4.4C)

TRPM4 is another calcium activated, monovalent cation permeable membrane channel that resides on the membranes of endothelial cells that has structural and functional similarity to
TRPM5\textsuperscript{5-9}. To ensure that the phenotypes observed are solely due to the inhibition of TRPM5, transcript values of both TRPM4 isotypes were analyzed. TRPM4a/b transcript values did not significantly change in response to TRPM5 inhibition, suggesting that TRPM4a/b do not genetically compensate for TRPM5 in loss of function conditions (Fig 4.3B)

4.2.4 TRPM5 is involved in endothelial cell motility and mural cell recruitment

The inhibition of TRPM5 seems to be mediating two distinct phenotypes during development of the vasculature. One of these phenotypes is the reduction in endothelial cell motility. pErk1/2 is a transcriptional regulator that translocates to the nucleus to upregulate genes necessary for cell motility\textsuperscript{10-12}. To analyze pErk1/2 activity, zebrafish embryos were treated with 50uM of ketoconazole at 24hpf and fixed and immunostained for pErk1/2 at 78hpf (Fig 4.5A). Under control conditions, pErk1/2 nuclear patterning overlaps with fluorescently-tagged endothelial cells (Fig 4.5B Top). This shows that these endothelial cells are pErk1/2 positive. Zebrafish treated with the TRPM5 inhibitor showed no overlap with p-Erk1/2 nuclear activity, indicating that p-Erk1/2 is not actively transcribing genes necessary for motility resulting in motile cells (Fig 4.5B Bottom).

4.3 Discussion

In conclusion, TRPM5 is a blood flow-regulated gene (Fig 2.3). Inhibition of this membrane channel results in two phenotypes: 1) a decrease in pErk1/2 activity resulting in less cellular motility, and 2) an increase in mural cell-endothelial cell interactions due to an increase in endothelial PDGFb production (Fig 4.6).
4.4 Methods

Zebrasfish Lines

Transgenic zebrafish lines used for this study include: Fli:eGFP and Flk:mCherryCAAX;tagln:GFP.

Reagents

Reagents used in this study include: Ketoconazole, fish water, tricaine, p-erk1/2 antibody, all the qPCR probes, 4% PFA.

HUVECs

Remove media that is already in the T-25s. I added 5mL of PBS and rocked the flask to wash the cells. I then removed the PBS and added 1mL of trypsin which was then put in the incubator (temperature) for about a minute. The flask is then removed from the incubator and 1mL of FBS is added followed by 3mL of 1xM199. The cells and the media were then all transferred to a 15mL conical tube and placed in a centrifuge for 5 minutes at 1500 RPM. After the 5 minutes, the media was removed from the tube. 5mL of 1xM199 was then added to the tube and the pellet of cells was broken up. The cells were then spun again for 5 minutes at 1500 RPM and the media removed. 90uL of 1xM199 was then added and the cells resuspended. 50uL were then transferred to a new tube and gently mixed with 200uL of collagen gel which contained 353uL of collagen with a concentration of 10.58ng/mL, 58.5uL of 10xM199, 780uL of 1xM199, 2.5uL of NaOH, 6uL of SCF, 6uL of IL3, 3uL of SDF, and 3uL of FGF. 30uL of the gel+cell mix was then added to the wells of a 96-well plate. The collagen was allowed to polymerize for 20 minutes. 5mL of 1xM199, 10uL of IGF2, 50uL of AA, and 10uL of FGF were added to a tube
and 100uL of the mix were added to each of the wells containing the polymerized gel+cell mix. The cells were kept in the incubator for 48 hours before the media was removed and 100uL of 4% PFA was added.

**Zebrafish imaging of vasculature**

For imaging the cranial vessels, zebrafish embryos were exposed to Ketoconazole or control at 30hpf. At 54hpf and 78hpf, both water and drug were replaced. At 102hpf, fish were then fixed using 4% PFA.

For imaging of the dorsal aorta, zebrafish embryos were exposed to Ketoconazole or control at 30hpf. At 54hpf and 78hpf, both water and drug were replaced. At 102hpf, fish were then either fixed using 4% PFA.

For imaging of immunostained embryos, zebrafish embryos were treated at 24hpf with 50uM of Ketoconazole and fixed at 78hpf with 4% PFA for 30 minutes before adding being submerged in PBS.

All samples were mounted on slides with 0.1% agarose in fish water. All images were taken using W1 spinning disc, at magnification of 20x.

**Immunostaining**

After fixation, fish were rinsed with PBSTw (PBS + 0.1% Tween20) and milliQ water. The embryos were then permeabilized with PBSTw/1% Trixonx100 for 30 minutes. The embryos were then rinsed with water and Citrate buffer (C6H5NA3O•2H2O/0.05% Tween20) was added for 15 minutes. The fish were then heat treated in the citrate buffer for at 95 degrees Celsius for 20 minutes. The embryos were left to cool back to room temperature and then rinsed with
PBSTw. They were then blocked in PBSTw+5% donkey serum for 30 minutes. p-Erk1/2 antibody was diluted to 1:1000 in PBSTw and was added to the fish for an overnight incubation. The fish were then washed with PBSTw 5 times at 20 minute each. A 1:1000 dilution of RB 633 antibody in PBSTw was then added to the embryos and left to incubate overnight. The fish were then washed with PBSTw 5 times at 20 minute each followed by a rinse with PBS. 1:500 of HOECHST/DAPI in PBS was then added for 30 minutes. The samples were then rinsed 3 times with PBS.

RNA extraction and qPCR Analysis

RNA extractions of zebrafish embryos were completed following the Total RNA Mini Kit (Tissue) (IBI Scientific, IB47302).

cDNA was performed using 800ng of RNA from each sample up to 16uL. Water was added to bring the total up to 16uL in each tube. 4uL of reverse transcriptase was also added to each of the tubes. PCR machine settings include running the samples at 25 degrees Celsius for 10 minutes, then 50 degrees Celsius for 10 minutes, followed by 83 degrees Celsius for 5 minutes, and a 4 degrees Celsius hold.

1.5uL of each cDNA sample was diluted to 1:1 in milliQ water. 2uL of the diluted cDNA, along with 5uL of the 2xMM, 0.75uL of the Taqman primer of interest, and 2.75uL of milliQ water were added to the wells of a 96-well plate. The plate was then placed in the qPCR machine for analysis. The experiment type is Comparative CT and the run mode was set to Fast. Additional settings include running the plate at 95 degrees Celsius for 20 seconds, followed by 40 cycles of 95 degrees Celsius for 3 seconds and 60 degrees Celsius for 30 seconds.
4.5 Figures

Figure 4.1. HUVEC tube formation is reduced with the addition of the TRPM5 inhibitor Ketoconazole.  A) Structure of Ketoconazole, from MSG ERC.  B) Ketoconazole was added to HUVECs at 50uM and 25uM concentrations.  HUVECs were imaged at 48 hours.  C) Graphical representation of tube area under control or Ketoconazole conditions.  D) Diagram representing the HUVEC experiment performed where cells were seeded and either exposed to Ketoconazole or not for 48 hours.  p = <0.05
Figure 4.2. Zebrafish embryo cranial vasculature decreases with TRPM5 inhibition. A) Diagram representing area of the fish imaged. B) Quantification of branches between the MCeV and MsV. C) Zebrafish embryos were treated with Ketoconazole at 25μM and imaged at 78hpf. MCeV, middle cerebral vein; MsV, mesencephalic vein; p = <0.05
Figure 4.3. Transcript values in zebrafish embryos treated with Ketoconazole. A) Diagram showing the steps for qPCR analysis. (1) zebrafish samples were collected for (2) RNA extraction followed by (3) generation of cDNA. (4) qPCR was then performed using Taqman probes for genes of interest. This was then (5) ran on qPCR machine for a transcript value readout. B) 50uM of Ketoconazole was added to zebrafish embryos at 24hpf and RNA was extracted at 48hpf for qPCR analysis. C) Zebrafish embryos were treated with Ketoconazole at 12.5uM at 24hpf or 30hpf and fixed at 48hpf or 102hpf. All qPCR samples were normalized to GAPDH with a value of 1. p = <0.05
Figure 4.4. Arterial vascular smooth muscle cell recruitment increases with Ketoconazole. A) Diagram showing the area of the zebrafish that was imaged. B) Images of the dorsal aorta (magenta) and mural cells (arrows) in both the control and ketoconazole treated fish. C) Graphical representation of the number of mural cells recruited to the dorsal aorta or cardinal vein. p = <0.05
Figure 4.5. ERK1/2 expression decreases with Ketoconazole.  A) Diagram representing the area of the zebrafish embryo imaged.  B) Fli:eGFP transgenic labeled zebrafish embryos were treated with 50uM of Ketoconazole at 24hpf and fixed at 78hpf.  Panels from left to right show either endothelium or Erk1/2 staining single channels, the two channels merged, and a zoomed in area of the merged image.
Figure 4.6. Proposed pathways for TRPM5’s role in vascular development. TRPM5 may take part in two distinct pathways. The first through the activation of p-ERK1/2 which is a transcription factor mediating genes necessary for cell motility. The second pathway is through the upregulation of PDGFB which recruits vSMCs to blood vessels. PDGFB = platelet derived growth factor beta; vSMCs = vascular smooth muscle cells; p = <0.05; Figure was designed using BioRender.

4.6 References


Chapter 5: Conclusion

This chapter provides concluding comments and future directions for the project described in this thesis.
5.1 Concluding Comments

Throughout development, endothelial cells sense changes in blood flow to alter the structure of the vasculature. The different blood flow types—laminar versus pulsatile—recognized by endothelial cells influence the KLF2-PDGFb pathway. The production of PDGFb recruits mural cells, structural support-providing cells, to the endothelium. The Stratman Lab’s goal is to understand how endothelial cells sense changes in blood flow to mediate this developmental process using 3D in vitro culture modeling assays with human cells and zebrafish. Zebrafish provide a great model for studying such questions about vascular development due to their easy genetic modifiability, transparent appearance, lack of blood flow dependence for the transport of nutrients and waste up to five days post fertilization, and similarities to human vascular development.

In preliminary experiments, a pharmacologic inhibitor, BDM, was used to stop the beating of embryonic zebrafish hearts. Tagged ribosomes from endothelial or mural cells were collected and the mRNA associated with these ribosomes were purified and sequenced. Sequencing results showed transcriptional changes—an increase in endothelial cells and a decrease in mural cells—in the gene trpm5.

TRPM5 is a transmembrane channel that is activated by intracellular calcium and permeable to monovalent cations. This channel can be found on endothelial cells, mural cells, tuft cells, and pancreatic beta cells, but it is most characterized on the type two taste cell for its role in taste transduction. When sweet, umami, or bitter stimuli bind to G-protein coupled receptors on type two taste cells, IP$_3$ binds to its receptor on intracellular calcium deposits. Upon binding of IP$_3$ to its receptor, calcium is released and binds to TRPM5 which allows for an influx
of monovalent cations and depolarization of the cell. This depolarization leads to the release of ATP which serves as a neurotransmitter to neurons which transduce the message of taste to the brain for comprehension.

The role of TRPM5 in taste transduction is well characterized, but its role in vascular development is not well described. The scope of this thesis was to answer some of the fundamental questions of how TRPM5 alters endothelial and mural cell behavior and function. A pharmacological small molecule inhibitor—Ketoconazole—was first added to HUVECs, and the phenotype of a reduction in tube formation was observed. A similar phenotype was observed in the zebrafish embryo; the addition of Ketoconazole resulted in a decrease in cranial vessel formation. To investigate a potential mechanism for the lack of tube/vessel formation, the activation of p-Erk1/2 was analyzed. p-Erk1/2 is a transcriptional regulator that increases transcription of genes involved in endothelial cell motility. Immunostaining of zebrafish embryos for p-Erk1/2 showed a decrease in p-Erk1/2 activation in fish treated with Ketoconazole, insinuating that TRPM5 is involved regulating in Erk1/2 activity.

Increased mural cell recruitment to the dorsal aorta was also observed with inhibited TRPM5. qPCR showed an increase in pdgfb, the mural cell recruitment molecule. We think that TRPM5 also plays a role in promoting increased PDGFb production to recruit more mural cells to the vessels. We conclude that TRPM5 plays two roles in vascular development: 1) decreasing pErk1/2 activity resulting in less endothelial cell motility, and 2) increasing the production of PDGFb to recruit more mural cells.