FGF8a is Required for Proper Vascularization of the Zebrafish Retina

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ABSTRACT

Fibroblast growth factors (FGFs) are critical in many aspects of embryonic development and other cellular functions including apoptosis, cell adhesion, and proliferation. FGF8a, specifically, is known to initiate retinal ganglion cell (RGC) differentiation along with FGF3 early in retinal development (Martinez-Morales et al., 2005b). There has been little research into later roles for FGF8a in eye development. Here we show mRNA expression of *fgf8a* in the presumptive RGCs of 2 day-old zebrafish, past the time of RGC differentiation (28-48 hours) (Schmitt and Dowling, 1996). In addition, mRNA expression of putative receptor, FGFR1b, was localized outside the retina on the presumptive vasculature. Acerebellar (*ace*) mutants lacking FGF8a show mispatterned retinal vasculature and a lack of blood flow through the eye at 48 hpf. Further, we looked to see if this lack of blood flow had any effect on the developing neural retina. We found a significant reduction in the size of *ace* mutant eyes and also a reduction in total cell numbers in the retina starting at 48 hours post fertilization (hpf) suggesting a role for *fgf8a* in neurovascular signaling. The cause of the small eye phenotype was found to be due to a lack of proliferating cells and not an increase in cell death. We hypothesized if this phenotype was a result of a lack of blood flow to the retina. It has previously been reported that zebrafish survive and develop normally for 7 days without blood flow as the embryo receives nutrients by simple diffusion with its surroundings (Sehnert et al., 2002). To investigate the role that blood flow plays on the developing retina we utilized a silent heart mutant (*sih*) fish line, which lacks cardiac troponin t resulting in embryos without blood flow, as heart contractility does not initiate. To explore lack of blood flow to the retina as a cause for the observed *ace* mutant phenotype, *sih* mutant eye phenotypes were assessed. Retina cell counts from these embryos show a decreased eye diameter and a loss in total retina cell numbers due to lack of proliferation, phenocopying *ace* mutants. *sih* mutants also show a mis-patternning of their retinal vasculature with ectopic vessel branches similar to *ace* mutants. Our data support the small eye phenotype seen in both mutants is a result due to lack of proliferation. After morpholino knock down of the receptor, *fgfr1b*, we see mispatterend vasculature that phenocopies what we see in *ace* mutants. These finding led us to hypothesize that FGF8a, secreted by the RGCs, signals through its receptor, FGFR1b, on the retinal vasculature to promote cell growth and development. Further these data suggest that the retinal vasculature subsequently responds by secreting an unknown factor to support the proliferation and maintenance of the RGCs.
ACKNOWLEDGEMENTS

The completion of this degree is in large part thanks to numerous advisors, mentors, colleagues, friends, and family. First and foremost I would like to give my sincere gratitude to my advisor Dr. Alicia Ebert. She was able to take a very green college graduate and turn me into a scientist. She has continually supported me in my research goals with a patient and supportive attitude. From teaching me new techniques, editing my abstracts and posters, and listening to my ideas, to helping me deal with the stresses of everyday life in graduate school, Alicia has always been there for me to turn to. I would also like to thank Dr. Bryan Ballif who has provided me with numerous reagents, and countless ideas and words of wisdom in science and in ping-pong. The other members of my committee, Dr. Paula Deming and Dr. Rae Nishi also deserve a great deal of thanks. Both of them have been supportive of me and provided me with hours of advice in various aspects of this project.

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Finally, I would not have made it without the love and support from my amazing family who has seen me through tears of joy and tears of sadness over these past three years. To them, I will be forever grateful.
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LITERATURE REVIEW

Natural Habitat and Behavior

Since the early 1980’s *Danio rerio*, or zebrafish, have been building momentum as a preferred model organism for the study of developmental biology. Due to their small size, ease of rearing, large clutch sizes, and ease of genetic manipulation, many scientists began to use these versatile fish for research. Early ground work with genetic manipulation of zebrafish showed that it was possible to create homozygous mutant fish lines making them ideal to study development and embryology on a molecular scale (Streisinger et al., 1981). These studies along with others, propelled research in other areas such as disorders of the nervous system and descriptions of differentiation, further solidifying the zebrafish as a reliable model organism (Kimmel, 1989). With zebrafish having such potential in the world of developmental biology, Granato and Nüsslein-Volhard performed a large scale mutagenesis screen on the zebrafish generating over 4,000 mutations leading to the identification of 400 genes responsible for several aspects of development (Granato and Nüsslein-Volhard, 1996). In 2001, the Sanger Institute in Cambridge, Massachusetts sequenced the entire zebrafish genome, granting scientists the advantage of being able to easily locate and create mutations as well as make comparisons to the human genome. Today, the zebrafish model system is being used to help make strides in biomedical research (Brittijn et al., 2009; Dooley and Zon, 2000; Köhler et al., 2013), drug screens (Ali et al., 2011; Rubinstein, 2006), and modeling human disease (Barut, 2000; Berghmans et al., 2005; Santoriello and Zon, 2012).

The zebrafish is a common freshwater fish of the family Cyprinidae. These small fish are
typically less than 120 mm in total length and can be recognized by their distinctive color patternning (Spence et al., 2008). They have dark and light alternating stripes that may be broken into spots or bars. These fish can also be distinguished by their incomplete lateral line, which is a structure that runs along the side of the fish and is used to detect changes in water pressure allowing fish to detect prey and school properly. Along the lateral line are neuromasts, sensory organs composed of hair cells similar to those seen in the vertebrate inner ear (David et al., 2002). The lateral line, which normally extends the length of the body, does not extend beyond the pelvic fin in zebrafish (Barman, 1991).

Francis Hamilton first described the zebrafish in his book, *An Account of the Fishes Found in the River Ganges and its Branches* in 1822 and now there are 44 known Danio species that range from South east Asia to India with their highest density being in the north eastern part of India, Bangladesh, and Myanmar (Barman, 1991) (Figure 1.1). These fish are naturally found in 6-37°C, slow moving to standing water around the edges of streams adjacent to rice fields (Talwar, 1991). The reason for their location is speculated to be that fertilizer used in rice fields promotes the growth of zooplankton, a common staple in the zebrafish diet. The rice field habitat is also a safe haven from large fish that commonly reside in rivers and larger bodies of water, zebrafish also prefer to mate in the shallow waters provided by the rice fields (Spence and Smith, 2007). Zebrafish are omnivorous and in addition to zoo plankton, they enjoy a diet including insects, filamentous algae, invertebrate eggs, and vascular plant material (McClure et al., 2006).
Figure 1.1 Zebrafish geographical locations. This map indicates the areas that have reported wild zebrafish colonies (black dots). (Spence et al., 2008) Permission granted by John Wiley and Sons, license number: 3597160992837.
In laboratory conditions, zebrafish are maintained to breed year round. However, in the wild they follow a more regimented spawning cycle with the seasons. Males and females are stimulated to breed at the sign of first light (Darrow and Harris, 2004). Males instinctively chase the females to an appropriate spawning area and then begin to swim alongside of them. The male continues to nudge the female oscillating his body to trigger simultaneous oviposition and sperm release. Females will typically release eggs in spurts of 5-30 eggs with a potential to release over 200 eggs in one spawning. The number of eggs produced by a female is positively correlated with her age and size until the female is past her prime breeding age upon which time she begins to produce fewer eggs as she ages. As soon as the eggs come in contact with the water they are partially activated even in the absence of sperm. Initially, the egg undergoes a series of developmental steps in preparation for fertilization. Initially, the size of the egg is reduced and a separation of the chorion from the plasma membrane occurs. The egg will then undergo several fruitless cleavages but without sperm will not develop further (Lee et al., 1999). Once fertilized, the embryo, allowed to develop at 28.5°C undisturbed, will develop rapidly and will hatch out of its protective chorion between 48 and 72 hours post fertilization (hpf) (Darrow and Harris, 2004). In the wild the zebrafish are preyed upon by snakeheads (Channa) and garfish (Xenehtodon cancila) (Engeszer et al., 2007). The average lifespan for a zebrafish in captivity is around 2 years (Brand et al., 2002).
Scientific Classification:

Kingdom: Animalia - multicellular, eukaryotic organism
Phylum: Chordata - possesses a notochord
Class: Actinopterygii - ray finned fishes, subclass of the boney fishes
Order: Cypriniformes – order of ray finned fishes
Family: Cyprinidae – carps and minnows
Genus: Danio
Specia: rerio

**Developmental Timetable**

Zebrafish embryos are transparent and because of this, the developmental stages have been well characterized. The stage of development of an individual clutch of zebrafish may vary depending on the variation in time of fertilization of each egg. Each embryo however, will follow the same basic developmental stages (Table 1.1 and Figure 1.2).

The first cleavage of the embryo occurs around 40 minutes post fertilization. Before this time, the embryo is referred to as a zygote. Cell divisions in early fish embryos are meroblastic meaning they do not divide the entire embryo. Instead, they divide only the animal pole and leave the yolk undivided. Following the first cleavage, the cells or blastomeres of the embryo will divide once approximately every 15 minutes until the 128 cell stage. At this time, the embryo is now in the blastula stage and looks like a ball of cells on top of the yolk. During the blastula stage, the embryo undergoes the mid-blastula transition where it turns on its own gene transcription and is no longer reliant on maternally deposited mRNA. At this time a yolk syncytial layer is formed, a developmental step that is common among many fish. The yolk syncytial layer plays
Table 1.1 Zebrafish developmental timetable. (Kimmel et al., 1995) Permission granted by John Wiley and Sons, license number: 3597210234295.

<table>
<thead>
<tr>
<th>Period</th>
<th>h</th>
<th>Description</th>
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<tbody>
<tr>
<td>Zygote</td>
<td>0</td>
<td>The newly fertilized egg through the completion of the first zygotic cell cycle</td>
</tr>
<tr>
<td>Cleavage</td>
<td>¾</td>
<td>Cell cycles 2 through 7 occur rapidly and synchronously</td>
</tr>
<tr>
<td>Blastula</td>
<td>2¼</td>
<td>Rapid, metasynchronous cell cycles (8, 9) give way to lengthened, asynchronous ones at the midblastula transition; epiboly then begins</td>
</tr>
<tr>
<td>Gastrula</td>
<td>5¼</td>
<td>Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly</td>
</tr>
<tr>
<td>Segmentation</td>
<td>10</td>
<td>Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears</td>
</tr>
<tr>
<td>Pharyngula</td>
<td>24</td>
<td>Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development</td>
</tr>
<tr>
<td>Hatching</td>
<td>48</td>
<td>Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously</td>
</tr>
<tr>
<td>Early larva</td>
<td>72</td>
<td>Swim bladder inflates; food-seeking and active avoidance behaviors</td>
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Figure 1.2 Zebrafish development. Images of the average developmental stages and characteristics of zebrafish embryos from zygote (A) through 48 hpf (H, I). Adapted from (Kimmel et al., 1995). Permission granted by John Wiley and Sons, license number: 3597210552546.
many roles in early development including coordination of patterning and morphogenesis (Carvalho and Heisenberg, 2010). At the end of the blastula stage, the embryo then proceeds into epiboly, or the first coordinated cell movements to enclose the embryo around the yolk. Halfway through epiboly (5.25 hpf) the beginning of involution at the dorsal shield marks the initiation of gastrulation and formation of the three primary embryonic germ layers ending by 10 hpf. Somitogenesis occurs starting at 10.5 hours and continues until 24 hpf. These somites are paraxial mesoderm that form as segments along the body axis of developing vertebrate which will later form the spine and rib cage (Stickney et al., 2000). From 24 to 48 hpf the embryo is in the pharyngeal period, which is the most comparable stage between all vertebrates during development. During this time the embryo will develop fins, pigment, and tactile movement in the form of bursts of swimming. During the final early developmental stage, the hatching period from 48 to 72 hpf, the embryo develops stronger movement capabilities, more fully developed organs, and strong blood flow. By 3 days postfertilization (dpf) the majority of the embryo’s morphogenesis is complete and it continues to grow. The final stage includes inflation of the swim bladder. (Kimmel et al., 1995).

**Zebrafish as a Model for Development**

The zebrafish has many characteristics that make it an exciting and ideal model for developmental biology. Initially it is attractive because it is relatively easy to rear and maintain for a reasonable cost. Females are easy to breed and can produce upwards of 200 embryos at each spawning. This allows for quick turnover and large sample sizes for experiments. Not only are they functionally attractive, they are molecularly attractive as
well. There have been numerous genetic screens providing various mutant lines for study (Dooley and Zon, 2000). Along with this, genes are being rapidly discovered that control aspects of development which allows for the use of hundreds of mutant zebrafish lines (Granato and Nüsslein-Volhard, 1996). The zebrafish can be more useful than the traditional mouse model to study development because the zebrafish develops rapidly, is externally fertilized and is transparent (Dooley and Zon, 2000) (Figure 1.3). Along with the benefit of transparency, the zebrafish is also easy to manipulate genetically. This allows scientists to genetically engineer tags for various structures within the zebrafish that can then be visualized throughout development without needing to sacrifice the animal. This ease of genetic manipulation also allows for relatively easy development of mutant fish lines, and use of many biological tools such as morpholino knockdown, CRISPR and TALEN knock out and the cre-lox system.

**Zebrafish Eye Development**

The eye is an extremely important organ in most organisms. Without this method of light detection for vision, many organisms would struggle to survive with an impaired ability to search for food, shelter, or mates. They would also be left unaware of dangerous predators that would threaten them. For this reason, the eyes of many insects and vertebrates have evolved to be very large with a great deal of emphasis placed on the proper development and wiring of the eye.

The vertebrate eye, the most prominent organ in the head, is composed of three parts, the cornea, lens, and the retina (Graw, 2010). The zebrafish is a highly visual animal that
Figure 1.3 Zebrafish as a model for development. (A) Zebrafish are laid in large clutches, are transparent, externally fertilized and develop rapidly. (B) 72 hpf flk:GFP embryo with GFP-expressing vasculature. (C) Large genetic screens have resulted in numerous genetic mutants that can be utilized to study many genes of interest and their effects on development.
responds to light by 60 hpf and shows visual alertness as early as 72 hpf. The rapid completion of the visual system signifies the need and importance of visual system. By 5 dpf the embryo has smooth eye movements and is able to track illuminated stripes (Brockerhoff et al., 1995). For most vertebrates, eye development is not solely important for visual acuity, the eye has other roles designed to keep the organism thriving including the control of circadian rhythms. Especially important are the retinal ganglion cells (RGCs); these cells are not only responsible for relaying visual information to the brain via their projections as the optic nerve, they are also responsible for expressing melanopsin. Melanopsin is a photopigment that is responsible for regulating circadian rhythms and constriction of the pupil (Panda et al., 2003). The zebrafish makes a wonderful model for studying vertebrate eye development due to their rapid development, the lamination of the retina being completed by 72 hpf, and because they are highly related to humans allowing for research into potential sources of human function and disease. Due to the common function of the eye among vertebrates, the biochemistry, morphology, and physiology are highly conserved. The cell types and lamination patterning of the retina are consistent in vertebrates along with the initial developmental transcription factors as will be discussed below (Fadool and Dowling, 2008).

Development of the zebrafish eye is a complex orchestration of events that must happen in a specific fashion in order to achieve optimal results (Figure 1.4). Initially during gastrulation, a single eye field is established in the center forebrain area. During the establishment of the midline, the eye field is split in two in response to SHH release (Graw, 2010). Early ocular tissue is specified by signals from transcription factors Six3a
and Pax6. During neurulation in response to retinoic acid and the transcription factor Rx3 (Fadool and Dowling, 2008), the two evaginations form two optic pits as the lateral wall of the diencephalon bulges out and enlarges to form two optic vesicles connected to the forebrain by the optic stalk. Without Six3a and Pax6, the retinal precursors do not evaginate and this results in fish with no eyes (Fadool and Dowling, 2008). The optic vesicles begin to invaginate forming optic cups with two layers, the outer layer which will form the neural retina, and the inner layer which will form the retinal pigmented epithelium (RPE) (Graw, 2010; Schmitt and Dowling, 1994, 1999). As the optic vesicles bulge out, they come into contact with the surface ectoderm, the contact between these two tissues results in a thickening of the pre-lens ectoderm and forms the lens placode. At the other end of contact, the retinal disc is formed. Without these events, the retina will not become specified (Graw, 2010; Hyer et al., 2003).

The newly forming eyes must differentiate into the 6 neuronal cells types and 1 glial cell type of the neural retina. Early signals from paired box gene 6 (pax6), orthodenticle homolog 2 (otx2), and retina and anterior neural fold homeobox (ray) initiate the production of retinal precursor cells (RPCs). RPCs will continue to differentiate into the specific neuronal cell types of the retina based on specific transcription factor cocktails that they are exposed to as development continues (Bassett and Wallace, 2012). As the RPC goes through the various stages of the cell cycle, it follows a specific pattern of interkinetic nuclear migration. In this fashion, the cell will move its nucleus up and down in an apical to basal manner until it receives cues from its local signaling environment to exit the cell cycle and
Initially the eye field is split during the late gastrula stage. This is followed by the invagination of the lens placode, which will eventually close during the optic cup stage. At this time, the two placodes begin to differentiate into the lens and retina fields. Initially, the eye field is split in two.
differentiate. Following this pattern, RPCs always are in mitosis at the most apical surface (retinal pigmented epithelium or RPE) of the retina and are in S phase at the basal surface (lens) (Baye and Link, 2007) (Figure 1.5). These cells are organized within the retina in three major layers (Figure 1.5). The ganglion cell layer, closest to the lens, houses the retinal ganglion cells and some displaced amacrine cells. The ganglion cells are responsible for relaying visual information to the brain through their axonal projections, which leave the eye as the optic nerve. 100% of these projections then cross the optic chiasm and innervate the brain in the optic tectum. This is in contrast to mammals where only some of the RGC projections cross the optic chiasm while others remain ipsilateral depending on the location of the visual stimuli. Also, while fish retinal projections innervate in the optic tectum, in mammals there is an intermediate innervation in the lateral geniculate nucleus before reaching the tectum as well as other direct pathways. In mammals, there can be direct innervation of RGCs to the suprachiasmatic nucleus to control circadian rhythms or to the pretectum to control pupil adjustments (Wassle, 2004). In vertebrates the RGCs are the first neuronal cells of the eye to differentiate and in zebrafish this translates to cells exiting the cell cycle around 28-32 hpf (Schmitt and Dowling, 1994) (Figure 1.6 A). Following this, there is a wave of differentiation associated with expression of sonic hedgehog (SHH) from various cells. The wave of differentiation starts in an area referred to as the ventro-nasal patch and spreads around the retina from ventral nasal, to dorsal temporal (Das et al., 2003; Shkumatava et al., 2004). The other layers of the retina, the inner and outer nuclear layers, containing the bipolar cells, horizontal cells, amacrine cells, muller glia, rods and cones, are beginning to laminate by 48 hpf and are finished by 72 hpf (Schmitt and
Dowling, 1999) (Figure 1.6 B). The rods are among the last to differentiate with the first rod responding by 50 hpf (Schmitt and Dowling, 1996).
Figure 1.5 RPC interkinetic nuclear migration and organization of the retina. (A) RPCs migrate from apical to basal with each stage of the cell cycle. Mitosis occurs at the apical surface while S phase occurs at the basal surface. When the cell differentiates (red) it will find its place in the correct layer of the retina. (B) A cross section of a 3 dpf zebrafish retina indicates the lamination created by the neuronal cell types. The layer closest to the lens houses the retinal ganglion cells. The next proceeding layer is the inner plexiform layer. Then is the inner nuclear layer containing bipolar, horizontal, and amacrine cells. The next layer is the outer plexiform layer. And the final layer, the outer nuclear layer, houses the photoreceptors. Figure part A adapted from (Baye and Link, 2007), permission granted by the official journal of the society of neuroscience, license number: 3597220544197and part B adapted from https://groups.oist.jp/dnu/fy2011-annual-report.
Figure 1.6 Retinal neurogenesis. Retinal precursor cells give rise to all of the cell type of the retina at varying time points in development. While the order of neuronal birth is consistent amount vertebrates, the developmental time varies from species to species. Image adapted from (Bassett and Wallace, 2012). Permission granted from Elsevier, license number 3600411186779.


**Zebrafish Retinal Vasculature Development**

This project focuses not only on the proper development of the zebrafish retina, but also on how this is influenced and dependent on the proper patterning of the surrounding vasculature. It is commonly understood that in order for vertebrate cells to survive, divide, and even die properly they must be nourished by the vasculature. Lack of blood flow to the brain can lead to death within minutes for most vertebrates. How blood flow affects the development of the eye on the other hand is less well understood. Proper eye vascularization is imperative for proper vision with defects leading to various eye conditions including retinal pigmentosa and retinal degeneration. Proper vessel branching and the number of branches that result are crucial, and too few or too many can cause loss of vision (Gore et al., 2012).

In humans, the initial vasculature that supports the developing eye is the hyaloid vasculature. Unlike in zebrafish, human eye vascularization goes through a series of remodeling steps as the embryo develops. Initially, the hyaloid artery develops as a branch off of the carotid artery and enters the eye at the site of the primitive optic nerve. It then branches around the back of the lens forming the hyaloid vasculature and exits as an annular collection vessel at the front of the eye (Figure 1.7). This vasculature is eventually replaced by retinal vasculature by mid-gestation. Prior to any vessels developing, there is a framework set down by astrocytes allowing the eye and most of the brain to be vascularized through angiogenesis, or the branching of vessels off of already existing vessels (Fruttiger, 2007). Zebrafish on the other hand, show no evidence of this type of remodeling or scaffolding as seen in humans where it seems that hyaloid
**Figure 1.7 Eye vascularization.** Vessel development and remodeling as modeled in human development. (A) The hyaloid artery (ha) supplies the hyaloid vasculature (hv), which drains through the venous, choroidal net (ch). (B) The primary plexus (pp) replaces the hyaloid vasculature as it regresses. (C) The primary plexus gives rise to the deeper plexus veins. Adapted from (Fruttiger, 2007) Permission granted from Springer, license number 3597230910955.
regression and retinal angiogenesis is not required (Alvarez et al., 2007b).

Similar to humans, zebrafish vasculature must start out as endothelial and hematopoietic cells. In humans these cells are initially derived from blood islands however in zebrafish these same cells are derived from the intermediate cell mass initially from the mesoderm (Figure 1.8). Even though the cells are derived from different sources, in humans and in zebrafish, many of the genes responsible are held in common such as VEGFR2 (zebrafish ortholog flk1), a main receptor type for growth factors signaling vasculature development. In zebrafish, by 24 hpf the optic artery has already branched from the primitive carotid artery and begun to enter the eye at the area of the optic fissure to begin forming the hyaloid vasculature (Gore et al., 2012). By 48 hpf, cells labeled with flk:GFP are found between the lens and the retina and become distinguishable as vasculature by 2.5 dpf. These cells seem to initially be attached to the lens and then later begin to lose contact around 15 dpf. Sections from adult zebrafish eyes confirm that the hyaloid vasculature does come into direct contact with the RGCs and the inner limiting membrane (Alvarez et al., 2007b). The development of the lens also seems to play an important role in proper vascularization. Zebrafish that show lens development abnormalities show mispatterned hyaloid development with no disruption in trunk vessels. This seems to suggest that there may be signals specifically coming from the lens to help the vasculature develop properly around the eye (Alvarez et al., 2007a).

Various mutants exist for studying the vessel development in zebrafish including MAGP1, a loss of microfibril associated glycoprotein 1, and HS6ST-2, a loss of heparin sulfate sulfotransferase 2, mutants. These both show a lack of defined branches and
arrested development in the eye. More specifically, MAGP1 mutants suffer from dilated brain and caudal vessels with less hyaloid vascularization and thicker branches. HS6ST-2 mutants present with over-luminized vessels and incomplete caudal branches with less and mis-patterned hyaloid vasculature (Alvarez et al., 2007a).
Figure 1.8 Specification of endothelial cells during early embryonic development. (A) Endothelial precursors are specified in the ventral mesoderm. At (B) 14 and (C) 16 somites angioblasts are shown in green. (D, E) Live embryo fluorescent images (dorsal and lateral views respectively) showing endothelial cells in green at the same ages as B and C. Adapted from (Gore et al., 2012). Permission granted by Cold Spring Harbor Laboratory Press.
Fibroblast Growth Factors

Fibroblast growth factors (FGFs) have long since been known for many important biological functions the most well known being their roles in growth. In adult tissues they can have numerous functions in chemotaxis, cell migration, cell survival, and apoptosis. However, in development, their roles have been expanded to include an importance in mesoderm formation, gastrulation movements, neural induction, anterior-posterior patterning, and endoderm formation (Bottcher R, 2005).

FGFs are a large family of small, heparin binding, polypeptide growth factors that are found in vertebrates and invertebrates. There are 22 FGFs in humans and 28 in the zebrafish that are broken into subfamilies based on their structures (Bottcher R, 2005). These subfamilies are categorized into intracellular FGFs, hormone-like or endocrine FGFs, and canonical FGFs (Itoh and Ornitz, 2004). FGFs vary in size from 17-34 kDa in size but all share a conserved 120 amino acid region with 16-65% identify among the whole family (Eswarakumar et al., 2005). The majority of FGFs act by binding to and activating fibroblast growth factor receptors (FGFRs) of which there are 4 highly related members (Johnson and Williams, 1993). FGFs do have much lower affinity binding to other receptor classes such as syndecans and integrins and the results of these pairings are largely unknown (Murakami et al., 2008). FGFRs are single spanning transmembrane proteins with an extracellular binding domain, and an intracellular region with receptor tyrosine kinase activity. This intracellular domain also contains protein binding and phosphorylation sites and auto-phosphorylation sites (Schlessinger, 2000). The extracellular domain has 3 immunoglobulin like (Ig) domains denoted D1-D3. Hallmarks
of these domains include a positively charged docking site for heparin sulfates in the D2 region, and an ‘acid box’ in between D1 and D2 that acts as a linker. FGFRs are susceptible to alternate splicing which leads to various numbers and combinations of these Ig like domains. For proper binding and stabilization, the receptor requires specific ligand binding, heparin sulfate binding, and receptor dimerization (Eswarakumar et al., 2005). The binding of heparin sulfate is crucial for proper FGF binding to the receptor. This is an important level of regulation of the FGF/FGFR signaling cascade (Allen and Rapraeger, 2003). Heparin sulfates also protect the receptor from thermal denaturation, proteolysis (Gospodarowicz and Cheng, 1986), and limits the diffusion and release of the ligand (Moscotelli, 1987).

Proper binding of FGFs to their receptors allow for the activation of various pathways including the MAPK/ERK pathway which can lead to cell proliferation, the PI3K pathway supporting cell survival, or the PLC$\gamma$ pathway allowing for cell motility (Figure 1.9). Which pathway initiated is dependent on tissue specific signals, FGF/FGFR specific binding partners, splice variants of FGFs, and the specific heparin binding partners. The signaling cascade must also be controlled. There are various negative regulators of these pathways including members of the sprouty protein family and the sef protein family. There is also negative regulation through receptor uptake following activation. All of these factors can influence the strength and type of response initiated (Bottcher and Niehrs, 2005).
Figure 1.9. FGF signaling pathways. (A) Activation of the Ras/MAPK pathway that leads to increased cell proliferation. (B) Activation of the PI3K pathway that promotes cell survival. (C) Activation of the PLCγ pathway allowing for increased cell motility. Figure adapted from (Goetz and Mohammadi, 2013). Permission granted by Nature Publishing Group license number 3598840375176.
Mutant Embryo lines

Acerebellar (ace) mutant

FGFs are known to be influential growth factors in many aspects in development. This project focuses around the role of one specific fibroblast growth factor, FGF8a. More than 300 million years ago, there was a genome duplication during the evolution of the ray-finned fishes (Taylor et al., 2003). Over time, some of the duplicated genes were lost due to a presumed redundancy in function; others however were retained with believed new functions different from their original clone. This is the case for fgf8a and fgf8b. We choose to focus our study on fgf8a because of its expression pattern in the early retina. Fgf8b was not investigated because in situ hybridization studies show no expression in the eye at 48 hpf, the time point focus of this study (Figure 1.10). fgf8b does however share other overlapping expression patterns with fgf8a including the midbrain hindbrain boundary (MHB) and fin buds. In order to study this growth factor, we began by knocking down FGF8a by morpholino injection (see Appendix A). We then became aware of the acerebellar mutant fish line, which demonstrates strong to complete loss of FGF8a (Reifers et al., 1998). We switched to using this mutant for the consistency and ease of study that it would provide to us. However, morpholino based studies showed consistent results with those found using the mutant line (Appendix A).

FGF8 was originally found as an androgen induced growth factor but has since been found to be crucial in many aspects of development including a role in limb bud formation, forebrain development and tooth development. However, by far its largest role seems to be at the midbrain hindbrain boundary (MHB) organizing center. It is seen as early as
somitogenesis at this location in mice. The MHB helps derivate the optic tectum and the
tegmentum along with patterning of the midbrain and cerebellum (Reifers et al., 1998).
The use of zebrafish grants us an opportunity to study FGF8 in a faster and more cost
effective manner compared to mice. Zebrafish FGF8 shares 79% amino acid identity
with mice and human and 84% identity with chick (Figure 1.11). It also shows expression
in the same tissues as humans and mice including, mesodermal tissue, early MHB, and
several nervous system tissue sites (Reifers et al., 1998).

The mutation in zebrafish is considered to be strong to complete in its effectiveness of
depleting FGF8a. The loss of protein is due to a mutation of a guanine to an adenosine at
the 5' splice donor site following exon 2 (Figure 1.12 A). This point mutation leads to
inactivation of the splice site, skipping exon 2, and leads to a premature stop codon
(Figure 1.12 B). The amino acids from exon 2 and 3 that are eliminated due to this
mutation are responsible for the FGF binding and activating its receptor making the
shortened protein presumably non-functional. This mutant shows a weak phenotype
compared to the FGF8 mutant mouse, which does not complete gastrulation. The thought
is that zebrafish have more maternally derived FGF8, as detected by RT-PCR. This is less
available in mice since they have little maternal cytoplasm to draw from. The maternal
levels of fgf8a in zebrafish seem to be very low and undetectable by in situ hybridization
(Reifers et al., 1998).
Figure 1.10 mRNA expression of fgf8a and fgf8b at 48 hpf. Whole mount in situ hybridization shows the expression patterns of fgf8a and fgf8b overlap in areas such as the midbrain hindbrain boundary. Fgf8a shows strong expression in the retina (white star), while fgf8b shows no retinal expression at this time point (black star). Image from www.zfin.org
Silent heart (sih) mutant

In order to study the effect that blood flow plays on the development of the eye, we utilized the silent heart mutant fish line (sih<sup>tc300b</sup>). This fish line has the most severe mutation affecting heart contractility. This mutation, identified in a chemical gamma irradiation screen, results in a failure to the heart to initiate beating. This mutation is embryonic lethal by 7 dpf (Sehnert et al., 2002).
**Figure 1.11 fgf8a sequence alignment.** Amino acid sequence comparisons between zebrafish, chicken, mouse, and human. Arrows indicate exon boundaries, dashes indicate identical residues, and shared N-linked glycosylation sites are shaded. Adapted from (Reifers et al., 1998). Permission granted by Development, license number 3600820996681.
Figure 1.12 FGF8a mutation in acerebellar mutants. (A) Genomic structure and splice variants in wild type and acerebellar embryos. (B) A point mutation from a 100% conserved G at the splice site following exon 2 is replaced with an A leaving in exon 2. (C) acerebellar embryos lack exon 2 which causes a frame shift and a premature stop codon. Adapted from (Reifers et al., 1998). Permission granted by Development, license number, 3600820996681.
however it is reported to have minimal developmental deficiencies (Stainier et al., 1996). In a study to identify the protein responsible for the loss of contractility, it was determined to be cardiac troponin T (Tnnt2), verified by RT-PCR and morpholino knockdown (Sehnert et al., 2002). It was also reported that only the cardiac troponin was affected, influencing the assembly of thin filaments and improper sarcomere assembly only in the heart. Thick filaments however, were reported to be at wild type levels. As the mutant embryo develops it receives necessary nutrients and oxygen through diffusion with the water. However, these embryos begin to develop pericardial edemas around 30 hpf. As they continue to age the endocardium peels away from the myocardium and the sinus venosus and ventricle collapse by 3 dpf. The cause of this mutation is a point mutation where an adenosine is replaced by a guanine at the -2 position of the splice acceptor site in intron 2. As a result, a cryptic splice site is used resulting in a partial exclusion of exon 3 causing a frame shift and premature stop codon (Figure 1.13) (Sehnert et al., 2002). This mutant line gives us the perfect model to investigate questions regarding the influence of blood flow on development of the eye. These mutants show limited deficiencies up through 3 dpf.
Figure 1.13: Point mutation in *sih* mutant fish. *sih* embryos lack a functioning beating heart due to loss of cardiac troponin T (*tnnt2*). This mutation is due to a point mutation changing an adenine with a guanine at the -2 position of the splice acceptor site on intron 2. This causes a cryptic splice site to be used resulting in a partial inclusion of exon 3 leading to a frame shift and premature stop codon. Adapted from (Sehnert et al., 2002). Permission granted from Nature Genetics, license number, 3600811105575.
INTRODUCTION

How a single cell becomes a fully functional organism is a complicated orchestration of millions of cell signaling events and processes. If even a small event fails, this can lead to detrimental developmental effects. Vascular development is one of these key processes that must happen with precision and accuracy to ensure a healthy organism. The retina provides an ideal microenvironment to study the development of the vasculature and to investigate the overall effects on cell survival. The retina is supported by the hayloid, choroidal and retinal vasculatures in mammals. The hayloid is the first to form and it supports the retinal ganglion cells (RGCs). In mammals, the hayloid vasculature degenerates as the retina develops into a mature organ, this however does not happen in zebrafish (Kitambi et al., 2009). The retina is one of the most metabolically active tissues with an extremely high oxygen consumption rate (Alvarez et al., 2007b). Oxygen concentrations are critical to vasculature development in the retina. In adult zebrafish, when the eye is under hypoxic conditions, there is an increase in angiogenesis in the retina due to the induction of vascular endothelial growth factor (VEGF) through hypoxia-inducible factors (HIFs) (Cao et al., 2008).

Along with VEGF, fibroblast growth factors (FGFs) are intimately involved in angiogenesis by regulating the activation of endothelial cell proteases, which digest the basement membrane and allow for growth of new capillary-like structures (Antoine et al., 2005). FGFs are a large superfamily with 22 members in mammals and are instrumental in many developmental processing including pattern formation of the mesoderm, limb bud formation, and eye development (Bottcher R, 2005). Basic FGF (bFGF) was the first identified pro-angiogenic molecule and along with VEGF is responsible for development
and proliferation of endothelial cells (Cross and Claesson-Welsh, 2001). FGFs act by binding to one of four fibroblast growth factor receptors (FGFR) in mammals or one of five in zebrafish (Itoh, 2010). FGFRs are receptor tyrosine kinases that require heparin sulfate in order to bind FGF and initiate one of three signaling cascades (Eswarakumar et al., 2005). Tumor cells are known to express FGFs and VEGFs in order to promote angiogenesis for increased tumor growth and survival. This makes these two factors important targets for cancer drugs wherein these growth factors or their receptors are inhibited (Cross and Claesson-Welsh, 2001). While there is much known about FGFs in relationship to angiogenesis, its role in vasculature patterning of the eye is not well studied. During early eye development, members of the FGF family are important in optic vessel patterning, lens formation, and neuronal differentiation; FGF8, specifically, has been shown to be present in the developing optic stalk (Martinez-Morales et al., 2005b). FGF8, along with FGF3 trigger retinal progenitor cell differentiation into RGCs. Interestingly, loss of one of these factors is not enough to prevent differentiation (Martinez-Morales et al., 2005b). Proper retinal vascularization is also of key importance to neuronal survival and overall retinal health as it is responsible for providing nutrients and oxygen to the developing retina (Kitambi et al., 2009). Improper vascularization results in certain human eye diseases such as retinopathy of prematurity, diabetic retinopathy, and pathological retinal angiogenesis (Alvarez et al., 2007b; Gariano and Gardner, 2004). We demonstrate here, that FGF8a plays a crucial role in regulating proper retinal development and vasculature patterning. We hypothesize that FGF8a signaling from the RGCs promotes proper vasculature growth and patterning, which consequently nourishes the retina and controls further retinal development.
MATERIALS AND METHODS

Zebrafish Husbandry

All experiments were performed with approval of the University of Vermont Institutional Animal Care and Use Committee (Protocol #12-055). Breeding stocks were maintained and bred under standard conditions as previously described (Brand et al., 2002).

Mutant and Transgenic Fish Lines

The Acerebellar (ace) mutant fish line was a gift of Dr. Bruce Riley from the Biology Department at Texas A&M University. The Silent heart (sih) mutant tc300b fish line was a gift from the Burns laboratory at Massachusetts General Hospital Cardiovascular Research Center at Harvard Medical School. Mutant embryos were identified through mating and phenotypical analysis. The flk:GFP and flk:mCherry transgenic lines (also identified as kdrl:GFP and kdrl:mCherry) were a gift from Dr. Jau-nien Chen at UCLA and used for visualization of blood vessels. The isl2b:GFP transgenic line was a gift from the late Dr. Chi Bin Chien at the University of Utah and was used for visualization of retinal ganglion cells. ace and sih mutant lines were crossed onto flk:GFP and isl2b:GFP transgenic lines to create various combinations of mutant transgenic lines. The mutant lines were maintained through heterozygous carriers. Embryos were collected and maintained at 28.5 °C and staged according to Kimmel et al., 1995.
PCR and RNA Probe Creation

fgfr1a, fgfr1b, fgfr2, fgfr3, fgfr4 probes were created as described previously (Rohs et al., 2013). The fgf8a and fgf8b probes were created by PCR using the following primers:

fgf8a F – ATGAGACTCATACCTTCAC
fgf8a R – TCAACGCTCTCCTGAGTAG

fgf8b F – ATTTCACTCGCTCGCTTTTC
fgf8b R – AACATTGCTTTGCTGATG

RNA probes were made from 1 µg of linearized plasmid or 100ng/100bp of PCR product. 1 µg of template (volume calculated from original DNA concentration) was combined with 10 µL of 20 mM dithiothreitol, 1X transcription buffer (Promega), 1X RNA labeling mix (Roche), 2 mg/mL RNA inhibitor (Invitrogen), and either 19 U of SP6 polymerase (Promega) or 20 U of T7 polymerase (Affymetrix). 2 U of DNAse (Ambion) was added and incubated at 37ºC for 15 minutes. This was precipitated overnight at -20ºC after addition of 104.5 µL of 10 mM EDTA and 100 mM LiCl in 100% EtOH. The RNA was spun at 14,000x in an Eppendorf centrifuge 5415c for 30 minutes, washed with 70% EtOH, spun an additional 15 minutes at the same specifications, air dried and resuspended in 20 µL of sterile water. 80 µL hybridization buffer (50% formamide, 5X saline-sodium citrate (SSC), 5mg/ml tRNA, 0.1% Tween-20) was added and the probe was stored at -20ºC.
**In situ Hybridization**

Embryos were collected, manually dechorionated, and fixed overnight in 4% paraformaldehyde (PFA) (Alfa Aesar) in egg water at 4°C. They were then dehydrated in 100% MeOH overnight at 4°C. Embryos were rehydrated in five minute serial washes of MeOH and PBT and permeabilized in proteinase K (Promega Corporation) 1:250 in *in situ* blocking solution (Phosphate-buffered saline (PBT), 5% normal sheep serum (NSS), 1% BSA) for 30 minutes. Embryos were refixed in 4% PFA for 15 minutes at room temperature followed by three five-minute washes in PBT. 3 μL of specific probe (created as described above) in 300 μL of hybridization buffer was added and incubated overnight at 70°C. Embryos were equilibrated in 15 minute serial washes at 70°C in hybridization buffer and 2X SSC followed by two 30 minute washes in 0.2X SSC at room temperature. Embryos were rehydrated in 10 minute serial washes at room temperature in 0.2X SSC and PBT. Embryos were incubated with anti-digoxigenin-AP (Roche Diagnostics) 1:5000 in *in situ* block for 2 hours at room temperature. Anti-dig was removed and replaced with PBT and left at 4°C. Embryos were stained in 1mM nitro-blue tetrazolium/ 5-bromo-4-chloro-3'-indolyphosphate (NBT/BCIP) (Thermo Scientific). Staining was stopped by addition of 4% PFA overnight followed by PBT and stored at 4°C until sectioning and imaging.

**Whole Mount Measurements**

The embryos were reared to the desired time point, manually dechorionated, and anesthetized in 0.4% tricaine (0.2g (3.8 x 10^5 M) Tricaine (Western Chemical Inc.), 0.5g...
(0.07 M) Na$_2$HPO$_4$, 50 ml 1x E3 water). The embryos were oriented in the desired position in 4% methylcellulose (Fisher Scientific). Whole mount eyes were measured using SPOT software calibrated to the microscope. Measurements were graphed and statistically analyzed with student’s unpaired t-tests using GraphPad Prism version 6.0 software. Error bars represent SEM.

**Tissue Preparation and Resin Sectioning**

Embryos were reared to the desired time point, manually dechorionated, and fixed overnight in 4% paraformaldehyde (PFA) (Alfa Aesar). They were transferred to 100% EtOH for a minimum of 2 hours to overnight. Following dehydration in EtOH, embryos were embedded using a JB-4 embedding kit (Polysciences, Inc) as per manufacturer’s protocol. Embryos were manually oriented in a vertical position under a Nikon SMZ800 dissecting microscope to allow for transverse sections. Embryos were sectioned on a Leica RM2265 microtome at either 3 or 7µm as indicated. Sections were collected in serial order onto slides and stored at room temperature until imaging. Slides were coverslipped using Vectashield® mounting medium with DAPI (Vector Laboratories).

**H & E Staining**

Embryos were prepared and sectioned as described above. Slides were re-hydrated in water for 5 minutes followed by hematoxylin (0.5% (w/v) Hematoxylin, 6.0% (w/v) Aluminum Ammonium Sulfate Dodecahydrate, 0.2% (v/v) Methanol, and 4.8% (v/v) Ethanol) (Ricca Chemical Company) for 7 minutes. This was followed by 2, 2-minute
washes in tap water followed by Eosin (0.25% (w/v) certified Eosin Y, 54% (v/v) denatured alcohol, 3.6% (v/v) ACS reagent grade isopropanol, 1% (v/v) Methyl Isobutyl Ketone (MIBK), and 1% (v/v) ACS reagent grade glacial acetic acid) (Ricca Chemical Company) for 25 seconds. Finally, the slides were washed 2 times for 2 minutes each with tap water. Slides were stored at room temperature until imaging.

**Immunohistochemistry**

Embryos were fixed at the desired stage in 4% PFA for 2 hours then washed 4 times for 5 minutes in PBST (PBS + 1% Triton). Embryos were then transferred to immuno block (PBST + 10% NGS) for 1 hour at room temperature. The rabbit anti-pHH3 (Cell Signaling technology) (Ser10)(D2C8) antibody was diluted 1:1000 in immuno block and incubated on embryos overnight at 4°C. Embryos were washed 3 times for 1 hour in PBST followed by a 1:1000 dilution of anti-rabbit IgG Fab2 Alexa Fluor 555 secondary antibody (Cell Signaling Technology) for 2 hours at room temperature. Embryos were stored in PBST at 4°C until they were sectioned as described above and imaged.

**Morpholino Injection**

The *fgfr1b* morpholino was designed as previously described (Rohner et al., 2009). The morpholino was stored in a 2mM stock or in a 500µM working stock diluted in dH2O.

6 ng of morpholino was injected into the 1 cell stage of wild type (TL or AB) embryos using a microinjector. Injected embryos were stored in egg water at 28.5°C and staged according to (Kimmel et al., 1995).
Microscopy and Imaging

Bright Field and Fluorescent Imaging

All whole mount images were taken using a Nikon SMZ800 dissecting scope at the indicated magnifications under bright field light. Images were taken using a SPOT Insight 4 camera and SPOT software (Diagnostic Instruments Inc.).

H & E, resin sectioned DAPI slides, and immunohistochemistry slides were imaged at 20x on an Olympus IX71 fluorescent microscope using a SPOT Insight 4 camera. The images were then adjusted for brightness and contrast in Adobe Photoshop (Adobe systems Inc.). Images were used for cell counts by hand using ImageJ software cell counter (NIH).

Blood flow was observed whole mount in ace mutants using the inverted Olympus IX71 fluorescent scope, blood cells were counted manually under 20x magnification.

Confocal Imaging

To visualize the vessels of the developing retina in embryos, ace/flk:GFP and sih/flk:GFP transgenic embryos were anesthetized at the desired developmental time point and manually oriented to a lateral position in 1% low melt agarose (Affymetrix). Z stacks of one eye were taken on a Nikon Eclipse Ti confocal microscope at 4 µm steps. Images were captured using Nikon C2Si software. Images were then transferred to Photoshop where they were adjusted for brightness and cropped.
RESULTS

Expression of zebrafish \textit{fgf8a} in the developing retina.

As a first step in understanding the role of FGF8a in zebrafish retinal development we performed whole mount \textit{in situ} hybridization. During early retinal development, both \textit{fgf8a} and \textit{fgf3} are required for retinal ganglion cell (RGC) differentiation and loss of one alone does not inhibit the initiation of \textit{ath5}, a marker of differentiated retinal ganglion cells (RGCs) (Martinez-Morales et al., 2005b).

In agreement with these results, \textit{fgf8a} is expressed in the retinal precursor cells of the central retina at 24 hpf (Figure 2.3.1 A) and 36 hpf (Figure 2.3.1 C), consistent with its known role in RGC differentiation. At 48 hpf, the RGCs have differentiated forming the first layer of the retina closest to the lens. The expression of \textit{fgf8a} at this time point is located strictly in the presumptive RGCs (Figure 2.3.1 E). This expression pattern suggests a role for \textit{fgf8a} other than the previously described role in differentiation. \textit{fgf8b}, a paralog of \textit{fgf8a}, is not expressed in the eye at the examined time points (Figure 2.3.1 B,D, F) but is co-expressed in other developing tissues including the mid-brain hindbrain boundary (Figure 1.10) and fin buds.

We next proceeded to identify the mRNA expression of zebrafish FGFRs during the stage at which \textit{fgf8a} is expressed in the retina (Figure 2.3.2). The only receptor that shows strong mRNA expression in the eye at 48 hpf is \textit{fgfr1b} (Figure 2.3.2 B). This expression appears to overlap with the expression of \textit{flk} labeling the vasculature (Figure 2.3.2 F).
Figure 2.3.1 fgf8a is expressed in the early zebrafish retina at 24, 36, and 48 hpf. Transverse central retina sections of fgf8a and fgf8b in situ hybridization at 24 hpf (A, D), 36 hpf (B, E), and 48 hpf (C, F). (A, B) At 24 and 36 hpf fgf8a mRNA expression is located in the central retinal precursor cells. (C) By 48 hpf, fgf8a expression is isolated to the presumptive differentiated retinal ganglion cells (D-F) fgf8b is not expressed in the retina but is co-expressed with fgf8a in areas such as the mid-brain hindbrain boundary (Figure 1.10)
Figure 2.3.2 *fgfr1b* is expressed in a pattern consistent with retinal vasculature. Transverse central retina sections of (A) *fgfr1a*, (B) *fgfr1b*, (C) *fgfr2*, (D) *fgfr3*, (E) *fgfr4*, and (F) *flk in situ* hybridization at 48 hpf. (A, D) *Fgfr1a* and *fgfr3* show no retinal staining but show staining in surrounding brain tissue (C, E) *fgfr2* and *fgfr4* show mRNA expression in the tissue between the lens and retina. (B) *fgfr1b* shows strong mRNA expression similar to vasculature staining by *flk* shown at 72 hpf (F). Figure adapted from (Rohs et al., 2013).
Loss of fgf8a results in a mispatterned eye vasculature at 48 hpf.

Identification of fgfr1b mRNA expression in the presumptive vasculature led to the hypothesis that FGF8a signaling through FGFR1b leads to proper development of the retina vasculature. To investigate this hypothesis, we observed the vasculature patterning when FGF8a is knocked out. We utilized the acerebellar (ace\textsuperscript{XI5}) mutant which results in a complete loss of fgf8a in the entire embryo (Reifers et al., 1998). We also generated a line of ace x flk:mCherry fish used to visualize the retinal vasculature in ace mutant embryos.

Wild type embryos show characteristic vasculature development with one nasal in-flow vessel and two outflow vessels dorsal and ventral to the lens. They also show a complete double ring around the lens and classic hyaloid vasculature branching behind the lens (Figure 2.3.3 A). In all ace mutant embryos examined however, we observed an extra vessel branching off of the central lens ring between the nasal and the dorsal vessels and highly disorganized hyaloid vasculature (n=7) (Figure 2.3.3 B). We were curious as to whether this disorganized and mispatterned vasculature would affect blood flow through the eye. When embryos were viewed under the microscope at 48 hpf, blood flow was tracked through the whole body, including the eye and down through the tail. However, when ace mutants are viewed at 48 hpf, 93% (42 of 45) have beating hearts and blood flow through the tail but no blood flow through the eye while the remaining 7% were observed to have normal blood flow (3 or 45) (data not shown).
Figure 2.3.3 Loss of FGF8a results in mispatterned retinal vasculature. Confocal images of 48 hpf whole mount flk:mCherry (A) wild type and (B) ace mutant embryos. (A) Wild type retinal vasculature develops in two rings with a vessel around the lens with one nasal inflow vessel and two outflow vessels, dorsal and ventral. The hyaloid vessels develop in a branching network between the lens and RGCs. (B) In the ace mutant, the ring around the lens is incomplete (arrow) and has an extra vessel located between the nasal and dorsal vessels (star). Also, the hyaloid vasculature is not branched properly and is unquantifiable. Confocal images taken at 20x, images adjusted for brightness. Scale bar equals 50µm.
**Loss of fgf8a results in smaller eyes.**
We next wondered how this lack of blood flow through the eye would affect the development of the retina. As previously published, the *ace* mutant embryos do not have a cerebellum and they exhibit gross body curvature when compared to wild type siblings at 48 hpf (Figure 2.3.4 A and C). We also observed that *ace* mutants have smaller eyes as compared to wild type siblings (Figure 2.3.4 B and D). To rule out any gross structural abnormalities in the retina, we performed an H & E stain on transverse sections through the central retina at 48 hpf and observed no obvious defects in *ace* mutants when compared to wild type siblings (Figure 2.3.4 E and F). To quantify the size difference between wild type sibling and *ace* mutants we measured the area of the eye whole mount at 48 hpf. *ace* mutant eyes are statistically smaller at 48 hpf (Figure 2.3.4 G) (wild type n=21; *ace* mutant n=20; p value<0.0001 unpaired Student’s t-test) and 72 hpf (Figure 2.3.4 H) (wild type n=9; *ace* mutant n=8; p value<0.0001 unpaired Student’s t-test).

**ace Mutants have dimensionally smaller eyes with fewer total cells at 48 hpf.**
The size of the eye was quantified by measuring length (wild type n=18; *ace* mutant n=20; p value<0.02 unpaired Student’s t-test), lens area (wild type n=19; *ace* mutant n=19; p value<0.0001 unpaired Student’s t-test), and width (wild type n=18; *ace* mutant n=20; p value not significant; unpaired Student’s t-test) at 48 hpf (Figure 2.3.5 A, B, C). Both length and lens area were significantly smaller however there was no difference in the width of the retina between *ace* mutants and controls. Measurement details are outlined in Figure 2.3.5 E. To look more closely at the cells of the retina of the *ace* mutant fish to determine if it was fewer cells that was making the eyes smaller, we
utilized DAPI staining in order to visualize cell nuclei for cell counts (Figure 2.3.5 F). At
48 hpf, there are statistically fewer cells in the retina of ace mutants when compared to
wild type siblings (Figure 2.3.5 D) (wild type n=14; ace mutant n=26; p value<0.0005
unpaired Student’s t-test).

**ace mutant retinas have fewer proliferating cells compared to wild type siblings.**

We next looked to see whether the cells of the retina in ace mutants were not
proliferating as much as wild type siblings. To do this, we utilized the proliferation
marker phospho-histone H3 (pHH3) (Habberstad et al., 2011) and analyzed percent
proliferation of total DAPI positive cells. We observed pHH3 labeling at the apical
surface of the retina (Figure 2.3.6 A). At 48 hpf, there are fewer proliferating cells in ace
mutants compared with wild type siblings (Figure 2.3.6 B) (wild type n=22; ace mutant
n=28; p value<0.0001 unpaired Student’s t-test).

To investigate if cell death might also be playing a role in the size of the eye, we used
acridine orange to measure the number of dying cells in the retina. Whole embryos were
stained and dying cells were identified as acridine orange positive with green fluorescent
and were counted in the same plane of focus (Figure 2.3.7 A and B). There was no
statistical difference in the number of dying cells between wild type and ace mutant
embryos at 48 hpf (Figure 2.3.7 C) (wild type n=20; ace mutant n=23; p value<0.6;
unpaired Student’s t-test).
**Figure 2.3.4** *ace* mutants have a smaller eye. 48 hpf bright field images of (A,B) wild type and (C,D) *ace* mutant embryos, and H&E stained transverse sections of (E) wild type and (F) *ace* mutant. Quantification of eye area at (G) 48 hpf and (H) 72 hpf. (A-D) *ace* mutants have a slight body curvature and smaller eyes compared to wild type siblings. (E,F) H&E sections show no gross abnormality in general eye structure of *ace* mutants compared to wildtype siblings. (G, H) Whole mount eye measurements reveal that *ace* mutants have statistically smaller eye areas at 48 and 72 hpf. Student’s unpaired t-test assuming equal variance p value **** < 0.0001. Error bars represent SEM.
After recognizing that the ace mutant embryos have smaller eyes and a

**Figure 2.3.5 ace mutants have fewer cells in their eyes and smaller dimensions at 48 hpf.** Quantification (A) retinal length, (B) lens area, and (C) retinal width. (D) Quantification of (F) DAPI stained transverse sectioned retinal cell counts. ace mutants have statistically fewer total retinal cells, shorter retinal length and smaller lenses. Retinal width is not affected. (E) Retinal measurement diagram. Student’s unpaired t-test assuming equal variance p value, ** < 0.02, *** < 0.0005, **** < 0.0001. Error bars represent SEM.
Figure 2.3.6 ace mutants have fewer proliferating cells in the retina at 48 hpf. DAPI and pHH3 stained lateral sections of 48 hpf (A) wild type and (B) ace mutant retinas with (C) quantification of percent proliferation. ace mutant embryos have significantly fewer proliferating cells at 48 hpf compared to wild type siblings. Student’s unpaired t-test assuming equal variance p value, ****< 0.0001. Error bars represent SEM. Scale bar equals 50µm.
Figure 2.3.7 *ace* mutants show no increase in cell death at 48 hpf.

Acridine orange staining of (A) wild type and (B) *ace* mutants and (C) quantification of acridine positive cells per retina. There is no significant difference in the number of dying cells in wild type and *ace* mutant retinas. Error bars represent SEM. Scale bar equals 50µm.
Lack of blood flow negatively affects eye size at 48 hpf.

We wanted to see what effect, if any, blood flow plays on the proper development of the eye. For this, we utilized the silent heart mutant fish line. These fish carry a point mutation in cardiac troponin t and therefore have no heartbeat or blood flow (Figure 1.10). Without a beating heart to provide nutrients for survival, these mutants survive through simple diffusion with the water for around 7 days before they die. The mutant line is kept alive through heterozygous carriers (Stainier et al., 1996).

Initial observations show that at 48 hpf there is very little phenotypical variation between the wild type siblings and the *sih* mutants (Figure 2.3.7 A and C). Close up whole mount images reveal that *sih* embryos have an edema around their heart and a smaller eye (Figure 2.3.8 B and D). The whole mount areas of wild type and mutant eyes showed that *sih* embryos have significantly smaller eyes at 48 hpf (wild type n=20; *sih* mutant n=20; p value <0.0004 unpaired Student’s t-test) and 72 hpf (wild type n=20; *sih* mutant n=20; p value <0.0001 unpaired Student’s t-test) (Figure 2.3.8 G and H). At 24 hpf, embryos show no size difference in their eyes (data not shown). To verify that there were no structural abnormalities in the eyes of the mutants we preformed H & E stains on transverse sections of 48 hpf wild type and mutant embryos. These sections show that while the eye is smaller, there is no variation in basic retinal composition (Figure 2.3.8 E and F).
Figure 2.3.8 Silent heart mutant phenocopies ace mutant. 48 hpf bright field images of (A,B) wild type and (C,D) sih mutant embryos, and H&E stained transverse sections of (E) wild type and (F) sih mutant. Quantification of eye area at (G) 48 hpf and (H) 72 hpf. (A-D) sih mutants have slight pericardial edemas and smaller eyes compared to wild type siblings. (E,F) H&E sections reveal no gross abnormality in general eye structure of sih mutants compared to wild type siblings. (G,H) Whole mount eye measurements reveal that sih mutants have statistically smaller eye areas at 48 and 72 hpf. Student’s unpaired t-test assuming equal variance p value *** < 0.0004, **** < 0.0001. Error bars represent SEM.
Looking further into the size variation of \textit{sih} and wild type embryos we conducted a more in-depth study of the dimensions of the eye. We observed that the total eye diameter, retinal width, and lens diameter are all statistically smaller in \textit{sih} embryos compared to wild type siblings (Figure 2.3.9 A) (wild type n=5; \textit{sih} mutant n=5; p value <0.0001, 0.0029, and 0.03 respectively; unpaired Student’s \textit{t}-test). Measurement details are outlined in Figure 2.3.9 B. We also quantified the total number of cells in the retina of \textit{sih} and wild type siblings at 48 hpf. DAPI stained transverse sections of the central retina (Figure 2.3.9 D) showed that there are fewer total cells in the retina of \textit{sih} embryos compared to wild type siblings (Figure 2.3.9 C) (wild type n=35; \textit{sih} mutant n=45; p value <0.0001 unpaired Student’s \textit{t}-test).

\textit{sih} embryos have fewer proliferating cells in the retina at 48 hpf.

Similarly to \textit{ace} mutants, we wanted to understand why there were fewer cells in the eyes of the \textit{sih} mutants. Just as done for the \textit{ace} mutants, we labeled proliferating cells with a pHH3 antibody and all nuclei with DAPI (Figure 2.3.10 A). At 48 hpf, \textit{sih} mutants have statistically less proliferation in their retinas (Figure 2.3.10 B) (wild type n=21; \textit{sih} mutant n=20; p value <0.0004 unpaired Student’s \textit{t}-test).

Cell death studies were also performed using acridine orange. When compared to wild type siblings, \textit{sih} embryos show no increase in cell death in the eye at 48 hpf (Figure 2.3.11) (wild type n=17; \textit{sih} mutant n=20; p value <0.27; unpaired Student’s \textit{t}-test).
Figure 2.3.9 *sih* mutants have fewer cells in their eyes and smaller retinas at 48 hpf. Quantification of (A) retinal dimensions of *sih* embryos (n=5) as diagramed in (B) and (C) total retinal cell number, and (D) examples of DAPI stained sections used for retinal cell counts. (A,C) *sih* mutants had statistically smaller eyes in each dimension measured and fewer total cells in their retinas when compared to wild type siblings. Student’s unpaired t-test assuming equal variance p value **** < 0.0001, ** < 0.0029, * < 0.03. Error bars represent SEM.
Figure 2.3.10 *sih* mutants have fewer proliferating cells in the retina at 48 hpf. DAPI and pH3 stained lateral sections of (A) wild type and (B) *sih* mutant retinas with (C) quantification of percent proliferation. *Sih* mutant embryos have significantly fewer proliferating cells at 48 hpf compared to wildtype siblings. Student’s unpaired t-Test assuming equal variance p value, *** < 0.0004. Error bars represent SEM. Scale bar equals 50µm.
Figure 2.3.11 *sih* mutants show no increase in cell death at 48 hpf. Acridine orange staining of (A) wild type and (B) *sih* mutants and (C) quantification of acridine positive cells per retina. There is no significant difference in the number of dying cells in wild type and *sih* mutant retinas. Error bars represent SEM. Scale bar equals 50µm.
sih embryos have incomplete and disorganized retinal vasculature at 48 hpf and 54 hpf.

Wild type embryos show classic vessel branching at 48 hpf (Figure 2.3.12 A). The vasculature pattern of sih embryos is incomplete. The ring vasculature does not form a full ring in sih embryos (Figure 2.3.12 B) as seen in the wild type siblings (Figure 2.3.12 A). More interestingly, the vasculature that comes in direct contact with the retina, the hyaloid vasculature, is highly indistinguishable and mispatterned (Figure 2.3.12 B). This mispatterned hyaloid vasculature and lack of a complete ring was observed in all sih embryos examined (n=7). By 54 hpf, the retinal vasculature of sih mutants has developed ectopic branches in excess of the normal three (Figure 2.3.12 C and D).

Loss of fgfr1b phenocopies ace mutants

To support our claim that FGF8a is acting by binding to FGFR1b primarily and not one of the other receptors we used a morpholino. UIC control embryos show normal vessel branching at 48 hpf (Figure 2.3.13 A). The vasculature pattern for fgfr1b morphants phenocopies ace mutants. The morphants have an extra ectopic vessel branching off of the central lens ring between the nasal and dorsal vessel (Figure 2.3.13 B). They hyaloid vasculature does not seem to show the same drastic mis-patterning as seen in ace mutants.
Figure 2.3.12 *sih* mutant embryos have mispatterned eye vasculature at 48 and 54 hpf. Confocal images of whole mount flk:mCherry (A,C) wild type and (B,D) *sih* mutant embryos. (B) At 48h hpf *sih* mutants lack complete ring vasculature (arrow) and have mispatterned hyaloid vasculature behind the lens. (D) By 54 hpf, the ring vasculature in *sih* mutants has developed ectopic branches (stars). Confocal images taken at 20x, images adjusted for brightness. Scale bars equal to 50µm.
Figure 2.3.13 Loss of \textit{fgfr1b} phenocopies \textit{ace} mutants. Confocal images of 48 hpf whole mount flk:mCherry (A) wild type and (B) \textit{fgfr1b} morphants (A) Wild type retinal vasculature develops in two rings with a vessel around the lens with one nasal inflow vessel and two outflow vessels, dorsal and ventral. (B) In the \textit{fgfr1b} morphants, there is an extra vessel located between the nasal and dorsal vessels (star) similar to that seen in the \textit{ace} mutants. Confocal images taken at 20x, images adjusted for brightness. Scale bar equals 50µm.
DISCUSSION

In this study, we provide evidence that FGF8a, in zebrafish, is a key player in signaling to the developing eye vasculature. Our data support that FGF8a is required for the proper development and patterning of both the retina and its surrounding vasculature. Consequently, if FGF8a is lacking, the vasculature develops incorrectly and this therefore negatively affects the neurons of the retina, which rely on the vasculature for oxygen and nutrients for growth and survival. We can also conclude, through the use of the sih mutants, that lack of blood flow does not seem to affect the signaling factors responsible for the initial development of the vasculature. And therefore, the phenotypes seen with these mutants must be due to a lack of signal coming from the blood itself.

FGFs have long been known to play key roles in development. FGF8 specifically has many roles including limb bud development, cerebellum development, and eye development. Its role in the developing eye has previously only focused on differentiation of RGCs along with FGF3 (Martinez-Morales et al., 2005a). Here we focus on a potential role for FGF8a later in vertebrate eye development focused on its relationship with the synchronous development of the vasculature. FGFs have previously been shown to be important in vasculature development. Furthermore, human umbilical vein endothelial cells (HUVECs) express FGF8 and FGFR 1,2,3, and 5 (Antoine et al., 2005) supporting what we have found in our data.
RGCs are the first neurons to be born in the retina around 30 hpf (Schmitt and Dowling, 1996) as a result of signaling from FGF8a and FGF3. However, we first became interested in FGF8a’s later role in eye development when it was expressed in the presumptive RGCs in an in situ hybridization at 48 hpf. This was surprising because at 48 hpf, most of the RGCs have differentiated and would no longer require their differentiating signal. While an in situ only shows the mRNA expression and does not tell us where the protein is, we are confident that fgf8a is being expressed in differentiated RGCs (Figure 2.3.1). The only other cell type that is in this layer are displaced amacrine cells of which there are few in comparison to the RGCs and would not explain the strong staining that we see.

To understand the signaling of FGF8a in this area we also looked for in situ staining of all of the zebrafish FGFRs. We noticed that the FGFR showing the strongest and most interesting expression in the retina at this time point was fgfr1b. While there is in situ hybridization evidence to support that the other FGFRs are in the retina at this time, we argue that FGFR1b is primarily responsible for the signaling with FGF8a at 48 hpf. The staining of fgfr1b however, did not share the same pattern as the ligand fgf8a. When the receptor staining was compared to flk (vegfr2/4) staining, they show almost identical staining patterns leading us to believe that the FGFR1b is located on the endothelial cells not in the retina suggesting that FGF8a is acting in a non-autonomous fashion. This idea is supported by a previous study showing by in situ hybridization that fgfr1 was located in all endothelial cells of mid-trimester human fetuses (Gonzalez et al., 1996).
To further support our thoughts of the ligand and receptor locations we would plan to do double labeled *in situ* hybridizations to show that *fgf8a* and the RGCs co-localize as well as do *fgfr1b* and retinal endothelial cells. Along with this study, we are currently collaborating on an experiment to examine if cultured RGCs from zebrafish embryos *in vitro* are not stimulated by treatment with FGF8a while cultured endothelial cells are. These studies are in progress and we are optimistic that the results will be in agreement with our current hypothesis. Further, our preliminary studies looking at the effect that loss of *fgfr1b* has on the embryo phenocopy our *ace* mutant results. These results make us confident that the ligand receptor pair that is at play in the proposed system is FGF8a and FGFR1b. The knockdown that we used was through the use of a morpholino that has yet to be verified however we are optimistic that this is the correct receptor partner for FGF8a at the time point. We would like to further confirm the *fgfr1b* phenotype with more reliable knockout methods such as the creation of a CRISPR mutant.

We first provide supporting evidence that FGF8a is signaling through the vasculature showing that loss of FGF8a, with the use of the *ace* mutant, results in mispatterend eye vasculature. *ace* mutants were crossed onto the flk:GFP line so we would have mutant embryos with fluorescent vasculature. All wild type siblings observed showed the classical number of three vessels around the lens and a complete vessel ring. *ace* mutants all showed four vessels, with an additional vessel between the dorsal and nasal vessels, and an incomplete ring. The hyaloid vasculature, which is directly behind the lens, was also affected. In wild type siblings the hyaloid vasculature is a complex branched network that cups the lens and that are easily quantified and commonly show 5-8
branches. In *ace* mutants, these branches are so disorganized and undistinguishable that it makes them unquantifiable. When we quantified the blood flow in these mutants we found that 93% of embryos showed no blood flow in the eye while they still had beating hearts. Un-quantified, but seemingly normal blood flow was observed in the tail vessels of 47% of mutants suggesting that while vessel patterning and development are also affected in other parts of the body, however it is to a lesser degree than in the eye. We argue that this mis-patterning is due to the lack of FGF8a signal coming from the RGCs. It would be interesting to add FGF8a soaked beads directly to the area of the retina during their development to see if this would rescue the phenotype. The lack of vascularization could be leading to a hypoxic environment, which may be the reason for the extra ring vessel seen in all *ace* mutants. This is in line with a study that showed zebrafish in hypoxic conditions exhibit increased vessel branching (Cao et al., 2008). We need to look further at whether these retinas are in a hypoxic environment and plan to explore *in situ* for hypoxia-inducible factor 1 (*hif1a*). The opposite experiment could also be performed where we would infuse the water with extra oxygen to assess the increase in the number of branches as done by Cao et al.

Understanding that *ace* mutants have a mis-patterning of the eye vasculature led us to look at the effect that this could be having on the morphology and development of the retinal neurons and eye in general. Quantifying embryos whole mount indicates that there is an obvious size difference in the eye at 48 hpf (Figure 2.3.4). The gross phenotype of the *ace* mutant embryos also seems to affect the growth and curvature of the body, a lack of a mid-brain hindbrain boundary and cerebellum, and a larger yolk sac.
in agreement with previous reports (Reifers et al., 1998). When the various dimensions and size of the eyes of these mutants were measured, we found that they are significantly smaller compared to wild type siblings while also having fewer numbers of total retinal neurons. We then investigated if the eyes were smaller due to an increase in cell death using acridine orange staining, or a decrease in cell proliferation using pHH3. Our data showed no increase in cell death and a decrease in the percentage of proliferating cells. Acridine orange is understood to be a weak marker for cell death and we therefore would propose to confirm these results with other cell death markers such as activated caspase 3.

Alternatively, the decrease in proliferation may be explained due to lack of FGF8a signaling to the ciliary marginal zone. This area of the developing retina is home to retinal precursor cells and is responsible for the proliferation of the more mature retina. In situ hybridization shows that there is limited expression of fgfr4 in the ciliary marginal zone at 48 hpf. FGF8a could be signaling through this receptor and lack of the ligand could explain why there is less proliferation. Due to the limited expression of fgfr4 compared to the expression of fgfr1b this hypothesis was not pursued. We contend instead that this lack of size in the eye is due to a disruption in nutrient retrieval due to a lack of blood flow.

We found that blood flow did have a negative effect on the proliferation of the retinal neurons and the patterning of the vasculature. In order to assess how blood flow affects the development of the zebrafish eye we needed a fish line that had little or no blood flow.
to observe. This is possible because fish have the ability to hatch, survive, and develop for several days with a lack of blood flow due to their small size and ability to have gas exchange through the water (Burggren and Pinder, 1991). The *sih* fish provided the exceptional platform with which to observe the effects of blood on the development of the retina and eye.

Initially the eye size of the *sih* embryos was measured. We found that they had significantly smaller eyes in all dimensions tested and had fewer total retinal neurons phenocopying what we see in *ace* mutants. We have yet to verify which cell types are affected by the smaller eye size or if they are all affected equally. It is hypothesized that because the inner and outer retina are supported by different blood supplies that the mispatterend hyaloid would only effect the cells closest to it, the RGCs. We plan to perform cell specific immunostainings for each of the retinal cell types in both *sih* and *ace* mutant embryos. This information will be vital to give us a clearer picture into the full development of the eye and where lack of blood flow is taking its toll. Looking deeper into the similarities between *ace* mutants and *sih* mutants, we again looked to see if the smaller eye phenotype seen in *sih* mutants is a result of lack of cell proliferation or increased cell death. Just as seen in the *ace* mutants, there is a marked decrease in the percentage of proliferating cells in *sih* mutants while there is no difference in cell death numbers.

A lack of proliferation in the retina could suggest that the cells are arresting in one phase of the cell cycle. Because cell cycle arrest typically leads to cell death we would have
expected to see an increase in cell death, which we did not. The lack of increased cell
death in ace mutants is consistent with previous studies (Reifers et al., 2000). However to
test this idea further, we would propose to use flow cytometry as done in (Alex et al.,
2010). Using this technique we will be able to compare the DNA content of specific
retinal cell types over time to assess if they are arresting during any point in the cell
cycle.

Finally, we were curious as to how the vasculature develops in the sih mutants and
whether they also have mispatterned vasculature in the eye. When we looked at
sih/flk:GFP embryos at 48 hpf we found that they do not have ectopic vessels around the
lens however they do seem to have an incomplete ring vasculature and undistinguishable
hyaloid vasculature. When we observe these embryos at a later time point we find that at
54 hpf the number of ring vessels has increased anywhere from 4-5 branches. These
ectopic branches are not surprising because as reported previously (Cao et al., 2008), a
zebrafish embryo in a hypoxic environment will produce extra blood vessels. Presumably
this is in response to the lack of blood and nutrients and the addition of blood vessels is
an attempt to get more blood flow to the area. Because the sih embryos develop excess
vessels, this tells us that they are not lacking the signaling factors that lead to the growth
of vessels. This suggests to us that the phenotype observed in both sih and ace mutants is
due to factors being secreted from the blood.

These data further support that it may be the lack of blood flow in the ace mutants that is
causing their retinal phenotype. This is unique since we are proposing that the signal to
the developing vasculature is neuronally derived and therefore partially responsible for their own proliferation. Our data show that a lack of FGF8a causes a drastic retinal vasculature phenotype that directly affects the development of the retinal neurons. It is still necessary to more strongly connect the ace mutant phenotype to a lack of blood flow. Using S-Nitroso-N-acetylpenicillamine (SNAP) a potent vasodilator we tried to increase blood flow to ace mutants in an attempt to rescue the phenotype (Appendix B). While the replicates are small, these results showed no rescue. We are now considering if there is less blood in ace mutants and therefore nothing to pump through the veins however we currently hypothesize that the patterning of the vasculature is just too far compromised to allow for blood flow to the retina. With a gata:dsRed transgenic linea or o-dianisidine stain we could investigate the amount of red blood cells in ace mutants to verify that this is unchanged. Zheng et al., 2009, similarly employed this technique. Using live video imaging, we may be able to track the blood flow and determine if it is getting stuck or re-routed before reaching the eye to support our hypothesis that the vessel patterning is compromised.

We also need to determine what cell types of the retina are being affected and if they are affected equally. We reason that the cell type affected most readily will be the RGCs due to their close proximity to the hyaloid vasculature. Preliminary data using morpholino injections into the isl2b:GFP transgenics suggest there are fewer RGCs when FGF8a is knocked down. Attempts to cross this transgenic onto the ace mutants resulted in a loss of transgene expression as determined by loss of GFP expression in all typical cell types for this transgene including RGCs and Rohon Beard neurons. We are unsure as to why this
happens and reason that it may have something to do with full loss of FGF8a or the genetic manipulation of the mutant line. Due to this limitation, we plan to perform cell type immunohistochemistry on each of the retinal cell types to assess how each type is affected. It is hard to predict the effect that loss of FGF8a will have on the outer cell layers of the retina because these cells, the rods and cones, are supported by a separate vasculature, the chorodial vasculature. We have yet to study how the chorodial vasculature development is effected. However, we do not hypothesis that the mis-patterened hyaloid vasculature should effect the outer retinal layers due to limited contact (Schmitt and Dowling, 1994). This phenotype would also be interesting to look at in later time points to see if retinal lamination is affected or prevented. This would have to be done with caged morpholinos since the loss of FGF8a is so harmful that mutant embryos are too distorted to observe beyond 48 hpf.

FGFs are known to play critical roles in various aspects of development. Their angiogenic properties are well known and understood in many ways. However, here we propose a unique model that has FGF8a being secreted neuronally in the retina of Danio rerio. This neuronal secretion of FGF8a, we hypothesize, properly pattern the vasculature in the eye, this in turn, provides support for the continuing development of the retinal neurons. This information is important and provides us with a more detailed picture of how the eye develops in synchrony with the vasculature and the importance of their interplay together. The more we can understand the complexity of development the more tools we will have available to us for future work in understanding the complex diseases and problems that can occur within a developmental system.
APPENDIX A- Use of fgf8a morpholino

INTRODUCTION

Morpholino technology is a tool for gene-specific knockdown and is widely used in zebrafish. Morpholinos (MO) function by either blocking the translational start site of the RNA or causing improper mRNA splicing leading to no protein product or altered message. Specifically, morpholinos are synthetically designed oligonucleotides with a morpholine ring as compared to a ribose ring, which provides stability and protects it from nucleases. The use of morpholinos must be strictly monitored and there must be proper controls in place in order to draw appropriate conclusions from the data. This is due to the common occurrence for morpholinos to cause off-target effects sometimes making it hard to determine if the result is due to a loss of a specific gene or the mis-target effects. Due to this, various controls must be utilized. There are various methods for controlling morpholinos including designing a morpholino against a gene not present in the test animal, use of a different morpholino designed against the same gene, and showing phenotype rescue by RNA injection (Eisen and Smith, 2008).

This project initially started with the use of and fgf8a morpholino. The results obtained by using the mutant phenocopy those obtained by using the morpholino and the morphants data are reported in this appendix.
MATERIALS AND METHODS

Morpholino Design and injection

The *fgf8a* e2i2 morpholino antisense oligonucleotides were purchased from Gene-Tools LLC (Philomath, OR) with the following sequence: 5’-TAGGATGCTCTTACCATGAACGTCG-3’. This morpholino was designed to block the splice acceptor site on exon two resulting in excision of exon two and a frameshift in exon three. The morpholino was stored in a 2mM stock or in a 500µM working stock diluted in dH2O.

6 ng of morpholino was injected into the 1 cell stage of wild type (TL or AB) embryos using a microinjector. Injected embryos were stored in egg water at 28.5°C and staged according to (Kimmel et al., 1995). Measurements and data collected were done as described in chapter 2.2.

Full-length *fgf8a* RNA was also used to show morpholino rescue. *Fgf8a* RNA was injected at a 250 pg concentration mixed with 6 ng morpholino.

Morpholino Verification

Primers were designed for *fgf8a* and a control house keeping gene, *ef1α*. The primers were ordered from Integrated DNA Technologies. The primers were as follows:

Fgf8a F: GCCAGTTGACCTGTTCATCA
Fgf8a R: AAAGCTGGTAGGTCCGGATT
ef1α F: CGGTGACAACATGCTGGAAGG
 elfα R: ACCAGTCTCCACACGACCCA

The PCR program used was as follows:

2 minutes at 95°C; 30 seconds at 95°C; 30 seconds at 50°C; 45 seconds at 72°C; 5 minutes at 72°C; cycle 30 times then hold at 4°C.
RESULTS AND DISCUSSION

fgf8a MO verification.

In order to verify that our morpholino was knocking down the desired mRNA, morpholino verification was performed. cDNA was created from control and fgf8a MO injected embryos. Primers were designed against fgf8a in exons one and three and a housekeeping gene ef1α as a positive control. PCR was then used to verify that there was a loss of fgf8a in the morphant embryos (figure A.1). ef1α levels were strong and consistent in both the control and fgf8a morphant embryos. A band representing full-length fgf8a PCR product (306 bp) was present in uninjected control embryos but not in fgf8a morphants. In the morphant lane we expected to see a splice product of 269 bp indicating the loss of exon two, however, we saw no product implying the altered transcript was degraded by nonsense mediated decay. The results from this experiment demonstrated that our morpholino is altering splicing of our gene of interest.

Loss of fgf8a results in smaller eyes due to a lack of proliferation and can be rescued with full length RNA.

Our initial observations following the injection of the morpholino was the obvious difference in the size of the morphants eyes. Therefore, we began by quantifying the eye size in morphants. fgf8a morphants had smaller total eye area when compared to controls at 48 (UIC average= 43004 µm², n=4; fgf8a MO average= 31499.1 µm², n=10; P<0.01 unpaired Student’s t-test). This loss could be rescued by the injection of full length
zebrafish fgf8a RNA that cannot be targeted by the morpholino (Figure A.2) (UIC average= 43004 µm$^2$; n=4; fgf8a RNA rescue average= 46952.7 µm$^2$, n=10; P<0.84 unpaired Student’s t-test). Morphants also had had shorter body lengths at 48 hpf that could also be rescued by RNA injection (Figure A.3) (UIC average= 3906.5 µm, n=4; fgf8a MO average= 3475.4 µm, n=10; fgf8a RNA rescue average= 3742.6 µm, n=10; P<0.02 ordinary one-way ANOVA). The small eye size observed in the morphants was investigated by immunohistochemistry staining for anti-pHH3, a cell proliferation marker. When normalized to total retinal cell number, there were a smaller percentage of proliferating cells in the morphants when compared to the un-injected controls (Figure A.4) (UIC average= 5.54%, n=13; fgf8a MO average= 2.43%, n=6; P=0.003 unpaired Student’s t-test). These data are in agreement with the data previously presented in chapter 2.3 with use of the ace mutant.
**Figure A.1 fgf8a morpholino verification.** The expected 220 bp ef1α band is present in both control and morphants embryos. There is a band representing fgf8a in the control lane at the expected size of 306 bp, however, no band for fgf8a is observed in the morphant lane.
Figure A.2 fgf8a morphants have smaller eyes at 48 hpf. Total eye measurements of control, fgf8a morphant and fgf8a rescue embryos. fgf8a morphants have significantly smaller eyes than control embryos and this phenotype can be rescued by co-injection of full length fgf8a RNA. There is no significant difference between un-injected control and fgf8a rescued embryos. Student’s unpaired t-test assuming equal variance p value, ** < 0.001, * < 0.02. Error bars represent SEM.
Figure A.3 *fgf8a* morphants have shorter bodies at 48 hpf. Total body length of control, *fgf8a* morphant and *fgf8a* rescue embryos. *fgf8a* morphants had a shorter body length when compared with un-injected controls and this phenotype was rescued with co-injection of full length *fgf8a* RNA. There is no significant difference between the un-injected controls and the rescue embryos. Student’s unpaired t-test assuming equal variance p value, * < 0.02. Error bars represent SEM.
Figure A.4 *fgf8a* morphants have fewer proliferating cells at 48 hpf. Transverse retina sections of control and *fgf8a* morphant eyes labeled with DAPI and pH3. Wild type embryos have a higher percent of proliferating cells at 48 hpf than *fgf8a* morphants embryos. Student’s unpaired t-test assuming equal variance p value, ** < 0.001. Error bars represent SEM.
APPENDIX B – SNAP Data

INTRODUCTION

Angiogenesis, or the formation of new blood vessels off of already existing vessels is a process that is induced in development, wound healing, and in tumor growth. The mechanisms that allow for this process are complicated and require proper signaling from a variety of growth factors (Ziche and Morbidelli, 2000). Our data suggest that a lack of blood flow to the retina causes a retinal phenotype that presents as a decrease of cells in ace (fgf8a) mutant embryos at 48 hpf. We questioned if it would be possible to increase the amount of blood flow being delivered to the retina in these mutants and if that would rescue or partially rescue the phenotype.

To do this, we employed the use of S-nitroso-N-acetyl-penicillamine (SNAP), a nitric oxide donor. Nitric oxide is known to inhibit platelet aggregation, regulate neurotransmission, and be a potent vasodilator. Normally, nitric oxide is present in cells during periods of inflammation, which led to the idea that it may be important in angiogenesis due to the role that extra blood flow plays in the wound healing process. With this information, many studies have shown that there is a link between vasodilation and angiogenesis (North et al., 2009; Ziche and Morbidelli, 2000). For the current study, we allowed ace mutant embryos to develop in SNAP to see if this would improve the blood flow through the eye. Our results indicate that while SNAP was able to increase
vessel diameter in control embryos, it did not have the ability to rescue the blood flow in *ace* mutants.

**MATERIALS AND METHODS**

**SNAP treatment**

SNAP was added to the water of 24 hpf wild type flk:GFP or *ace* mutant embryos at a concentration of 50μM or 100μM. Egg water was used as a control. The embryos were allowed to develop normally at 28.5°C for 24 hours and the dorsal aorta of each fish was imaged under an inverted Olympus IX71 fluorescent scope and photos taken using a SPOT Insight 4 camera (Diagnostic Instruments Inc.). The diameter of each vessel was measured and data was graphed and statistically analyzed with student’s unpaired t-tests using GraphPad Prism version 6.0 software. Error bars represent SEM.
RESULTS AND DISCUSSION

Addition of SNAP increases the vessel diameter of control embryos at 48 hpf.

When flk:GFP embryos are treated with SNAP for 24 hours from 24 hpf to 48 hpf, there is an increase in the diameter of the dorsal aorta. The increase happens in a dose dependent manner with the increase from control average vessel diameter of 27.6 µm to 30.7 µm average diameter with 50 µM SNAP treatment, and 33.9 µm average diameter with treatment of 100µM SNAP (Figure B.1) (control, n=20; 50 µM SNAP, n=11; 100 µM SNAP, n=18; p<0.0004 ordinary one-way ANOVA). This data was in support of research preformed by (North et al., 2009) who show that there is an increase in vessel diameter when SNAP is applied to living zebrafish. With support that the vessels could be dilated with addition of SNAP, we then applied the drug to ace mutant embryos. Our hypothesis was if the blood vessels were dilated then more blood would reach the area and this would increase proliferation and total eye size.

SNAP treatment does not rescue the ace mutant phenotype.

ace mutants were treated with SNAP for 24 hours from 24 hpf to 48 hpf. We observed no increase in the area of their eyes at 48 hpf with a p value of 0.08 when ace mutants with and without SNAP were compared with a student’s unpaired t-test (control average area=
39121.4 \( \mu \text{m}^2 \), n=8; \textit{ace} mutant on 100 \( \mu \text{M} \) SNAP average area= 24851.7 \( \mu \text{m}^2 \), n=6; \textit{ace} mutant with no SNAP average area= 29260 \( \mu \text{m}^2 \). There was a difference between control embryos not treated with SNAP and \textit{ace} mutants treated with SNAP with \textit{ace} mutants having smaller eyes (Figure B.2) (p value< 0.0001, student’s unpaired t-test). There is also a statistical difference between controls and \textit{ace} mutants not treated with SNAP, again, \textit{ace} presenting with smaller eyes (p value= 0.003, student’s unpaired t-test). While the SNAP did not rescue the size of the eyes, we have not determined if this is because the SNAP is not dilating the vessels of the \textit{ace} mutants, if the amount of red blood cells is fixed and therefore increasing vessel diameter would not increase the amount of blood, or if there is an increase in blood flow, but it is not helping to rescue the phenotype. Currently, we argue that the development of the vessels is too compromised, as shown in Figure 2.3.3, and the increased size of the blood vessels does not fix the circuitry of the system and therefore, no more blood is delivered to the area. The sample size for the current experiment was low and with repeat experiments we will be able to make a more reliable analysis. An increase in the sample size could change our statistics. We would like to do more drug tests to look at the size of the vessels to verify that they are dilating as observed in control embryos. We would also like to video and quantify blood flow to see if more blood flow is reaching the retina.
Figure B.1 SNAP increases dorsal aorta vessel diameter of control embryos at 48 hpf. Flk::GFP embryos treated with SNAP for 24 hours. There is no difference in the diameter of control vessels and embryos treated with 50µM SNAP. However, there is an increase in vessel diameter between control and embryos treated with 100µM SNAP. Student’s unpaired t-test assuming equal variance p value, *** < 0.0001. Error bars represent SEM.
Figure B.2 SNAP does not rescue the *ace* mutant phenotype at 48 hpf. Quantification of eye area in control and *ace* mutants with and without SNAP treatment. There is no increase in the area of the eye of *ace* mutant embryos after treatment with SNAP. Student’s unpaired t-test assuming equal variance p value, **** < 0.0001. Error bars represent SEM.
BIBLIOGRAPHY


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