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Investigating the Role of FGF8 Signaling in Neurogenesis of the Developing Zebrafish Eye

Benjamin A. Flinn
University of Vermont

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Benjamin Flinn
Undergraduate Honors Thesis
College of Arts and Sciences
Department of Biochemistry

Advisor: Paula Deming, PhD
CAS Co-Advisor: Alicia Ebert, PhD

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Abstract: In the embryonic zebrafish, the fibroblast growth factor 8a (FGF8) signaling network is essential for proper development and maintenance of retinal ganglion cells (RGCs) as well as the hyaloid vasculature, the vessels that supply the eye with nutrients during development. Disruption of FGF8 signaling via knock down of FGF8 or pharmacologic inhibition of FGF receptors (FGFRs) results in extensive abnormalities throughout the developing eye. Our preliminary data indicated that in developing zebrafish, mRNA expression of *fgf8a* is present exclusively in the RGCs, while the fibroblast growth factor receptor 1 (*fgfr1b*) is expressed exclusively in the area of the hyaloid vasculature. These results led us to hypothesize that FGF8 signals from the RGCs to the vasculature of the developing eye, and that this signaling network is essential for proper eye development. In order to test this hypothesis, we demonstrated the ability to detect downstream phosphorylation events in response to acute FGF8 stimulation in cells that expressed FGFR1 using Western blot and immunofluorescence (IF). Next, we established a zebrafish eye explant culture system to treat the cells of the developing zebrafish eye *in vitro*. Using transgenic zebrafish lines expressing green fluorescent protein (GFP) tags in either the differentiating RGCs or the vascular cells of the eye, we attempted to identify the specific cells capable of responding to FGF8. Our data indicate that recombinant FGF8 is capable of activating detectable intracellular signaling cascades, such as ERK phosphorylation, in cultured endothelial cells. Furthermore, FGF8 is capable of inducing signaling in some of the cells from the developing zebrafish eye, but not in the RGCs. These findings support our proposed model in which FGF8 signals from the RGCs to the hyaloid vasculature, resulting in the activation of signaling pathways that are necessary for proper development of the hyaloid vasculature and RGCs.

I. Background and Significance

A. Cell Signaling: Proper cell growth, survival, proliferation, and differentiation during development are facilitated by the presence of various signals available to the cell within its environment. Typically, the first step of this process involves the interaction between a soluble protein signal such as a growth factor and a receptor located on the surface of a cell. Cell-surface receptors generally consist of a ligand-binding domain to recognize a signaling molecule, a transmembrane domain to cross the plasma membrane and anchor the receptor within the membrane, and lastly a cytosolic domain that interacts with various intracellular proteins (1). Through the use of specific soluble signals, surface receptors, and numerous complex interactions between proteins within the cell, cells are capable of responding to paracrine or autocrine signals (2) in the specific ways necessary to facilitate the cell's intended function. Cellular responses downstream of growth factor signals include modulation of the biological activity of various proteins, changes in gene expression, or fluctuations in the concentration of various components of the cytoplasm, which drive processes such as migration, proliferation, differentiation, and survival.

When a receptor is bound to its ligand, it undergoes a conformational change that alters how it interacts with proteins within the cell compared to when the receptor is in an unbound state. This results in a process known as "signal transduction", during which a protein is altered by the activated receptor, and subsequently alters numerous other proteins in the cell, which often go on to do the same to other proteins, and so forth. This results in a broad amplification of an initial small signal by many downstream secondary messengers. One common mechanism used by cells to transmit signals is phosphorylation, in which a phosphate group from ATP in the

cytoplasm is added to serine, threonine, or tyrosine residues within a protein (3). Because of its negative charge, addition or removal of a phosphate group is capable of changing the shape of a protein, resulting in a change in its stability, biological activity, or proteins that it can interact with in the cell.

B. Fibroblast Growth Factors (FGFs): FGFs are peptide growth factors with important roles in nervous system development (4). In vertebrates, 22 FGFs have been identified (5, 6) and they signal through four FGF receptor tyrosine kinases (FGFR1-4) (7, 8). *In vitro* studies have characterized interactions between different FGF and FGFR isoforms, demonstrating significant selectivity of individual FGFRs for particular FGFs (9). Our unpublished observations indicate the importance of FGF8a in RGC growth and development, with knock down of FGF8a or pharmacologic inhibition of FGF receptors (FGFRs) causing extensive abnormalities throughout the developing eye (Ebert, unpublished). FGF8 has been shown to bind to and activate FGFR1, FGFR2, and FGFR3 (9, 10) which then causes the activation of several biochemical signaling pathways resulting in a host of cellular responses, including proliferation and survival (11). Many studies in model systems have shown that fibroblast growth factors are important for proper neural development, including that of the eye; however the precise FGF8-mediated signaling events that drive neurogenesis in the eye remain elusive. Moreover, although a number of individual FGF8-mediated signaling events have been described in the literature, a global analysis of the events occurring immediately downstream of FGF8-stimulated FGFR1 activation (e.g. binding partners) has not been reported.

C. The MAPK Pathway: The MAPK pathway is one of the most important pathways for cell growth and survival (12). The MAPK pathway can be defined as a phosphorylation cascade, as it is initially activated at a receptor tyrosine kinase (RTK) that leads to the phosphorylation of

another kinase in the pathway, allowing it to subsequently phosphorylate the next enzyme in the pathway, and so forth. Phosphorylation cascades such as the one seen in the MAPK pathway are very important for both the amplification and propagation of a signal, as not only does one kinase pass on the signal through the phosphorylation of other proteins, but the kinase also can phosphorylate many downstream kinases before it becomes inactivated (13). The final kinase in the canonical MAPK pathway is known as extracellular signal-regulated kinase (ERK). It becomes activated when it is phosphorylated to become phospho-ERK (pERK) by MEK directly upstream in the pathway. ERK has been identified as one of the signaling intermediates to become activated downstream of FGFR1 (14), as well as other RTKs such as the platelet-derived growth factor receptor (PDGFR) and the receptors for nerve growth factor (NGF) (15,16). Once ERK becomes activated, it is capable of phosphorylating numerous substrates to facilitate numerous cellular responses, including increases in expression for various genes, inhibition of apoptosis, cell growth and proliferation, and cell migration. Depending on the cell type and context, ERK phosphorylation has been implicated in neuronal survival (17) and cell death (18). As many growth factor receptors can activate the MAPK pathway, ERK phosphorylation serves as a common means to detect growth factor signaling in cells.

D. Eye Development & The Zebrafish Model: Within the eye, retinal ganglion cells (RGCs) are the final recipients of the electrical signal formed when the photoreceptors of the retina are stimulated by incoming light. They extend their axons out from the eye forming the optic nerve, which sends information to the centers of the brain responsible for receiving and processing visual information (19). The hyaloid vasculature, a network of vessels that branches around the lens, is responsible for supplying blood to the lens and the adjacent RGC layer during retinal development and neuronal differentiation (20). Both the hyaloid vasculature and the RGC layer

can be easily visualized in the zebrafish embryo through the use of transgenic lines that express fluorescent proteins specifically within the cells of these structures.

Zebrafish display numerous characteristics that make them ideal for studies involving the embryonic development of the nervous system (21). Embryos are abundant and externally fertilized making them amenable to surgical and genetic manipulation. Moreover, rapid development of the embryo leads to a functional visual system within 72 hours post-fertilization (hpf). The zebrafish genome is fully sequenced, and importantly, the mechanisms of visual system development are highly conserved in all vertebrates (22).

E. Research Objective & Rationale: Unpublished observations from *in situ* hybridization studies in the Ebert Laboratory have identified mRNA expression of *fgf8a* in the area of the differentiated RGCs in the eye of the developing zebrafish at 48 hours post fertilization (48 hpf). The only cells found to express any FGFRs at this stage were the suspected endothelial cells in the hyaloid vasculature (supplemental figure 1A). Moreover, an antisense morpholino oligonucleotide (MO) to inhibit mRNA expression of FGF8 (knockdown of FGF8) resulted in decreased RGCs (supplemental figure 1B). Effects on the hyaloid vasculature were also characterized, with inhibition of FGF8a via MO resulting in extensive mis-patterning of the hyaloid vasculature, thinner vessels, abnormal branching, and fewer overall vessels (supplemental figure 2A). The above two phenotypes were also observed in Acerebellar (*ace*) mutant zebrafish, which lack FGF8 expression (supplemental figure 2B). These data suggest that the developing RGCs provide an FGF8a signal that is required not only for their own growth and maintenance but also for that of the endothelial cells in the nearby vasculature. This study aims to investigate this essential signaling network and establish a model for FGF8a signaling in the eye that may help define its role in proper embryonic eye development.

Although FGF8/FGFR1 signaling is implicated in RGC growth and maintenance, very little is known about the mechanism by which FGF8 might regulate RGC development. This project bridges the studies of eye development in the Ebert lab with the research focus in the Deming laboratory, which is to understand the signaling pathways downstream of peptide growth factors including FGFs. Given that the RGCs produce FGF8a and the cells of the vasculature express FGFR1, we hypothesize that FGF8a signals from the RGCs to the vasculature of the developing eye, and that this signaling network is essential for proper eye development (figure 1). Although the RGCs do not express any FGFRs, it is possible for FGFs to signal independent of FGFRs through molecules such as syndecans (23). Therefore, there is a possibility that the RGCs can directly utilize the FGF8 signal that they produce in order to activate necessary signaling pathways. The overall goal of this research is determine which cells in the eye are activated by FGF8 by monitoring ERK phosphorylation following treatment with purified FGF8. This will allow us to gain a better understanding of how FGF8 supports the growth and development of RGCs.

G. Significance: As the mechanisms involved in visual development are highly conserved among vertebrates, and zebrafish are a popular model organism for the study of nervous system development, these and other similar studies are crucial in developing a better understanding of embryonic eye development. Additionally, the data obtained will lead to a greater understanding of FGF8-mediated signaling, on which less work has been done than other members of the FGF family.

II. Experimental Design

We began with Western blot analysis of whole cell extracts (WCEs) harvested from cultured human umbilical vascular endothelial cells (HUVECs), which express FGFR1, 2, and 3

(24), in order to demonstrate the ability to detect signaling events following treatment with purified, recombinant FGF8 in cells known to express FGF receptors. HUVECs were treated with FGF8 for increasing lengths of time in order to demonstrate its ability to stimulate ERK phosphorylation, as well as to determine the dosage time that provided peak levels of pERK. Once the efficacy of our FGF8 treatment was demonstrated, we progressed into the establishment and optimization of a protocol for the observation of cells plated to coated coverslips and monitoring of ERK phosphorylation using IF. These experiments began with HEK 293 cells (instead of immediately commencing with zebrafish explant cells) as HEK 293 cells are easier to grow and plate in large quantities.

Once we had established an IF protocol that was effective at discerning signaling events (such as ERK activation), we began our investigation into these signaling events in the RGCs and endothelial cells from the developing zebrafish eye. To do this, we utilized two different transgenic strains of zebrafish: the isl2b:GFP line which expresses green fluorescent protein (GFP) in differentiating RGCs and other neurons, and the flk:GFP transgenic line which uses GFP to fluorescently label the endothelial cells of the vasculature (25, 26). Whole eye explants were harvested and cultured from 48hpf embryos from both transgenic strains. These cultures were treated with purified FGF8 along with vehicle (DMSO) and positive controls (for ERK activation) including *phorbol*-12-myristate-13-acetate (PMA), nerve growth factor (NGF) for experiments utilizing the isl2b:GFP line, and platelet-derived growth factor (PDGF) for experiments utilizing the flk:GFP line. Levels of ERK phosphorylation were then monitored by IF using an anti-phospho-ERK (α-pERK) antibody to detect signaling activity, an anti-GFP antibody to identify the RGCs or the vascular endothelial cells via their GFP "tag", and DAPI, which stains DNA with a blue signal (27) in order to provide an identifier for all cells on the

slide. The number of cells responding to the various treatments was calculated using the cell counts as described in methods.

III. Materials and Methods

A. Antibodies and Other Reagents: Primary antibodies were obtained from Santa Cruz Biotechnologies (anti-GFP #J0813, anti-actin #1615), Cell Signaling Technologies (anti-phospho-ERK #9101, anti-total-ERK #9102), and Millipore (anti-phosphotyrosine (4g10) #05-321). Secondary antibodies were obtained from Invitrogen (anti-rabbit-alexa-fluor-594, #A11037 and anti-mouse-alexafluor-488, #A11029), as well as Jackson Immunolaboratories (anti-rabbit-hrp, #211-035-109). Also obtained from Invitrogen was the DNA stain 4',6-diamidino-2-phenylindol (DAPI, #D1306).

Cell culture supplies included poly-L-lysine (PLK) (Sigma #P2636), laminin (corning #354232), and collagen (Upstate Biotech catalog # 08-110) for coverslip preparation. Other cell culture supplies included a 0.1% trypsin solution in PBS (Gibco #15090-046) and a soybean trypsin inhibitor (STI) solution prepared with 25 mg STI (Sigma #T9003), 500 µL albumin (Sigma #A9205 (30% solution in 0.95% sodium chloride), 300 µL of 1M $MgSO_4$, and 10 mg of DNase (Sigma #DN15) dissolved in 50 mL Hank's Balanced Salts Solution (HBSS) (Gibco #14025-092). Various media used in cell culture included neurobasal media (Gibco #21103049) supplemented with 1X B27 (Gibco #17504044), 100 units penicillin/100 µg streptomycin (Life Technologies #15140-122), 500 µM glutamine (Sigma #G7513), and 25 µM glutamic acid (Sigma #G2128), as well as F12K serum-free medium (ATCC #30-2004) and Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Fisher, #03-600-511)). Cells were treated with PMA (sigma P8139), FGF8 (Peprotech #100-25), or NGF (Sigma #N1408).

B. Western Blot Analysis of HUVEC and HEK 293 cells: Human Embryonic Kidney 293 cells (HEK 293 cells) obtained from American Type Culture Collection or HUVECs obtained as a gift from K. Lounsbury (University of Vermont) were plated onto sterile 60 mm dishes and allowed to grow until they were approximately 90 confluent. Cells were then serum starved for either 6 hours (HEK 293 cells) or 24 hours (HUVECs), followed by treatment with DMSO, EGF (100 ng/mL), FGF8 (50 ng/mL) or PMA (4 μM) for varying amounts of time. WCEs were prepared and quantified (BCA Assay, Pierce) and 20 μg from each sample were subjected to SDS-PAGE analysis. Proteins were then transferred to nitrocellulose membranes and blocked in 1% bovine serum albumin (BSA) in TBST for 1 hour. Membranes were then incubated in rabbit anti-pERK antibody or anti-phosphotyrosine (pY) (4G10) diluted in Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 1% BSA O/N at 4°C. The next morning, membranes were washed three times in TBS-T, and then incubated with anti-rabbit-hrp secondary antibody diluted in TBS-T /1% BSA. Blots were washed three times in TBS-T and then subjected to chemiluminescence which was detected with photofilm. For loading control purposes, the above methods were repeated utilizing a rabbit antibody directed against total ERK (tERK) that is capable of binding to ERK regardless of its phosphorylation state (for lysates originally blotted for pERK) or against actin (for lysates originally blotted for phosphorylated tyrosine).

C. Coverslip Preparation: Two protocols were utilized in separate experiments for the preparation of sterile coverslips. First, glass coverslips were sterilized in 100% ethanol for 15 minutes and placed on edge within a 12 well plate to air dry. Next, the coverslips were coated with 1 mg/mL poly-L-lysine (PLK) in phosphate-buffered saline (PBS) for 5 minutes at room temperature (RT) followed by one PBS wash and 3 hour coat with 2 mLs of 10 μg/mL laminin in 1:50 collagen at 28.5°C. Alternatively, coverslips were also coated using the same concentrations

O/N (16 hours) at 4°C for PLK followed by ten 30 second washes with PBS and 5 hours at RT for laminin/collagen while eye explants were harvested. In both protocols, the coverslips were washed with 1X PBS before adding cells.

D. HEK 293 Immunofluorescence: Human Embryonic Kidney 293 cells were plated (200,000 cells/well) on acid-washed coverslips that were coated using the extended coverslip preparation protocol described above and maintained in DMEM-FBS. The cells were serum starved for 6 hours, then treated with DMSO, PMA, or 100 ng/mL epidermal growth factor (EGF), fixed with formaldehyde, permeabilized, and then blocked in either 5% NGS or 1.5% bovine serum albumin (BSA) in PBS. After blocking, the cells were treated with varying concentrations of a rabbit anti-pERK antibody for either 2 hours at RT or O/N at 4°C, washed four times with PBS, and treated with anti-rabbit-alexa-fluor-594 secondary antibody for 1 hour at RT. The cells were then washed four times with PBS, once with ddH_2O to remove salts, and mounted onto glass slides using Vector Vectashield Hard-Set Mounting Medium with DAPI (Vector, H-1500). Fluorescence was monitored using an epifluorescent microscope (Nikon Eclipse E600) housed in the Deming laboratory, and images were captured using a Nikon Digital Sight DS-Qi1Mc camera mounted to the microscope.

E. Zebrafish Embryo Preparation: All procedures involving zebrafish were approved by the UVM Institutional Animal Care and Use Committee (IACUC) protocol #12-055. Adult zebrafish were split into sets containing three females and two males, and each set was placed in a breeding tank 24 hours prior to embryo collection, with a clear divider separating males and females. To release the fish and promote breeding, the zebrafish were transferred to new breeding tanks with shallow water and the divider removed. After mating, the adult zebrafish were returned to their feeding tanks and embryos were collected. The embryos were transferred to clean glass dishes and incubated at 28.5 °C overnight (O/N). At 24 hpf, the embryos were

dechorionated and screened against improper development and lack of visible fluorescence, then incubated for another 24 hours at 28.5°C.

F. Dissection and Culture of Zebrafish Eye Explants: At 48 hpf, the embryos were transferred to sterile egg water. Two sets of sterile, fine-pointed forceps were utilized to remove the head from each embryo, followed by removal of the eyes from the head. Eye explants were retrieved using a pipette and then placed in a 1.5 mL tube containing sterile PBS. The explants were digested with 1 mL 0.1% trypsin at 28.5°C for five minutes and then centrifuged. The trypsin was removed and then 500 µL of soybean trypsin inhibitor was added. Following centrifugation, the soybean trypsin inhibitor was removed. The eyes were then resuspended in supplemented neurobasal media. The resuspended cells were split into wells with prepared coverslips and supplemented neurobasal media was added to each well to a final volume of 1.5 mLs. The cells were then incubated at 28.5 °C O/N.

G. Treatment of Explant Cultures: After O/N (16 hour) incubation in supplemented neurobasal media, cultures were placed in nutrient-free media (F12K media, ATCC) for 5 hours, and then treated for 15 minutes with DMSO vehicle control, purified FGF8 (50-100 ng/mL), and various positive controls 1-4 µM PMA, 50 ng/mL NGF, or 50 ng/mL PDGF.

H. Immunofluorescence of Explant Cultures: After treatments, cells were fixed onto the coverslips with1 mL of 4% paraformaldehyde and permeabilized with1 mL 0.1% Triton X-100 in PBS. Before staining, cells were blocked with either 1.5% BSA or 5% normal goat serum (NGS) in PBS either O/N at 4°C or for 1 hour at RT. The coverslips were removed from the wells and incubated with primary antibodies (diluted in 1.5% BSA or 1% NGS in PBS) O/N at 4ºC. A mouse antibody directed against activated phosphorylated ERK1/2 and a rabbit anti-GFP antibody were utilized (both diluted 1:50). Following four 5-minute PBS washes, the cells were

incubated with fluorescently-tagged secondary antibodies (anti-rabbit-alexa-fluor-594 and anti-mouse-alexafluor-488) and DAPI to stain nuclei. Secondary antibodies were diluted in 1.5% BSA or 1% NGS in PBS and incubated for 1 hour at RT. The coverslips were then washed, mounted, and observed with an epifluorescent microscope.

I. Quantification of ERK phosphorylation: Aside from qualitative observation, cells were quantified for each slide in two ways. First, every GFP+ cell on each slide was counted and the percentage of GFP+ cells displaying a red pERK response was determined. Second, 100 cells were counted using DAPI, and the percentage showing a red pERK signal (denoting response to PMA or FGF8), the percentage of green (GFP-expressing) cells (RGC or endothelial cells, depending on the transgenic line), and the percentage of cells displaying both red and green signals (indicating signaling in the tagged cells) were determined.

IV. Results

A. FGF8 stimulation results in increased levels of ERK and pan-tyrosine phosphorylation in HUVECs: In order to confidently use our purified, recombinant FGF8 as a treatment in experiments using cells from the zebrafish eye, we first aimed to demonstrate the effectiveness of our FGF8 treatment to activate FGFR-mediated signaling events in a human umbilical vascular endothelial cell (HUVEC) line, which expresses FGFR1, 2, and 3 (22). Specifically, we measured the ability of FGF8 treatment to stimulate increased activation (phosphorylation) of ERK, as well as increased global levels of phosphorylation within the cell, measured by phosphorylated tyrosine residues. Following acute stimulation with purified, recombinant FGF8 for 0, 10, 20, or 30 minutes, WCEs were subjected to Western blot analysis and probed for pERK and pY to detect signaling, as well as tERK and actin for loading control purposes (Figure 2). Our results indicate that treatment with FGF8 results in steadily increasing levels of pY as

dosage time increases, whereas the level of pERK appears to peak between 10 and 20 minutes followed by a slight decrease by 30 minutes. Using these findings, we estimated a peak level of ERK phosphorylation to arise 15 minutes post-treatment, and thus 15 minute treatments were utilized for later experiments using IF. These findings demonstrate the effectiveness of our FGF8 treatment to promote ERK phosphorylation in cultured cells.

B. Establishment of a protocol for the detection of ERK phosphorylation via IF: Once we demonstrated that our FGF8 treatments were successful at stimulating ERK activation in cells expressing FGF receptors, we established and optimized a protocol for the detection of ERK phosphorylation in specific cells using IF. HEK 293 cells were cultured on coverslips coated in laminin, collagen, and PLK and serum starved for five hours. Cells were then treated with DMSO and PMA and processed for IF as detailed in methods. We observed that PMA treatment was capable of producing a detectable level of ERK phosphorylation when treated cells were incubated in anti-pERK primary antibodies overnight at 4°C, whereas signaling could not be detected when cells were incubated at RT for 2 hours (figure 3A). These results were crucial for the optimization of the culture and IF protocol utilized to treat and observe zebrafish eye explant culture cells in later experiments. We had previously demonstrated the ability to detect ERK phosphorylation via Western blot analysis in HUVECs, however Western blots were also performed using HEK 293 cells in order to further demonstrate the ability of PMA to activate ERK phosphorylation in these cells. HEK 293 cells were serum starved to cease any basal level of MAPK signaling, then treated with DMSO for 20 minutes, PMA (4 μM) for 10 or 20 minutes, or EGF (100 ng/mL) for 10 or 20 minutes. The Deming lab has already previously demonstrated the ability of EGF to stimulate ERK phosphorylation via Western Blot, and therefore it was included in these experiments as a positive control for ERK activation beside PMA. Whole cell extracts were obtained from each of the treated cell cultures and subjected to Western Blot

analysis. It was observed that both EGF and PMA were capable of producing ERK phosphorylation in HEK293 cells following acute stimulation at the time-points examined (figure 3B).

C. RGCs do not Phosphorylate ERK in Response to Acute FGF8 Treatment: Having established and optimized a culture and IF system for detecting ERK phosphorylation following acute stimulation, we next set out to determine which cells in the developing zebrafish eye respond directly to FGF8a stimulation. As we hypothesized that the RGCs of the developing eye are the cells responsible for producing the FGF8 signal, we began by investigating whether the RGCs could utilize the FGF8 signal that they produce themselves, or if other cells in the eye are stimulated by FGF8 treatment instead. Whole eye explants were removed from 48 hpf isl2b:GFP zebrafish embryos and cultured as described in methods. Cells were treated with FGF8 as well as positive (PMA, NGF) and negative (DMSO) controls for 15 minutes, and then processed for IF as described in methods. Upon IF observation of all RGCs (detected by a green signal) present in each experiment, RGCs displaying elevated pERK levels were nearly absent in response to FGF8 treatment. As seen in Figure 5, a total of 406 GFP+ cells were counted across five trials, with only 4 (0.65%) showing an elevated response to FGF8 treatment. Despite the inability of FGF8 to stimulate ERK phosphorylation in RGCs, increased pERK signal could be detected in GFP+ cells in response to NGF treatment, with nearly half of all RGCs (45%) responding to NGF treatment (figure 5A). Representative photos of NGF treated cells from the zebrafish eye are provided in figure 4 I-L, which display the ability of NGF to provide an intense red signal in GFP+ cells, denoting ERK phosphorylation within RGCs. The results obtained from the NGF trials were of particular importance, as they demonstrated the ability of the GFP-tagged RGCs to produce increased levels of pERK in response to a provided signal. These data suggest that

RGCs within the developing zebrafish are capable of activating the MAPK pathway normally, resulting in phosphorylation of ERK; however they are unable to do so in response to FGF8.

D. FGF8a is Capable of Stimulating Non-Retinal Ganglion Cells in the Eye: As we were able to conclude that FGF8 treatment is unable to stimulate the RGCs, cell counts were also performed to investigate the capability of FGF8 to stimulate ERK phosphorylation in other cells in the eye. 100 cells were counted via DAPI and their possible signaling combinations were recorded, noting whether each cell produced a red signal (indicating elevated levels of phosphorylated ERK), a green signal (indicating the cell is GFP+ and therefore a RGC), or both (indicating the cell is an RGC that successfully responded to the treatment administered with a pERK response). As we observed while examining the RGCs alone, FGF8 was unable to produce a pERK response in any GFP+ cells, however it was capable of stimulating approximately 12.2% of all other cells in the eye (figure 6). These results are also shown in figure 4 E-H, which shows intense pERK signaling in two of the cells within the field in response to FGF8 treatment, but shows no detectable pERK signal in the lone GFP-tagged RGC seen in figure 4F. Therefore, we can conclude that the FGF8 treatment was effective and successfully administered to the cultured cells, producing a response in cells that could interact with it. Remarkably, PMA was unsuccessful as a positive control for ERK activation in RGCs, but was able to stimulate ERK phosphorylation in 31% of the cells in the eye.

V. Discussion

We have established a retinal explant cell culture model system that can be used for studying signaling events in specific cells of the zebrafish eye. Using this system, we have demonstrated the capability of the RGCs within the zebrafish eye to respond to growth factor signaling via a detectable pERK response, and have concluded that FGF8 is not capable of

producing a significant increase in ERK phosphorylation in these cells (figure 5B). Additionally FGF8-mediated ERK phosphorylation can be detected in other non-RGC cells in the eye. Lastly, we have outlined multiple modifications to established tissue explant and RGC culture protocols that may help improve future studies that aim to use these techniques.

FGFs have been widely shown to have key roles in proper embryonic development. The results of the present study, as well as others being conducted in the Ebert laboratory, are beginning to help elucidate a potential role for FGF8a in a unique signaling loop between the cells of the retina and of the vasculature. Through the use of morpholino injection and examination of mutant zebrafish, unpublished observations from the Ebert laboratory have well characterized the significant abnormalities that can arise following the disruption of FGF8a signaling. The data obtained from the present study indicate that the only cells producing FGF8a in the developing zebrafish retina (the RGCs) do not directly utilize FGF8 for their own signaling events, whereas other cells present in the eye do in fact respond to soluble FGF8. Given that the expression pattern for FGF receptors in the eye was restricted to FGFR1b expression in the area of the hyaloid vasculature, these responding cells are likely vascular in nature. These results heavily support the model that we have proposed, in which neuronally-secreted FGF8a signals to other cells in the eye, evoking a response in those cells that in turn provides reciprocal support to the neurons within the retina.

Although this study did not specifically identify the cells capable of responding to FGF8a as endothelial cells, many sources have documented the capability of FGF8 to signal from one cell in an organism to another in order to facilitate proper embryonic development (28). As shown in supplemental figure 1A, *in situ* hybridization for *fgfr1b* provided nearly identical staining compared to flk staining directed against the vasculature. Although this does not outright

confirm that *fgfr1b* is expressed within the vasculature of the zebrafish eye, it is clear that *fgfr1b* is expressed in cells outside the retina, providing sufficient basis for the possibility of FGF8a signaling from the retina to the presumptive hyaloid vasculature. There is evidence that *fgfr1* is expressed specifically in vasculature cells, including a previous study identifying the presence of *fgfr1* in endothelial cells throughout the developing human fetus (29). Using the flk:GFP transgenic line, experiments such as those described in the present study will allow for detection of signaling within GFP-tagged endothelial cells. If we observe that these cells are capable of responding directly to FGF8a, those findings would further support the idea that *fgfr1b* is expressed specifically within the vasculature cells of the developing eye.

Although disruption of FGF8a signaling results in less RGCs and dramatic developmental abnormalities to the structure of the vasculature within the eye, we have yet to determine the specific essential response that is stimulated following FGF8a signaling, to what we presume to be the vasculature cells themselves. Future studies within the Deming and Ebert laboratories will aim to further elucidate the specific responses within the eye to FGF8 signaling that are crucial for development of the vasculature and the developing RGCs. Unpublished observations from the Ebert laboratory have been performed to investigate the possibility that the decrease in RGC numbers when FGF8a is absent, despite the inability of the RGCs to respond directly to FGF8a, is due to lack of nutrient delivery resulting via abnormal development of the hyaloid vasculature. The lack of proper hyaloid vasculature development would lead to decreased blood flow to both the RGCs, resulting in a hypoxic environment within these structures. FGF8a knockout using both MO and the *ace* mutants demonstrated abnormal branching and patterning of the hyaloid vasculature, and studies have shown that hypoxic conditions can cause an increase in vessel branching (29) similar to the phenotype seen with

FGF8a signal disruption. Should the abnormal vascularization phenotype resulting from FGF8a signaling disruption be identified as the cause for decreased RGC development, these findings would contend with previously reported findings in the field of zebrafish eye development, which have concluded that zebrafish are capable of receiving all necessary nutrients and gasses through simple diffusion with the embryo's surroundings (31).

The present study also provides useful information to researchers who aim to culture RGCs or other neurons obtained from various tissue explants, as well as for the proper IF observation of signaling events in these cultured cells. A particular difficulty surrounding the culture of cells from zebrafish embryos is the presence of bacterial contamination within the environment of the zebrafish embryo during development. Various antimicrobials are utilized in aquaculture, including ciprofloxacin and methylene blue (32), and these have largely been shown to eliminate the growth of some, but not all, sources of contamination with mold, bacteria, and other unicellular organisms. Despite the presence of methylene blue in the egg water utilized in the present study, bacterial contamination was an extremely prevalent complication during initial studies. Various measures that proved effective at further reducing the prevalence of contamination in cultured cells from eye explants included the filter-sterilization of egg water using a vacuum filter containing 0.22μm PVDF membrane, followed by the transfer of embryos to the filter-sterilized egg water multiple times prior to explant retrieval. In addition, autoclave sterilization of plates and pipette tips used, as well as ethanol sterilization of the forceps utilized to remove the eye explants all led to decreased incidences of contamination in later experiments. Additionally, information gained from our optimization studies should be useful for future studies. Specifically, we found that an increase in incubation times for PLK, laminin, and collagen when preparing coverslips for the addition of explant cells aided in the number of cells

present for IF observation. Moreover, increasing the incubation time for anti-pERK primary antibodies allowed for improved monitoring of ERK phosphorylation using IF. Initial protocols were designed to manufacturer specifications, however we found the coverslip coating times for the proteins listed above to be wholly insufficient for proper culturing and adherence of RGCs to glass coverslips. Recent protocols have been released for the use of the pERK antibody utilized in the present study that call for permeabilization with methanol, followed by the use of Triton X-100 in solutions containing either primary or secondary antibodies for IF detection of ERK phosphorylation (33). Future studies will be performed using methanol permeabilization in order to determine if this alteration to our IF protocol will allow for more intense or specific staining against pERK.

The signaling events that occur during embryonic development are extraordinarily complex, and data such as these allow a better understanding of how various cells and structures interact to provide the correct environment for the proper development of the organism. Over a dozen congenital diseases have been identified in humans that result from mutations to FGFs or their receptors (34), further demonstrating the importance of proper FGF signaling, not only in the developing zebrafish but in other mammals as well. Thus, by helping elucidate the specific cellular origin and destination for soluble FGFs during development, the present study contributes to the body of knowledge surrounding the various signaling networks relevant to human disease. Further investigation into the specific FGF8a signaling network in the developing eye in order to better describe the interplay between nervous and vascular systems during development may increase our understanding of the development of congenital diseases within the eye, as well as our understanding of eye development in the zebrafish itself, which is

crucial towards its continued use as a model organism for studies investigating nervous system development.

VI. Figures

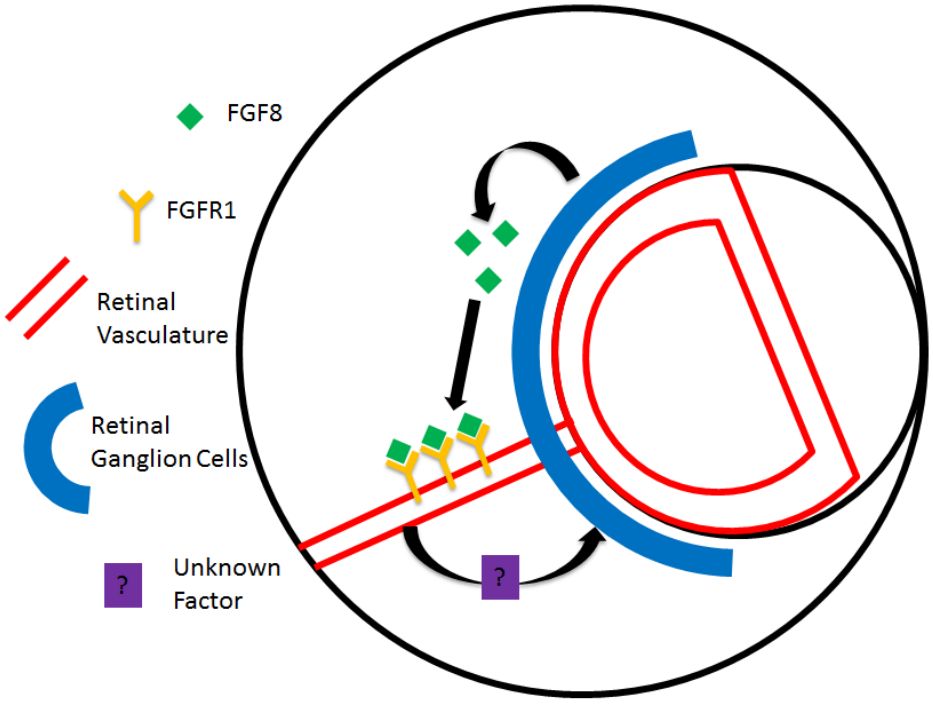

Figure 1. Proposed model for FGF8a signaling from the RGCs to the hyaloid vasculature. We propose a model that involves the production of an FGF8a signal that travels from the RGCs to the hyaloid vasculature, activating signaling events that are crucial for the proper development and growth of the vasculature, which subsequently results in the production of an unknown signal from the vasculature to the RGCs that is essential for their survival and proliferation.

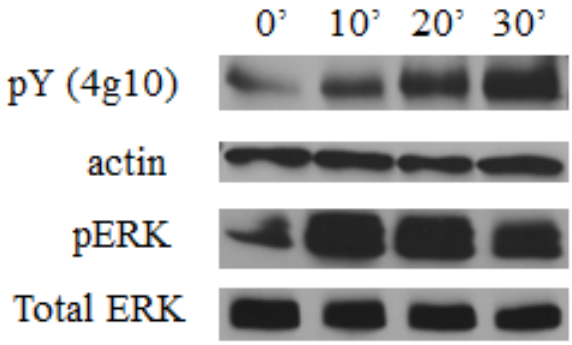

Figure 2. Acute FGF8a stimulation results in increased levels of ERK and total tyrosine phosphorylation. HUVEC cells were serum starved for 24 hours, then stimulated with 50 ng/mL FGF8 for 0, 10, 20, or 30 minutes and cell lysates were harvested with RIPA lysis buffer. Whole cell extracts were separated by SDS-PAGE and subjected to Western blot analysis using antibodies directed against phosphorylated tyrosine (4G10) and phosphorylated ERK to detect signaling, and actin and total ERK for loading control purposes. Representative band chosen for phosphotyrosine blot.

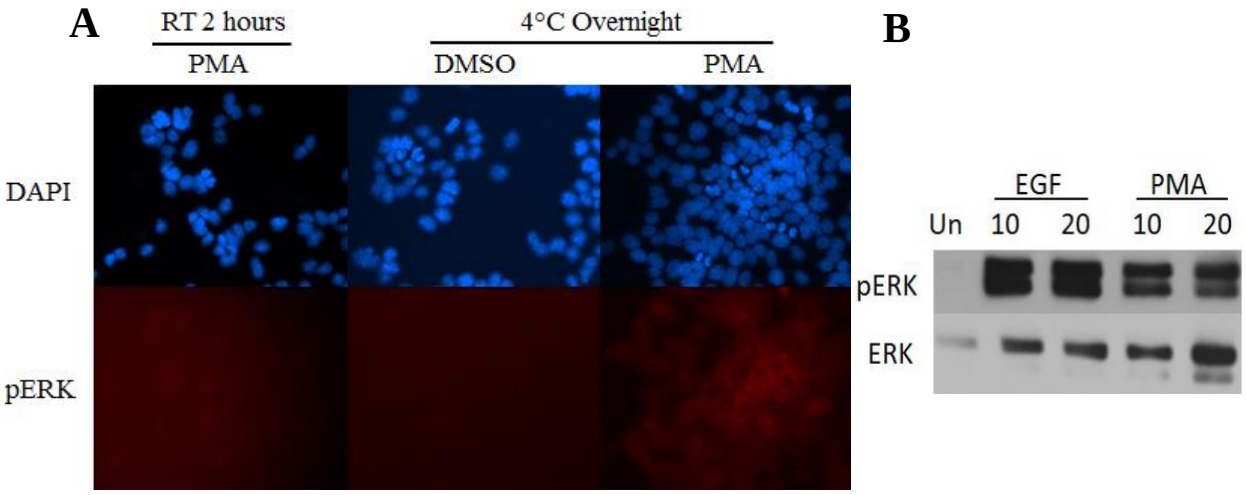

Figure 3. Detection of pERK in HEK 293 cells via immunofluorescence. (A) HEK293 cells were serum starved for 6 hours, then treated with DMSO or 4 µM PMA for 15 minutes. Cells were then blocked in 5% NGS or 1.5% BSA and treated with anti-pERK antibody diluted either 1/50 or 1/25 in blocking solution either at RT for 2 hours or at 4°C O/N. Cells were then incubated with fluorescently tagged anti-rabbit secondary antibodies and DAPI, mounted, and observed using an epifluorescent microscope at 20X and 40X. (B) HEK 293 cells were serum starved for 5 hours, then treated with DMSO for 20 minutes, 100 ng/mL EGF or 4 µM PMA for 10 or 20 minutes, and cell lysates were harvested with RIPA lysis buffer. Equal amounts of whole cell extracts from each treatment were separated by SDS-PAGE and subjected to Western blot analysis using antibodies directed against phosphorylated ERK to detect signaling, and total ERK for loading control purposes.

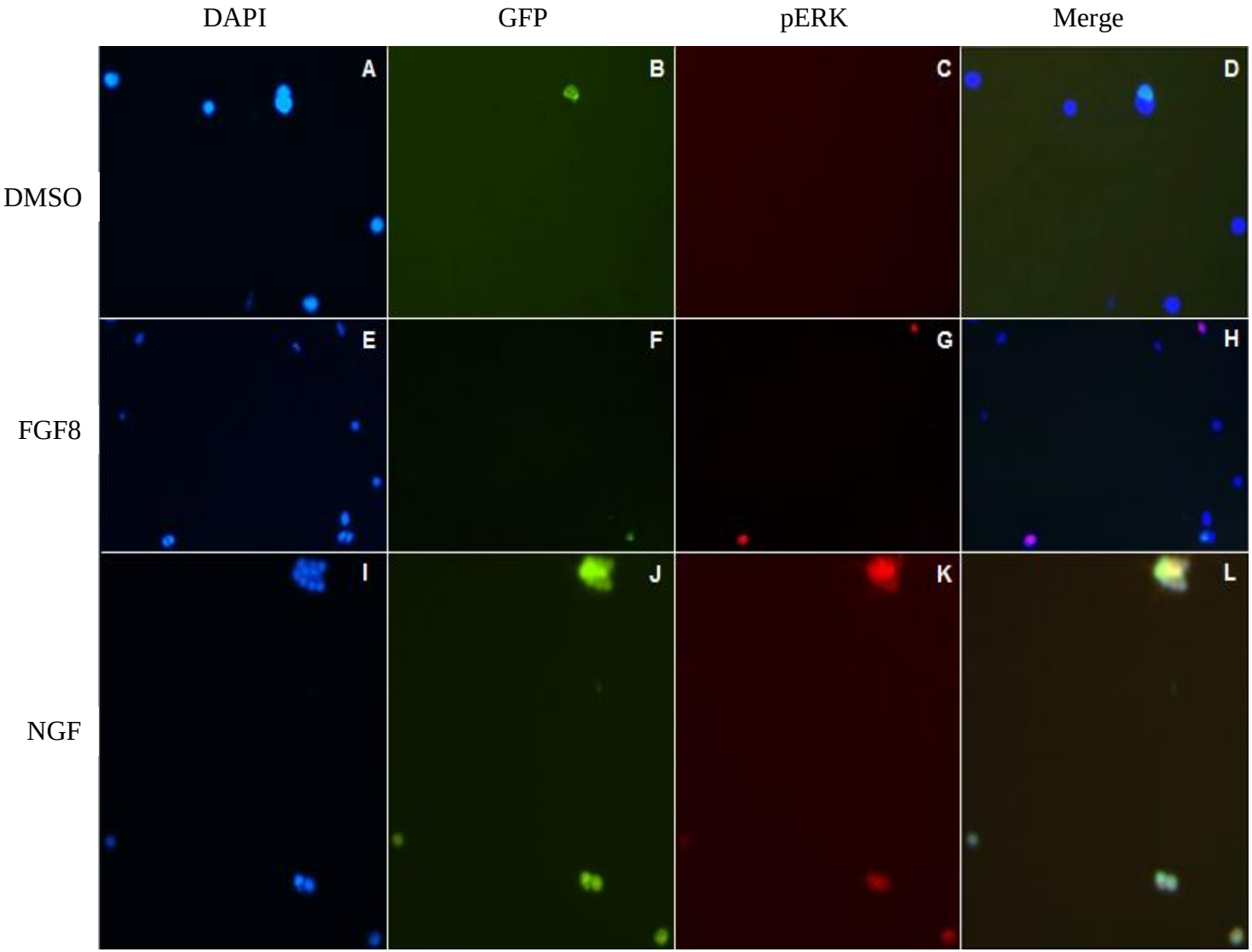

Figure 4. Acute FGF8a treatment cannot stimulate ERK phosphorylation in RGCs, but can in other cells within the eye. Cells were obtained from isl2b:GFP zebrafish were cultured, treated, and observed via IF as described in methods. Photographs were taken at 40X using an epifluorescent microscope. Images have been adjusted equally for brightness.

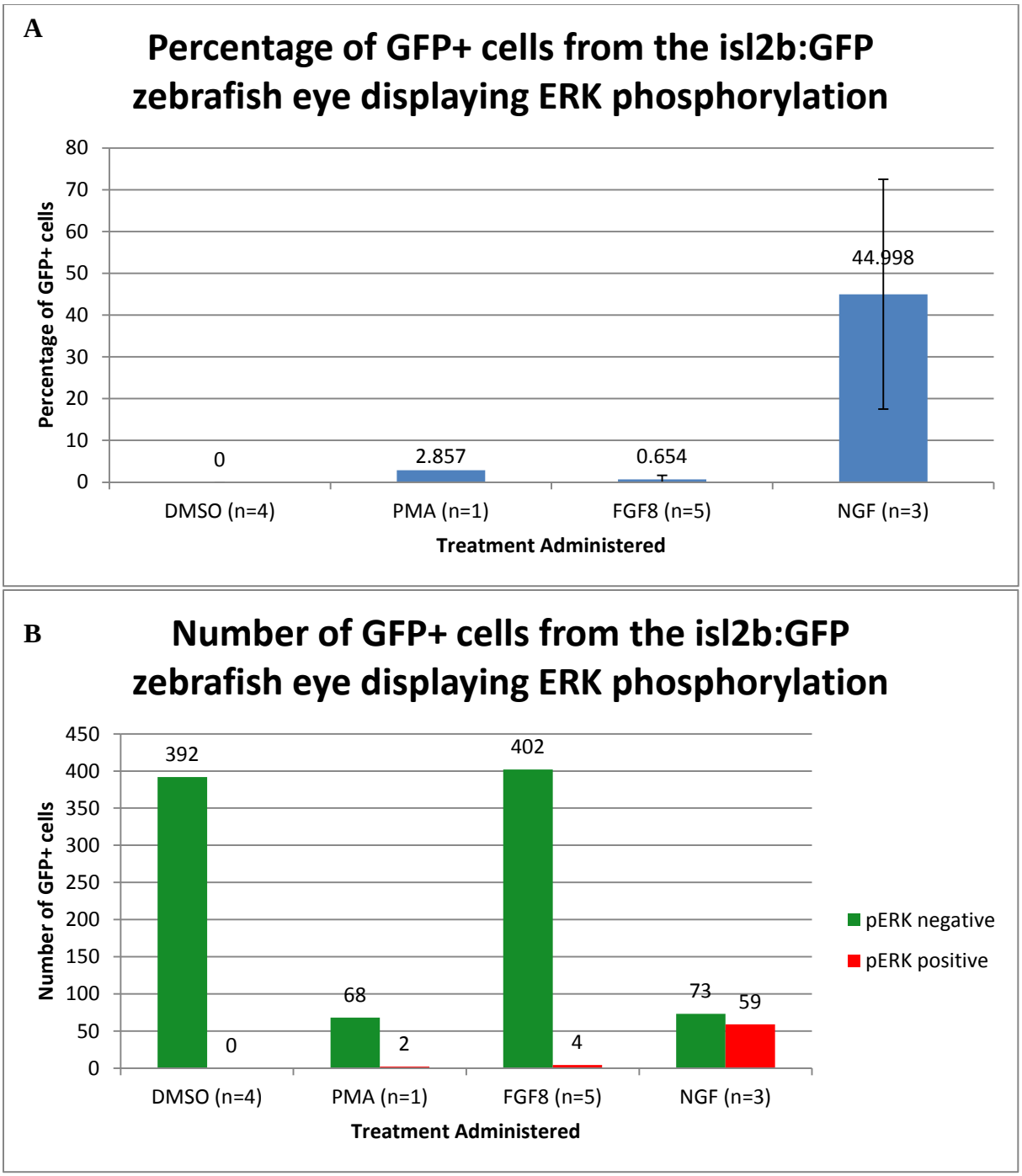

Figure 5. Acute FGF8a treatment is unable to stimulate ERK phosphorylation in RGCs. Cells obtained from whole zebrafish eye explants from isl2b:GFP zebrafish were cultured, treated, and observed via IF as described in methods. (A) Every GFP+ cell present on the slide (identified by a green signal) was counted and the percentage demonstrating elevated levels of ERK phosphorylation (identified by a red signal) was calculated. Error bars represent StdDev. (B) Number of GFP+ cells counted that were positive or negative for ERK phosphorylation across all experiments (n=number of experiments performed).

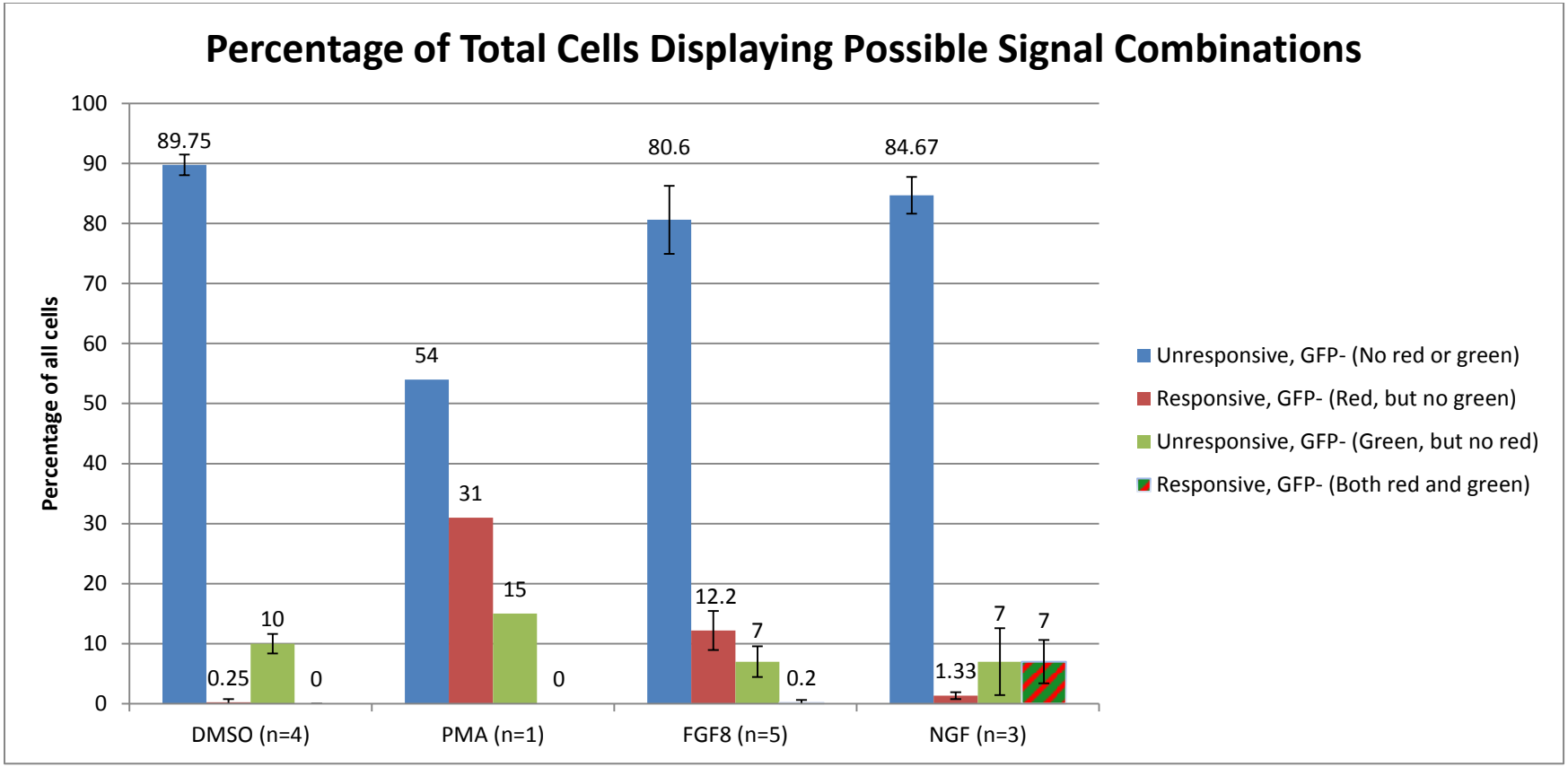

Figure 6. Acute FGF8a treatment is able to stimulate ERK phosphorylation in non-RGC cells within the eye. Cells obtained from whole zebrafish eye explants from isl2b:GFP zebrafish were cultured, treated, and observed via IF as described in methods. 100 total cells were counted and the percentage of each that displayed a green signal (identifying them as RGCs), a red signal (denoting ERK phosphorylation), both signals, or neither signal was quantified. Error bars represent StdDev.

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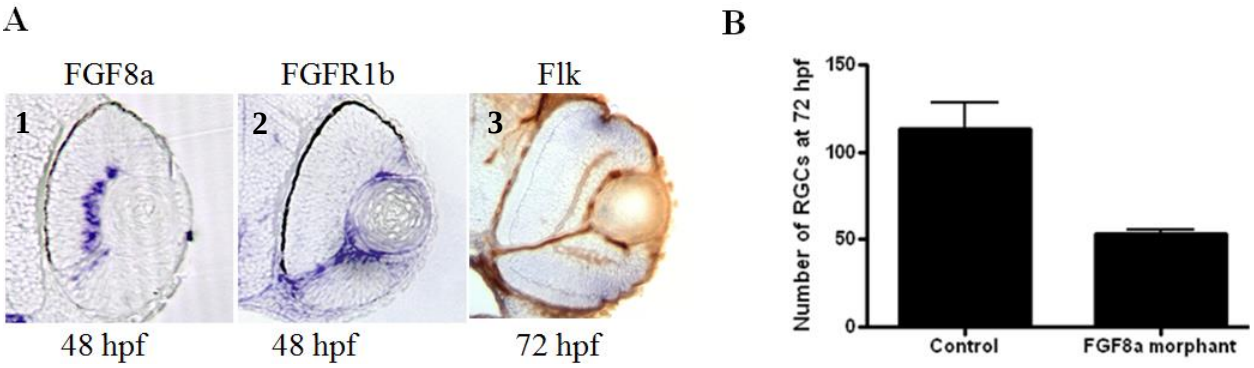
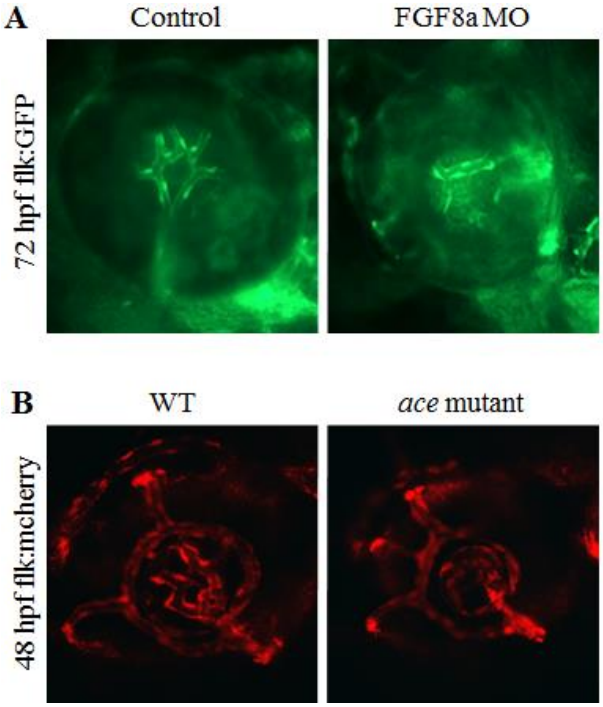


Supplemental Figure 1. Characterization of FGF8a signaling in the zebrafish embryo. Transverse sections were obtained from zebrafish eyes and subjected to either *in situ* hybridization against FGF8a or FGFR1b at 48 hpf (A1,2) or flk at 72 hpf (A3). At 48 hpf FGF8a expression is isolated to the area of the differentiating RGCs, whereas strong mRNA expression can be detected for FGFR1b in a similar pattern shown by flk staining at 72 hpf. Disruption of FGF8a signaling using MO results in a decreased number of RGCs at 72 hpf (B). Figure adapted from (35) as well as from unpublished images from the Ebert laboratory (1A, B).



Supplemental Figure 2. Disruption of FGF8a signaling results in abnormal vascularization. Confocal images of (A) 72 hpf flk:GFP and (B) 48 hpf flk:mCherry *ace* mutant embryos. (A) FGF8a MO results in mispatterning of the hyaloid vasculature including abnormal branching and thinner vessels. (B) In the *ace* mutant which lacks FGF8a, abnormal branching and an additional vessel are present within the hyaloid vasculature. Images taken at 20X magnification and adjusted for brightness. Figure created using unpublished images from the Ebert laboratory.