TARGETING *FGF8A* PROMOTER FOR GENE EXPRESSION

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Targeting *FGF8a* Promoter for Gene Expression

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In this paper, I will be assisting in developing a new method of characterizing communication between the enhancers and promoter within the *fgf8a* gene in vertebrates, specifically in zebrafish. The *fgf8a* gene controls important growth functions and regulates developmental processes in zebrafish. Currently, there is no understanding on how the enhancers function within the *fgf8a* gene, thus my research will further describe gene regulation and the role of these enhancers. My expected outcomes are to create specific engineered CRISPR RNA that will target the gene promoter and to potentially visualize the gene promoter within the nucleus. Previous research studied the role of enhancers in deceased samples and our research will develop an approach for imaging the promoter portion of the gene in real-time. Targeting the promoter is the first step to identifying the interaction between the promoter and enhancers in the *fgf8a* gene. Two CRISPR RNA sequences developed to target the *fgf8a* promoter were inserted into zebrafish embryos to test against a control. We conducted an inexpensive, rapid genomic extraction method with a high-resolution melt assay that is more sensitive and allows early detection of CRISPR-induced indels in zebrafish. The results showed that the CRISPR RNA

sequences caused a mutation in genetic expression and confirmed targeting of the *fgf8a* promoter. These findings will lead us to a step closer in visualizing the interaction of the enhancers and promoters of the gene to further understand its regulation. Demonstrating and defining how this gene expresses itself in zebrafish can advance our understanding of gene expression in human development.

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Contributors

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Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

Finally, thanks to my fellow classmates for their encouragement and to my fiancée for his patience and love.

The data and materials analyzed/used for Targeting *fgf8a* Promoter for Gene Expression were provided by Alvin Yeh, Bruce Riley, Sreeja Sarasamma, the Texas A&M University BioAquatics Facilities, and the Texas A&M University Department of Biomedical Engineering. The analyses depicted in Targeting *fgf8a* Promoter for Gene Expression were conducted in part by the Tissue Microscopy Lab under Dr. Alvin Yeh, the Texas A&M University BioAquatics Facilities, and the Texas A&M University Department of Biomedical Engineering and these data are unpublished.

All other work conducted for the thesis was completed by the student independently.

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1. INTRODUCTION

The *fgf8a* gene controls important growth functions within zebrafish. It regulates a variety of developmental processes from early to late development (Komisarczuk et al., 2009). The gene specifically has a fibroblast growth factor receptor binding function and a growth factor activity function that signals these developmental processes. How the *fgf8a* gene is regulated is still undetermined but necessary to understand since the gene is crucial for cellular responses. In my research, I will assist in developing a new approach to identify how enhancers found on the gene communicate with the promoter portion.

1.1 The Importance of Gene Communication

Our research wants to further understand how genes are affected by the interaction between enhancers and promoters. Enhancers can promote transcription and can influence how the gene is transformed into products, which increases the need to understand the element. It is essential to know the role of enhancers on the promoter portion of the gene because it affects the proteins that are created. As proposed in Furlong and Levine (2018), the complexity of the communication between enhancers and promoters should begin to be visualized for further exploration. Our lab has begun the process of attempting to image the communication by identifying promoter portions of different genes. For this paper, the *fgf8a* gene promoter will be targeted to eventually image and observe its relationship with nearby enhancers.

1.2 *fgf8a* Gene Selection

Craniofacial development, and the signaling pathways involved, in zebrafish is similar to the processes found in human brain morphogenesis (Mork & Crump, 2015). Furthermore, it has been discovered that a zebrafish's craniofacial skeleton, midbrain, hindbrain, midbrain-hindbrain

boundary (MHB), and many other organ systems are dependent on *fgf8* signaling for induction, development, and subdivision. These particular organs and their formation, especially the specifications and patterning of the MHB, is crucial to the developing brain (Gibbs et al., 2017). The *fgf8a* gene has been previously identified to affect the MHB and its development. By manipulating the gene and its promoter site, visible effects would be potentially seen. Furthermore, the *fgf8a* gene has also been previously used to investigate the effects of enhancers. Thus, the *fgf8a* gene is an excellent option to investigate genes at a transcriptional level, specifically in regard to the communication between the enhancer and promoter.

1.3 Targeting the *fgf8a* Gene with CRISPR

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) was the gene-editing tool used for the research. More specifically, a CRISPR-associated (Cas) endonuclease (a.k.a. enzyme) called Cas9 was used to generate double-stranded breaks of DNA (Dominguez et al., 2016). The Cas9 localizes the genomic sites with the usage of a programmed RNA-guided endonuclease (RGEN), which will be further discussed in the later portions of the paper. Once the site-specific DNA strand is broken, the DNA can restore itself with homology-directed repair (HDR) or nonhomology end-joining (NHEJ) (Dominguez et al., 2016). HDR is a repair method that occurs when the Cas9 enzyme is attached to a DNA template that can be used to either correct or alter a gene. NHEJ is a repair method that occurs when there is no DNA template present and the ends of the delete sequences are joined together. The NHEJ repair method will introduce insertion-deletion of the gene as there is no template provided and the gene will have to repair itself (Dominguez et al., 2016).

In our research, we are utilizing the CRISPR/Cas9 system to confirm localization of the promoter portion of the *fgf8a* target gene. Once the location is confirmed, we will implement the

deactivated Cas9 (dCas9) instead of the Cas9 enzyme to attach a fluorescent label to the verified site. The CRISPR/dCas9 system can be used to manipulate the transcription process without altering the genomic sequence. The dCas9 enzyme binds to DNA instead of cleaving it (Dominguez et al., 2016).

1.4 A New Approach to Visualize Enhancer and Promoter Communication

Previous research has investigated how the *fgf8a* gene is expressed by multiple enhancers and therefore we are continuing this research by introducing a method that will focus on the promoter portion of the gene to evaluate how it is regulated. For instance, Komisarczuk et al. conducted an *fgf8* gene study in zebrafish and determined that *fgf8a* gene regulation is dependent on multiple regulatory elements (Komisarczuk et al., 2009). Our research will lead to further investigation of the roles of these elements, specifically enhancers, by allowing the promoter portion of the gene to be monitored. Furthermore, Marinić et al. describes how enhancers may interact with the *fgf8* gene regulation in mice depending on gene position (Marinić et al., 2013). Our research will build upon this assumption as well and determine a way to investigate how enhancers communicate with the promoter of the *fgf8a* gene in vertebrates.

A key difference between previous research and our research is how we want our new method to approach the uncertainty of the enhancers' role. More recent techniques that were used in previous research, made measurements on dead samples which had no time measurements or genomic assays. Our approach involves adding a real-time imaging aspect of the gene. Our research will hopefully determine if this new method can characterize enhancer communication with the target *fgf8a* gene.

1.5 Objectives of The Paper

The expected outcome from my research is a sensitive probe that targets the promoter of the *fgf8a* gene and related enhancers without interfering with endogenous function. I will essentially begin the first step needed to visualize the enhancers and promoters of the gene directly. Afterwards, my research will be passed on to our laboratory group to edit the probe for imaging. The probe preparation will involve attaching fluorescent proteins to enhancers and promoters located on the genome. A high resolution, selective plane illumination microscopy will then be used to visualize the communication between the two regulatory elements over time.

My research is essential to begin the analysis of gene enhancer and promoter communication through live imaging.

2. METHODS

2.1 Obtaining Sequences

To begin the research, sequences from previous studies were utilized to potentially target the *fgf8a* promoter. Using the University of California- Santa Cruz zebrafish genome browser, the sequences referenced in Long et al. (2015) were input to find the sequences that are located within the position of the *fgf8a* gene promoter. The position had to be between 28,365,067 and 28,371,535 in chromosome 13 with a GRCz10/danRer10 genome assembly. Afterwards, using the CRISPR-Cas9 Synthetic Guide RNA (gRNA) design checker on the Integrated DNA Technologies (IDT) website, the sequences were input to determine the potential off-target effects. The website aided in determining which sequences would work most efficiently to introduce double stranded break at specified location. Two gRNAs found in Long et al. (2015) were potentially located at the fgf8a gene promoter portion and were estimated to have low off-target effects. The following table contains the gRNAs selected for the research.

Table 2.1: Sequences used to potentially locate the promoter portion of the fgf8a gene (Long et al., 2015, Supplementary information, Table S1).

Sequence Label	Sequence Information (5'→ 3")
fgf8a-1	GAGCATAGTAGCAAAACGCAAAG
fgf8a-2	GGTTCGGCTGTAAAGCTGGT

2.2 Preparation of guide RNA

The gRNA sequences above were ordered under the ALT-R™ CRISPR Cas 9 System from the IDT website. The system is used to produce double-stranded breaks in DNA by the

Cas9 enzyme (Integrated DNA Technologies [IDT], 2018). Furthermore, the system also contained instructions on how to prepare gRNA complexes to effectively activate the Cas9 enzyme for alteration when they are both combined and inserted. Successful alterations can introduce insertions and deletions of the sequences that demonstrates effective targeting of the *fgf8a* promoter.

The ALT-R™ CRISPR Cas 9 System contained two RNA molecules, the ALT-R™ CRISPR Cas 9 CRISPR RNA (crRNA) and the ALT-R™ CRISPR Cas 9 transactivating crRNA (tracrRNA), for each specific sequence ordered. The crRNA contains the particular DNA target sequence and is the customizable portion of the gRNA that will guide the complex to the *fgf8a* gene. The tracrRNA binds and activates the Cas9 nuclease to functionally edit/cut the DNA sequence. The gRNA complex is formed by combining the crRNA and the tracrRNA. When the crRNA and tracrRNA for each sequence arrived, both were suspended in an IDT nuclease-free IDTE buffer to obtain a final concentration of 100 µM each. The IDTE buffer is used to resuspend single-stranded RNA (IDT, 2018).

To achieve a 3 µM gRNA solution, the following parts were mixed together: 3 µL of the crRNA, 3 µL of the tracrRNA, and 94 µL of an IDT nuclease-free duplex buffer. The duplex buffer is used to store duplexed strands. The mixture was then heated at 95°C and removed after five minutes. The mixture was put on the benchtop to cool down to room temperature (about 20°C). The final concentration of the crRNA and tracrRNA was 36 ng/µL and 67 ng/µL, respectively.

The gRNAs for both sequences were then stored at -20°C for later usage (IDT, 2018).

2.3 Zebrafish Embryo Collection

Using the Texas A&M University Fish Facilities, the embryos were collected from wild-type zebrafish (*Danio rerio*) with the Tüpfel long-fin (TL) strain intercrossed with the AB strain. The embryos were collected directly from the in-lab zebrafish. The zebrafish used to breed the embryos were stored and raised in tanks at 28.5° C with an alternating light and dark 12-hour cycle. The zebrafishes were kept in tanks by gender and regularly fed by lab workers. The water in all the tanks came from one central watering system. The same type of zebrafish was used throughout the entirety of the experiment.

To collect embryos, one male zebrafish and one female zebrafish were placed in a smaller container with a separator overnight. The fishes are isolated together overnight to increase chance of fertilization because their breeding is photoperiodic and create embryos in the morning (Westerfield, 2000). A reusable filter is also placed between the fish and the bottom of the tank to collect embryos. To increase the likelihood of fertilization, food was added to the smaller tanks before departing the fish for their overnight isolation. After one night, the separator between the male zebrafish and female zebrafish was taken out early the next day. After fifteen minutes, any successful fertilization would result in embryos being filtered out to the bottom of the small container.

The embryos can only be utilized for injection at the one-cell stage which only lasts for 0.2 hours (~12 minutes) (Westerfield, 2000). Due to how fast zebrafish embryos develop, the zebrafishes were quickly located into another container and the embryos were collected with a net as soon as they were visible. The embryos were then placed carefully in a petri dish with fish water to transport for microinjections. The number of embryos present would range from about twenty to fifty. Since not all zebrafish pairs will produce embryos, at least eight pairs of

zebrafish were placed overnight to further increase the possibility of having an abundant number of embryos available for microinjection.

2.4 Microinjection Procedure

2.4.1 Gene Material Preparation

The Alt-R™ CRISPR-Cas9 System was referenced to prepare the ribonucleoproteins (RNP) that would be microinjected into the zebrafish embryos. The system essentially requires that the Cas9 protein be paired to the gRNA to insert altogether in the embryos. The process of combining the gRNA with the Cas9 protein must occur on the day of injection for increased stability and efficiency.

Having already prepared the gRNAs, the microinjection preparation began with the dilution of the 10 µg/µL Cas9 protein with a provided Cas9 working buffer. The desired concentration for the Cas9 protein was 0.5 µg/µL which called for 9.5 µL of the Cas9 working buffer to be mixed in 0.5 µL of the 10 µg/µL Cas9 protein.

To form the RNP complexes, 3 µL of the 0.5 µg/µL Cas9 protein was combined with 3 µL of the gRNA complex and was then incubated at 37°C for ten minutes (IDT, 2018).

2.4.2 Microinjection Preparation

An agarose gel with semicircular grooves, or capillaries, is prepared inside a petri dish to place the embryos for injection. The capillaries are 1 mm wide and are depressed enough for embryos to be stationed still during the microinjection. The embryos are gently held to the capillaries with minimal risk of embryo damage or death. Fish water is also prepared for embryos to be placed in. The microinjector in the Texas A&M BioAquatics facility was used for the injections.

2.4.3 Microinjection of Embryos

The embryos collected were transported to the laboratory room nearby in petri dishes and fish water. The embryos were gently placed in the capillaries of the agarose gel formulated previously using a pipette. Embryos are placed down the capillaries within the grooves. The embryos were positioned in a line in each capillary with no stacking whatsoever. No forceful fitting occurred as it would damage the embryos or manipulate the agarose gel. Only a portion of the embryos were placed in the agarose gel while others were set aside in a tube with fish water as a control to compare against the injected embryos.

The agarose gel with the embryos was placed under a Nikon SMZ1500 Stereo Microscope. Due to rapid growth of the zebrafish embryos, quick administration of the RNP mixture is required. The embryos each had to be injected with 1-2 nL of the mixture of 50 ng/µL of the Cas9 protein and 30 ng/µL of the gRNA. To inject this small amount of mixture to each embryo, the Narishige IM-300 Pneumatic Microinjector was utilized along with a micromanipulator for increased precision. The injection needles used were sterilized and sharpened beforehand for proper injections. The needle, micromanipulator, and electric-controlled microinjector were all connected to allow pressure to uptake about a 1 mL of the mixture and to release 1-2 nL into each embryo. The microinjector was connected to a foot pedal that could be pressed to release the small volume of the mixture into the chorion. The apparatus was then used to inject each embryo.

The needle was pushed through the egg yolk towards the chorion of the cell with the usage of the micromanipulator. After the needle was placed in the correct position within the embryo, the foot pedal would be pressed. This method was repeated for all the embryos in the

agarose gel. After injections, the embryos were then transported into a tube with fish water for incubation in the lab.

Since two different sequences were being observed, each sequence was manipulated in thirty embryos and two controls were used for separate comparison.

2.4.4 Post-Injection Care of Embryos

The control and injected embryos were placed into corresponding petri dishes with fish water to incubate at 28.5°C for four days. There was a total of four petri dishes which were two control groups and the groups with differing target sites. The water in each petri dish was changed daily for optimized growth. The injected embryos were checked at the following post-fertilization times for visible toxicity: eight hours, one day, two days, and four days.

2.5 DNA Extraction

After four days of post-fertilization, the DNA had to be extracted from the embryos for testing. The DNA was isolated using the Invitrogen PureLink® Genomic DNA Mini Kit for mammalian cells.

2.5.1 Lysate of Embryonic Cells

To extract the DNA from the embryos, the cells have to go through a lysis process that will break down the cell without degrading the DNA and proteins found within the embryos. The contents of the lysed cells, or lysate, was prepared by following the mammalian cell lysate procedure found in the PureLink® Genomic DNA Mini Kit.

An average of fifteen embryonic cells was collected and put into individual, corresponding tubes. The water was completely drained from each tube as well. After separation, the embryos were suspended in 200 µL of Phosphate Buffer Saline, 20 µL of Proteinase K, and 20 µL of RNase A and incubated for two minutes at room temperature (Life Technologies,

2012). Once incubated, 200 µL of PureLink® Genomic Lysis/Binding Buffer was added and vortexed together. The solution was then placed in a water bath at 55°C for 10 minutes to dissolve the cell membrane (Life Technologies, 2012). The embryos were not completely dissolved afterwards and was continuously vortexed and placed in the water bath at 5 minutes intervals until completely dissolved. Finally, the solution was vortexed with an additional 200 µL of 96-100% ethanol.

2.5.2 Binding the Embryonic DNA

The PureLink® Genomic DNA Mini Kit allows for selective binding of DNA to a silica-based membrane with the presence of chaotropic salts in the column. This method of DNA binding allows the salts to disrupt cells and allow nucleic acids to bind to the silica. The mentioned buffers in the lysate will allow for residual RNA to be removed.

To bind the DNA, the lysate collected was inserted in a PureLink® Spin and centrifuged at 10,000 x g for 1 minute at room temperature. The contents leftover in the collection tube was discarded and the spin column with the bound DNA was placed in a clean PureLink® Collection Tube. Afterwards, 500 µL of Wash Buffer 1 was added to the column for DNA washing and elution (Life Technologies, 2012).

2.5.3 Washing the Embryonic DNA

The bound DNA in the column was washed with buffers to remove further contaminants and increase binding. The process involved centrifuging the bound DNA with the Wash Buffer 1 added at 10,000 x g for 1 minute. Afterwards, the contents left in the collection tube was discarded and the spin column was placed in a clean PureLink® Collection Tube. With the clean tube, 500 µL of Wash Buffer 2 was placed in the column. The column was centrifuged at a

maximum speed of 16,000 x g for 3 minutes at room temperature. Lastly, the contents left in the collection tube was discarded along with the tube itself (Life Technologies, 2012).

2.5.4 Eluting the Embryonic DNA

After washing the DNA of impurities, the DNA was eluted by releasing itself from the silica membrane using a low salt elution buffer. To complete this process, the spin column with the DNA was placed in a sterile 1.5 mL microcentrifuge tube and labelled correspondingly. An amount of µL of PureLink® Genomic Elution Buffer was added to obtain a higher/lower concentration. The spin column was incubated at room temperature for 1 minute and was centrifuged at 16,000 x g for 1 minute (Life Technologies, 2012). The centrifuge tube then obtained purified genomic DNA and the column was discarded.

2.6 High Resolution Melting Assay

For analysis of the embryonic DNA, a high-resolution melting assay (HRM) was performed to detect the mutations within the DNA. A real-time polymerase chain reaction (PCR) is performed beforehand to amplify the targeted DNA region. The amplified DNA is then heated until the double strands are broken apart. A fluorescent dye is inserted in the sample during the HRM analysis that only binds to double-stranded DNA. Whenever the dye binds to the double-stranded DNA, high fluorescence is measured. As the DNA melts apart, the fluorescence of the dye starts to decrease (Xing et al., 2014). A plot of how the fluorescence of the dye changes as the temperature increases is the output of the HRM analysis.

2.6.1 Primer Design for Target Sequences

The real-time PCR technique used before obtaining a HRM analysis requires forward and reverse primers to copy and amplify the original DNA strands. The primers were designed on the

Primer3Plus to locate the target. The following tables demonstrates the forward and reverse primers selected for both *fgf8a* sequences.

Table 2.2: Forward primer sequences designed to multiply the single DNA strands during PCR.

Forward Primer	**Sequence Information (5'→ 3")**
fgf8a-1	GCGCATTAACAGCTTTGCTT
fgf8a-2	TTCAGTCCCCGCCTAATTTT

Table 2.3: Reverse primer sequences designed to multiply the single DNA strands during PCR.

Forward Primer	**Sequence Information (5'→ 3")**
fgf8a-1	TTGCTCTCAAATATCGATTCCG
fgf8a-2	CAACAAGAAAATCAACGCCA

2.6.2 Polymerase Chain Reaction Preparation

A MicroAmp™ EnduraPlate™ Optical 96-Well Clear Reaction Plates with Barcode was used for the DNA to be inserted in for the real-time PCR and HRM analysis. The plate is made from propylene and prevents DNA from binding (Applied Biosystems™, 2020). Four columns, A-D, of the 96-well were utilized for the analysis. Before inserting DNA or primers into the wells, 5 µL of a Precision Melt Supermix was inserted into each of 9 wells in the four rows. The Precision Melt Supermix is a reagent used for real-time PCR specificity and contains EvaGreen dye for HRM analysis (Xing et al., 2014).

The embryonic DNA of the first and second target sequences were inserted into two separate rows. A volume of 1 µL was inserted into each well for 9 columns. The two controls of embryonic DNA that had no injections were also inserted into rows next to the corresponding

target sequences. A volume of 1 µL was also inserted into each well for 9 columns. Along with the embryonic DNA, the two types of forward and reverse primers were inserted in a control and their corresponding target sequences for comparative PCR and HRM analysis. A volume of 1 µL of a corresponding forward and reverse primer was inserted in each well. Finally, each single well was filled up with an estimated volume of 7 µL of water or until the well was filled up to 10 µL. The well plate was then sealed and vortex to remove all the bubbles present within the wells.

2.6.3 High-Resolution Melting of Embryonic DNA

After preparing the well plate with the required components, the plate is placed in the QuantStudio™ 5 Real-Time PCR System for analysis. The system was selected to run a two-step EvaGreen real-time PCR protocol that began heating the well plate at 95°C for two minutes. Afterwards, the system ran 40 cycles of the well plate being heated at 95°C for 10 seconds and at 60°C for 30 seconds, followed by being heated at 95°C again for 20 seconds and 60°C for 60 seconds. The temperature was then raised to 95°C once more at a rate of 0.02°C per second and was held for 10 seconds. The system then cooled the well plate down to 40°C. During this analysis, a real-time plot of the melt data is obtained for each separate sample. The curves on the plot were analyzed using a QuantStudio Real-Time PCR Software System (Version 1.5.1). The analyzed plots were then exported.

3. RESULTS

The results should display evident mutations that indicates the target location of the *fgf8a* gene promoter was targeted. The results of the research include the HRM analysis and the visualization of the embryos over four days of post-fertilization.

3.1 High-Resolution Melting Analysis

The following plots were obtained from the real-time PCR and HRM analysis that depicts the differences between the controls and the manipulated embryonic DNA. Using the QuantStudio™ 5 Real-Time PCR System, the data was able to be placed correctly on the following plots to display the change of normalized fluorescence against increasing temperature.

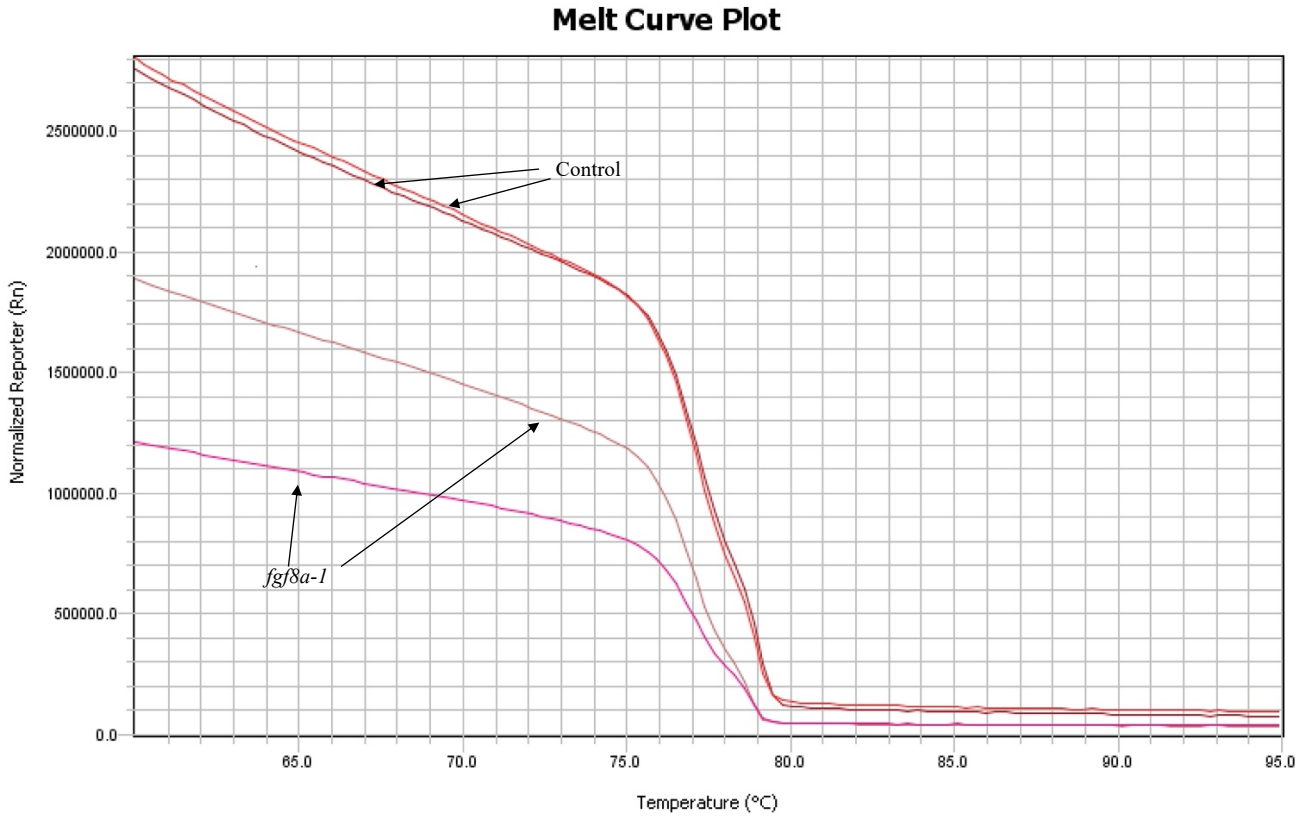

Figure 3.1.1: The following figure is a plot of normalized fluorescence melting curves of the embryonic DNA that was injected with the fgf8a-1 sequence and of un-injected embryonic DNA.

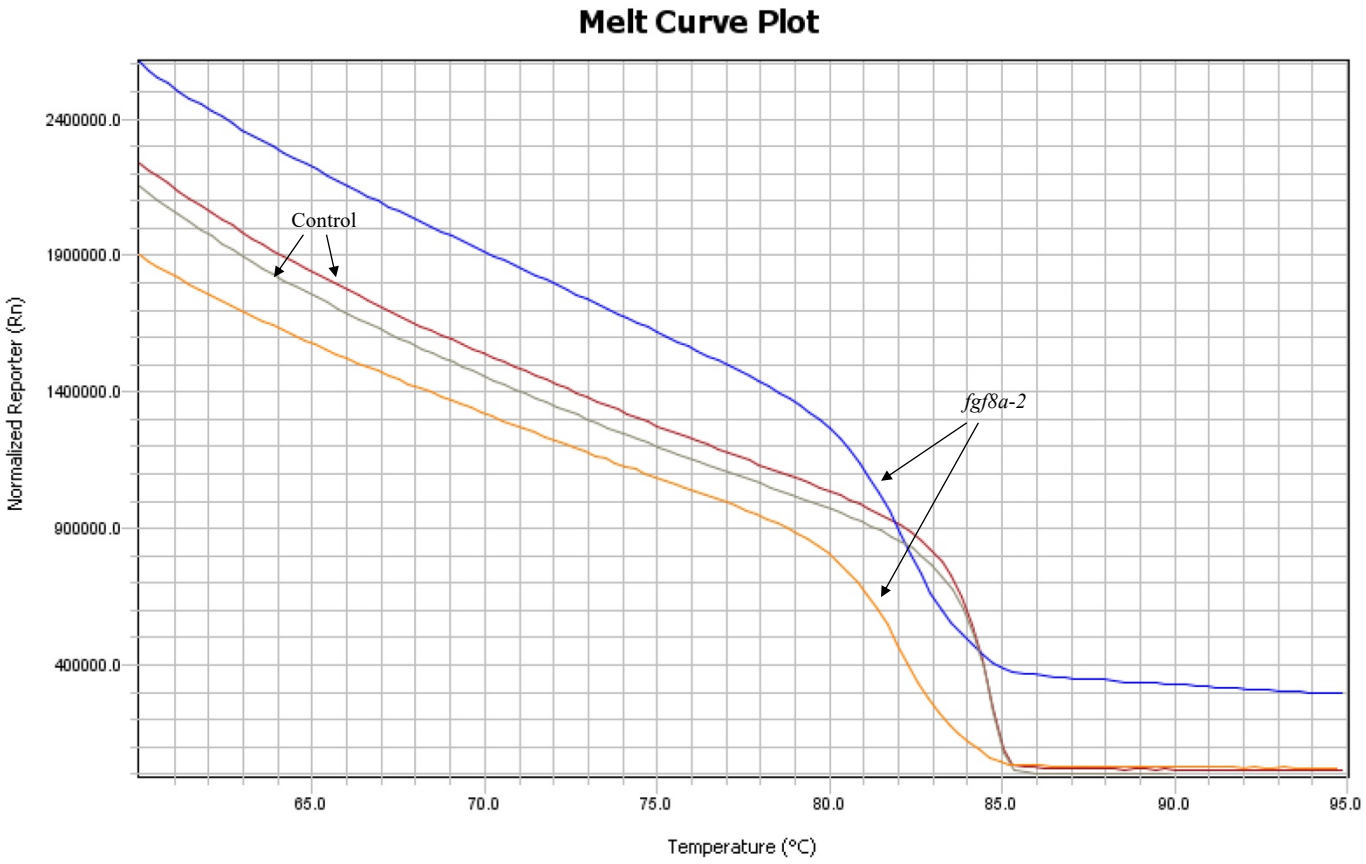

Figure 3.1.2: The following figure is a plot of normalized fluorescence melting curves of the embryonic DNA that was injected with the fgf8a-2 sequence and of un-injected embryonic DNA.

Figures 3.1 and 3.2 contain melt curve plots from embryonic DNA that showed the least impurities and/or faulty readings. The QuantStudio™ 5 Real-Time PCR System software was able to assist in finding the discrepancies in the PCR and HRM data.

To better understand the melt curves that need to be analyzed before the sudden slope drops in the figures, a derivative of the plots was also taken. The following figures display corresponding derivative fluorescence melt curve plots that can further illustrate the melting temperatures and the behavior of the dissociating double-stranded DNA.

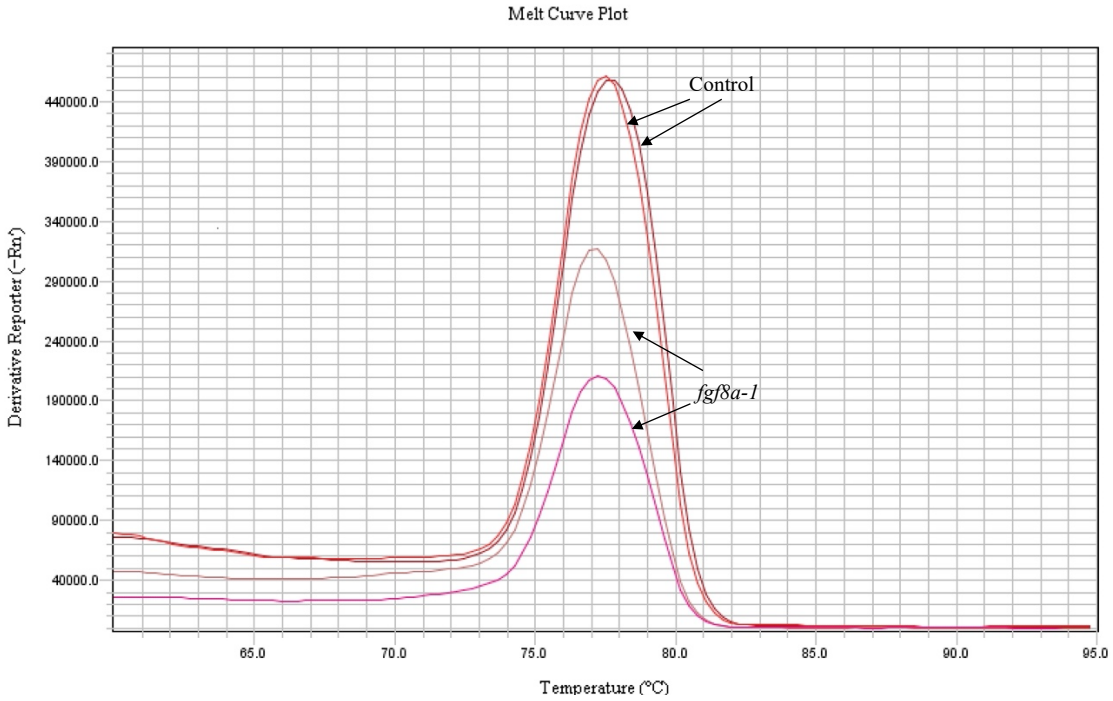

Figure 3.1.3: The following figure is a melt curve plot of the embryonic DNA that was injected with the fgf8a-1 sequence and of un-injected embryonic DNA.

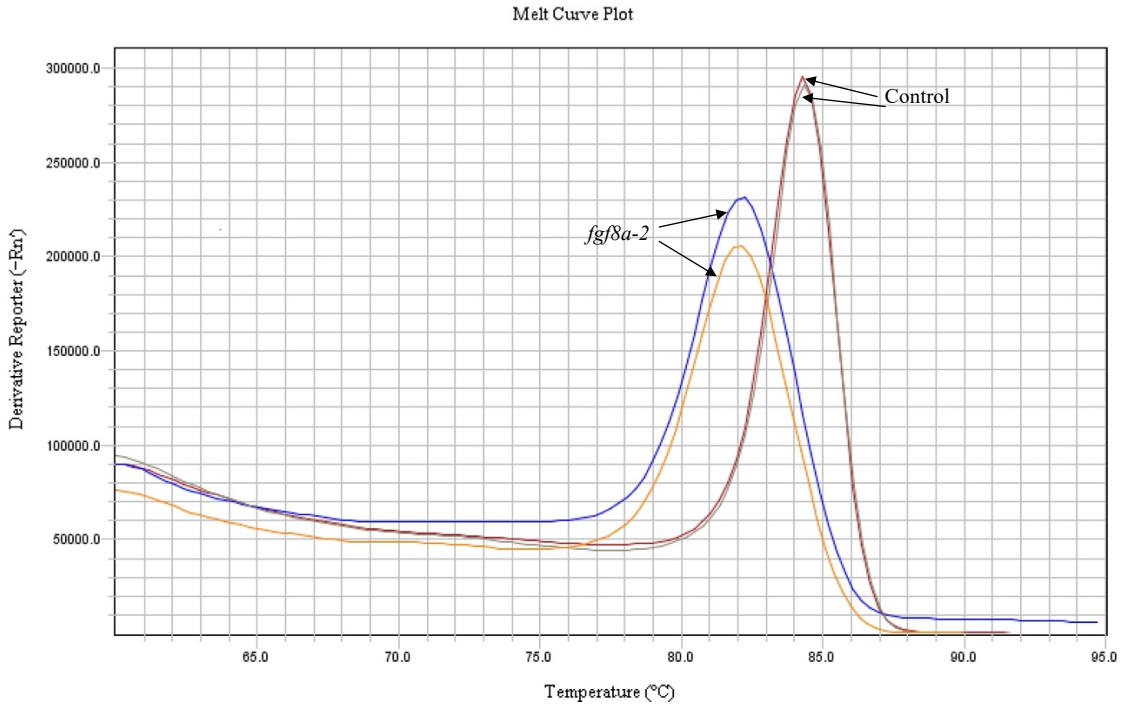

Figure 3.1.4: The following figure is a melt curve plot of the embryonic DNA that was injected with the fgf8a-2 sequence and of un-injected embryonic DNA.

From Figure 3.3 and 3.4, the melting temperature is evident between the un-injected and injected embryos. Table 3.1 demonstrates the averages of the melting temperatures of the double-stranded DNA from the injected and un-injected embryos.

Table 3.1: Melting temperatures of the double-stranded DNA found in the zebrafish that were injected with both sequences and those that were not injected.

Zebrafish Type	**Melting Temperature, T_m**
fgf8a-1 Control	77.59°C
fgf8a-1 Mutation	77.14°C
fgf8a-2 Control	84.34°C
fgf8a-2 Mutation	82.12°C

Apart from the melting temperatures, Figures 3.3 and 3.4 shows the variations of the melt curve plots that can't be easily seen in Figures 3.1 and 3.2. However, all four figures are essential in determining whether successful alteration of the corresponding genes occurred from the usage of our designed sequences.

3.2 Visualization of Abnormalities

The figures below were taken of the embryos after 7 days post-fertilization by Sreeja Sarasamma. These images were used as a qualitative reference to the data found in the HRM analysis. These images were taken by a fluorescent microscope present in the lab. The Appendix contains more imaging of the two types of injected embryos and the permission statement of photograph usage from Sreeja Sarasamma.

The differences that are evident in the images is the spinal abnormalities found between all three types of embryos. The un-injected embryo exhibits the expected spinal growth of any

zebrafish embryo while the other two types of embryos showed curved spines that caused swimming difficulties. These observations were also observed at 72 hours after fertilization.

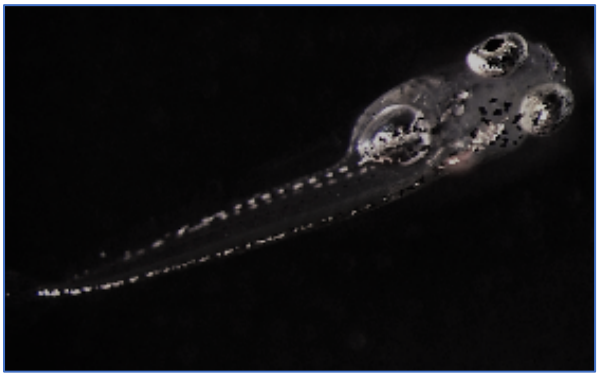

Figure 3.2.1: Fluorescence image of an un-injected embryo 7 days after fertilization. (Images taken by Sreeja Sarasamma and used with permission.)

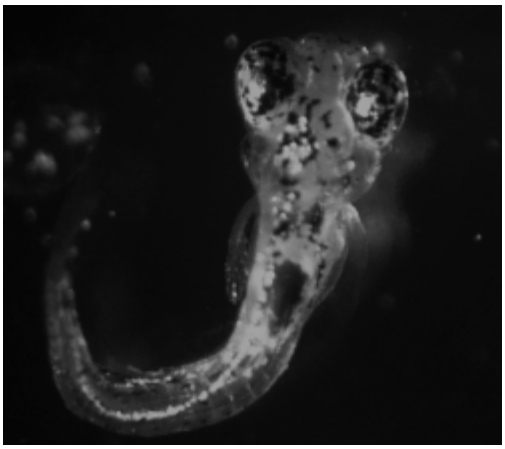

Figure 3.2.2: Fluorescence image of an fgf8a-1 injected embryo 7 days after fertilization. (Images taken by Sreeja Sarasamma and used with permission.)

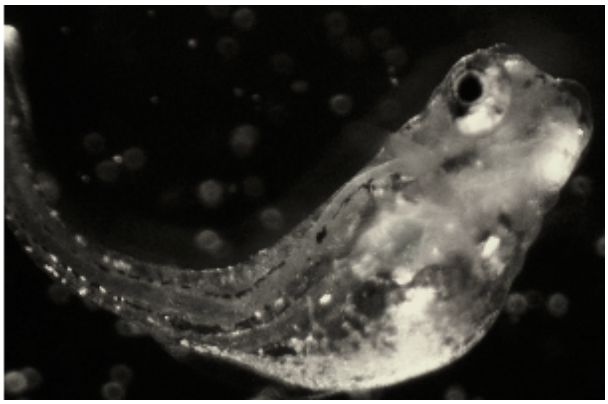

Figure 3.2.3: Fluorescence image of an fgf8a-2 injected embryo 7 days after fertilization. (Images taken by Sreeja Sarasamma and used with permission.)

4. CONCLUSION

4.1 Targeting Confirmation of *fgf8a* Promoters

The results from the HRM analysis were able to confirm that the *fgf8a* promoters were targeted for both sequences. We were able to confidently derive this conclusion by investigating the melting temperatures and the shape of the melt curves before double-stranded dissociation.

Any deviation in the melting temperatures between an un-injected embryo and a corresponding injected embryo is an identifier of indels (i.e., gene deletions or insertions). From Table 3.1, both injected embryos on average have a lower melting temperature than those that were not injected. The melting temperature of the zebrafish with the *fgf8a-2* sequence deviates the most from its respective control. This inspection is also evident in its derivative plot from Figure 3.1.4 as the melting temperature values of the injected embryos are visibly shifted. Although the difference between the melting temperatures of injected/un-injected *fgf8a-1* embryos were not as significant as the difference observed in the *fgf8a-2* embryos, both sequences showed an overall decrease in melting temperatures which is expected because of the decrease of guanine-cytosine content that results from gene alteration.

To further illustrate the confirmation of sequence targeting for the injected embryos, a visual inspection of the normalized plots found in Figures 3.1.1 and 3.1.2 can demonstrate the variations between the un-injected and injected embryos. The pre-melt phases of the plots between the injected and un-injected embryos vary in shape for both sequences. The injected embryos clearly display different profiles than the un-injected embryos. These visual distinctions from the normalized plots as well as each melting temperature demonstrate that there was an alteration within the gene.

The abnormalities found in the injected embryos as they were growing after the injection also supports the conclusion of a gene alteration in both sequences. The injected embryos displayed curved spine structures rather than the typical straight-line structures found in un-injected embryos. These abnormalities exhibit loss of function phenotypes of the targeted gene. These observations further support the conclusion of successful targeted gene alteration.

The sequences used in the thesis can now be used for further enhancer-promoter communication studies in the lab.

4.2 Significance of Findings

The sequences utilized were able to target the *fgf8a* promoter by demonstrating successful alteration of the portion of the *fgf8a* gene. These sequences can now be developed into a probe that will attach a marker (i.e., fluorescent protein) to the gene for live imaging. The probes will be used to monitor and observe the communication between nearby enhancers and the target promoter of the *fgf8a* gene.

Being able to observe the enhancer-promoter communication and their interactions are essential to understand gene regulation and cell differentiation. The findings from studying the enhancer-promoter relationship in the *fgf8a* gene can be related to other genes and assist us in further understanding how phenotypes come to be in humans.

4.3 Future Directions of Experiment

The study was conducted to find useful targeting probes in the *fgf8a* gene and will be replicated as needed in the lab to have a variety of sequences to choose from. Further investigation of the sequences should be performed to find more target locations to investigate. Furthermore, future studies should utilize HRM to identify induced indels because it is significantly easier to operate, reliable, and a less expensive method. However, it would be

suggested to utilize a secondary genomic examination to confirm the products from the HRM by analyzing its size with gel electrophoresis.

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APPENDIX A: IMAGE USAGE CONSENT

The written consent received from Sreeja Sarasamma to utilize the fluorescent images she captured is attached below.

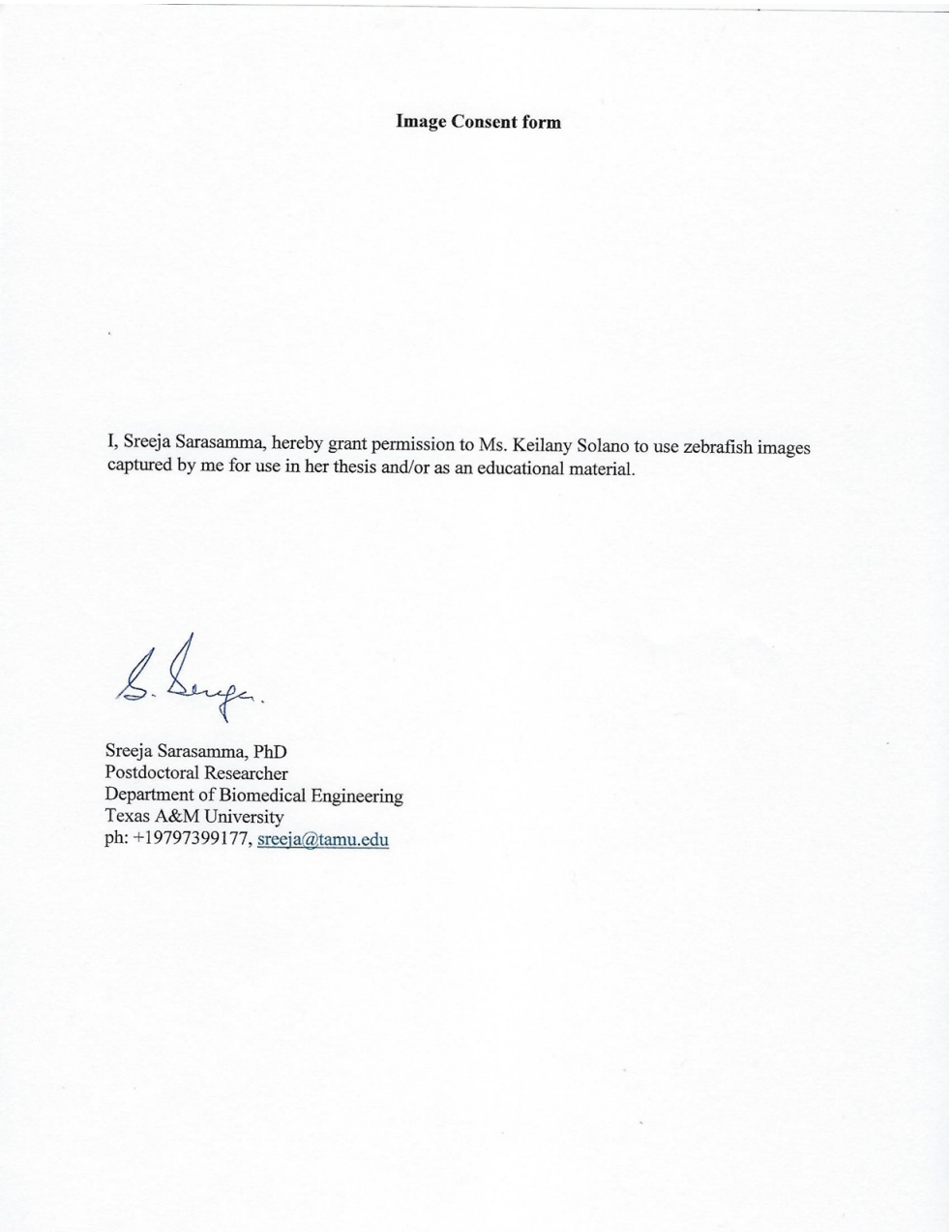

Image Consent form

I, Sreeja Sarasamma, hereby grant permission to Ms. Keilany Solano to use zebrafish images captured by me for use in her thesis and/or as an educational material.

Sreeja Sarasamma, PhD
Postdoctoral Researcher
Department of Biomedical Engineering
Texas A&M University
ph: +19797399177, sreeja@tamu.edu

Figure A.A.1: Written consent to use images captured by Sreeja Sarasamma for thesis.

APPENDIX B: FLUORESCENT IMAGES OF INJECTED EMBRYOS

The following fluorescent images are the embryos injected with the *fgf8a-1* at 7 days post fertilization. The images were taken by Sreeja Sarasamma.

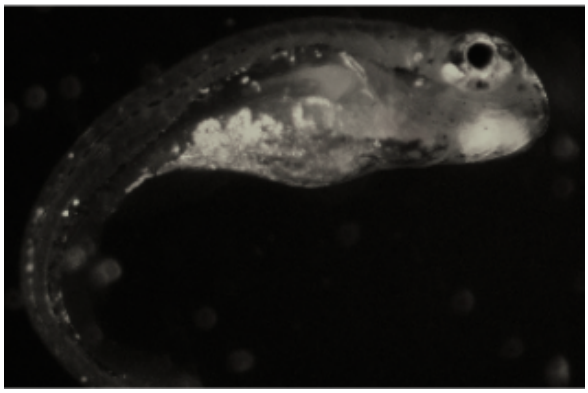

Figure A.B.1: A fluorescent image of an embryo that was injected with the fgf8a-1 sequence at 7 days post fertilization. (Images taken by Sreeja Sarasamma and used with permission)

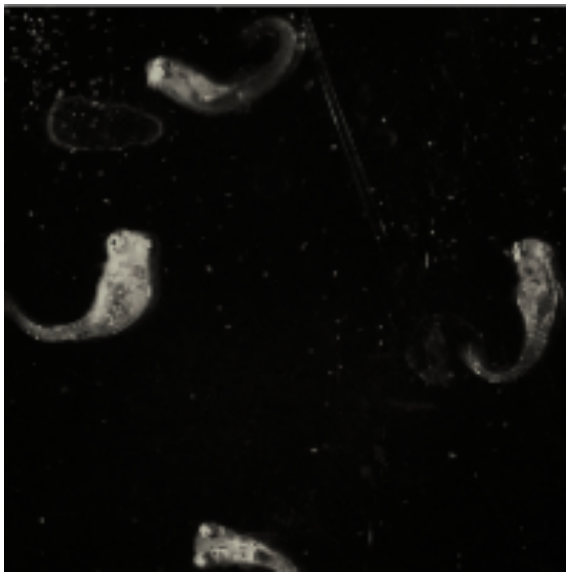

Figure A.B.2: A zoomed out fluorescent image of multiple embryos injected with the fgf8a-1 sequence at 7 days post fertilization. (Images taken by Sreeja Sarasamma and used with permission)

The following fluorescent images are the embryos injected with the *fgf8a-2* at 7 days post fertilization. The images were taken by Sreeja Sarasamma.

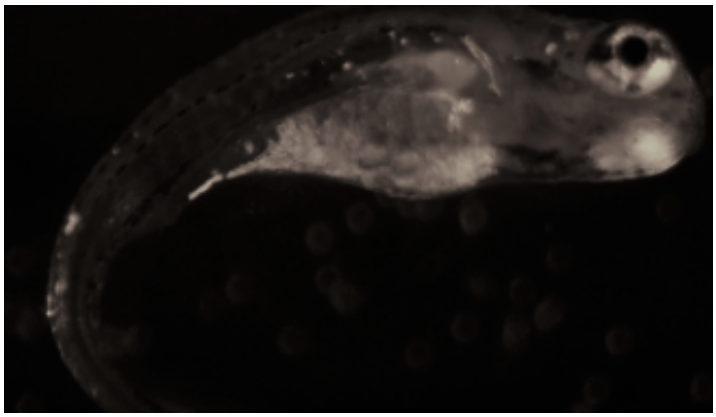

Figure A.B.3: A fluorescent image of an embryo that was injected with the fgf8a-2 sequence at 7 days post fertilization. (Images taken by Sreeja Sarasamma and used with permission)

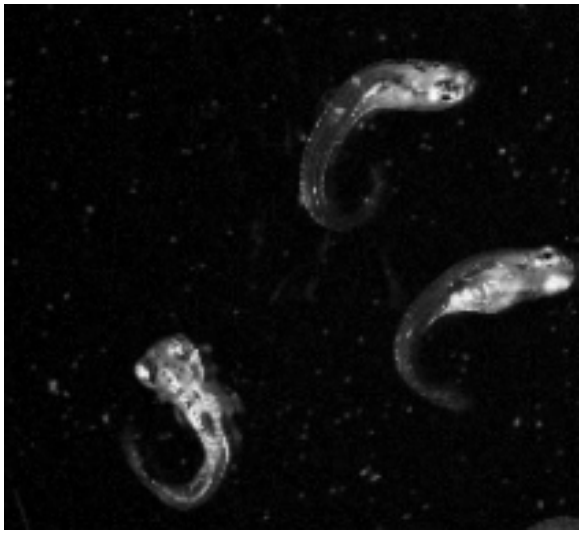

Figure A.B.4: A zoomed out fluorescent image of multiple embryos injected with the fgf8a-2 sequence at 7 days post fertilization. (Images taken by Sreeja Sarasamma and used with permission)