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Functional Analysis of Protease ADAMTS 6 during Vertebrate Eye Formation

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Abstract

Extracellular matrix (ECM) remodeling and epithelial sheet fusion occur during the development of many different tissues, including the vertebrate eye (Lu et al., 2011) (Pai et al., 2012). During morphogenesis of the eye, the ventral hemisphere of the developing retinal tissue forms an opening called the optic fissure which must undergo epithelial fusion (Pai et al., 2012). Failure of optic fissure fusion leads to a congenital blinding disorder called coloboma (Gregory-Evans, 2004). A long standing question pertaining to the mechanism of optic fissure fusion is exactly which ECM remodeling enzymes are actively involved in dismantling the ECM to allow for fusion to occur. I hypothesize that ADAMTS 6 has some function in the fusion of the optic fissure. The expression pattern of ADAMTS 6 was characterized to determine whether it has the spatiotemporal capacity to act as an ECM remodeler for epithelial sheet fusion. Through use of RNA *in situ* hybridization, the expression of ADAMTS 6 was compared at chronological time points in the development of zebrafish embryos. Findings seem to contradict previous work in the lab that suggested ADAMTS 6 has little to no function in the optic fissure during fusion of the retina (Taylor et al., 2017). The comparison of ADAMTS 6's expression pattern in wildtype and Pax2noi mutant embryos at certain time points had previously showed no changes in the optic fissure suggesting that there was no function (Taylor et al., 2017). However, optic fissure fusion of embryos injected with mRNA producing protein overexpression appears to be disrupted. Deeper analysis with different tests will need to be performed to determine the how of this mechanism.

Introduction

The formation of the eye in early developmental stages in vertebrates is crucial for the organisms' visual function. When complications arise, the ability for the organism to see could be severely impacted. The fusion of the retina during development is of particular interest to many researchers because the mechanisms of this fusion event can be applicable to other epithelial sheet fusion events in the body such as in the heart or the urethra (Figure 1 & 2) (Baum and Georgiou, 2011) (Lu et al., 2011) (Morrisey and Sherwood, 2015) (Ocutech, 2017) (Pai et al., 2012). How the extracellular matrix (ECM) is remodeled in epithelial sheet fusion events in the body is also of particular interest. When the ventral hemisphere of the retinal tissue fails to fuse during development, an opening called the optic fissure is made and coloboma can result. (Pai et al., 2012) Coloboma is a spectrum disorder, meaning that there is a varying degree of blindness in individuals affected (Gregory-Evans, 2004). The appearance of an eye affected by coloboma resembles something of a 'keyhole' phenotype (Figure 2) (Morrisey and Sherwood, 2015) (Ocutech, 2017). To determine how specifically coloboma occurs, the genes involved in the mechanism must be characterized. The ECM remodelers expressed at the optic fissure fusion site are the initial candidates.

The epithelial sheets are surrounded by an ECM that will need to be remodeled in order to form cell to cell contact in the optic fissure. ECM remodeling involves the removal of the extracellular matrix from the fusion site (Lu et al., 2011). Figure 1 shows that components such as laminin, perlecan, collagen IV, and nidogen are removed from the ECM during the process of fusion in order to form the spheroid eye (Baum and Georgiou, 2011). This is accomplished by ECM remodeling enzymes. By analyzing embryonic expression patterns of ECM remodeling

enzyme genes in the optic fissure, the genes involved in the mechanism of ECM remodeling in the optic fissure fusion can be elucidated.

Through prior work in the lab, a gene expression screen of possible ECM remodeling enzymes was conducted in zebrafish embryos and narrowed down from about seventy candidates to six genes. These genes are called matrix metalloproteinases (MMPs) because they are involved in the degradation of the extracellular matrix proteins (Vu and Werb, 2000). The effects of MMPs on such components during epithelial sheet fusion of the optic fissure play a key role in regulating this process (Vu and Werb, 2000). The matrix metalloproteinases determined to be expressed in the optic fissure prior to fusion were both MMPs and ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) (Kelwick et al., 2015): MMP 9, MMP 14a, MMP 24, ADAMTS 6, ADAMTS 18, and ADAMTS 22. The work done with these enzymes has narrowed the components thought to be necessary in the optic fissure fusion within this study.

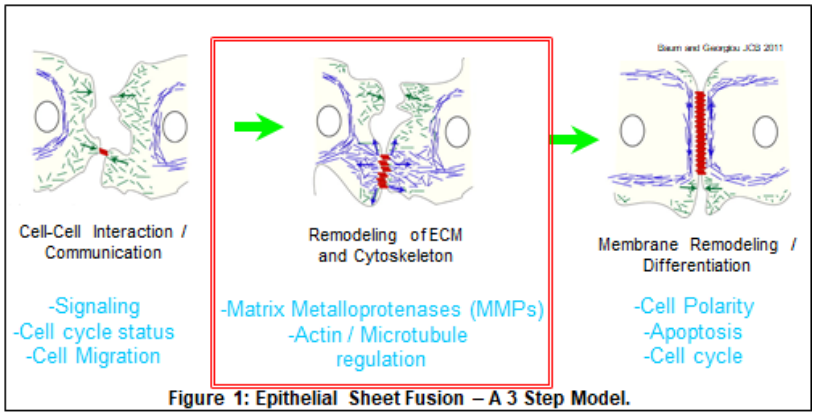

Figure 1: Within epithelial sheet fusion, there are three main steps of the mechanism. The cells first interact with each other through physical and chemical cues that signal for fusion to occur. Next and the focus of this study, the enzymes and components involved in extracellular matrix remodeling begin to be removed to undergo this process. Last, the fused epithelial sheet establishes a uniform layer of cells spanning the choroid gap. (Baum and Georgiou, 2011)

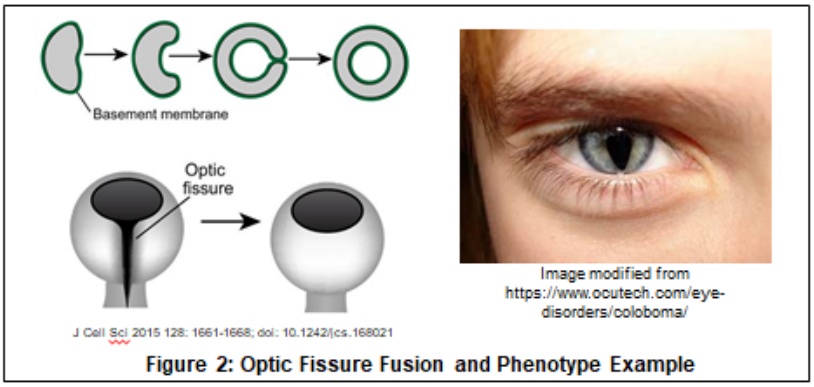

Figure 2: A simplistic view of the optic fissure fusion event is seen here. The fusion site extends from the optic stalk to the retina. The degree of blindness that the individual lives with depends on a number of factors. One of which is the variation in the fusion along the optic stalk to the retina of the optic fissure. The image of the individual with coloboma is one example of the fusion failure being at the retina end of the optic fissure. (Morrisey and Sherwood, 2015) (Morrisey and Sherwood, 2015)

Due to the conservation of eye development across all vertebrates, it was decided to use the zebrafish (*Danio rerio*) as the experimental organism in this study. The function and development of zebrafish eyes to human eyes is conserved as in most vertebrate species (Dooley and Zon, 2000). Overall, about seventy percent of DNA is shared between humans and zebrafish (Dooley and Zon, 2000). Since zebrafish have large clutch sizes, their embryos are easily reared for research as a model organism. Their eggs are laid outside of the mother before fertilization allowing the development processes to be observed by researchers through the clear chorion surrounding the developing embryo (Figure 3) (Dooley and Zon, 2000) *(Holterhoff et al., 2009)*. Their bodies are also mostly transparent so detection of colored antibodies is easily seen.

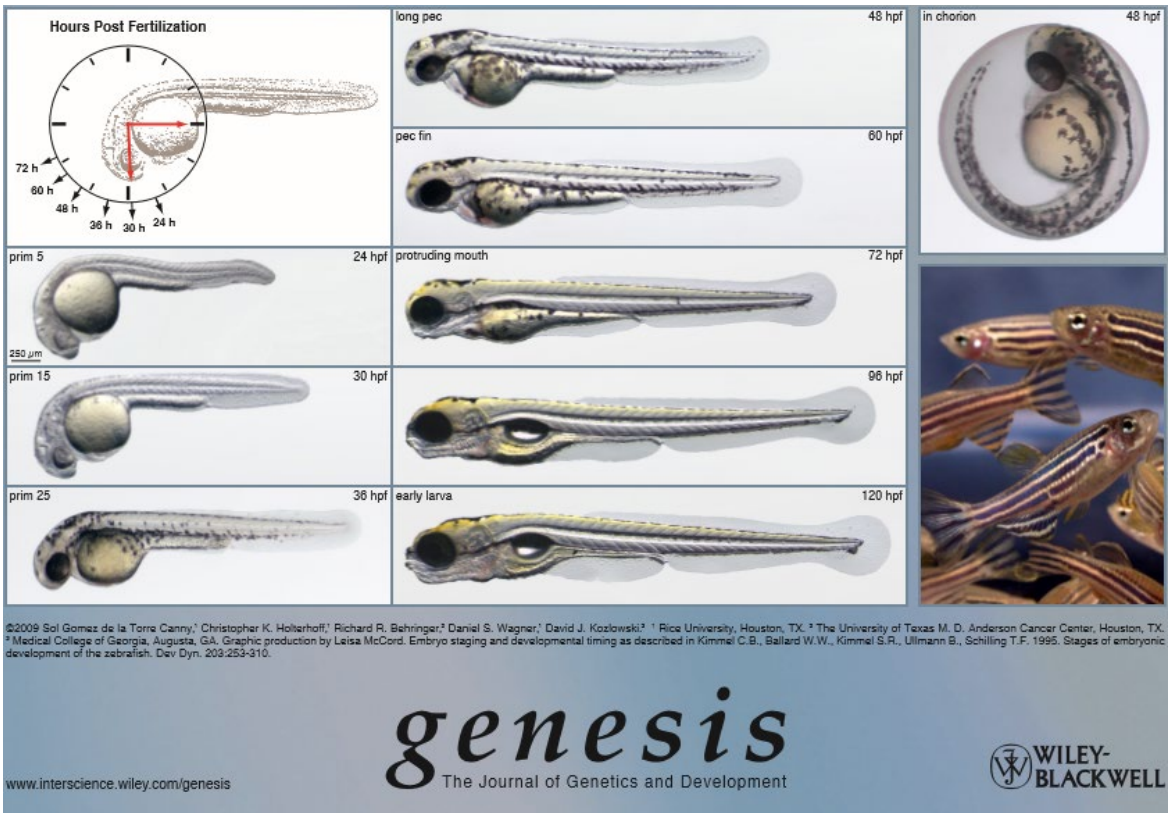

Figure 3: A timeline of the development of zebrafish embryos provided by "genesis". The chorion can be seen in the upper right photograph. Normal zebrafish embryos at many of the major time points utilized in this study can be seen. The transparency of the embryos is of one particular interest as use as a model organism in this study (Holterhoff et al., 2009)

Previously in the lab, the use of RNA whole-mount *in situ* hybridization on zebrafish embryos on six matrix metalloproteinases known to be expressed in the optic fissure were further narrowed down to the possible genes involved in the matrix remodeling of optic fissure fusion (Taylor et al., 2017). The expression patterns were observed at different time points of developing wildtype zebrafish embryos. These time points were 24, 32, and 48 hours post fertilization (hpf). From this gene expression screen, ADAMTS 6, MMP 9, and MMP 24 were observed to be expressed in the optic fissure during fusion of the retina (Figure 4) (Taylor et al., 2017). ADAMTS 18, ADAMTS 22, and MMP 14a showed no expression in the optic fissure at any time point observed. These three matrix metalloproteinases were excluded from further

study. The other three enzymes were characterized to determine if there were any functional contributions involved in the fusion event and not merely general expression.

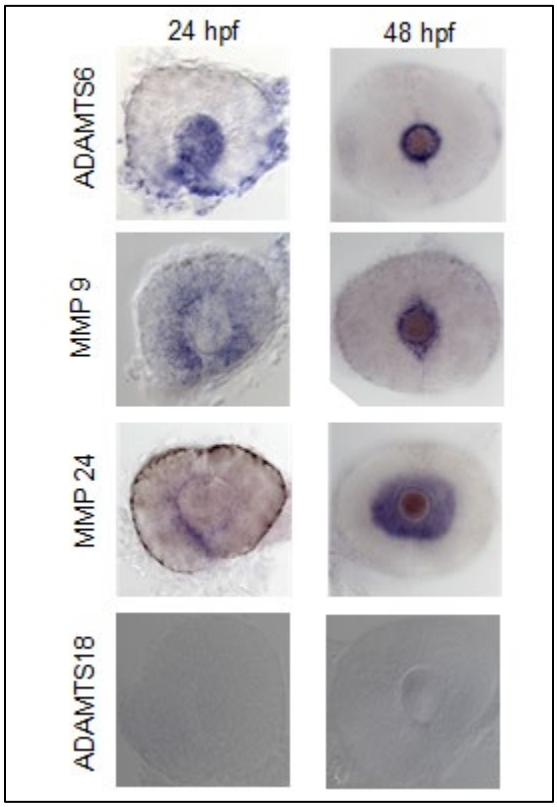

FIGURE 4: Wild type zebrafish embryos that have undergone whole-mount in situ hybridization probed for ADAMTS 6, MMP 9, MMP 24, and ADAMTS 18 expression. Eyes of 24 and 48 hpf embryos were dissected and imaged using DIC microscopy. ADAMTS 6, MMP 9, and MMP 24 display expression within the eye as well as within the optic fissure at 24 hpf. At 48 hpf, the expression of ADAMTS 6, MMP 9, and MMP 24 becomes restricted to the periphery of the lens. As time goes on, it appears that the presence of the probes diminishes. This would be representative of a protein necessary for early eye development being shut off as the embryo grows. ADAMTS 18 is shown as an example of a protease that does not exhibit expression within the eye or the optic fissure. (Taylor et al., 2017)

Other previous experiments in the lab characterized ADAMTS 6, MMP 9, and MMP 24's gene expression by RNA *in situ* hybridization in a zebrafish mutant line, Pax2^{noi}, coloboma model to find any functionality of these enzymes in the fusion event (Taylor et al., 2017). These expression patterns were compared between wildtype (WT) and Pax2 mutant embryos at 24, 30,

and 48 hpf. Staining indicated that expression of MMP 9 and MMP 24 is reduced in Pax2 mutant embryos (Figure 5), while previous results on ADAMTS 6 expression appear unaffected by loss of Pax2 function (Figure 6) (Taylor et al., 2017). Pax2 mutant zebrafish will not live past 5 days post fertilization due to a heart defect (Avanesov and Malicki, 2010). This phenotype is not present until 48 hours post fertilization and since Pax2 mutants only account for 25% of offspring in a heterozygous in-cross it is difficult to separate the embryos before the developmental stage of interest. Genotyping assays were performed on Pax2 mutant embryos to correlate *in situ* hybridization results with Pax2 genotypes. In order to genotype, genomic DNA was isolated from embryos and then amplified in a PCR reaction (Lee et al., 2016). The amplified DNA was used in a restriction fragment length polymorphism (RFLP) assay for the $Pax2^{noi}$ mutations (Figure 7) (Taylor et al., 2017).

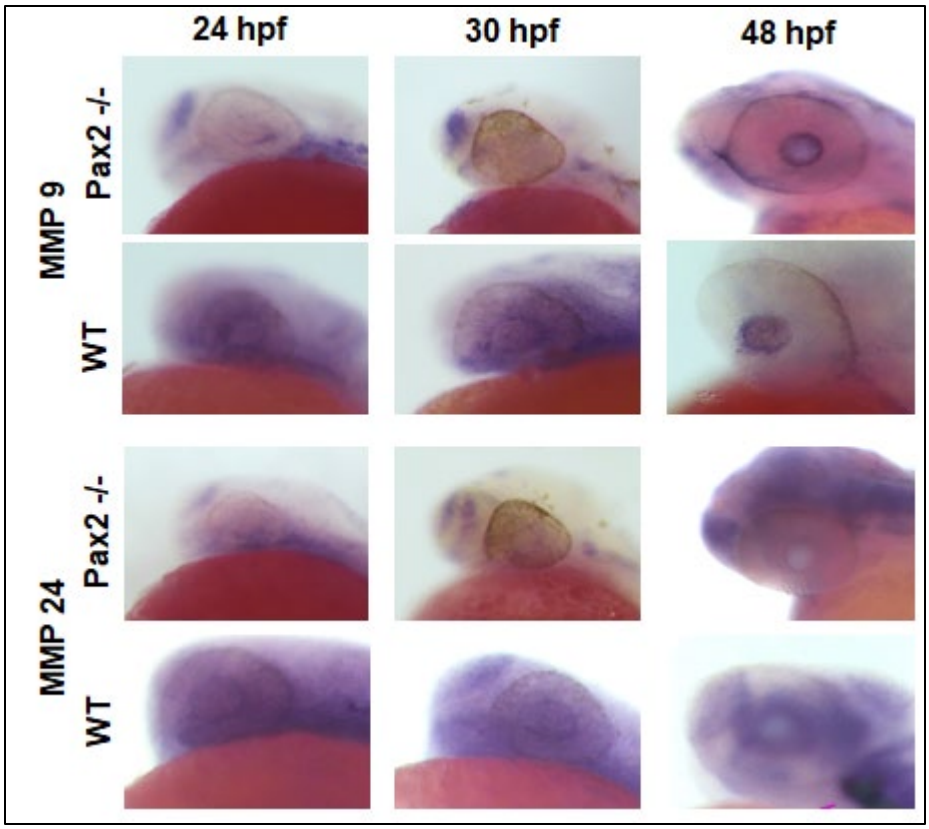

Figure 5: Expression of MMP 9 and MMP 24 observed using RNA whole-mount in situ hybridization in WT and Pax2noi/noi embryos at 24, 30, and 48 hpf. Staining suggests that expression of both MMP 9 and MMP 24 is diminished in the eyes and forebrain of Pax2 mutant embryos. (Taylor et al., 2017)

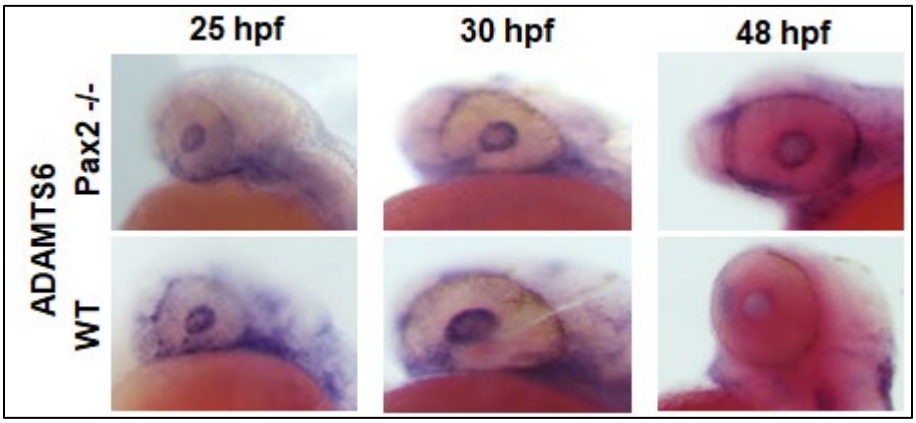

Figure 6: Expression of ADAMTS 6 observed using RNA whole-mount in situ hybridization in WT and Pax2noi/noi embryos at 25, 30, and 48hpf. Staining indicates little to no difference in the expression patterns of ADAMTS 6 between WT and Pax2 mutant embryos. This may suggest that ADAMTS 6 has no functional role in the fusion of the optic fissure. Further analysis of this enzyme is necessary to exclude it from the candidate list. (Taylor et al., 2017)

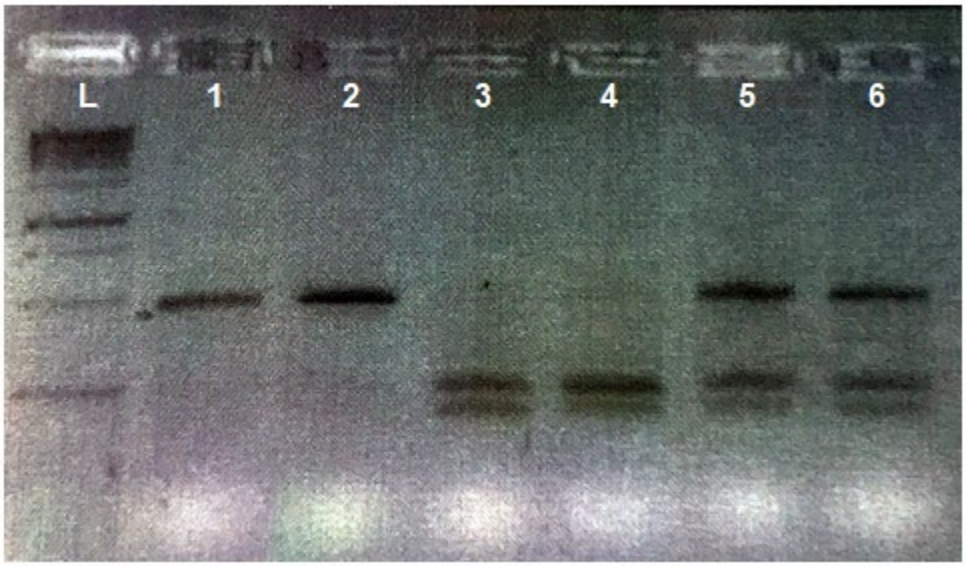

Figure 7: Genotyping of Pax2^{noi}. Genomic DNA isolated from embryos was used to amplify the Pax2^{noi} amplicon which was subsequently subjected to an RFLP assay using the Taq1 enzyme (Lee et al., 2016). WT sequence of Pax2 results in digestion yielding 2 bands of ~300 and 200bp. The noi mutation abolishes the Taq1 restriction site resulting in a DNA fragment of 500bp. Lanes 1 and 2 indicate noi/noi genotypes, 3 and 4 indicate WT, and 5 and 6 indicate noi/+.(Taylor et al., 2017)

These previous findings suggest that the failure of epithelial fusion in the optic fissure may result from the misregulation of ECM remodeling enzymes MMP 9 and MMP 24 (Taylor et al., 2017). The response of fissure fusion to gain and loss of function mutations of these two enzymes is currently being undertaken by a graduate student in the lab. mRNA microinjections and CRISPR/ Cas9 mutagenesis will be used to determine the functional relevance of these enzymes in the development of the eye. Further functional analysis of ADAMTS 6 utilizing the same protocols is also in the process of being completed. This is to ensure whether or not the observed expression in the optic fissure from prior *in situ* hybridizations allow the enzyme to be excluded from the study. This latter functional analysis is the area of focus for this current study.

The main question in this current study is whether or not the protease, ADAMTS 6, has a function in the fusion of the optic fissure. If so, what is it? More trials of RNA whole-mount *in situ* hybridization at 24, 28, and 48 hpf embryos were performed using the ADAMTS 6 probe. Embryos at 24 and 48 hpf in this study were wildtype. Embryos at 28 hpf were of the heterozygous in-crosses (het-in) for the ADAMTS6 recessive allele. This is assumed to be a nonsense mutation about half way through the gene that results in a loss of function. The expression patterns were compared between these two groups. The expected ratios of embryos for this het-in cross are 25% homozygous dominant for the ADAMTS6 allele, 50% heterozygous, and 25% homozygous recessive for the ADAMTS6 allele. The 25% homozygous recessive embryos are the mutants in this experiment. These cross ratios are expected to produce differences in the expression patterns of the embryos.

A different set of embryos at the 48 hpf time point were injected with mRNA to overexpress the protein. The injections were either 100 pg or 200 pg of mRNA. These embryos did not undergo *in situ* hybridization, but were imaged to visualize any phenotypic changes

between a control group and the two different injection amounts. The expression patterns were compared and scored. Analysis of ADAMTS 6 function in the optic fissure was completed with these trials.

Methods

RNA Whole-Mount in situ Hybridization

RNA whole-mount *in situ* hybridization used in this study utilized sequences of RNA of ~1000 base pairs (bps) that are complementary to the sequence of mRNA of interest (Darnell et al., 2010). It will bind to the specific complementary code of the endogenous mRNA. Once the sequences are bound together, staining can be done on the antibodies that are attached to the RNA sequence to locate the specific tissue that is expressing the staining. This allows the researcher to determine the tissue types that express the gene of interest. For the purposes of this study, mRNA sequences of the MMPs and ADAMTSs were utilized to determine if any are present in the optic fissure during fusion of the retina.

In order to carry out the RNA whole-mount *in situ* hybridization, there must be probe containing a complementary sequence to the endogenous mRNA of interest (Darnell et al., 2010). A polymerase chain reaction (PCR) was performed using primers specific to a cDNA sequence corresponding to the probe template needed (Mullis, 1990). PCR allows researchers to make genetic sequences in short periods of time. The key component of a PCR is the Taq polymerase which is utilized due to its stability at high temperatures. Taq polymerase was isolated from *Thermus aquaticus*, an extremophile bacterium (Mullis, 1990). Taq polymerase is the only polymerase effective at high temperatures which allows it to synthesize the genetic sequence of interest by using the primers made previously as a starting point. Once the sequence

has been made, amplified, and purified, a reverse transcription reaction is done to produce the RNA probes that will hybridize with the endogenous mRNA in the RNA *in situ* hybridization protocol (Darnell et al., 2010).

Now that the probe is ready, the zebrafish embryos must be prepared. Once the embryos have been fertilized, they are grown up to desired time points corresponding to major changes in the development of the eye, specifically the fusion of the optic fissure. The chorion must be removed in order to allow the solutions to have contact with the embryonic tissues, to position the embryos later for imaging, and for access to the eye of the individual. To remove the chorion, fine point forceps are used to pry the tissue apart. The embryos can now undergo staining through the RNA whole-mount *in situ* hybridization protocol.

The *in situ* hybridization used in this study utilized a four day protocol (Famulski, 2015):

On day one, embryos are grown up to the desired time point before being fixed with paraformaldehyde (PFA) overnight. On day two, embryos are washed with phosphate buffered saline with tween 20% (PBST) to remove any remaining PFA solution. The embryos are dechorionated before being washed with more PBST. Proteinase K was added to make the embryonic cells permeable (Fabbro et al., 2015). The next important step is the addition of the RNA probe made using PCR and an overnight incubation at 65°C. On day three, the first five washes are done at 65°C. They contain varying percentages of saline-sodium citrate (SSC) buffer with either hyb or 0.1% tween. Two more washes are done at room temperature with decreasing concentrations of SSC and increasing concentrations of PBST. An overnight wash with blocking buffer containing anti-DIG antibody at 4°C is then done. An antibody conjugated to alkaline phosphatase binds to the probes that are bound to the sequence of interest. On day four, the

embryos are rinsed of the anti-DIG antibody containing blocking buffer in PBST. The antibody conjugated with the alkaline phosphatase3 converts the BCIP/NBT substrate (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) used in the washes from a yellow precipitate to a blue precipitate through an enzymatic reaction. Any possible gene expression in the embryo will appear blue/purple. Once desired expression is reached, a stop solution is added which is just PBST at a higher pH in order to stop the enzymatic reaction. If too much staining is obtained so that visualization is difficult, then washes containing decreasing concentrations of methanol can be performed in order to remove background staining that is not needed for better clarity. The embryos are then stored at 4°C in PBST.

Once expression is obtained, the embryos are almost ready for imaging. First, the yolk of the embryo that provides nutrients in vivo needs to be removed. When large parts of the yolk are left, the proteins and lipids reflect light under the camera and can fluoresce ruining the clarity of the images. Once the yolk is removed, preliminary images showing expression in the eye and portions of the body are taken to characterize the gross expression pattern of the probe of interest from the RNA whole-mount *in situ* hybridization. The eyes of the embryos are then removed using dissection needles. They are mounted on slides to be imaged on a scope using DIC microscopy. The optic fissure is more precisely imaged and the expression of individual cells along the fusion site is easier to visualize. At this point the eyes of the zebrafish embryo can be stored for future use if necessary.

Genotyping

Genomic DNA must be extracted from the embryos in order to genotype the individual fish. Phenotypic differences observed during *in situ* hybridization place the embryos

into various groups based on probe expression. Two embryos from each group were used in genotyping. The tissue is digested in 50 mM NaOH for 5 minutes at 95° in a PCR machine, vortexed, and then heated again at the same temperature for 10 minutes. 10 µL of 1 M Tris-HCl pH 8.0 is added then vortexed again. The region of interest is amplified from the gDNA in a PCR reaction then cut with a restriction enzyme (Lee et al., 2016). The mixture is run on a gel to determine which embryos are heterozygous and homozygous. This is done by a restriction fragment length polymorphism (RFLP) analysis (Lee et al., 2016). RFLPs are loci-specific, allowing them to distinguish the differences in homologous DNA sequences (RFLP, n.d.). The RFLP analysis shows either cut bands or whole bands depending on the particular way the analysis is completed.

For the gel seen in this report, if one dark band is obtained in a lane at ~500 bps, the embryo is considered mutant homozygous. If two lighter bands close together are obtained in a lane at~200 and ~300 bps, the embryo is considered wildtype homozygous. If the two lighter bands and one dark band are both obtained in a lane at the corresponding base pairs, the embryo is considered heterozygous. The two different types of homozygous traits are the wild type and mutant phenotypes. To distinguish between the two, the number of bands seen near each other is important. The one dark band corresponds to a mutant embryo, while the two lighter bands correspond to a wildtype embryo. The gel is compared to the scoring completed on the embryo phenotypes. Depending on the comparison, the scoring system may need to be updated to reflect the genetic bases of the phenotypes.

Plasmid Purification for the Purpose of mRNA Synthesis

The mRNA generated from a plasmid template is utilized for the analysis of the gain of function responses in the optic fissure fusion enzymes of interest. cDNA sequences are inserted into vectors for amplification of the sequence (Engebrecht, 2001). The vector used in this experiment is pCS2 which contains an ampicillin resistant gene. The ampicillin resistance gene allows the bacterial vector carrying the inserted cDNA to grow unhindered in media while any bacteria without the pCS2 gene would die in the ampicillin containing media (Engebrecht, 2001). When the bacterial cells are lysed, large quantities of plasmid are extracted to be used as a template for mRNA synthesis of the particular gene of interest (Engebrecht, 2001). Once the mRNA is synthesized through additional steps, it can be used for mRNA microinjections.

mRNA Microinjection

mRNA was injected into embryos at the one cell stage of development to simulate gain of function mutations. The injected mRNA overexpresses the protein in the whole embryo. In this case, overexpression of ECM remodeler enzymes is expected to degrade the ECM and produce morphological differences in the optic fissure (Yuan and Sun, 2009).

Results and Conclusion

The chronological expression patterns of ADAMTS 6 can be seen in Figure 8. ADAMTS 6 appears to present in the optic fissure during all time points. Previous work with $Pax2^{noi}$ embryos had suggested that ADAMTS 6 had no expression and thus no probable functionality in optic fissure fusion, but the current expression patterns in the wildtype embryos and the mRNA injected embryos suggest that ADAMTS 6 actually has functionality in the fusion of the optic fissure (Taylor et al., 2017). The prior loss-of-function analysis with the $Pax2^{noi}$ mutants should be repeated in order to make sure the initial inferences are accurate.

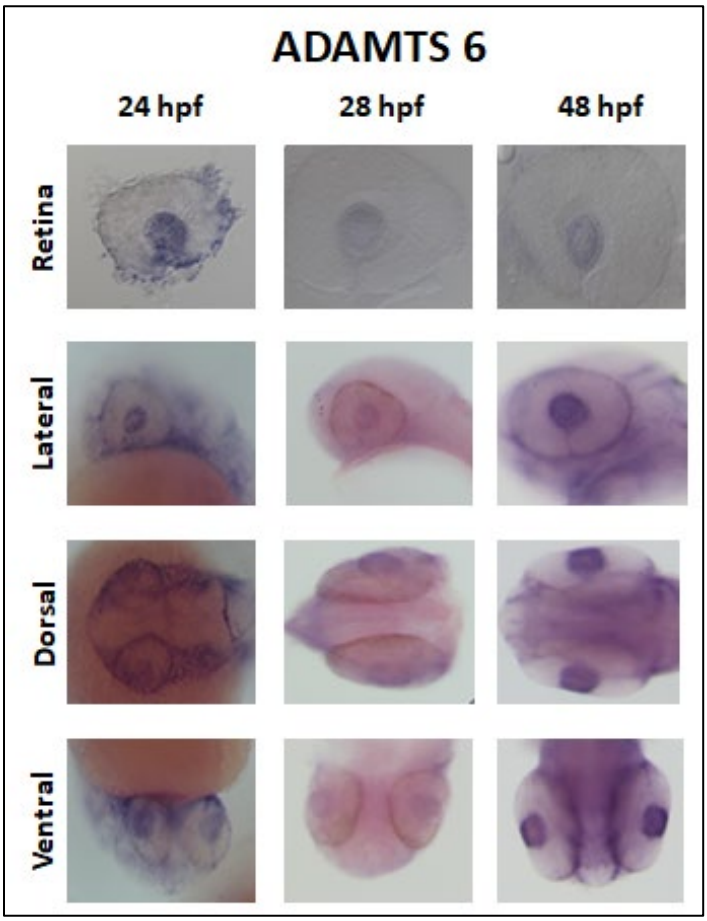

Figure 8: *Wild type zebrafish embryos that have undergone RNA whole-mount in situ hybridization probed for ADAMTS 6 expression. Eyes of 24, 28, and 48 hpf embryos were dissected and imaged using DIC microscopy. All other views were taken during dissection. ADAMTS 6 displays expression within the optic fissure at all time points. At 28 hpf, expression can be seen, but the staining could be allowed to go longer in future trials for clearer images.*

With an overexpressed ECM remodeler enzyme like ADAMTS 6, the ECM holding the two ends of the epithelial sheets may cause morphological differences in the optic fissure (Yuan and Sun, 2009). Since the overexpression of this degradation enzyme would be expected to result in a possible decrease in time to remove the ECM, the fusion event could be available to fuse together sooner. When mRNA was injected into the embryos during gain-of-function analysis, the optic fissure appears to have not fused as completely as in the control in 48 hpf embryos.

When 100 pg of mRNA was injected, ~13% of embryos appeared to have reduced function in the optic fissure. When 200 pg of mRNA was injected, ~29% of embryos appeared to have reduced function in the optic fissure. The fusion of the dorsal fissure also appears to be affected when ADAMTS6 is overexpressed. Further investigation of the ECM in older embryos would be necessary to determine what leads to these morphological differences. These results seem to be the opposite of the expected function of mRNA overexpression of the protein (Yuan and Sun, 2009).

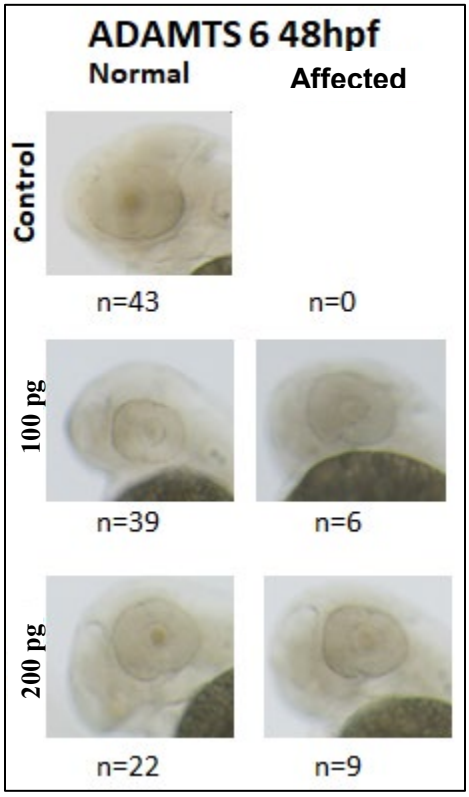

Figure 9: The phenotypes of ADAMTS 6 wildtype embryos were imaged and scored based on the amount of mRNA injected into the embryos during development. With a doubling of mRNA the percentage of disrupted optic fissure fusion increased by a little more than double.

Future Directions

Since the quantity of data in the ADAMTS6 analyses is limited, it would be good to complete the experiments over again with a larger number of embryos. This would allow the genetic ratio of 25% homozygous recessive individuals for the ADAMTS6 gene to possible be obtained and imaged for comparison since the embryos in this study appeared to have expression in all embryos imaged. The use of mRNA microinjection to show overexpression of the protein used in this experiment resulted in some confusing data. A better quantification system of the embryos injected for the morphological differences of their phenotypes should be utilized to produce a more accurate result. Again, the number of embryos involved in the analysis should be increased. Once these suggestions have been completed, the next step for the mechanism analysis can be reconsidered.

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