Differential Gene Expression in *Danio rerio* during Optic Nerve Regeneration

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Katherine E. Saul, B. S.

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Differential Gene Expression in *Danio rerio* during

Optic Nerve Regeneration

Committee Members Approved:

Dana M. García, Thesis co-Chair

Joseph R. Koke, Thesis co-chair

Nihal Dharmasiri

Timothy Raabe

Approved:

J. Michael Willoughby
Dean of the Graduate College

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The inspiration for this project was fueled by a spinal cord injury that left my brother, Devon, partially paralyzed. Devon, thanks for not listening to the doctors who said you'd never walk again. You've beaten the odds and given hope to all of us.

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ABSTRACT

Spinal cord injuries and neurodegenerative diseases in mammals result in a loss of function due to the failure of neurons in the central nervous system (CNS) to survive and regenerate their axons. Unlike mammals, fish and amphibians possess the ability to regenerate their CNS following damage. To gain a better understanding of the factors necessary for successful CNS regeneration, I conducted a temporal analysis of the changes in gene expression in the retina caused by optic nerve injury to identify genes specifically involved in regeneration. Dual color oligonucleotide microarrays were used to compare total RNA harvested from retinas of sham-operated and optic nerve-injured fish at 3, 24 and 168 hours following surgery. Statistical analyses identified 722 genes differentially expressed by at least 1.5-fold at one or more time points, and 142 genes with at least a 2.0-fold difference. Based on microarray fold differences and gene ontology analysis, six genes were selected for further analysis using qRT-PCR. The results of qRT-PCR identified noggin 2, activating transcription factor 3, and beta-tubulin 5 as genes that showed significantly increased expression in the injured fish as compared to sham; therefore, these genes' products may play an important role in optic nerve regeneration in zebrafish. These results support the hypothesis that an analysis of gene expression between optic nerve injured and sham-operated fish will reveal genes specifically involved in regeneration.

CHAPTER I

INTRODUCTION

Spinal cord injuries and neurodegenerative diseases in mammals result in a loss of function due to the failure of neurons in the central nervous system to survive and regenerate their axons. At some point during the development of mammals, the central nervous system (CNS) neurons lose their ability to regenerate axons and reestablish functional connections after axotomy (Cho *et al.*, 2005). The retina and optic nerve are developmentally and functionally part of the brain (and CNS), and because of its accessibility, optic nerve injury has become a standard model system for studies of nerve regeneration in the CNS.

The axons that make up the optic nerve originate from ganglion cells in the retina and project primarily to the optic tectum of the brain. In mammals, damage to the optic nerve results in wallerian degeneration of axons distal to the injury site (towards the brain) and apoptosis of cell bodies in the retina. Growth of any new neurites that may sprout from the nerve stump is attenuated by both physical barriers, specifically the formation of the glial scar (Goldberg and Barres, 2000; Ries *et al.*, 2007) and molecular barriers such as myelin inhibitory protein, semaphorin 3A, and chondroitin sulfate proteoglycans (Cao *et al.*, 2008).

In contrast, it has been known for many years that fish and amphibians possess the ability to spontaneously regenerate axons in the central nervous system following axotomy. Fish possess the same cellular and molecular players that prevent regeneration in mammals, but following nerve injury, the outcome is much different. Regenerating neurites sprout from the nerve stump and functionally re-enervate the brain (Attardi and Sperry, 1963; Gaze and Keating, 1972; Gaze *et al.*, 1972; Veldman *et al.*, 2007).

Why does this perplexing difference between fish and mammals exist? To approach this question and gain a better understanding of the factors necessary for successful CNS regeneration, an examination of the changes in gene expression during optic nerve regeneration in zebrafish was conducted. The working hypothesis for this experiment was that observing differences in gene expression between optic nerve injured and sham-operated fish will reveal genes involved in regeneration. A better understanding of the genetic mechanisms behind this remarkable capability will provide understanding of the requirements for functional recovery after nerve trauma.

Zebrafish (*Danio rerio*) are an excellent model system for studying nerve regeneration for several reasons: they are inexpensive, easily maintained, and their genome is fully sequenced. To observe changes in gene expression during optic nerve regeneration, RNA extracted from injured and sham-operated zebrafish eyes was compared using oligonucleotide microarray analysis. Microarrays allow the simultaneous examination of the expression of a large number of genes - in this case approximately 9,000 (14,067 unique ~50-mer probes representing 8,839 genes) at each time point. The results of the microarray assays were statistically analyzed using

bioinformatics software, and the change in expression of genes identified as suspects was further analyzed using quantitative PCR.

CHAPTER II

MATERIALS AND METHODS

Fish maintenance

Wild-type zebrafish were obtained from a local pet store (Animal Wonders, San Marcos, TX). Fish were conditioned on a 12 hour light/dark cycle for a minimum of 14 days before use. All protocols were approved by the Texas State IACUC (approval # 0703_0122_07).

Experimental Design

The experimental design of this analysis compared three interventions designated: the injured, sham-operated, and control. The surgical procedures for each are described in the section ”Optic Nerve Injury.” For this experiment, I used a dual-color microarray to compare between the sham-operated and optic nerve-injured fish. Previous studies of this nature have examined injured retina as compared to control retina. With this approach genes involved in general tissue restoration and immune response may also show significant temporal change and thus confound the analysis of gene expression changes important to nerve regeneration. By comparing injured to sham-operated fish, I attempt to dissect out the “noise” of non-neuronal tissue repair and inflammatory response, while emphasizing gene responses specific to neural injury and repair.

In general, a fold increase in an experimental sample as compared to a control would indicate an up regulation in gene expression. The experimental design employed in this study compares the experimental to the sham instead of a control; therefore, I am unable to determine whether the gene expression ratios greater than 1 are due to an increase in gene expression in the injured fish, decreases in gene expression in the sham-operated fish, or a differential increase or decrease in both. Thus, genes of interest selected from the microarray analysis were further analyzed by qRT-PCR where samples from experimental and sham retinas were compared to control retinas.

Total RNA was isolated from sham-operated and optic nerve-injured retinas at 3 time points. I selected 3 hours, 24 hours and 168 hours to compare early changes in gene expression (3 and 24 hours) to subsequent changes in expression as regenerating axons are first observed synapsing in the brain (168 hours) (Bernhardt *et al.*, 1996).

Optic Nerve Injury

Optic nerve injury was performed as described below using a method modified from Q. Liu and R. L. Londraville 2003 (Liu and Londraville, 2003). The zebrafish were anesthetized in 0.2% Finquel® tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, Washington) dissolved in tank water. The zebrafish were wrapped in a wet paper towel exposing only the head, and placed on a stereomicroscope for dissection. Surgical tools were sterilized with 70% ethanol. By separating the dorsal connective, cutting the lateral rectus muscle, and then angling the eye rostrally we are able to expose the optic nerve. Taking care not to damage the ophthalmic artery, the optic nerve was partially severed (~90%) using 3mm microscissors (EM Sciences, Hatfield, PA) (Figures 1A and 1B). The eye was placed back into the socket and the fish

revived by being placed in aerated aquarium water. Sham operations were identical except the optic nerve was not severed. Control fish were un-operated.

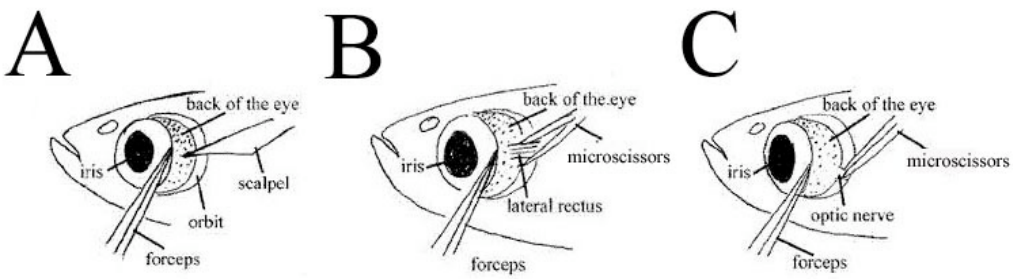

Figure 1A. Optic Nerve Injury Methods. First, the connective tissue surrounding the dorsal aspect of the eye was separated using a scalpel (A) and the lateralis muscle of the eye was cut using microscissors (B). The eye was angled slightly to expose the optic nerve which was then severed approximately 90% using microscissors (C) (see also Figure 1B below). This illustration adapted from Liu and Londraville (2003).

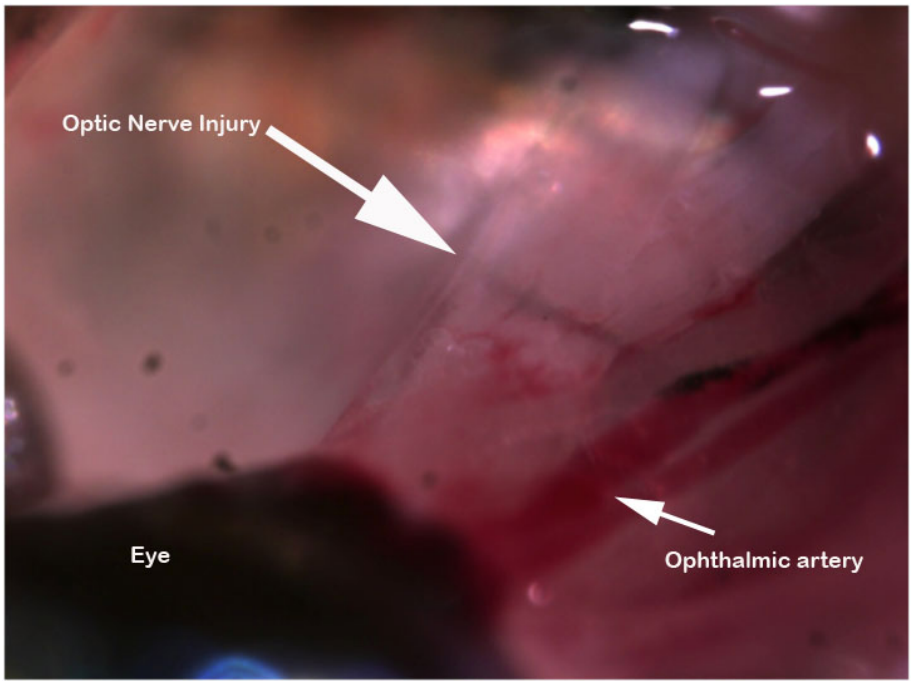

Figure 1B. Optic Nerve Injury Methods. The extent of optic nerve injury (arrow) is shown *in vivo* in this image. The optic nerve travels from the retinal ganglion cells of the eye to the brain. During the optic nerve injury procedure, care was taken not to damage the ophthalmic artery. Image was captured using a Nikon SNZ 1500 dissecting scope equipped with a Nikon DXM1200C digital camera.

RNA Extraction

All fish were sacrificed at midday to avoid any gene expression differences associated with diurnal rhythm. Following euthanasia by overdose in MS-222, whole eyes were removed from the fish 3 hours, 24 hours, and 7 days after optic nerve injury or sham operations and immediately placed in RNA later (Ambion; Austin, TX). To achieve 10 µg of total RNA required for the microarray, the retinas from 10-15 identically treated fish were pooled. The sclera and the lens were removed, and the remaining eye tissues (retina, RPE, and choroid) were placed in 1 ml of TRI-Reagent (Ambion; Austin, TX). Samples at each time point were collected in triplicate. The tissue was homogenized by trituration with a 27 gauge needle and syringe, and total RNA was isolated by organic extraction and isopropanol precipitation. The RNA isolated from RPE is often contaminated with pigment (Malik *et al.*, 2003, Invest Ophthalmol Vis Sci, 44, 2730-5) and this was confirmed in this study. RNA clean-up was performed using RNeasy spin columns (QIAGEN, Valencia, CA), resulting in a pigment free product. RNA quality and integrity was assessed using a Nanodrop spectrophotometer (Thermofisher Scientific, Waltham, MA) and glyoxal gel electrophoresis with ethidium bromide staining to detect the 18S and 28S rRNA bands (Sambrook and Russell, 2001). Samples comprising intact RNA as indicated by a lack of smearing on gels were sent to Michigan State University's Core Genomics Facility for an additional quality check using the Agilent BioAnalyzer and subsequent microarray analysis.

Microarray Analysis

Microarray analysis was performed by Dr. Jeff Landgraf at Michigan State University as follows. Labeling of the RNA for the oligonucleotide array was performed

using the Amino-Allyl MessageAMP II aRNA Amplification Kit (Ambion; Austin, TX). During this procedure, total RNA is reverse transcribed using an oligo(dT) primer bearing a T7 promoter using ArrayScript™ as reverse transcriptase. The resulting cDNA undergoes second strand synthesis in the presence of RNase H and subsequent purification to serve as the template for *in vitro* transcription (IVT). During IVT, modified nucleotides, 5-(3-aminoallyl)-UTP (aaUTP), are incorporated into the antisense RNA (aRNA) during amplification. The aaUTP contain a reactive amino group on the C5 position that can be coupled to N-hydroxysuccinimidyl ester-derivatized dyes (Cy3 and Cy5).

First strand cDNA synthesis was carried out on 1000 ng (1 µg) of total RNA at 42ºC for 2 hours. After second strand synthesis at 16°C for 2 hours, cDNA was purified through a filter cartridge. IVT was carried out for 12 hours at 37ºC, and the resulting aRNA was purified through a filter cartridge. The aRNA samples were coupled to either Cy3 or Cy5 dye. The dyes were swapped between sham and experimental sample replicates to control for any dye bias. This means that in one replicate sample, the sham was labeled with Cy3 while the injured was labeled with Cy5, and in two replicates the labels were switched. Each aRNA sample was fragmented using Ambion's RNA Fragmentation Reagents, added to the hybridization solution (Ocimum Biosolutions, Indianapolis, IN), heated to 95ºC for 3 min, cooled on ice for 3 minutes, and spun briefly. Hybridization was performed under a 40mm x 22 mm LifterSlip (Erie Scientific) by the addition of 240 µl of labeled solution to Zebrafish 14K OciChip™ (Ocimum Biosolutions). Slides were scanned using an Affymetrix 428 ArrayScanner and analyzed with the GenePix Pro 3.0 software (Axon Instruments, Sunnydale, CA).

Array normalization and statistical analysis were performed using the "limma: Linear Models for Microarray Data" library module (version 2.2.0) of the R statistical package (version 2.2.0). Signal intensities close to the background are considered unreliable data (Korenberg, 2007); therefore, all signal intensities of less than 1000 in all times points were removed from the analysis. A one-way analysis of variance (ANOVA) was performed on the ratios of the triplicate time points to determine whether statistically significant differences existed among gene expression between sham and experimental (statistically different from a ratio of 1). Slide intensity data were normalized using the global LOWESS (locally weighted scatter plot smoothing) method with the least squares method used for the linear model fit. The purpose of normalization is to remove technical variation while still retaining biological signal (Gentleman *et al.*, 2004).

Gene Ontology Analysis

Gene ontology provides a computational approach to answering questions such as, "what is known about the biological function of these genes?" Gene ontology is a controlled vocabulary that is used to describe knowledge and implications of the biological process, molecular function, and cellular localization of gene products (Korenberg, 2007 and Ashburner *et al.*, 2000). The biological process is particularly useful because up- or down-regulation of a set of genes within the same process provides evidence that a specific cellular event has been activated. All genes that displayed greater than 1.5-fold change in at least one time point were individually analyzed for known functional characteristics and gene ontology, using the bioinformatics software GeneSifter ® (VizXLabs, Seattle, WA), the web-based search engine GeneTools (Beisvag *et al.*, 2006), and ontology information provided by the chip manufacturer. In

the case of GeneSifter and GeneTools, ontology analysis was accomplished based on gene accession number.

Quantitative PCR

Corroboration of the results of the microarray analysis was sought by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Based on microarray fold-differences and gene ontology, seven genes were selected for validation (Table 1). Primers for these genes were designed observing the following criteria: primer length 18-25 bases long, 50-60% GC content, and with melting temperatures within 5^{o}C of each other. The gene product size for qRT-PCR should lie between 80 and 250 bases long (Bustin, 2004), and this was confirmed using UCSC's In-Silico PCR (http://genome.ucsc.edu/). Secondary structures and self-complementarity were assessed using an online oligo-nucleotide calculator (http://www.basic.northwestern.edu) (Table 1).

Table 1. Gene Selection and Primer Design. Genes selected for validation using qRT-PCR and their respective forward and reverse primers. The annealing temperatures used in the PCR program cycle for each of the primer pairs are given (T_{anneal}).

Gene	Forward Primer 5'→3'	Reverse Primer 5'→3'	T_{anneal}
Noggin 2	CGCTTCTGAAGTTCCGATTG	CTGAGCAATGAGGCTCCAGC	56.2^{O}C
Lunatic Fringe	GCGTCTCATAGCAATGGCG	GGCATAGTGATGTCCAACTG	53.7^{O}C
Hox A-11	CTCGGTTCTCTACCACTCC	TGTCCACCGGATGCTCAGTC	55.5^{O}C
ATF3	CCTTGTCATCTCCACGTCCAC	CAGACCTTCCTGCTCACAGC	53.7^{O}C
TF YY1	AGACGACGACGAGCACCA	CTTGCCAGACACGGTCAC	55.3^{O}C
ß-Tubulin	AAACCGCCGTCTGCGATATTCC	ACTACCACCTCCCCAAAACACC	59.0^{O}C
KLF7A	CATTACGTCTCCTCTGTTGG	AAAGATTGGGATTGCTGGCTTG	55.3^{O}C
GAPDH	CAAGGGGTCACATCTACTC	TGGGTGCTGGTATTCTCTC	53.0^{O}C

Quantitative RT-PCR was performed using the Express SYBR® GreenER™ One-Step qRT-PCR Universal Kit (Invitrogen, Carlsbad, CA) on 10 ng of total RNA with a total reaction volume of 20 µl. The following cycling program was executed using an Eppendorf Realplex^2 Mastercycler (Hamburg, Germany): 5 min at 50ºC for cDNA synthesis, 95ºC for 2 min, 40 cycles [95ºC for 15 sec, gene specific annealing temperatures (Table 1) for 15 sec, 20 sec for extension at 60ºC], followed by a melt curve analysis. A gradient analysis was performed on each primer pair to determine the optimum annealing temperatures. The gradient temperature ranged from 0.5ºC below the lower recommended T_m to 60ºC. The optimal temperature was determined by examination of the amplification plots and selection of the temperature that with the lowest C_T value, indicating the most efficient reaction.

Analysis of qRT-PCR results was completed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where:

$$\Delta\Delta C_T = (C_T, \text{Target} - C_T, \text{HKG})\text{Injury} - (C_T, \text{Target} - C_T, \text{HKG})\text{Sham}$$

or

$$\Delta\Delta C_T = (C_T, \text{Target} - C_T, \text{HKG})\text{Injury or Sham} - (C_T, \text{Target} - C_T, \text{HKG})\text{Control}$$

By using the $2^{-\Delta\Delta CT}$ method, the data are represented as the fold change in gene expression normalized to an endogenous reference gene (HKG, or house-keeping gene) and relative to a control (or sham in this instance). The reference gene chosen was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

CHAPTER III

RESULTS

Microarray Analysis

Total RNA was isolated from sham-operated and optic nerve injured retinas at 3 time points. I chose 3 hours, 24 hours and 168 hours post surgery to compare early changes in gene expression (3 and 24 hours) to subsequent changes in expression as regenerating axons are first observed forming terminal arborizations in the optic tectum (168 hours) (Bernhardt *et al.*, 1996). The Zebrafish 14K OciChip™ Oligo-nucleotide Array (Ocimum Biosolutions) comprises 14,067 unique ~50-mer probes representing 8,839 genes. Statistical analyses identified 722 genes differentially expressed by at least 1.5-fold in one or more time points, and 142 genes with at least a 2.0-fold difference. Table 2 illustrates the changes in differentially expressed genes (1.5-fold or more) at each time point. The 20 most differentially expressed genes at each time point are displayed in Tables 3 through 8 (Appendix).

Table 2. Genes Differentially Expressed by 1.5-fold or more. Ratios were expressed as Injury/Sham so that genes that displayed more intense signal intensity in the injury model vs. the sham model would result in a ratio greater than 1. "Genes Up" refers to genes that showed greater expression in the injured vs. the sham-operated fish (ratio > 1.5) "Genes Down" refers to genes that showed greater expression in the sham vs. the injured fish (ratio < 0.67).

Time Point	Genes Up	Genes Down
3 Hrs	50	17
24 Hrs	112	86
168 Hrs	217	191

All genes that displayed greater than 1.5-fold change in at least one time point were individually analyzed for known functional characteristics and gene ontology using bioinformatics software (Figure 2 and Tables 9 through 14).

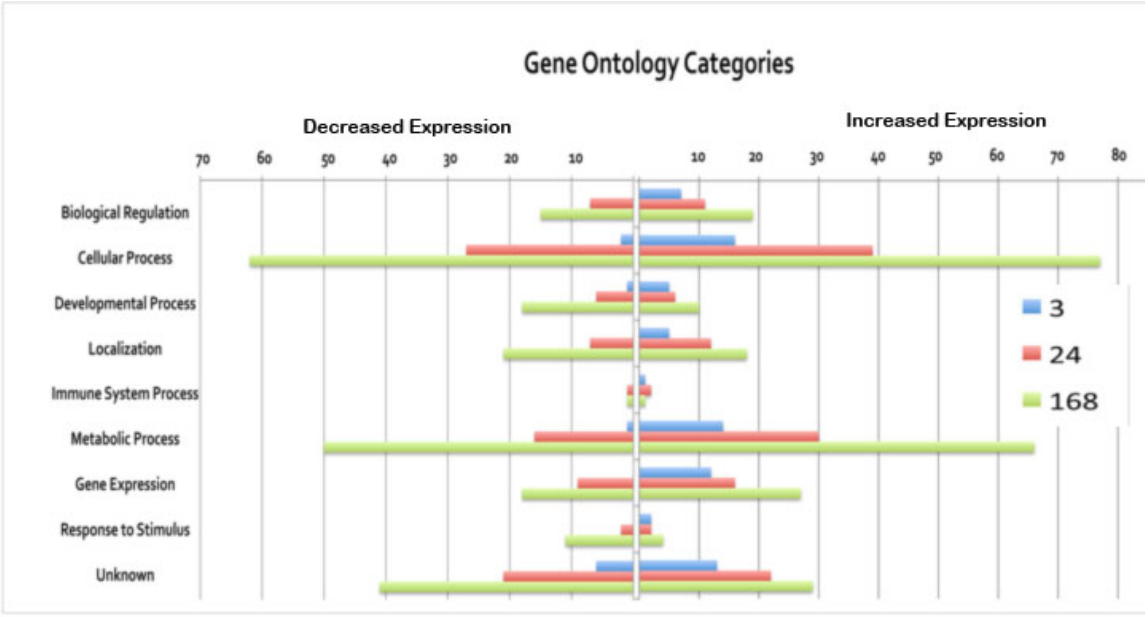

Figure 2. Temporal analysis of gene ontology during optic nerve regeneration. Total number of genes differentially expressed at least 1.5-fold within each ontological category. The smallest category represented was from the immune system response category that indicated the largest number of differentially expressed genes occurred at 24 hours.

At all time points, those genes that were differentially expressed were primarily in the ontology category of cellular process, metabolic process, and gene expression. Cellular process is a broad category including actions such as cell adhesion, communication, homeostasis, and proliferation. Metabolic process includes genes involved in biosynthetic and catabolic processes, generation of precursor metabolites and energy, and primary and secondary metabolic processes. The category of gene expression includes genes that are involved in nucleic acid binding, or regulate transcription or translation.

Overall, there is an increase in expression of genes associated with cell growth and proliferation (Table 9) as well as genes associated with axon extension (tubulins) and guidance (neural adhesion molecule L1.2 and ephrin a4b) (Table 10) in the injured fish as compared to the sham-operated fish. Examples of genes up-regulated in the cell proliferation and differentiation categories included ATF3, jun B proto-oncogene (a member of the AP-1 transcription factor family), and fibroblast growth factor 24. There was an increase in expression in the injured fish of genes associated with central nervous system development (Table 11), specifically YY1, frizzled 2 (fzd2), noggin 2 (nog2), tumor protein p63 (tp63), and lunatic fringe (*lfng*). Of the nine main categories of gene ontology listed in figure 2, the immune system process category had the least number of genes represented. The largest representation in the immune response category occurred at 24 hours with 2 genes showing increased expression in the injured fish as compared to the sham-operated fish.

A temporal analysis of the gene ontology categories indicates differences between early and late changes in gene expression. Differential gene expression that occurred

during the early time points (3 hours and 24 hours) consisted of genes involved in cell differentiation and proliferation (hoxa11b, YY1, and p63), developmental process (fzd2, YY1, insulin-like growth factor binding protein 2, Dicer1, p63, nog2, *lfng*), and also cytoskeletal genes (annexin A2a and tubulins). In addition, there was an increase in the number of genes involved in basic physiological processes such as protein metabolism, gene expression, and localization. Differential gene expression that occurred during the later time point (168 hours) consisted of 51 genes identified as representatives of protein metabolism, 32 within translation, 18 within localization, and 10 within development. There 57 genes differentially expressed that were involved in cell growth and proliferation (inhibitor of growth family, member 3, tubulins, and YY1) and 7 genes implicated in differentiation (tumor protein p63-like, zgc:103619, baculoviral IAP repeat-containing 5a, neural adhesion molecule L1.2, transcription factor AP-2 alpha, fibroblast growth factor 24, and hematopoietically expressed homeobox). In addition, sixteen genes associated with phototransduction were differentially regulated between the injured and sham-operated models (Table 14). All but one, retinaldehyde binding protein (rlbp1), were down regulated at 168 hours (Figure 3); however, rlbp1 expression was essentially unchanged between the sham-operated and injured models with an expression ratio of 1.19.

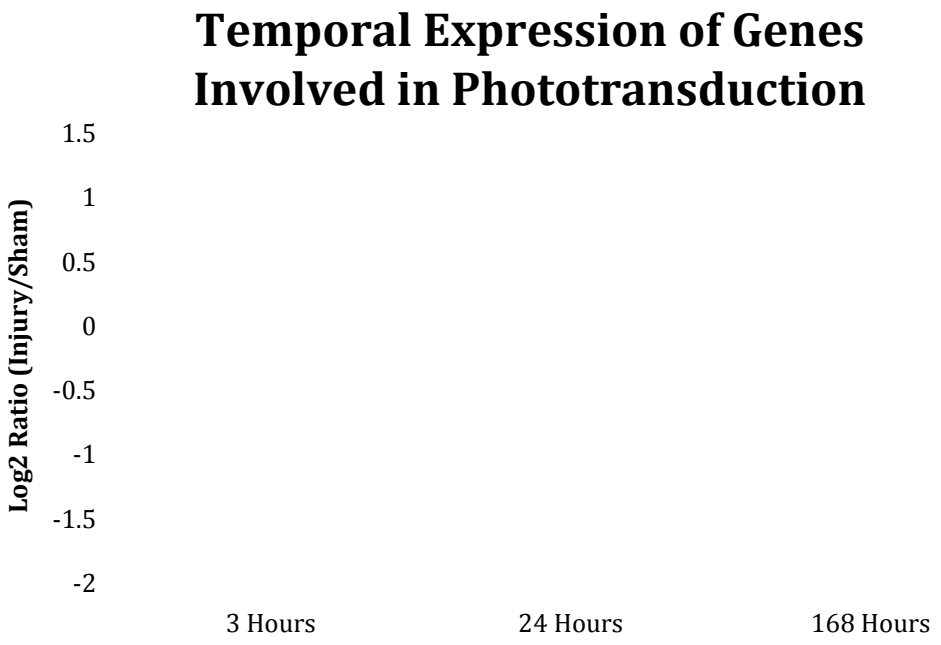

Figure 3. Temporal expression of genes involved in phototransduction. Log_2 of the ratio (injury/sham) shows a decrease in expression by 168 hours.

qRT-PCR Results

Quantitative RT-PCR was conducted on triplicate samples to validate the microarray data on 6 genes represented on the microarray and one gene (KLF7a) that was determined to be essential to nerve regeneration by Veldman *et al.* (2007). For the purposes of the qRT-PCR discussion comparisons between injured and sham-operated fish will be designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C).

Lunatic fringe was chosen for further investigation based on its 2.31-fold difference at 24 hours (I *vs.* S) on the microarray, and its known role as a regulator in the notch-signaling pathway during the segmentation phase of development (Lai, 2004). At 3 hours, the microarray results were insignificant, however, qRT-PCR results show a 1.6--fold change (I *vs.* S) (Table 15). When compared to the control, there is a marked

increase in expression in both the sham-operated (23.1-fold) and the injured (37.9-fold) fish (Table 15). At 24 hours, the microarray indicated a 2.31-fold difference (I *vs.* S). The results of qRT-PCR did not agree with the microarray; it revealed a 1.3-fold difference (I *vs.* S). Both sham-operated and injured fish show significant (>1.5) increase when compared to control, 1.5-fold and 2.0-fold respectively. At 168 hours, microarray data was insignificant as was the qRT-PCR results (1.4-fold decrease I *vs.* S). Both sham-operated and injured fish were significantly increased, 2.4-fold and 1.7-fold respectively, when compared to control; however, the sham-operated had a larger increase than the injured, giving the overall I *vs.* S ratio a value less than one.

Table 15. Lunatic Fringe qRT-PCR Results. The results of qRT-PCR do not reflect the same 2.31-fold change at 24 hours as the microarray. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	Microarray (I *vs.* S)	qRT-PCR (I *vs.* S)	qRT-PCR (S *vs.* C)	qRT-PCR (I *vs.* C)
3 hours	--	1.6 ± 0.11	23.1 ± 0.82	37.9 ± 3.41
24 hours	2.31	1.3 ± 0.12	1.5 ± 0.02	2.0 ± 0.22
168 hours	--	-1.4 ± 0.05	2.4 ± 0.15	1.7 ± 0.04

Hoxa11b was chosen for further investigation based on its 1.85-fold differential expression (I *vs.* S) at 3 hours on the microarray. Additionally, I was interested in this gene because it is within a family of homeobox genes that code for transcription factors involved in axial patterning and development. Hoxa11b has been shown to play a role in cell differentiation and pattern formation during development and limb and tail

regeneration of newts (Beauchemin *et al.*, 1994) and in the regenerating fin of adult zebrafish (Geraudie and Borday Birraux, 2003). Perhaps this gene is also involved in optic nerve regeneration. The qRT-PCR results indicate that at 3 hours there is a non-significant fold change of 1.18 (I *vs.* S) (Table 16). However, when we look at how the expression compared to the control, it is apparent that both the sham-operated and injured fish showed a greater than 2-fold increase in expression. This suggests that this gene may be responding to the general stress and wound and not to the neuronal repair. At 24 hours there is an observed change of 1.94-fold (I *vs.* S), which is due to the increased expression of hoxa11b in the injured model (1.7-fold I *vs.* C). At 7 days, we see no significant differential expression (I *vs.* S) on the microarray or in qRT-PCR. There is a 30% decrease in expression (I *vs.* S), however, both sham-operated and injured fish showed increased expression of hoxa11b when compared to the control.

Table 16. Hoxa11b qRT-PCR results. The results of qRT-PCR do not reflect the same 1.85-fold change at 3 hours as the microarray. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	**Microarray (I *vs.* S)**	**qRT-PCR (I *vs.* S)**	**qRT-PCR (S *vs.* C)**	**qRT-PCR (I *vs.* C)**
3 hours	1.85	1.2 ± 0.07	2.2 ± 0.02	2.7 ± 0.18
24 hours	--	1.9 ± 0.14	-1.1 ± 0.04	1.7 ± 0.17
168 hours	--	-1.3 ± 0.10	1.7 ± 0.13	1.3 ± 0.08

ATF3 was chosen for further investigation because it was among the most differentially expressed genes on the microarray; it indicated fold increases at 24 hours (3.6 I *vs.* S) and 168 hours (2.13 I *vs.* S). It also was selected because its gene ontology indicated that the gene products play a role in cell differentiation and proliferation. Previous studies have shown that ATF3 showed an increase in expression in axotomized retinal ganglion cells and in axotomized peripheral nerves of rats (Hunt *et al*., 2004). At 3 hours the microarray showed non-significant results, however, qRT-PCR showed a 2.5-fold change (I *vs.* S) (Table 17). This is primarily due to an increased expression of ATF3 in the injured model, however the 2.5-fold change (I *vs.* S) is slightly exaggerated by a decrease in expression in the sham-operated fish as indicated by the 20% decrease when sham is compared to control (-1.2-fold S *vs.* C). At 24 hours the microarray showed a differential expression of 3.60-fold (I *vs.* S). The qRT-PCR results show a 2.1-fold (I *vs.* S) increase. When compared to control, it is apparent that this difference in expression is due to an increase in ATF3 expression in the injured fish (1.7-fold I *vs.* C). Similar to the 3 hours sample, the 2.1-fold change (I *vs.* S) is slightly exaggerated by a decrease in expression in the sham-operated fish as indicated by the 20% decrease when sham is compared to control (-1.2-fold S *vs.* C). At 168 hours, the microarray revealed a 2.13-fold change. qRT-PCR results show a different story at 168 hours: the results show essentially no differential expression between the injured, sham, or control model.

Table 17. ATF3 qRT-PCR results. The results of qRT-PCR reflect a similar fold change at 24 hours, but not at 168 hours when compared to the microarray. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	Microarray (I *vs.* S)	qRT-PCR (I *vs.* S)	qRT-PCR (S *vs.* C)	qRT-PCR (I *vs.* C)
3 hours	--	2.5 ± 0.16	-1.2 ± 0.04	2.1 ± 0.04
24 hours	3.60	2.1 ± 0.3	-1.2 ± 0.04	1.7 ± 0.12
168 hours	2.13	1.3 ± 0.10	1.0 ± 0.08	1.2 ± 0.01

YY1 was chosen for further investigation based on the microarray data indicating a 1.85-fold (I *vs.* S) increase at 24 hours and a 2.62-fold (I *vs.* S) increase at 168 hours. Additionally, gene ontology indicated its involvement in cell proliferation, gene expression and differentiation. YY1 codes for a ubiquitous and multifunctional zinc finger transcription factor protein that can activate or repress gene expression depending on its binding partners (e.g., histone deacetylase 1). It is in the GL1-Krüeppel gene family, and it has been shown that YY1 regulates the expression of diverse genes (e.g., p53, c-myc, IFN-β, CREB) that are important for cellular activity (Kurisaki *et al.*, 2003). At 3 hours the microarray data were not significantly differentially expressed (I *vs.* S) and this was confirmed with the qRT-PCR (-1.1 I *vs.* S) (Table 18). However, when compared to the control model, both injured and sham show a marked increase in expression (17.8 and 19.5-fold respectively). At 24 hours the microarray data revealed a

1.85-fold difference (I *vs.* S) that was not supported by the qRT-PCR results as indicated by -1.4-fold change (I *vs.* S). However, this change is not due to a decrease in expression in the injured fish. Both the sham-operated and injured fish showed an increase in expression of YY1 when compared to the control; the (S *vs.* C) fold change of 2.0 was greater than the (I *vs.* C) fold change of 1.5, thus giving the (I *vs.* S) ratio a value less than 1. At 168 hours the microarray data showed a 2.62-fold difference (I *vs.* S), and similar to 24 hours, was not supported by the qRT-PCR results as indicated by -1.2-fold change (I *vs.* S). At all time points, YY1 was expressed at a higher level in the sham-operated fish than in the injured.

Table 18. YY1 qRT-PCR results. The results of qRT-PCR do not reflect the 1.85-fold change at 24 hours (I *vs.* S) and the 2.62-fold change (I *vs.* S) at 168 hours reveal on the microarray. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	Microarray (I *vs.* S)	qRT-PCR (I *vs.* S)	qRT-PCR (S *vs.* C)	qRT-PCR (I *vs.* C)
3 hours	--	-1.1 ± 0.04	19.5 ± 1.13	17.8 ± 0.87
24 hours	1.85	-1.4 ± 0.08	2.0 ± 0.09	1.5 ± 0.23
168 hours	2.62	-1.2 ± 0.11	1.7 ± 0.17	1.3 ± 0.03

Nog2 was chosen for further investigation because of the 2.57-fold change at 24 hours (I *vs.* S) on the microarray. This gene is of further interest because it codes for a protein that inhibits the bone morphogenic protein during development to result in the formation of neural tissue. At 3 hours there was no significant microarray data, however

a 2.0-fold change (I *vs.* S) was revealed by qRT-PCR (Table 19). When compared to the control model we see that both injured and sham-operated fish were up regulated, however, the injured shows 200% more expression of this gene than the sham-operated fish. At 24 hours the microarray data revealed a 2.57-fold difference (I *vs.* S). qRT-PCR showed a similar 1.6-fold difference (I *vs.* S), which was due to an increase in the expression of nog2 in the injured model (1.6-fold I *vs.* C and -1.1-fold S *vs.* C). At 168 hours, the microarray showed non-significant results and this was confirmed with qRT-PCR (1.1-fold I *vs.* S). There was no differential expression between injured and sham-operated, however, both sham-operated and injured models showed an increased expression of 1.4 and 1.5-fold respectively.

Table 19. Nog2 qRT-PCR results. The results of qRT-PCR reflect a similar fold change at 24 hours. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	Microarray (I *vs.* S)	qRT-PCR (I *vs.* S)	qRT-PCR (S *vs.* C)	qRT-PCR (I *vs.* C)
3 hours	--	2.0 ± 0.06	1.9 ± 0.07	3.8 ± 0.04
24 hours	2.57	1.6 ± 0.17	-1.1 ± 0.05	1.6 ± 0.23
168 hours	--	1.1 ± 0.09	1.4 ± 0.15	1.5 ± 0.11

Tubb5 was selected for further investigation based on its differential expression on the microarray at 168 hours (3.5-fold I *vs.* S) and its previous implications in nerve regeneration (Cameron *et al*., 2005 and Veldman *et al*., 2007). The up-regulation of tubulin mRNA's is often reported in nerve regeneration studies. At 3 hours qRT-PCR

showed a 3.1-fold difference between injured and sham models (Table 20). When compared to control, both the sham-operated and injured fish showed a significant increase in expression (6.4-fold and 9.5-fold, respectively). At 24 hours qRT-PCR showed an unexpected 2.1-fold decrease (I *vs.* S). When compared to control, it reveals that this is a true decrease in tubb5 expression in the injured fish (-1.6-fold I *vs.* C). At 168 hours the microarray data indicated a fold difference of 3.5 (I *vs.* S). The results of qRT-PCR revealed a much more exaggerated 28.5-fold difference (I *vs.* S). When compared to the control, we see this is due to a large increase in expression of the injured as compared to control. The 2.3-fold decrease in sham-operated fish is unexpected, however this marked up regulation in the injured model suggests that this gene may play a role in optic nerve regeneration.

Table 20. Tubb5 qRT-PCR results. The results of qRT-PCR reflect an exaggerated fold change at 168 hours as compared to the microarray. The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	Microarray (I *vs.* S)	qRT-PCR (I *vs.* S)	qRT-PCR (S *vs.* C)	qRT-PCR (I *vs.* C)
3 hours	--	3.1 ± 0.20	6.4 ± 0.52	19.5 ± 1.40
24 hours	--	-2.1 ± 0.79	1.4 ± 0.09	-1.6 ± 0.07
168 hours	3.50	28.5 ± 2.1	-2.3 ± 0.4	61.4 ± 6.68

Krueppel-like factor 7a (KLF7A) was chosen for qRT-PCR based on research out of the Goldman lab at University of Michigan in which they showed that KLF6a and KLF7a were required for axonal sprouting in retinal explants and for optic nerve

regeneration by use of morpholino knockdowns (Veldman *et al.*, 2007). This gene was not represented on the microarray however I wanted to evaluate how their results compared when using a sham model. In general, KLF7a showed an increased expression (I *vs.* S) at all time points with the largest fold change of 4.0 at 24 hours (Table 21). At all time points the increase in expression is due to an increase in the injured model as confirmed by (I *vs.* C). Veldman *et al.* conducted a time course study spanning 0-24 days in which they reported 7 days to be the peak expression of KLF7a in the injured fish as compared to the control (2007). The results presented here indicate a peak at 24 hours, and therefore, do not concur.

Table 21. KLF7a qRT-PCR results. The results showed an increased expression (I *vs.* S) at all time points with the largest fold change of 4.0 at 24 hours. At all time points the increase in expression is due to an increase in the injured model as confirmed by (I *vs.* C). The numbers represented for qRT-PCR reflect the fold change calculated with the $\Delta\Delta C_T$ method employing GAPDH as the housekeeping gene. The standard error is also reported (± standard error). Comparisons between injured and sham-operated fish are designated (I *vs.* S), comparisons between sham-operated and control fish (S *vs.* C), and comparisons between injured and control fish (I *vs.* C). Ratios with values <1 were converted to fold change by inversion and indicated by a negative sign preceding the number.

Time Point	**Microarray (I *vs.* S)**	**qRT-PCR (I *vs.* S)**	**qRT-PCR (S *vs.* C)**	**qRT-PCR (I *vs.* C)**
3 hours	NA	1.7 ± 0.16	-1.1 ± 0.04	1.5 ± 0.10
24 hours	NA	4.0 ± 0.40	-1.2 ± 0.02	3.5 ± 0.42
168 hours	NA	1.7 ± 0.14	1.1 ± 0.09	1.8 ± 0.06

CHAPTER IV

DISCUSSION

The experimental design of this project aimed to reveal changes in gene expression during optic nerve regeneration that may provide a better understanding of the mechanisms required for successful regeneration of damaged neurons in the CNS. There is a general increasing trend in the number of genes differentially expressed throughout the time course, which suggests that earlier genes may be initiating signaling pathways leading to the response of additional genes later. Genes expressed within the first 24 hours included genes encoding transcription factors, genes involved in chromatin remodeling and genes implicated in developmental pathways. Genes expressed at the later time point (168 hours) corresponded to transcription factors, tubulins, ribosomal subunits, and genes involved in cell metabolism. The complex changes in gene expression observed support my hypothesis that observing differences in gene expression between optic nerve injured and sham-operated fish will reveal genes specifically involved in regeneration.

In several cases, the qRT-PCR did not agree with the microarray data. This is most evident with YY1 in which the microarray indicated a differential expression of

1.85-fold at 24 hours and 2.62-fold at 168 hours and qRT-PCR resulted in a differential expression of -1.4-fold and -1.2-fold (I *vs.* S) respectively. These data may call in to question the quality of the microarray. Reports from the Core Genomics Facility at Michigan State University indicated the microarray chips had a high background fluorescence atypical of microarrays commonly used, which may suggest that the arrays provided by Ocimum Biosolutions were not of the highest quality. However, the discrepancies between qRT-PCR and the microarray analysis can be explained by the sensitivities of the two methods. qRT-PCR is a more direct and sensitive method than microarray analysis and can be considered more reliable.

By comparing the experimental models to a control fish, it was determined that lunatic fringe and YY1, showed a large increase in expression in both models. This suggests lunatic fringe and YY1 may show increased expression as a result of stress or inflammation instead of as a result of neural repair, and it is unlikely that they contribute to the regeneration of the optic nerve. This is not surprising for YY1 considering that it codes for a ubiquitously expressed transcription factor (Kurisaki *et al.,* 2003).

There are data for nog2, tubb5, and ATF3 that may suggest a role in nerve regeneration. Nog2 showed a greater than 1.5-fold increase in expression (I *vs.* S) for both 3 hours and 24 hours, but not 168 hours suggesting that this may be an early response gene. At both 3 hours and 24 hours, it was determined that the fold change reflected an increase in the injured fish. Nog2 is important in the formation of the neural plate during development, working as an antagonist to bone morphogenic proteins (BMPs). BMPs have many actions in the nervous system including cell proliferation, patterning, cell fate determination, and apoptosis (Mabie *et al.*, 1997). The actions of

noggin favor the formation of neural tissue by inhibiting BMPs from interacting with their receptors (Trindade *et al.,* 1999). The increase in expression of nog2 in the injured fish may be required to stimulate retinal ganglion cell formation from the retinal stem cell population. This proposal is substantiated by a study conducted by Setouchi *et al.* showed that noggin induced neural precursor cells to differentiate into neurons and oligodendrocytes (Setoguchi *et al.,* 2004). Most recently, noggin genes have been shown to promote significant regrowth in corticalspinal tract of mammals when injected at the spinal cord lesion site (Matsuura *et al.,* 2008).

ATF3 showed >2.0-fold difference (I *vs.* S) at 3 hours and 24 hours, but no difference at 168 hours. ATF3 mRNA levels increase greatly in cells when exposed to stress signals (Hai et al., 1999), which may explain the increase in expression at the early time points and not at 168 hours. ATF3 codes for a bZIP leucine zipper transcription factors that bind cAMP response elements to regulate cell proliferation and differentiation (Hai and Hartman, 2001; Hai *et al.,* 1999). It has also been reported to serve as an anti-apoptotic and growth-promoting factor for neurons in culture (Nakagomi *et al.,* 2003). Perhaps the increased expression of ATF3 is promoting retinal ganglion cell survival and growth after optic nerve injury.

Tubb5 showed a very large increase in expression (I *vs.* S) at both 3 hours and 168 hours by qRT-PCR. Tubulins are the principle subunits of microtubules, which are essential to the growth and maintenance of axons (Mitchison and Kirschner, 1988). In addition to tubb5, several alpha-tubulins represented on the array showed increased expression in the injured model as compared to sham.

I believe that the experimental design of comparing the injured fish to the sham-operated fish on the array had several benefits. Firstly, we successfully limited the number of genes that were differentially expressed in the immune system response category of gene ontology. The gene ontology analysis revealed the immune response category as one of the smallest represented at all time points. We were also able to show that in some instances, differences do exist between the sham and control fish (*lfng*, YY1, nog2, and tubb5), while for some genes, the sham-operated and control fish show very similar expression (KLF7a and ATF3).

The microarray data support ways to tie together results from other researchers investigating regeneration in a variety of models. There are some researchers who believe CNS regeneration in fish and amphibians is possible due to the re-activation of developmental pathways. During limb regeneration in newts, the patterning and formation is regulated by many of the same genes that controlled its initial development, and the re-expression of these genes is essential to successful regeneration (Candinouche *et al.*, 1999). The results presented here suggest that the pathways that govern developmental axon growth and repression may also control regenerative axon growth. It is possible that the initiation and potentiation of axon regeneration is accomplished through the reactivation of developmental pathways. However, although aspects of the developmental pathways are the same, previous studies have indicated that some signaling mechanisms differ between development and regeneration (M.Z.A. Candinouche *et al.*, 1999; Goldman and Ding, 2000; Udvadia *et al.*, 2001). I observed differential expression of genes involved in 3 developmental pathways: BMP pathway, notch signal pathway and the Wnt signaling pathway. During the discussion of the qRT-

PCR results we suggested the possibility that lfng (notch signaling pathway) showed increased expression due to inflammatory response and was not specific to neural repair; therefore, I will not discuss the notch-signaling pathway. The role of nog2 in the BMP signaling pathway was discussed previously, so I will limit the discussion to the Wnt signaling pathway.

Wnt Pathway, fzd2 and N-Myc

Wnt proteins are secreted signaling molecules that bind to Frizzled family cell surface receptors to activate signaling pathways that result in gene transcription (ß-catenin/Wnt pathway), cell polarization (planar polarity pathway), or an increase in intracellular calcium (Wnt/Ca^{2+} pathway). Activation of the canonical Wnt pathway results in transcription of genes such as the *Myc* family of transcription factors; including *c-myc* which codes for proteins that are transcriptional activating factors, well known stimulators of cell growth and proliferation. *N-myc* is another gene targeted for transcription by the Wnt pathways. *N-myc* expression has been correlated to undifferentiated cells in the embryonic kidney, skin and brain; and further differentiation of these cells requires down regulation of *N-myc* (Mugrauer *et al.*, 1988; Moens *et al.*, 1992). There were two *N-myc* related genes, *N-myc* downstream regulated gene 1 and *N-myc* downstream regulated family member 3a, differentially expressed -1.63-fold at 24 hours and -2.23-fold at 7 days respectively. Recently, activation of Wnt signaling pathways by application of Wnt3a or inhibitors of GSK-38 has been shown to promote neural regeneration in mammalian retina through proliferation of Muller glia-derived progenitor cells (Osakada *et al.*, 2007). Perhaps down-regulation of *N-myc* through Wnt signaling pathway is required for differentiation of the retinal stem cell population into

new retinal ganglion cells. This conjecture is not far-fetched considering the role of Wnt signaling in the intestinal stem cell population. In the small intestine of mammals, the cells of the epithelium are constantly being replaced as a result of cell division from a population of stem cells in the crypts of the villi. Wnt signaling is responsible for keeping the stem cells proliferative and the differentiating cells quiescent (Clatworthy and Subramanian, 2001).

Some scientists believe that the ability of retinal ganglion cells to regenerate results from the resident stem cell population in the retina. Fish and frogs possess a proliferative region called the ciliary marginal zone (CMZ) that contains multipotent stem cells and progenitor daughter cells (Hitchcock and Raymond, 2004). After 60 hours post fertilization, retinal growth, with the exception of rods and cones, occurs at the CMZ by the addition of new cells to the retina in concentric rings as long as the eye is growing (Wehman *et al.*, 2005; Hitchcock *et al.*, 2004). Mammals also possess stem cells at the CMZ, but their regenerative potential remains unknown. Several studies have researched the regenerative potential of Müller glia-derived progenitor cells, which have the ability to differentiate into few retinal cell types (Fausett and Goldman, 2006). I observed differential regulation between injured and sham fish of several genes (Dicer1 and *N-myc*) that are recognized as regulators of stem cell populations in the skin and gut (Moens *et al.*, 1992; Clatworthy and Subramanian, 2001) respectively that possibly play a role in the differentiation of the CMZ into retinal ganglion cells.

Gene Ontology Analysis

The analysis of ontological categories of the differentially expressed genes sheds light on the general physiological processes involved in optic nerve regeneration. The

incomplete annotation is a limiting factor in this analysis; of the 722 differentially expressed genes with a change of at least 1.5-fold, 132 genes had an unknown ontology. In addition, the oligonucleotides array covers only a portion (14,067 unique probes representing 8,839 genes) of the approximate 25,000 genes in the zebrafish genome. Despite these limitations, some conclusions can be made from the available data.

In general, we observe an increase in the number of genes recruited over time (Table 2). Increased protein synthesis (Sikora-VanMeter *et al.*, 1987) and stimulation of cytoskeletal genes (Bisby and Tetzlaff, 1992) are characteristic of regenerating neurons. These observations are confirmed in this study. There is an increase in the number of genes involved in protein metabolism, particularly those involved in translation machinery; 70 ribosomal protein genes showed increased expression in the injured as compared to sham on the microarray. Genes related to cytoskeleton (tubulins and annexin) also showed an enhanced expression in the injured fish as compared to the sham.

There was a general temporal decline in expression of genes associated with phototransduction in the injured model as compared to sham, with the exception of rlbp1 which is essentially not differentially expressed at 168 hours (ratio of 1.19). Interestingly, there was a -2.08-fold differential expression between experimental and sham in rhodopsin. Rhodopsin is a rod-specific visual pigment in the retina that initiates signal transduction when excited by light. This change in expression is similar to results reported in 2007 by Veldman *et al.* (Veldman *et al.*, 2007), which showed a fold change of -1.73 at 3 days when comparing isolated RGC's from optic nerve crush injuries to control fish. However, Cameron *et al.* used rhodopsin as the reference gene for qRT-

PCR when comparing control and injured (by patch removal) retinas, which may have skewed their data (2005). This general decline in genes associated with phototransduction may result from a decrease in the number of photoreceptors as a result of apoptosis. It would be interesting to correlate these changes in gene expression to retina morphology and behavioral studies.

Conclusions and Future Directions

Why is there a difference between mammals and fish in the regenerative capacity of the optic nerve? In the scope of this project, this question will remain largely unanswered. The data presented here aimed to observe gene expression changes to gain a better understanding mechanisms involved in nerve repair in the CNS. The mechanisms governing optic nerve regeneration are very complex and may require the activation of developmental pathways for successful axon growth and re-enervation. Whether retinal ganglion cells in zebrafish become apoptotic after axotomy or survive to sprout new neurites from the existing cell body is yet to be conclusively determined, although there is one report of a 20% decline in retinal ganglion cells during regeneration of the zebrafish optic nerve (Zhou and Wang, 2002). It would be interesting to examine the cell survival in the zebrafish by TUNEL after optic nerve injury to address that question. Further inquiry into nog2 and ATF3 would be useful in determining the role of these genes in optic nerve regeneration in zebrafish. Fluorescence *in situ* hybridization studies can be performed to identify the localization of expression within the retina.

Additionally, we observed up-regulation in the injured fish of several genes that play a role in epigenetic modification, specifically CXXC1 and histone deacetylase 1 (Lee and Skalnik, 2005; Yamaguchi *et al.*, 2005). Future studies can focus on the role of

epigenetics in nerve regeneration; particularly whether the differences in chromatin or histone modifications that exist between mammals and zebrafish offer an explanation as to why zebrafish have the ability to spontaneously access and regulate genes necessary for nerve regeneration while mammals do not.

APPENDIX

Table 3. Microarray Results: Genes Showing Increased Expression 3 Hours. At 3 hours there were 50 genes that showed increased expression in the injured fish as compared to sham-operated fish. The table below lists the top 20 genes.

3 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-value**
NM_200931.1	zgc:56065	6.65	0.04
NM_200570.1	selenium binding protein 1	3.35	0.05
NM_200333.1	CXXC finger 1 (PHD domain)	3.09	0.04
AY929292.1	Danio rerio phosphatidylinositol 4-kinase III alpha (pi4kIII alpha)	3.02	0.04
NM_001012262.1	crystallin, gamma S2	2.51	0.02
XM_696168.1	hypothetical protein LOC402822	2.27	0.02
BC076530.1	tumor protein p73-like	2.19	0.02
NM_214773.1	acid phosphatase 5, tartrate resistant	2.06	0.05
NM_001007383.1	zgc:101832	2.05	0.00
AL627263.6	clone RP71-1L9 in linkage group 14 Contains part of a novel gene similar to ATP8B1 (ATPase, Class I, type 8B, member 1), part of a novel gene similar to MCF2 (MCF.2 cell line derived transforming sequence) and two CpG islands	2.05	0.00
NM_200319.1	transmembrane protein 57	1.99	0.02
NM_214716.1	heat shock protein 4, like	1.97	0.03
AF028724.1	zgc:91934	1.96	0.03
XM_682300.1	similar to KIAA0523 protein	1.93	0.04
BX248503.7	clone CH211-232M7 in linkage group 10	1.85	0.04
NM_131147.1	homeo box A11b	1.85	0.05
XM_697090.1	hypothetical protein LOC554904	1.80	0.05
U49412.1	frizzled homolog 2	1.80	0.03
NM_212952.1	ribosomal protein L36	1.77	0.01
NM_212588.1	solute carrier family 20 (phosphate transporter), member 1	1.77	0.02

Table 4. Microarray Results: Genes Showing Increased Expression 24 Hours. At 24 hours there were 112 genes that showed increased expression in the injured fish as compared to sham-operated fish. The table below lists the top 20 genes.

24 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-Value**
NM_200964	activating transcription factor 3	3.60	0.00
NM_001024811	GTP binding protein 1, like	3.09	0.03
NM_130992	noggin 2	2.57	0.05
NM_130971	lunatic fringe homolog	2.31	0.05
NM_001001399	signal sequence receptor, beta	2.23	0.05
AY178796	annexin A2a	2.19	0.00
XM_687162.2	clone CH211-81A5 in linkage group 19	2.16	0.05
BX000434	CH211-2E18	2.12	0.05
NM_201293	S-adenosylhomocysteine hydrolase-like 1	2.11	0.05
NM_001045083.1	clone DKEY-161L11 in linkage group 2	2.06	0.01
XM_001346372.1	nuclear receptor-related 1	2.04	0.04
XM_692347	similar to Bardet-Biedl syndrome 1	2.00	0.03
NM_207060	transmembrane protein 49	1.98	0.02
XM_691572	similar to BRCA1 interacting protein C-terminal helicase 1	1.94	0.02
XM_695077	similar to conserved hypothetical protein	1.93	0.01
NM_205690	retinaldehyde binding protein 1	1.93	0.02
NM_200281	sarcoma amplified sequence	1.93	0.03
BX511080	clone DKEY-237N7 in linkage group 3	1.92	0.01
NM_131105	alpha-tropomyosin	1.92	0.00
AL928556	clone DKEY-63M7	1.91	0.03

Table 5. Microarray Results: Genes Showing Increased Expression 168 Hours. At 168 hours there were 217 genes that showed increased expression in the injured fish as compared to sham-operated fish. The table below lists the top 20 genes.

168 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-Value**
NM_200937.1	inhibitor of growth family, member 3	3.96	0.02
BX640466.9	clone CH211-138A11 in linkage group 2	3.52	0.02
NM_198818.1	tubulin, beta 5	3.50	0.00
XM_683892.1	zgc:103738	3.19	0.00
NM_213062.1	ubiquitin-activating enzyme E1 (A1S9T and BN75 temperature sensitivity complementing)	3.12	0.03
CR356231.12	clone CH211-232N7, complete sequence	2.78	0.01
NM_001002378.1	zgc:92066	2.72	0.00
NM_212617.1	YY1 transcription factor	2.62	0.00
NM_200093.1	ORM1-like 1 (S. cerevisiae)	2.59	0.02
NM_130921.1	nonspecific cytotoxic cell receptor protein 1	2.56	0.01
XM_694574.1	similar to ATP-binding cassette, sub-family A member 1	2.54	0.00
NM_131098.1	apolipoprotein Eb	2.54	0.01
NM_200751.1	zgc:73213	2.54	0.01
CR848747.8	clone DKEYP-77H1 in linkage group 16	2.51	0.00
NM_212758.1	peptidylprolyl isomerase A (cyclophilin A)	2.49	0.00
AY391434.1	ribosomal protein SA	2.49	0.00
NM_001004679.1	zgc:103619	2.45	0.02
NM_001007105.1	apoptotic chromatin condensation inducer 1a	2.42	0.02
NM_212756.1	zgc:111860	2.38	0.00
AY394971.1	tubulin, alpha 8 like 4	2.34	0.00
BX548026.10	clone CH211-193D9	2.28	0.02

Table 6. Microarray Results: Genes Showing Decreased Expression 3 Hours. At 3 hours there were 17 genes that showed decreased expression in the injured fish as compared to sham-operated fish. The table below lists those 17 genes.

3 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-value**
NM_200751.1	zgc:73213	0.44	0.02
NM_200751.1	zgc:73213	0.46	0.01
BX323035.8	clone DKEYP-94H10 in linkage group 2	0.46	0.01
NM_131568.1	transient receptor potential cation channel, subfamily C, member 4 associated protein b	0.47	0.02
NM_213506.1	zgc:63491	0.49	0.03
NM_200090.1	WD repeat domain 75	0.52	0.05
NM_200048.1	arginyl-tRNA synthetase	0.56	0.01
NM_001007063.1	membrane associated guanylate kinase, WW and PDZ domain containing 1	0.58	0.01
NM_205695.1	zgc:77282	0.63	0.04
XM_680501.1	similar to plasma membrane calcium ATPase	0.63	0.02
NM_001017721.1	zgc:112171	0.64	0.05
XM_689534.1	similar to mKIAA0306 protein	0.64	0.05
NM_201471.1	aldehyde dehydrogenase 9 family, member A1 like 1	0.64	0.05
NM_182877.1	solute carrier family 34 (sodium phosphate), member 2b	0.64	0.05
BX649516.8	clone DKEY-51D8 in linkage group 14	0.66	0.03
NM_180965.4	claudin g	0.67	0.04
CR847897.15	clone DKEY-90L8 in linkage group 8	0.67	0.05

Table 7. Microarray Results: Genes Showing Decreased Expression 24 Hours. At 24 hours there were 86 genes that showed decreased expression in the injured fish as compared to sham-operated fish. The table below lists the top 20 genes.

24 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-Value**
NM_200410	zgc:64089	0.41	0.02
XM_686959	similar to CG9590-PA	0.44	0.05
XM_679775	similar to cerebellin 2 precursor	0.47	0.01
NM_131594	beta-catenin-interacting protein	0.47	0.02
NM_199946	male germ cell-associated kinase	0.48	0.05
NM_200711	calbindin 2, (calretinin)	0.48	0.00
NM_213364	proteasome (prosome, macropain) subunit, beta type, 3	0.48	0.00
NM_212866	zgc:77051	0.49	0.00
NM_212809	phosphorylase, glycogen; brain	0.49	0.01
BX004766	jagged 2	0.50	0.03
NM_213000	chimerin (chimaerin) 1	0.50	0.01
AF273890	immunoglobulin heavy variable 1-1	0.52	0.00
CR788254	clone DKEY-82K12 in linkage group 2	0.53	0.00
NM_200910	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	0.54	0.02
NM_213442	serine/arginine repetitive matrix 1	0.54	0.01
NM_131641	paired box gene 6b	0.55	0.03
NM_199926	transcriptional adaptor 3 (NGG1 homolog, yeast)-like	0.55	0.02
NM_001013293	zgc:110753	0.56	0.01
NM_001002299	protein tyrosine phosphatase, receptor type, U	0.56	0.02
NM_001007376	zgc:101877	0.56	0.00

Table 8. Microarray Results: Genes Showing Decreased Expression 168 Hours. At 168 hours there were 191 genes that showed decreased expression in the injured fish as compared to sham-operated fish. The table below lists the top 20 genes.

168 Hours			
Accession	**Description**	**Ratio (I *VS.* S)**	**P-value**
AY050506.1	phosphodiesterase 6G, cGMP-specific, rod, gamma	0.30	0.00
NM_194384.1	aldolase c, fructose-bisphosphate	0.36	0.01
NM_131868.2	guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	0.36	0.02
NM_001007160.1	phosphodiesterase 6A, cGMP-specific, rod, alpha	0.36	0.00
NM_212609.1	guanine nucleotide binding protein (G protein), beta polypeptide 1	0.36	0.00
NM_212755.1	wu:fb12g05	0.36	0.00
NM_131838.2	ATPase, Na+/K+ transporting, beta 2b polypeptide	0.37	0.01
BC091819.1	hypothetical protein LOC553339	0.37	0.03
NM_213202.1	guanine nucleotide binding protein (G protein), beta polypeptide 3	0.37	0.02
BX511094.6	clone CH211-137G12 in linkage group 19	0.39	0.00
XM_686878.1	similar to SI:dZ75P05.1 (novel protein similar to human spindle pole body protein (SPC98P, GCP3))	0.39	0.00
NM_213149.1	FK506 binding protein 5	0.39	0.00
BC076174.1	phosducin 2	0.39	0.00
BC076120.1	opsin 1 (cone pigments), long-wave-sensitive, 2	0.39	0.01
NM_200784.1	coiled-coil-helix-coiled-coil-helix domain containing 2	0.40	0.00
NM_200719.1	ADP-ribosylation factor-like 3, like 2	0.40	0.00
NM_212609.1	guanine nucleotide binding protein (G protein), beta polypeptide 1	0.41	0.00
NM_152955.1	dachshund a	0.41	0.00
NM_001030061.1	transient receptor potential cation channel, subfamily M, member 7	0.41	0.03
BC060894.1	opsin 1 (cone pigments), short-wave-sensitive 1	0.42	0.00

Table 9. Gene Ontology: Cell Proliferation. Of the genes differentially expressed at least 1.5-fold or more, 16 were representatives of the cell proliferation category. The ratios at each time point are reported as injury/sham.

Cell Proliferation	GenBank Accession	3 Hours	24 Hours	168 Hours
activating transcription factor 3	NM_200964	1.04	3.60	2.12
annexin A2a	AY178796	0.96	2.19	2.26
baculoviral IAP repeat-containing 5a	AY057057	0.95	1.17	2.12
CCAAT/enhancer binding protein (C/EBP), alpha	NM_131885	0.92	1.57	1.67
Dicer1, Dcr-1 homolog (Drosophila)	AY386319	0.91	1.63	0.76
E2F transcription factor 4	NM_213432	1.51	1.43	0.85
eukaryotic translation initiation factor 3, subunit 3 (gamma)	NM_001003763	0.97	1.22	1.48
fibroblast growth factor 3	NM_131291	1.03	0.66	0.93
guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	NM_131868	0.90	0.69	0.36
guanine nucleotide binding protein (G protein), beta polypeptide 1	NM_212609	0.87	0.63	0.36
histone deacetylase 9	NM_200816	1.40	1.25	1.85
neurogenic differentiation	NM_130978	0.89	1.21	0.63
similar to Ubiquitin carboxyl-terminal hydrolase 13 (Ubiquitin thiolesterase 13) (Ubiquitin-specific processing protease 13) (Deubiquitinating enzyme 13) (Isopeptidase T-3) (ISOT-3)	XM_681175	1.12	1.70	0.96
SWI *VS.* SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	NM_181603	1.01	0.75	0.66
TNF receptor-associated factor 6	NM_199821	0.95	0.79	0.60
tumor protein p63	BC076530	2.19	1.49	1.53

Table 10. Gene Ontology: Axon Extension and Guidance. Of the genes differentially expressed at least 1.5-fold or more, 5 were related to axon extension and guidance. The ratios at each time point are reported as injury/sham.

Axon Extension and Guidance	GenBank Accession	3 Hours	24 Hours	168 Hours
cadherin 2, neuronal	NM_131081	1.17	1.33	1.48
Ephrin a4b (epha4b)	NM_153658	0.87	0.65	0.71
fasciculation and elongation protein zeta 1 (zygin I)	NM_213396	0.85	0.62	1.01
neural adhesion molecule L1.2	NM_131361	1.14	1.25	1.81
tubulin, alpha 8 like 3	NM_001003558	1.26	1.70	2.12
tubulin, beta 5	NM_198818	1.15	1.01	3.50
tubulin, alpha 8 like 4	NM_200185	1.00	1.50	2.05

Table 11. Gene Ontology: Embryonic Development. Of the genes differentially expressed at least 1.5-fold or more, 8 were representatives of the embryonic development category. The ratios at each time point are reported as injury/sham.

Embryonic Development	GenBank Accession	3 Hours	24 Hours	168 Hours
abl-interactor 1	NM_200738	1.72	0.83	1.27
apolipoprotein Eb	NM_131098	1.46	1.07	2.54
beta-catenin-interacting protein	NM_131594	0.93	0.47	0.81
frizzled 2	U49412	1.80	0.98	0.88
homeo box A11b	NM_131147	1.85	1.08	1.06
jun B proto-oncogene	NM_213556	1.20	1.28	1.78
neurogenic differentiation	NM_130978	0.89	1.21	0.63
noggin 2	NM_130992	0.93	2.57	0.79
tumor protein p63	BC076530	2.19	1.49	1.53

Table 12. Gene Ontology: Immune System Process. Of the genes differentially expressed at least 1.5-fold or more, 5 were associated with immune system process. The ratios at each time point are reported as injury/sham.

Immune System Process	Accession	3 Hours	24 Hours	168 Hours
CCAAT/enhancer binding protein (C/EBP), alpha	NM_131885	0.92	1.57	1.67
CXXC finger 1 (PHD domain)	NM_200333	3.09	1.03	1.13
Invariant chain-like protein 1	NM_131590	1.19	1.63	1.05
LIM domain only 2	NM_131111	0.78	1.02	0.67
Zgc:91843	NM_001003997	0.91	0.66	0.87

Table 13. Gene Ontology: Neuron Differentiation. Of the genes differentially expressed at least 1.5-fold or more, 9 were representatives of the neuron differentiation category. The ratios at each time point are reported as injury/sham.

Neuron Differentiation	GenBank Accession	3 Hours	24 Hours	168 Hours
CCAAT/enhancer binding protein (C/EBP), alpha	NM_131885	0.92	1.57	1.67
epha4b	NM_153658	0.87	0.65	0.71
neural adhesion molecule L1.2	NM_131361	1.14	1.25	1.81
neurogenic differentiation	NM_130978	0.89	1.21	0.63
roundabout homolog 3	AF304131	1.00	0.86	0.65
similar to Bardet-Biedl syndrome 1	XM_692347	0.99	2.00	1.13
similar to Krueppel-like factor 15	XM_688679	1.36	0.62	1.36
similar to plexin C1	XM_685667	0.86	0.62	0.70
similar to SLIT and NTRK-like family, member 4	XM_681309	0.83	0.76	0.42

Table 14. Gene Ontology: Phototransduction. Of the genes differentially expressed at least 1.5-fold or more, 16 were associated with photoreception. All but retinaldehyde binding protein are significantly down regulated at 168 hours post injury. The ratios at each time point are reported as injury/sham**.**

Phototransduction	**GenBank Accession**	**3 Hours**	**24 Hours**	**168 Hours**
ATP-binding cassette, sub-family E (OABP), member 1	AY391404	0.96	0.82	0.65
crystallin, gamma S2	NM_001012262	2.51	0.92	0.70
guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	NM_131868	0.90	0.69	0.36
guanine nucleotide binding protein (G protein), beta polypeptide 1	NM_212609	0.87	0.63	0.36
guanylate cyclase activator 1B	NM_131871	0.96	1.10	0.56
opsin 1 (cone pigments), long-wave-sensitive, 2	BC076120	1.00	0.94	0.39
opsin 1 (cone pigments), short-wave-sensitive 1	BC060894	1.00	0.65	0.42
opsin 1 (cone pigments), short-wave-sensitive 2	NM_131192	1.07	0.97	0.50
phosducin 2	BC076174	0.83	0.64	0.39
phosphodiesterase 6A, cGMP-specific, rod, alpha	NM_001007160	0.96	0.63	0.36
phosphodiesterase 6G, cGMP-specific, rod, gamma	AY050506	0.95	0.67	0.30
retinal degradation slow 4	NM_131567	0.84	0.88	0.46
retinal homeobox gene 2	NM_131226	1.04	0.86	0.56
retinaldehyde binding protein 1	NM_205690	0.68	1.93	1.19
retinol binding protein 4, like	NM_199965	0.99	1.02	0.63
Rhodopsin	BC063938	0.88	0.89	0.48

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VITA

Katherine Elizabeth Saul (Katie) was born to Mary and Gary Saul in 1981. She was raised in Austin, Texas graduating from James Bowie High School. Katie eagerly entered Texas State University as a music major in 1999 with the dream of playing cello alongside Yo-Yo Ma. Her focus shifted to biology in 2002 when her brother was partially paralyzed from a spinal cord injury. She graduated from Texas State in 2006 with a B.S. in Biology. Katie's thesis research has focused on nerve regeneration with the hope of contributing towards the understanding of mechanisms behind reestablishing functional connections after injury. She is an active member of the community, volunteering her time as a trainer and equipment manager for a local wheelchair rugby team.

Permanent Address: 4013 Eskew Drive

Austin, TX 78749

This thesis was typed by Katherine E Saul.