AN INVESTIGATION OF THE GENETIC BASIS FOR SPINAL CORD REGENERATION OF LARVAL ZEBRAFISH

by

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Recent studies by the Center for Disease Control (CDC) show that central nervous system injury represent 30% of all injury related deaths in the United States, and that rates of traumatic brain injury-related emergency room visits have risen by nearly 50% over the last six years alone. These statistics serve to showcase human's extremely poor ability to recover from central nervous system injury or disease and represent the limitations to our current therapeutic techniques for the same. In comparison, zebrafish (*Danio rerio*) possess vast regenerative ability not only of the central nervous systems but of many of their organs, and are able to recover rapidly and effectively from injuries that would otherwise lead to permanent disability in humans. In the following thesis, the spinal cord of zebrafish was used as a model for studying the genetic basis of central nervous system regeneration. Spinal cord regeneration requires a myriad of different skills from cells including the ability to recognize that an injury has occurred, a shift from normal growth and development to regenerative healing, and the ability to grow in the correct direction to bridge the injury and eventually heal the organ in question. Although some genetic patterning and expression of the developing nervous system has been previously characterized, it is largely unknown if these genetic expression patterns are present utilized during spinal cord regeneration after initial central nervous system (CNS) development. In this thesis we develop an experimental pipeline for evaluating candidate genes necessary for spinal cord regeneration and carried a gene of interest, vimentin (vim), through this pipeline and although upregulation was seen through genetic screening, preliminary immunohistochemistry

analysis through suggested that there was not a correlation of vim upregulation with spinal cord regeneration. Although the finding represented a negative result the project generated an exciting list of further candidate genes for further study. Furthermore although vim had been implicated in other forms of zebrafish regeneration, there was an absence of established literature characterizing the gene's full body expression past three days post fertilization (dpf) and this thesis served to contribute a greater understanding of vim's expression during development. We anticipate that this project will set the basis for future genetic screens into the genetic basis of spinal cord regeneration and further our understanding of how the cells of the zebrafish spinal cord transition from development to regeneration.

Acknowledgements

I would like to thank Professor Miller and Dr. Farnsworth for their mentorship throughout this process, and for helping me to fully explore both the subject of neurodevelopment and the process of research itself. Furthermore I would like to thank Rachel Lukowicz, Abbey Lasseigne, and Jen Michel for their time and support in educating me on different protocols and technical concepts of lab work, and for encouraging me in my research experience. I would like to acknowledge that the depth of my research could not have been possible were it not for the generous support of the Summer Program for Undergraduate Research, which sponsored my full time contribution of research over the course of a summer.

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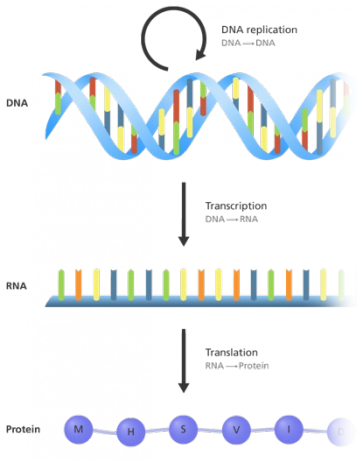
INTRODUCTION

We live in a world that is unimaginably biologically complex. Our earth teems and thrives with life, as simple as a single-celled bacterium and as complex as a human being presenting scientific research. The level of diversity between the biology of different creatures is nearly unfathomable, however the language of this biology is beautifully simple, composed of only 4 letters: A, T, C, and G. These letters make up the blueprints of every living creature and plant on our planet. Over millennia, the mechanisms for altering and reading these blueprints have become increasingly complex and specialized, allowing to differential gene expression as unique as the creatures they code for. So that is the DNA of the world, but what about the DNA of you? How do such simple blueprints produce a living creature? Furthermore if DNA is largely stagnant, how is it that you can react to the world around you instead of simply being a large and complex human statue? Specifically to this project, how does the reading of this genetic blueprint change in response to an injury to allow for cells to alter their functions and structures to recover from inflicted wounds? If cells communicate in combinations of As, Ts, Cs and Gs how is it that a cell can recognize when an injury has occurred, alter its own fate to adjust for this disruption, and coordinate with its cellular neighbors to not only grow but to grow in the correct direction to bridge a gap as is the case for spinal cord regeneration?

The first crucial concept is that DNA is generally identical in every cell of your body. The DNA of the cells of your hair is the same as the DNA of your eye which is the same as the DNA of the cells

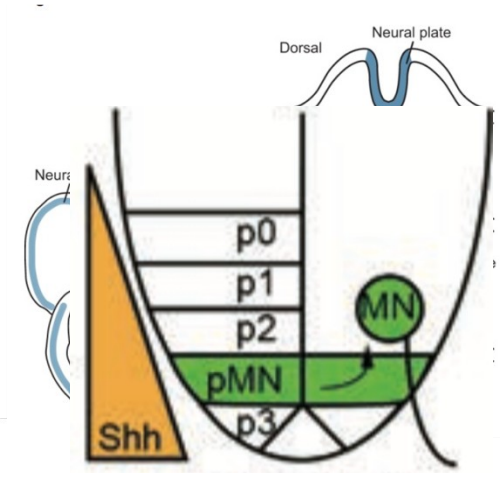


of your skin. However, since every cell contains the same set of genetic instructions, other mechanisms must be utilized to account for the vast diversity of cellular types and to adjust for the plasticity of their functions throughout their lifespan. Overall, the major mechanism underlying differential genetic expression is known as the Central Dogma of Molecular Biology. The Central Dogma describes the multi-step process of the transfer of genetic information from DNA to RNA, and ultimately, proteins. Depending on what is needed in each cell, different portions of DNA in the nucleus of are translated into another form of genetic information called RNA. RNA then acts as a messenger, carrying genetic instructions out of the nucleus and into the cytoplasm of cells, where it is then translated into proteins. Proteins act as the functional units of genetic material, carrying out tasks and building structures that fit the identity of the cell in which they were made. The capability of this process to produce such a vast array of expressions patterns lies in the multitude of regulatory mechanisms that alter transcription and translation to ultimately affect protein expression. DNA can be inactivated by being wound into histones, thereby eliminating the possibility that RNA is translated from it. Transcription factors, or transient proteins that promote production of RNA from DNA, can also alter DNA expression, and can be the result of environmental factors outside of the cell itself. Furthermore, not all RNA is transported out of the nucleus for transcription. Instead segments of RNA, called introns, are cut out and discarded while other segments called exons are then stitched together and exported into the cytoplasm, where the final mRNA strand is transcribed into proteins by ribosomes. Not even proteins represent the end to this regulation. Enzymes can further

cut, reshape, remodel, and redistribute assembled proteins to further differentiate their functions.

Though all cells contain identical genetic instructions, regulatory mechanisms enable the activation of necessary genetic components. Once cells reach an age of maturity and obtain equilibrium, this does not render transcription a static process. When this homeostasis is disrupted (as in the case of injury, resource withdrawal, or immune attack) regulation can alter phenotype through changes in gene expression. Hypothetically, this is the case in spinal cord regeneration. Although the overarching mechanical processes of spinal cord regrowth have been characterized, the underlying genetic mechanisms remain a mystery.

Although the focus of this research is regeneration and not development, a thorough understanding of spinal cord genesis forms an excellent foundation for predicting the mechanisms of spinal cord reformation. Early in zebrafish development, the neural plate is formed. This plate then folds and fuses to form the neural tube, which will eventually develop and differentiate into the central nervous system. Once formed, this neural tube subdivides into discrete domains which give rise to specific neural and glial cell types. These domains house stem cells, which give rise to multiple different cell subtypes. Their activity is controlled by the concentration gradient of various morphogens which diffuse ventrally



through the neural tube and act in a concentration dependent manner to assign identity to progenitor domain. The pMN domain, when activated extracellular ligands such as sonic hedgehog (shown above) begins to express the gene olig2, and further gives rise to motor neurons and oligodendrocytes. Motor neurons are cells which carry information from the spinal cord into muscles and allow for movement, thereby representing a crucially important bridge between the central and peripheral nervous system. This importance in animal movement, as well as their specific olig2 expression and rapid regenerative ability make them prime candidates for further investigation into the mechanisms underlying spinal cord regeneration.

In order to identify candidate genes which are aiding in motor neuron regeneration the genetic expression of regenerating olig2 expressing motor neurons had to be somehow characterized. To do this motor neurons were lesioned and their RNA expression was recorded 48 hours into regeneration. This data implicated a gene called vim as being highly upregulated in regenerating motor neurons. Vim codes for a protein called vimentin, an intermediate filament protein located throughout the nervous system early in development including the spinal cord, midbrain, and axis. Previous research by Cheng et al. used various genetic knockouts to show that vimentin protein is necessary for keratinocyte differentiation and fibroblast proliferation during wound repair. This in itself suggests that vimentin protein is not only necessary to structurally allow for cellular regeneration but that it is a part of a larger process with which other filament proteins build on. Furthermore, research performed by Lebert et al. identifies vimentin as necessary for other forms of zebrafish regeneration including caudal fin healing following heat ablation and suggest that its transcription could be activated by reactive

oxygen species (ROS) generated during wound repair. Interestingly, although the authors of this paper predicted that vimentin would aid in wound repair through the migration of previous vim^+ cells to the wound site the actual results shows novel vim activation in previously vim^- cells which then extend their internal filament framework to regrow the caudal fin. These papers combine illustrate a pathway of vimentin controlled regeneration processes in zebrafish. The majority of the body of research done to characterize vimentin has taken place in larval fish 3 DPF or younger, demonstrating a current gap in knowledge. This paper serves to further our current understanding of the expression pattern of vimentin in 5 and 7 DPF larval zebrafish.

Zebrafish were chosen as research subjects for a number of reasons. To begin with, they generally make excellent research subjects because they reproduce quickly and easily, allowing for research to be done with high N values. Furthermore, zebrafish embryos develop in isolated and transparent eggs instead of inside their mother like mammals. This makes studying embryological development and intervening early in development easier. Research into zebrafish genetics is also highly applicable to humans. Zebrafish share 70% of DNA with humans, and 85% of disease-causing DNA, making them excellent research subjects for studying the genetic basis of disease in humans. Finally zebrafish are excellent models for regenerative research because they can regenerate many of their organs. Although this thesis will focus on spinal cord regeneration, zebrafish are also capable of regenerating their hearts, caudal fins, and retinas.

EXISTING LITERATURE

Though the genetic basis and mechanisms underlying spinal cord regeneration in larval zebrafish is not fully understood, the standard development of this species has been extensively investigated and characterized. In their paper “From Cells to Circuits: Development of the Zebrafish Spinal Cord.” Doctor’s Lewis and Eisen outline the development of the zebrafish spinal cord from the single cell stage to the developed animal. Specifically, they describe how spinal cord cell specification originates from progenitor domains that become mature following the closing of the neural crest into the neural tube. These progenitor domains are determined by diffuse protein concentrations throughout the dorsal axis of the neural tube, and each act as the differentiation sites to various neuronal cells. This project focuses specifically on the pMN domain which is located slightly ventrally in the neural tube, and is differentiated by Olig2 expression leading to the determination of motor neurons and oligodendrocytes. Because all mature cells that emerge from the pMN domain retain this Olig2 expression, Olig2 represents an excellent marker gene to track origin of these cell types (Lewis, Eisen)

Furthermore, genes have previously been implicated in driving regeneration in specific cell types, lending credence to our hypothesus that we can predict specific genes involved in regeneration. The paper “Genome Wide Expression Profiling during Spinal Cord Regeneration Identifies Comprehensive Cellular Responses in Zebrafish.” establishes precedent that Zebrafish cell differentiation results from characterized and coordinated gene expression throughout the development of the animal and that wide-scale suppression of gene expression hinders the animal’s ability to regenerate organs

such as the spinal cord. This paper establishes that genetic expression is crucial in regeneration and provided methods for large scale genetic screens and quantification of regeneration.

RESULTS

Gross Regeneration of the spinal cord can be observed 48 hours post lesioning

I first sought to characterize gross spinal cord healing in a wild type (WT) zebrafish. 5 days post-fertilization (DPF) WT larvae were lesioned using an insect needle and imaged immediately at 0 days post lesioning and 2 days post lesioning (DPL). Bright field imaging of 2 DPL larvae demonstrated significant repair of the gross anatomy of the fish spine following lesioning (Fig. 1).

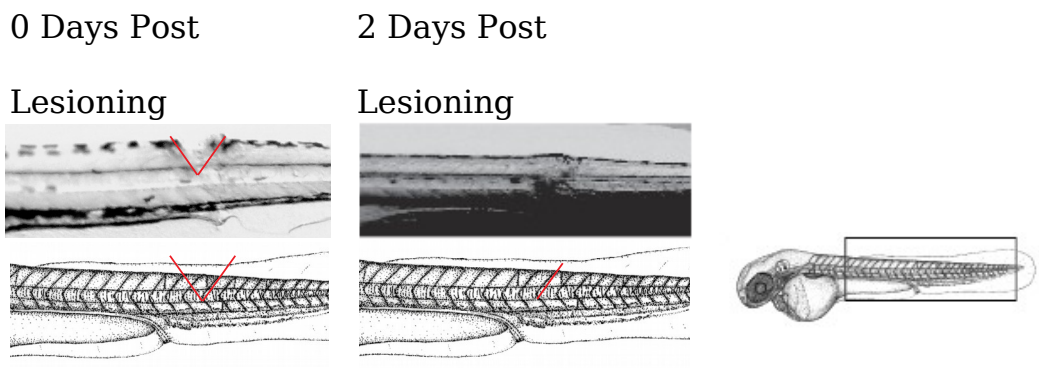


Fig 1: Brightfield imaging of gross regeneration.

Brightfield images of zebrafish 0 and 48 hours post lesioning taken at 10x zoom showed significant healing of the visible tissue along the tail of the zebrafish following mechanical lesioning.

This finding confirmed previous research as to the rapidity of zebrafish spinal cord healing, and outlined the time frame with which further experiments were carried out. It also established a consistent and easily repeatable assay for initiating spinal cord regeneration which was used throughout future experiments. Once it was established that gross spinal cord healing occurs after 48 hours the investigation was narrowed to the cells of interest, motor neurons.

Olig2 expression shows significant regeneration of motor neurons following lesioning of the spinal cord

Before the genetic basis for motor neuron regeneration could be more fully investigated the process of WT regeneration needed to be characterized. In order to do this Olig2:GFP transgenic larvae were lesioned at 5 days post fertilization and their spinal cords imaged using immunohistochemistry. Expression of Olig2 was monitored through visualization of fluorescent antibodies using a confocal microscope. Unlesioned larvae demonstrate extensive branching of motor neurons from the cell bodies at the spinal cord and into the adjacent musculature as well as consistent expression of Olig2 throughout the cell (Fig. 2a). Immediately following injury lesioned larvae demonstrate complete disruption of this neural network and branching at the site of lesioning (Fig. 2b). 2 DPL larvae showed bridging of motor neurons across the lesion site and an apparent increase in olig2 expressing cells at the lesion site itself. (Fig. 2c and Fig. 3).

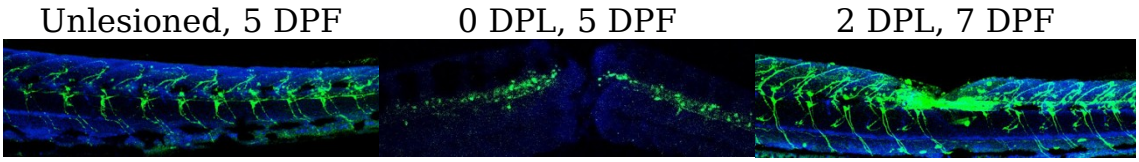


Fig 2a: Confocal imaging of motor neurons throughout the tail of Olig2:GFP transgenic fish stained with GFP and GFAP antibody

A significant increase (P = 3.838 x 10-5) in Olig2 expression was observed in lesioned spinal cords compared to non-lesioned larvae. Furthermore, the observed increase in Olig2 expression was not detectable in more posterior and anterior regions of the spinal cord which were not subject to injury, suggesting that this upregulation of

olig2 expression is specific to regenerating cells and not a process occurring throughout the entire animal.

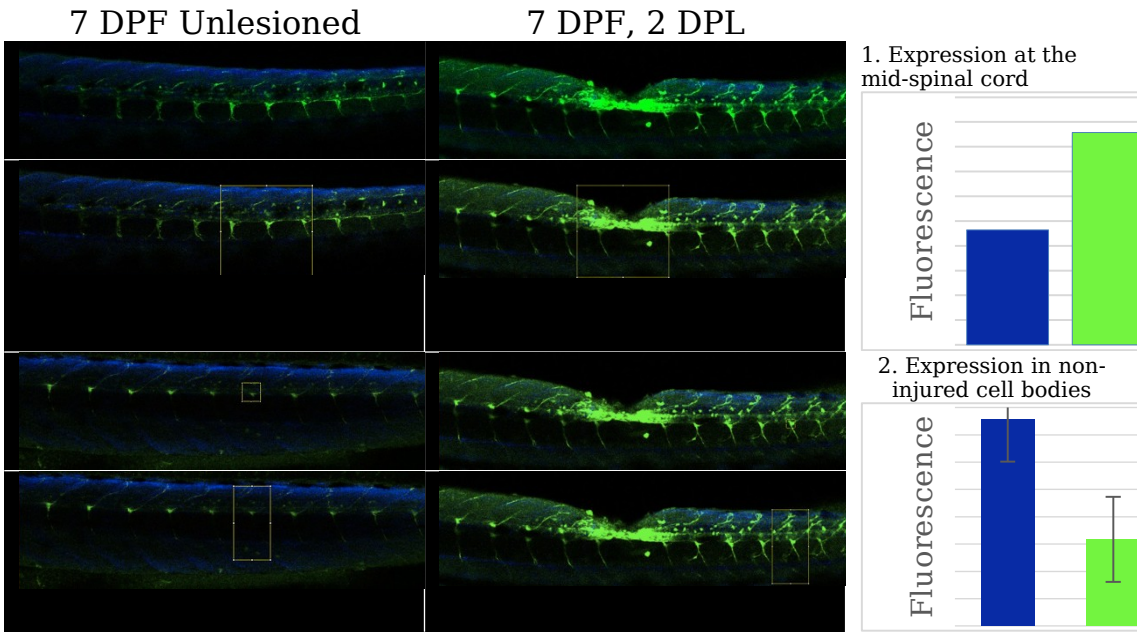


Fig 3: Quantifying motor neuron regeneration

Area of quantified fluorescence is shown above in yellow squares. Area of measured fluorescence around the lesion: 200x200. Area of measured fluorescence of cell body: 20x20. Area of measured fluorescence of tissue surrounding and including an individual cell body: 80x200. Quantification of fluorescence shown at the right with the blue bar representing WT GFP expression and green bar representing Lesioned GFP expression. The zebrafish were lesioned at the mid-spinal cord, using the end of the yolk stack extension as a reference point.

Cells within the regenerative site are actively dividing

Cells within the lesion site were evaluated for mitotic identity through the use of EdU staining. EdU is a chemical which incorporates into the DNA of cells undergoing mitosis, and is therefore an excellent marker for cellular division. This assay was used to confirm if lesioned spinal cord neurons were simply injured cells lengthening over

the lesion site, or new cells that were dividing off and being “reborn” to heal this injury. Confocal imaging of the lesion site 2 days after regeneration showed a visible increase in EdU expression at the lesion site, suggesting that the site of spinal cord regeneration contains mitotic cells (Fig 4).

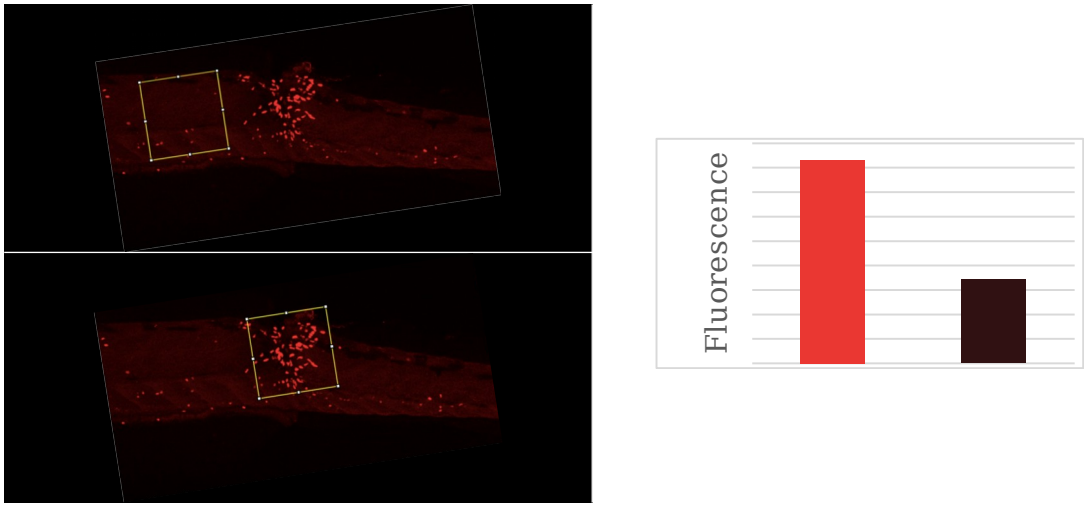


Fig 4: EdU expression shows the mitotic nature of regenerative cells

This image shows EdU expression is increased at the lesion site when compared to the rest of the neural tissue of the animal. (100x100 pixel area measured). Quantification shown in the graph on the right, with the red bar representing EdU fluorescence at the lesion site and the dark bar representing EdU expression outside of the lesion site. Areas of measured fluorescence are marked with yellow squares

Single Cell RNA sequencing showed elevation of the novel gene vim in regenerating motor neurons

Once characterization of motor neuron regeneration was established a genetic screen was performed by Dr. Farnsworth on the regenerating cells to identify candidate genes involved in the regenerative process. Single cell RNA sequencing (scRNA seq) was used to characterize the gene expression of the entire lesion site of olig2:GFP

positive fish. In the figure below each dot represents a cell in which its entire transcriptome is known. Clusters (groups of cells which in the below image are similarly colored and numbered) were then formed through a computer algorithm by ranking the similarity of gene expression. It is worth assuming logically that clusters of cells with extremely similar gene expression likely represented groups of similar cells. The use of marker genes allowed us to identify many of these clusters. A partial annotation of the plot is shown below.

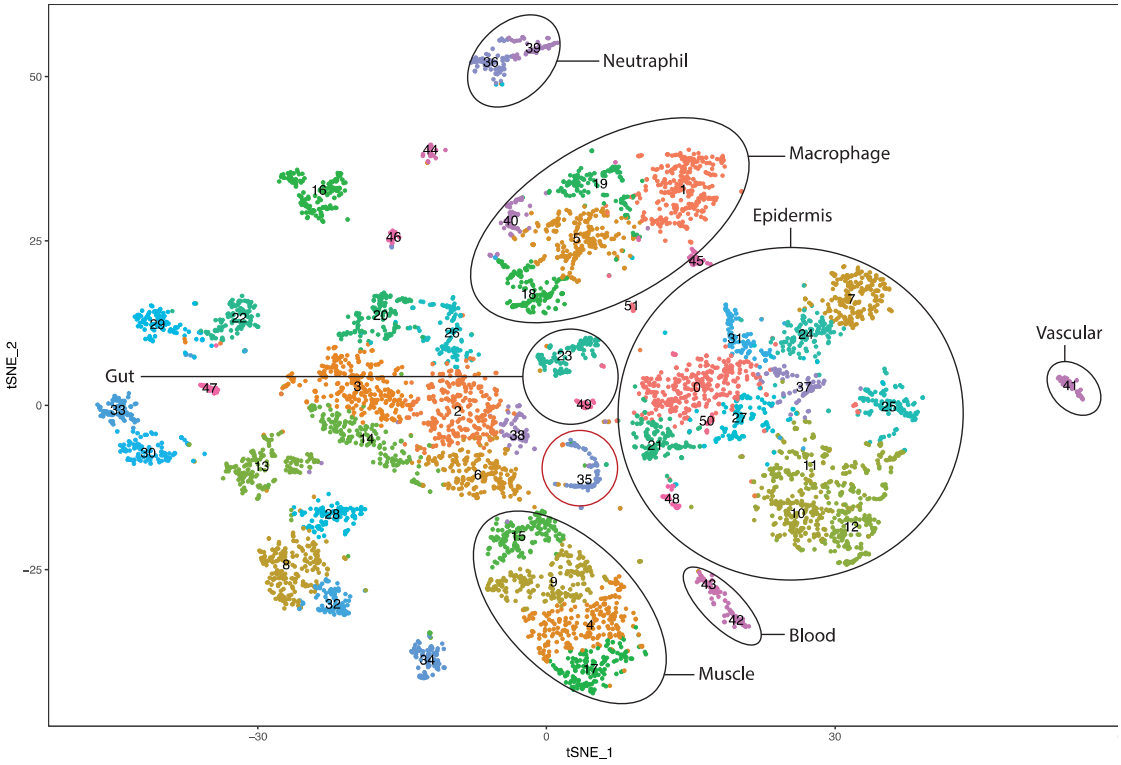


Fig 6: tSNE chart illustrate the transcriptome of cells of regenerative tissue

A cross section of 50 lesioned zebrafish was taken and the cells were dissociated and suspended in solution before being sequenced. A partial annotation of the chart has been performed through the use of marker genes. Cells expressing neuronal marker genes as well as olig2 and GFP were identified in cluster 35 which is circled in red above. For further explanation as to methods see methods section.

Specific motor neuron marker genes included snap25a and elavI3, and it was these genes that were used to identify neurons in our cell sample. The expression of these genes was primarily found in a small number of cells located in cluster 35, and further investigation showed co-expression of olig2 and GFP in a number of these cells, further identifying them as motor neurons.

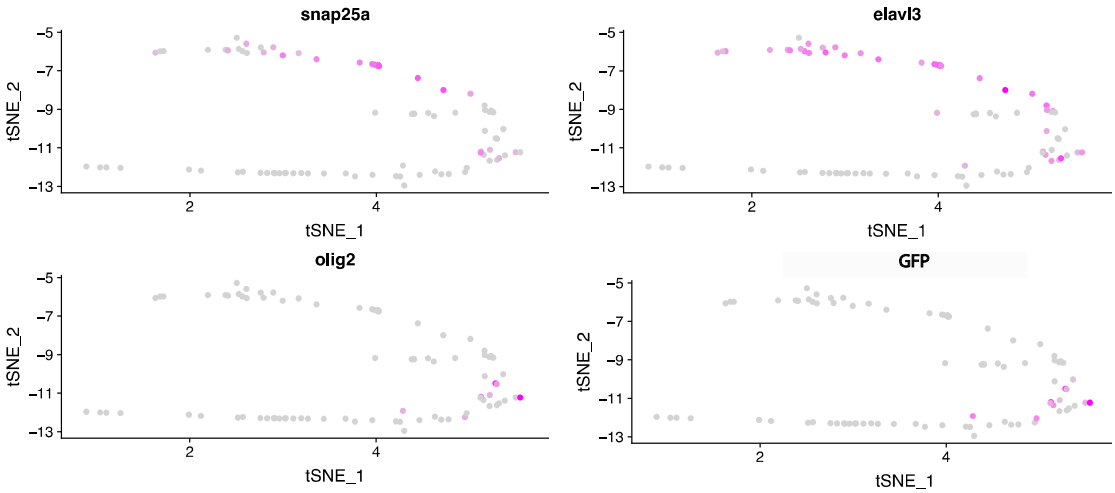


Fig 7: Motor neurons of interest are present in the final scRNA data

Co-expression of marker genes for neurons snap25a and elavI3 with target genes olig2 and GFP.

Upon identification of motor neurons, differential gene expression patterns were observed in a variety of neuronal-specific genes. The extensive list of genes was ordered by specificity of expression (measured by the ratio of expression in our cluster of interest vs. all other clusters). A sample of this data is shown in Table 1 below

Gene	p_val	avg_logFC	pct.1	pct.2	p_val_adj	pct.1-pct.2
vim	3.58E-238	1.97590487	0.716	0.029	7.75E-234	0.687
tuba1a	2.88E-	1.192919	0.531	0.013	6.24E-	0.518

	249	05			245	
rtn1a	5.74E-71	1.19215563	0.58	0.071	1.24E-66	0.509
stmn1b	2.37E-202	1.7220071	0.506	0.016	5.14E-198	0.49

Table 1: Comparative genetic expression of olig2 expressing motor neurons

pct.1-pct.2 shows the specificity of the expression of the genes. The higher the number the more specifically they are expressed in our cells of interest. Vimentin was the most specifically expressed gene in our cells of interest. Pct.1 is defined as the fraction of cells in the cluster of interest which express the gene. Pct.2 is defined as the fraction of all sampled cell which expressed the gene. Avg_logFH is shows the amount of upregulation in the cells

By focusing on genes that were specifically expressed in our cells of interest and investigating previous research into the possible regenerative implications of the different genes a short list of genes was generated for further investigation. The gene vim was the most highly and specifically expressed protein in olig2 positive cells in our sample (Table 1) and therefor represented a promising gene to direct motor neuron regeneration. The co-expression of vimentin in our cells of interest is shown below. Based upon the high occurrence of vimentin expression in our cells of interest, the lack of vimentin expression outside of neuronal cells, and its novel nature, vimentin expression was investigated further.

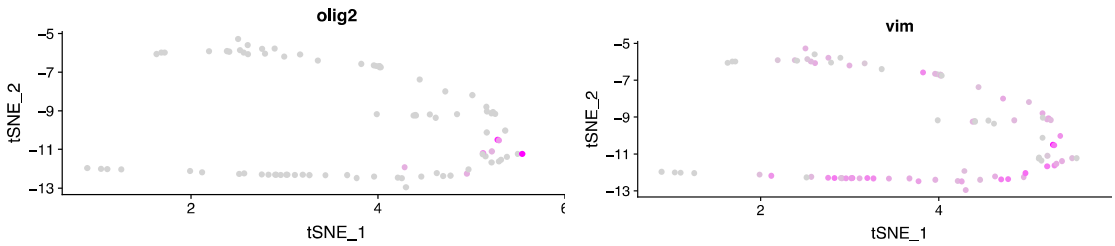
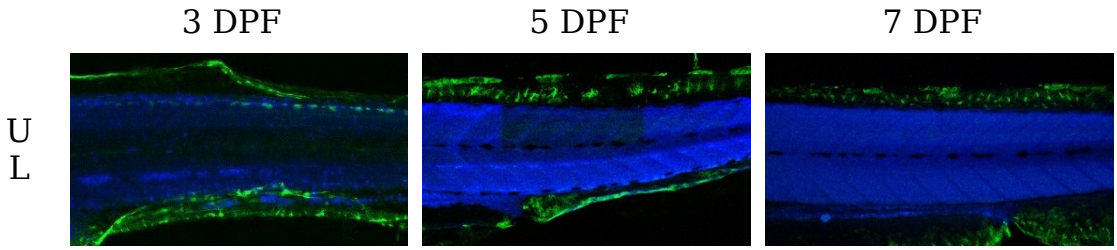


Fig 8: Olig2 and Vim expression

Olig2 shows some co-expression with the novel gene vimentin. Purple color indicates gene expression, while grey indication no detected expression.

Confocal imaging showed no change in vimentin protein presence in regenerating motor neurons

Next, the expression of vimentin protein in the spinal cord was characterized. Previous characterization of vimentin demonstrated presence in the spinal cords of 3 DPF larvae, as well as a characteristic branching of vimentin filament proteins in the fins of the fish. Confocal imaging of zebrafish at 3 DPF was consistent with previous literature, in that there was vimentin presence in the spinal cord and in the fins. Transgenic vim:GFP (reference, acknowledgement) fish were lesioned at 5 DPF and allowed to regenerate for 2 days before being taken down at 7 DPF. Characterization of vimentin presence in 5 and 7 DPF fish showed no vimentin presence in WT, and this result was not altered by the process of regeneration following a lesioning event. Spinal cord expression could be seen in 3 DPF fish, which confirms previous research and represented a positive control for the experiment.



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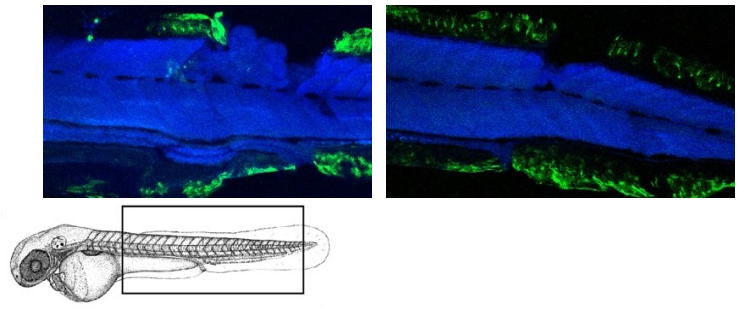


Fig 9: Characterization of vimentin expression

Vim:GFP transgenic fish 5-7 DPF were stained with GFP and RMO44 antibodies. Spinal cord expression can be seen in 3 DPF fish, as well as in the fins of fish at every age point, confirming the success of the stain.

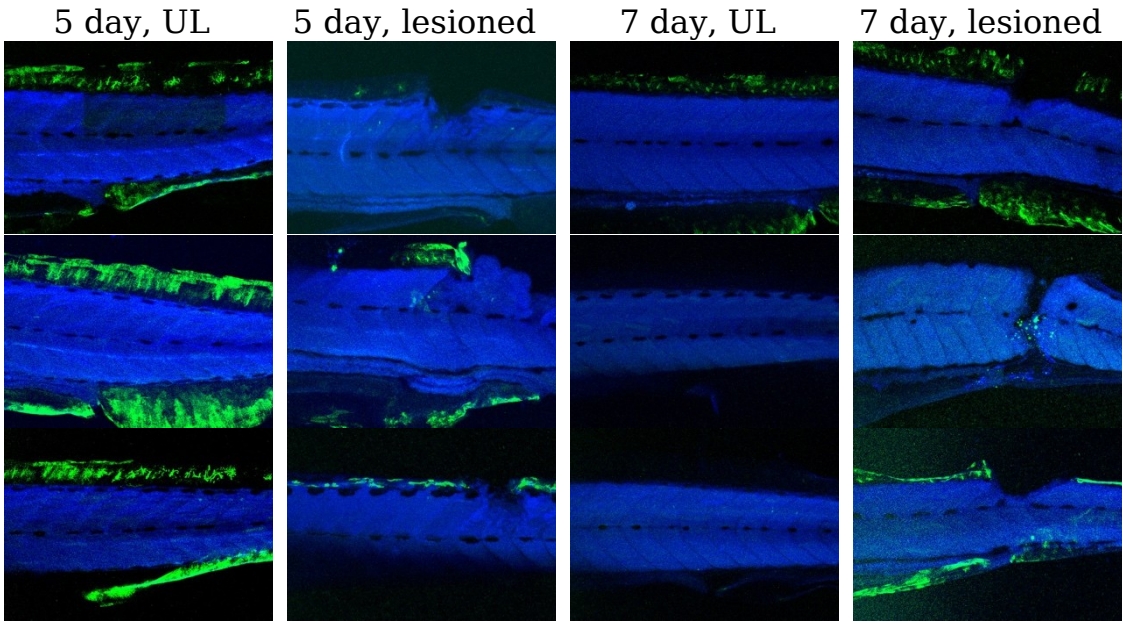


Fig 10: Collated vim expression data

Collated immunohistochemistry stains of vimetin expression in WT vs. regenerative tissue showed no change in vim expression in regenerating spinal cords.

DISCUSSION

Understanding the genetic mechanisms which allow cells to transition from WT development to regeneration holds immense potential for our understanding of wound repair, spinal cord injury, and aging. Although higher order animals such as humans have little regenerative ability a deeper understanding of the processes that allow for regeneration in other species could hold the key to therapeutic treatments for nervous system injury or diseases that currently have no cure. In this thesis an assay was designed to lesion the spinal cord of larval zebrafish and WT motor neuron regeneration was characterized through the use of immunohistochemistry. Following this a large scale genetic screen was performed on regenerating tissue, producing a list of promising candidate genes for spinal cord regeneration. Vim was chosen as a gene of interest due to its specific expression in regenerating cells and its implication in other forms of regeneration in zebrafish, however further investigation did not show upregulation of vim expression with spinal cord regeneration through antibody staining.

Olig2:transgenic fish were raised and a protocol was designed to initiate spinal cord lesioning at 5 days post fertilization. Significant regeneration of motor neurons could be seen bridging the lesion site 48 hours post fertilization as measured by quantification of immunofluorescence. Furthermore this increase in fluorescence was specific to the lesion site and was not seen in either the anterior or posterior direction.

Single cell RNA sequencing of the regenerating tissue allowed for a comprehensive analysis of the genetic expression of healing cells. Multiple cell types were identified in this pipeline including vascular cells, muscle cells, and immune cells.

Neurons were identified with the marker genes elavI3 as well as snap25a and motor neurons were further specified through the identification of olig2 and GFP positive cells. T While overarching evaluations in changes of gene expression (such as single cell RNA sequencing) are an effective way to make predictions about candidate genes that allow for regeneration, this method does not provide enough information to fully make a statement about specific genes. Instead it was used as a starting point for identifying genes of interest which were then further investigated through scholastic research to determine if they are worth future evaluation. RNA expression data of these cells showed specific high levels of expression of the gene vim which encodes for an intermediate filament protein called vimentin.

Characterization of vim expression was achieved through the use of a vim:GFP transgenic line. Immunohistochemistry showed characteristic branching of vimentin filaments in the fins of zebrafish at all ages imaged as well as spinal cord presence at 3 DPF which disappeared at 5 and 7 DPF. Although we hypothesized based on the scRNA data that regeneration would cause an upregulation of vim represented by higher fluorescence within the regenerating spinal cord no such increase was seen.

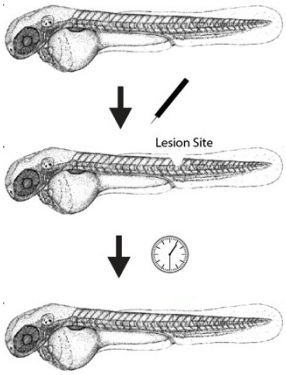
Although preliminary analysis of vimentin's role in spinal cord regeneration showed that it was not found to be upregulated as had been previously predicted, this result still represents an exciting step forward into research on spinal cord regeneration. Through this project regeneration was successfully initiated and the cellular mechanisms governing this process were further characterized through immunohistochemistry stains of Olig2 and Vim genes. Data collected from the single cell RNA sequencing of regenerating spinal cord tissue implicated numerous genes in

motor neuron regeneration, and a pipeline was established to screen these genes for changes in expression during regeneration. This pipeline was used successfully to show that vim showed a lack of increased expression with regeneration, and can be used in future studies to explore other gene's involvement in the regenerative process.

METHODS

Lesioning

Fish were first anesthetized with enough MESAB to stop any reflexive swimming. This involved adding 15 drops of MESAB to the

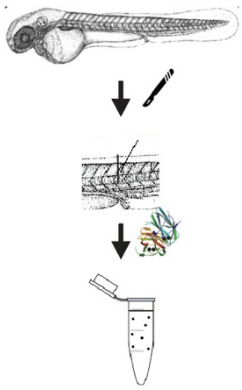


petri dish, waiting 10 seconds, and then tapping the bottom of the dish with forceps. If the fish reflexively swam away 5 more drops were added and the process was repeated. Fish were removed from their EM dishes and moved into a petri dish containing 1x Pen/Strep in EM as well as enough MESAB to keep them anesthetized (15 drops). A pipette was used to suck up the fish head first, making sure to include as little water as possible. Fish were injected into tubes filled with 1.4% low melt agar in EM, heated to 42 C. The fish were then sucked up again with enough agar that when they are ejected onto a petri dish lid situated under a microscope, they are suspended in a droplet of agar. A bent insect needle was used to guide the fish to the top of the agar droplet and the droplet was allowed to cool and harden at room temperature for about 5 minutes (during which time this process was repeated for more fish). Once the agar was hardened a straight insect needle was used to lesion their spinal cords directly after the yolk sac extension. To do this the pin was inserted just above the

neural tube and then jerked upwards sharply to lesion the spinal cord. After lesioning, the fish were allowed to recover for 3 minutes while a solution of 1x Pen/Strep in Ringer solution is made up in a petri dish. Following this a droplet of the Pen/Strep in Ringer solution was placed on each droplet of agar and needle nosed tweezers were traced around the larva to loosen and finally them from the agar. The fish were moved into a dish containing 1x Pen/Strep Ringer and allowed to recover in a warm room.

Embryo Dissociation for Fluorescence-Activated Cell Sorting and scRNA Sequencing

In a 24-well plate fill use a 0.6 mL pipet to fill wells with 1.2 mL of 0.25% Tryptophan Protease solution. Each well can hold approximately 30-40 embryos. Next warm the plates in a 28 C incubator. At this stage the fish tissue was added. To isolate the site



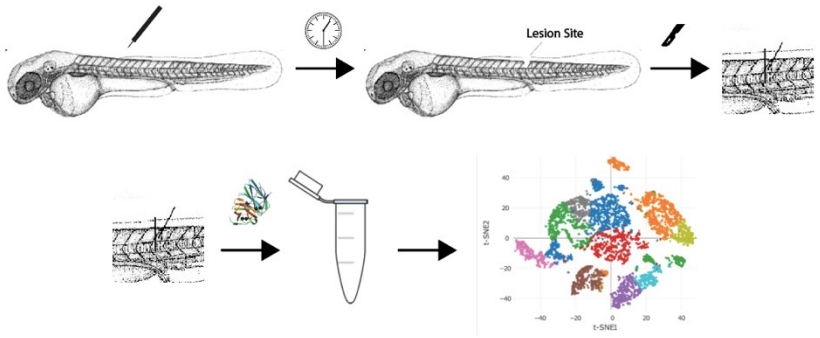
of interest fish were euthanized by exposing them to high doses of MESAB before being translocated onto a dry plate and laid on their sides. A scalpel was used to cut on either side of the lesion site to isolate the tissue of interest (hereafter referred to as a “steak”). Once the steak had been freed it was moved into a siliconized 1.5 mL microcentrifuge tube filled with EM

and spun in a centrifuge at 1 min/2000 RPM to localize the steaks to the bottom of the tube. The steaks were transferred to the pre-warmed protease solution by removing the majority of the EM from the centrifuge tubes (leaving approximately 0.1 mL at the bottom) and then cutting off the end of a plastic pipette tip to allow for a rinse-repeat style transfer of steaks from the centrifuge tubes to the wells. Once all of the steaks were in the wells 27 uL of collagense P in HBSS was added to each well. The plates were incubated at 28 C for 15 minutes. During this incubation the fish were homogenized every 5 minutes by being repeatedly pipetting in and out of a P1000 pipet. After 15 minutes the plates were removed from the incubator and 200 uL of 6x stop solution was added to each well and mixed in a similar manner that was described above. This resulted in 1.5 mL of solution per well. Next the cells were transferred into centrifuge tubes and centrifuged down for 5 minutes at 350 x g at 4 C. This condensed the dissociated cells into a pellet at the bottom of the tube. Liquid was removed from the tube, leaving about 100 uL of solution covering the pellet to prevent disturbance. 1 mL of chilled suspension solution was added and the tubes were centrifuged again. Next the liquid was removed in a similar manner to the procedure described previously. The cells were resuspend in chilled suspension solution, vortexed, and strained through a 40 micrometer

strainer. The final cells were stored on ice. Finally add BSA to final concentration of 0.04%.

Single Cell RNA Sequencing

In order to perform scRNA seq I first lesioned larval zebrafish as described in the methods section “Lesioning” and allowed them to regenerate for 2 days. Next I isolated cells from the lesion site by cutting out the cross section of the fish which had been lesioned (approximately 4 somites long). This cross section will be further referred to as a “steak”. This steak was then dissolved and the resulting live cells were fed into the single cell RNA sequencing pipeline. Analysis of this data involved identifying our cells of interest based on marker genes, and candidate genes involved in regeneration were chosen based on their levels of expression within these cells, the specificity of their expression, and scholastic research which showed they produced proteins whose function may indeed contribute to regeneration. A methods diagram is shown below for clarity.



Antibody Staining

Before staining the fish are euthanized through high doses of MESAB. 10-25 fish are then sucked into a glass pipette and ejected into a 2 mL tube, waste solution is removed. Add approximately 1 mL of 4% PFA in PBS to each tube and rock for 4 hours at room temperature. Remove PFA and wash the fish 5x5 in 1x PBST. Next the fish are blocked in Western Block with Sodium Azide+PBS for 1 hour at room temperature. After they have been blocked the Western Block was removed and the primary antibody solution was added. The fish rocked in this solution overnight. The next day the primary antibody solution was removed and the fish were washed 5x30 in PBST to remove any primary antibodies free-floating in solution. Next secondary antibody solution was added and the fish were again allowed to rock overnight at room temperature. The next day the secondary antibody solution was removed and the fish were washed 5x5 in PBST to remove any primary antibodies free-floating in solution. Finally the fish were dehydrated through a step-wise dehydration in glycerol. To do this the final wash of PBST was removed and 25% glycerol in PBS was added to the tubes. Once the fish sank to the bottom the 25% glycerol solution was removed and a 50% solution was added, followed by a 75% solution. The fish were then stored in this solution until mounting.

EdU Staining

Fish were incubated with 0.1 mg/ml of 5-ethynl-2-deoxyuridine (Thermo Fisher) in embryo media for 2 hours prior to harvest. Harvested embryos were euthanized in a manner previously described, fixed in 4% PFA in PBS overnight at 4°C, then washed 3 x 10 min in PBS. Embryos were then dehydrated through a methanol series of 25%, 50%, 75%, and two washes of 100% methanol, and stored at -20°C for at least 24 hours in 100% methanol. Embryos were rehydrated into PBS containing 0.1% Tween-20 prior to performing EdU Click-It reactions (Thermo Fisher) as described by manufacturer.

Click-it reaction cocktail was made by combining 435 uL of 1x Click-it reaction buffer, 5 uL 500 uM Alexa Fluor 555 PCA solution, 10 uL $CuSO_4$+chelating mix, and 50 uL of 1x Click-iT buffer additive in the order listed. The tubes were then wrapped in tin foil to avoid light interactions and incubated at room temperature for 30 minutes. The cocktail was removed and the fish were washed 5x5 in PBS. Depending on the next stage of imaging for these larva the concentrations of CuSO4 vs. Chelate were moderated as shown below.

Component	Free Copper Level										
	Low			Medium				High			
CuSO4 (uL)	0	1	2	3	4	5	6	7	8	9	10

Copper Protectant (chelate)	10	9	8	7	6	5	4	3	2	1	0
	High (100-80%)			Medium (70-30%)					Low (20-0%)		
	Copper Protectant Level										

FUTURE DIRECTIONS

Although vimentin did not prove to be driving gene for motor neuron re-proliferation following spinal cord injury, our initial pipeline identified the subset of neurons responsible for this proliferation, that these motor neurons are actively dividing, and identified a large list of potential target genes for future investigation. These other candidate genes should be investigated in a similar matter to our vimentin investigation.

If it is found that other genes of interest are indeed correlated to spinal cord regeneration the next step is to determine if this gene is related to causation, in other words: does the expression of the gene of interest cause spinal cord regeneration? Determining causation will involve either developing or obtaining fish that are null mutants for genes of interest. These fish will then be lesioned and allowed to regenerate, and the amount of regeneration will be quantified, either by confocal imaging, swim tests, or dextran dye backfilling. Lastly, causation can be tested through the overexpression of the gene of interest to determine whether this gene on its own is sufficient for the induction of neuronal cell mitosis.

GLOSSARY

Central Nervous System: The brain and the spinal cord

Peripheral Nervous System: The nerves and receptors outside of the brain and spinal cord, that control movement and collect information from the outside world.

DNA: Deoxyribonucleic acid, self-replicating genetic material stored in the nuclei of eukaryotic cells.

Genes: A pattern of nucleotides located on DNA which encodes for mRNA

Marker Genes: Genes that are only expressed in certain cell-types, and can therefor be used to identify cells

Mitosis/Mitotic: Mitosis is the process of cell division resulting in one cell becoming two identical cells. This is the main method of cellular generation throughout the human body.

Morphogen: a diffusible chemical which acts in a concentration dependent manner to effect tissue development

Neural Tube: A hollow tube which develops into the central nervous system

Neural Plate: An undifferentiated structure which will later develop into the neural tube

Null mutant: A mutant that is completely missing the gene of interest

RNA: Ribonucleic acid, another form of genetic material which is often translated from DNA in eukaryotic organisms

Transcriptome: All of the mRNA expressed in a cell

Transcription factor: a protein that alters the genetic expression of another gene

Wild Type: A research subject that has not been exposed to experimental conditions, such as a genetic mutation or environmental change; used as a control

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