HOW DOES Wnt SIGNALING POSTERIORIZE THE NEURAL PLATE?

An Undergraduate Research Scholars Thesis

By

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Submitted to Honors and Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Arne C. Lekven

May 2013

Major: Molecular and Cell Biology

TABLE OF CONTENTS

Page

TABLE OF CONTENTS.....1

ABSTRACT……2

DEDICATION……3

ACKNOWLEDGMENTS……....4

NOMENCLATURE……....5

CHAPTER

I INTRODUCTION.....6

II METHODS.....9

Plasmid Construction.....9
Transgenic Line Generation.....10
In Situ Hybridization.....10

III RESULTS.....11

Plasmid Construction.....12
Transgenic Lines.....14
Results from transient expression assays.....15
New Reporter Constructs and Their Expression.....16

IV CONCLUSIONS…….....19

REFERENCES.....21

ABSTRACT

How does the Wnt signaling posteriorize the neural plate? (May 2013)

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Early in vertebrate development, the neural plate, from which the central nervous system forms, is subdivided into four gross domains through a process called patterning. Defects in neural plate patterning can have severe effects on brain development and can cause an array of catastrophic birth defects such as anencephaly and midbrain-hindbrain malformations. Thus, understanding how neural plate patterning occurs is crucial for understanding the causes of an array of human birth defects. The genes *sp5* and *sp5l* are thought to mediate neural plate patterning in response to signaling by the Wnt signal transduction pathway, but direct evidence connecting Wnt signaling to *sp5* and *sp5l* gene expression in the neural plate has not been identified. In order to obtain such evidence, we are generating transgenic *sp5* and *sp5l* reporters and comparing their expression in Wnt gain and loss of function conditions. For each reporter, we made mutations to predicted transcription factor binding sites to determine how the reporters are controlled by the Wnt pathway. In so doing, we will test the hypothesis that the regulation of *sp5* and *sp5l* is influenced by Wnt signaling during neural plate patterning. We will present data regarding transgene construction and characterization of the reporter expression patterns.

DEDICATION

I am dedicating this thesis to Dr. Arne C. Lekven, a tremendous professor and mentor.

It has been a great privilege to research under your guidance and consistent support. You have created such an exceptional learning environment in your laboratory that it encouraged me to give my fullest efforts in my research work as well as developing my character. I took many of your patent and empathetic approach to heart whenever I was encountered with a challenge. You have allowed me to become a better student, inspiring me to see the “light” in science, and my gratitude for all that you have done for me inspires me to make you prouder by exceeding expectations. Thank you.

ACKNOWLEDGMENTS

I would first like to acknowledge my parents, Song and Charles Park, and my brother Jared Lee. Their constant love and support showed no flaw. Without them, none of this would have been possible.

Thank you to the current and former members of the Lekven lab: Dr. Laura Beaster-Jones, Jo-Ann Fleming, Kevin Baker, Anand Narayanan, Amy Whitener, Holly Gibbs, and Adam Stewart. All of your advice, support, and patience made a great environment to learn and grow as a researcher.

NOMENCLATURE

EGFP	Enhanced Green Fluorescent Protein
mRFP	monomeric Red Fluorescent Protein
hpf	hours post-fertilization

CHAPTER I

INTRODUCTION

During early vertebrate development, determination of the two main axes, the antero-posterior axis and the dorsoventral axis, is crucial. Pattern formation, that is, the establishment of cellular identity in the correct place and time, in each axis is regulated by gene networks and the activities of these gene networks are essential for the formation of morphological features in the embryo. The expression patterns of genes within these networks are regulated by intercellular communication between cells, which promotes proper gene expression at the correct time and location. One such intercellular communication pathway important to both anteroposterior and dorsoventral axis patterning is the Wnt signaling pathway.

Wnts are gene family of paracrine factors that play an important role in development by influencing cell differentiation (1). In the "canonical" Wnt pathway, Wnt proteins bind to a specific receptor on the surface of a target cell, and its binding causes a series of reactions within the cytoplasm of the responding cell. The ultimate step of canonical Wnt signaling results in the nuclear localization of the protein β-catenin, which induces transcription by binding to Lef/Tcf family transcription factors (1). Thus, Wnt signaling likely controls patterning by regulating gene expression in responding tissues, but how responding tissues measure Wnt levels to respond with appropriate changes in gene expression is not understood.

An important tissue that is patterned by Wnt signals during development is the vertebrate neural plate. During this process, the neural plate, from which the central nervous system forms, is subdivided into four gross domains in the anterior-posterior axis; forebrain, midbrain, hindbrain and spinal cord. This process, also called “neural posteriorization”, is hypothesized to be driven by a posteriorizing gradient of Wnt signaling, where differential Wnt signaling is hypothesized to induce differential neural fates. More specifically, levels of Wnt signaling are hypothesized to be higher in more posterior regions of the neural plate, and Wnt signaling is proposed to induce posterior fates (1). In zebrafish, the model organism of our interest, the responsible ligand is Wnt8a (2). It is not understood how potential differences in Wnt signal levels are translated into different neural fate domains.

Two important target genes regulated by Wnt signaling that might play important roles in neural patterning are *sp5* and its paralog *sp5-like* (*sp5l*). Previous studies have shown that *sp5* is expressed in the posterior neural plate during gastrulation, consistent with a role in neural posteriorization (3). It is regulated by *Fgf* and Wnt signaling, and it is required for normal *pax2a* expression in the mid-hindbrain development (3). *Sp5l* is also known for similar functions. Previous studies showed that *sp5l* is a direct Wnt pathway target, where its overexpression represses forebrain gene expression, and it is induced by *Fgf* signaling (4,5). Even though *Fgf* signaling helps regulate *sp5* and *sp5l*, it is not responsible for neural posteriorization on its own (6). *Sp5l* is a direct target of Wnt/β-catenin signaling and, together with *sp5*, acts as a required mediator of *wnt8a* during nervous system patterning (4). To help understand how Wnt signaling controls posterior neural patterning, we are analyzing how Wnt8a signaling controls the spatial transcriptional patterns of *sp5* and *sp5l*.

To study how *sp5* and *sp5l* are regulated by Wnt8a signaling, we constructed reporter plasmids that comprise the *sp5* or *sp5l* regulatory regions fused to the coding sequence for EGFP (Enhanced Green Fluorescent Protein). We also constructed two variants that lack potential Lef/Tcf binding sites and should thus be unresponsive to Wnt signaling. The EGFP reporter genes will help visualize the expression patterns driven by the wild-type and mutant regulatory regions. With stable transgenic lines bearing these different reporter genes, we will be able to test the relationship between Wnt signaling and *sp5*/*sp5l* expression in the neural plate by comparing the expression patterns of the mutants to the wild-types.

CHAPTER II

METHODS

Plasmid Construction

The plasmid containing a mutant variant of the *sp5*1kb control region fused to EGFP was constructed. The plasmid containing *sp5*1kb control region fused to EGFP (pSp51kb) was provided by the Lekven lab and was used as the template to create the mutant variant. p*Sp5*1kb plasmid is a Tol2 transposon-based vector that has an ampicillin marker. p*Sp5*1kb plasmid was used as a template in an inverse PCR reaction with a forward primer and a reverse primer that had matching restriction site sequences in their 5' ends. The PCR primers match the sequence flanking an individual consensus Lef/Tcf binding site such that the new restriction site will replace the Lef/Tcf binding site sequence after PCR amplification. The PCR amplified plasmids were purified using the QIAGEN® PCR purification kit. The purified product was digested with the restriction enzyme matching the sequence in the primers, then treated with ligase to ligate the linearized DNA strands to form circular plasmids. The plasmids were then transformed to produce colonies. The colonies were picked randomly to raise cultures of bacteria. DNA miniprep experiments were performed to recover the mutated plasmid from the bacterial cultures. The miniprep products were tested with specific restriction enzymes to recover the plasmid with proper mutation. These series of steps were followed for each of the Lef/Tcf binding sites.

The *cmlc2* constructs were made in a similar fashion with minor changes. A vector provided by the Lekven lab containing both RFP and EGFP coding sequences was fused to the *cmlc2* promoter sequence such that the RFP gene will be expressed under the control of the cmlc2

promoter. This new plasmid (pT2GRC) was used as a recipient vector for the new *sp5* and *sp5l* constructs. The EGFP reporter gene was excised from pT2GRC with ApaI and ClaI, and the vector portion was purified from an agarose gel. The sp5mut:EGFP and sp5lmut:EGFP cassettes were gel purified from their respective plasmids by ApaI and ClaI restriction digestion and agarose gel electrophoresis, and then ligated into the pT2GRC vector. The steps for ligation and transformation were similar to what was mentioned previously. Resulting plasmids were named pT2GRC-sp5mut and pT2GRC-sp5lmut.

Transgenic lines

Once *sp5mut* was constructed, psp51kb, psp5l, psp5mut, and psp5lmut were injected into 1 cell stage zebrafish embryos. The injection solution included transposase RNA, pure plasmid DNA, food coloring, and distilled water. The embryos were screened at 24hpf for green fluorescence. The fluorescent embryos were raised to adulthood. The adult fish from the injection were crossed to wild-type zebrafish. The offspring were screened for green fluorescence. The fluorescent embryos are heterozygous for the transgene, and they were raised to maturity to establish a stable line.

In Situ Hybridization

In situ hybridizations were performed according to protocol as described in Ramel at al. with a GFP probe (2). Proteinase K treatment on 24hpf embryos was performed for 5 minutes.

CHAPTER III

RESULTS

The genes *sp5* and *sp5l* are thought to mediate neural plate posteriorization in response to *wnt8a* signaling. The two genes are paralogs, meaning that these two genes encode related transcription factor proteins, and they may share similar gene regulation mechanisms (Figure 1). Previous studies have shown that *sp5* and *sp5l* are regulated by Wnt signaling, but it is unknown whether Wnt signaling turns *sp5* and/or *sp5l* on or off in the posterior neural plate.

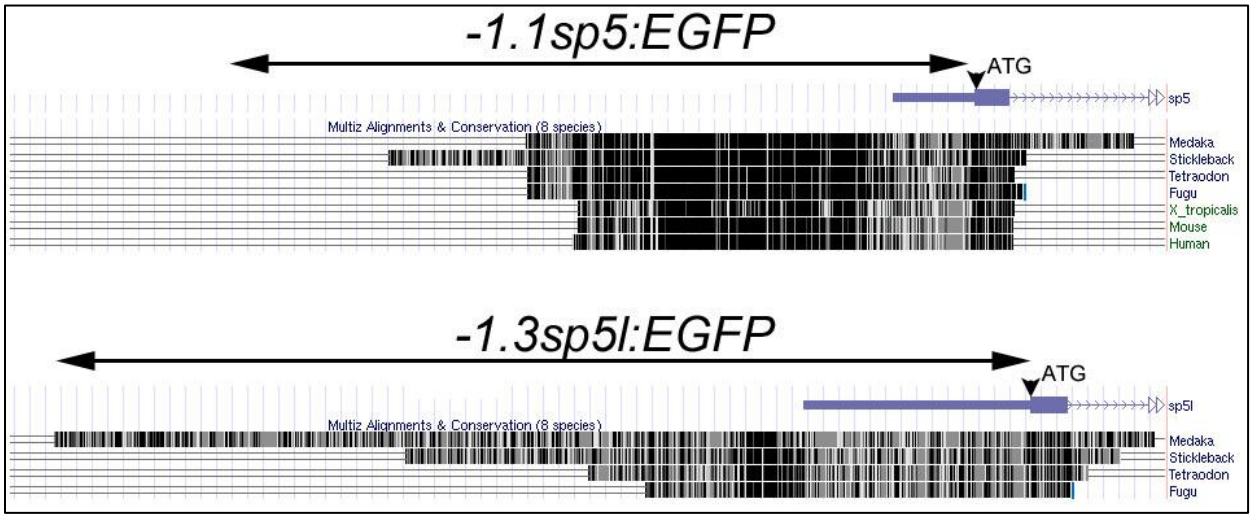

Figure 1. *sp5* (above) and *sp5l* (bottom) diagrams from UCSC genome browser (4). The double arrows indicate the promoter regions ligated into reporter plasmids for this study. The blue boxes represent the transcripts, and ATG indicates the translation start codon. Below each double line is a measure of the conservation between sp5 and sp5l promoter sequences in different vertebrate species. Note that the sp5 promoter is very highly conserved among vertebrates.

The promoter regions from each gene (Figure 1, double arrows) were isolated and used in reporter assays to determine how *sp5* and *sp5l* regulatory regions respond to Wnt signaling in vivo. The construction of the genes fused to EGFP and their mutant variants are described below.

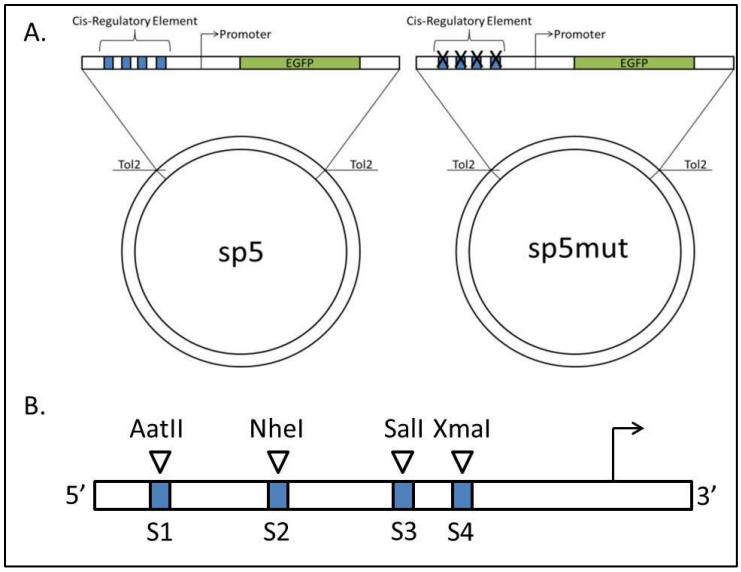

Figure 2. Diagram for *sp5* (left) and *sp5*mut (right) plasmids (A). The region to the left of the promoter is the control region, and the green box indicates the EGFP gene. The blue boxes indicate Lef/Tcf binding sites. Black X's indicate Lef/Tcf binding sites replaced by restriction enzyme sites. The whole reporter gene is flanked by Tol2 sequences, which enable easy transgenesis. (B) The control region of *sp5* gene. The blue boxes indicate Lef/Tcf binding sites, and above each is the restriction enzyme sequence used to replace that specific Lef/Tcf binding site.

Initial Reporter Plasmid Construction

To test our hypothesis that *sp5* and *sp5l* are regulated by *wnt8a* signaling during neural plate patterning, we constructed chimeric plasmids comprising the *sp5* or *sp5l* promoter regions fused to the EGFP gene. These plasmids were then mutated to replace Lef/Tcf binding sites with restriction enzyme sites.

The EGFP reporter plasmids for *sp5*, *sp5l*, and *sp5lmut* were previously made in the Lekven Lab. The reporter plasmid, p*sp5*mut, was constructed through a series of steps. The plasmid with the wild-type *sp5* promoter region fused to EGFP was used as a template to generate the mutant variant (Figure 2.A). First step was to use inverse PCR using the primers with restriction enzyme sites to replace Lef/Tcf binding sites (Figure 2.B & Figure 3). After PCR, the linear DNA was ligated back together and transformed into bacterial cultures for plasmid amplification. The bacterial cultures from transformation were collected for miniprep DNA preparations. The miniprep samples were digested with the relevant restriction enzymes and observed using gel electrophoresis for verification that the desired restriction enzyme site was introduced by the inverse PCR approach. This approach was repeated for all four Lef/Tcf binding sites.

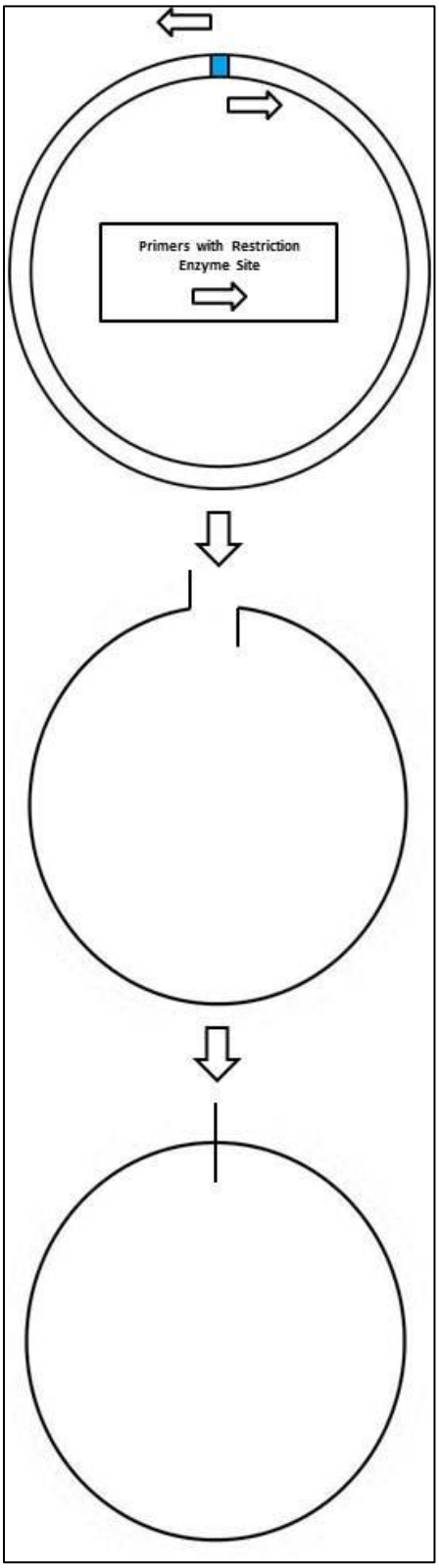

Figure 3. Inverse PCR Diagram. Blue box indicate Lef/Tcf binding site. The primers create new strands with restriction enzyme site instead of the Lef/Tcf binding site.

Once all four mutations were made, the final product was tested using restriction analysis and DNA sequencing to confirm the replacement of the Lef/Tcf sites with restriction enzyme sites. The restriction enzymes used for gel

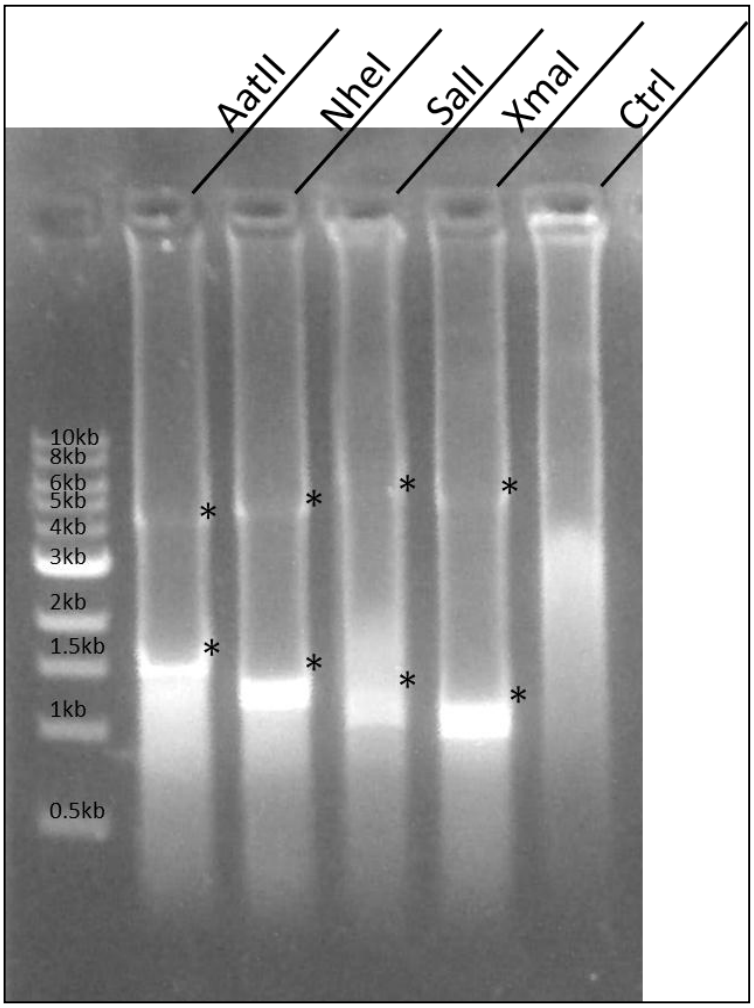

Figure 4. Restriction digests of *sp5*mut on Gel Elctrophoresis. Fragments from each lane add up to ~5.4kb. Each restriction enzyme digested two locations on the plasmid to give two fragments. The background smear comes from RNA in the samples.

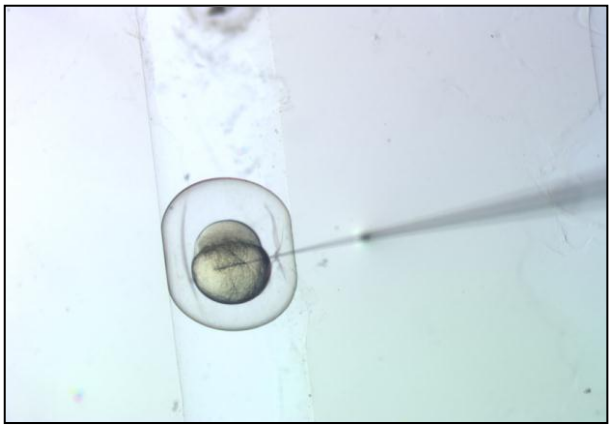

Figure 5. Injection of a one-cell staged embryo. The needle filled with the plasmid DNA solution is poked into the embryo just below the animal hemisphere.

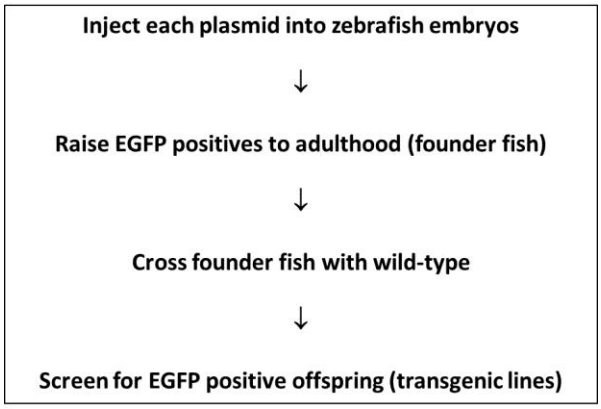

Figure 6. Flow chart for transgenesis.

electrophoresis were AatII, NheI, SalI, and XmaI, and each enzyme was used in a double digest with a second enzyme that cuts at the 3' end of the EGFP gene. These restriction enzymes targeted the mutations made in the plasmid. If successful, these double digests should each produce two fragments, with the smaller fragments in each digest differing in size as predicted as observed in Figure 4. After verification of mutations, the mutant sample was sequenced and confirmed for successful mutation.

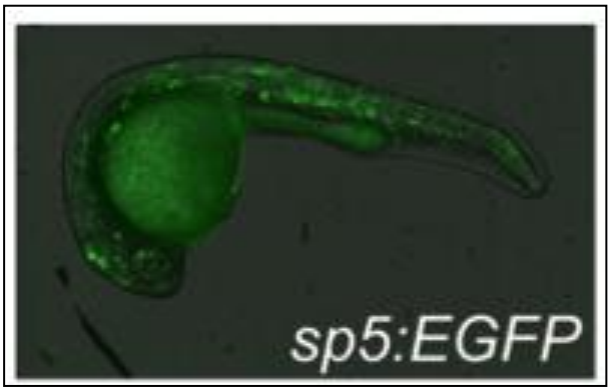

Figure 7. Image of EGFP fluorescence in a ~24 hour old injected zebrafish embryo. The green fluoresence can be seen in the nervous system. The green fluorescence in the yolk is due to autofluorescence.

Transgenic Line Production

The transgenesis process for acquiring stable transgenic lines for the four plasmids is diagrammed in Figure 6.The four plasmids were injected into one-cell staged zebrafish embryos for transgenic line generation (Figure 5). The steps taken for transgenesis of the four plasmids are diagrammed in Figure 6. The injection solution contained purified DNA, mRNA that codes for the transposase enzyme for transgenesis, food coloring, and distilled water. Once injected, the

transposase enzyme is translated and catalyzes transposition of the DNA construct into the zebrafish genome. If incorporated correctly, EGFP expression from the DNA construct will be visible under blue light (Figure 7). The embryos showing green fluorescence were raised to adulthood and these fish are termed founder fish. Once they reached adulthood, the founder fish were crossed with wild-type fish. The offspring from the cross were screened for green fluorescence, which indicates successful stable transgenesis. These steps were repeated for each of the plasmids.

Results from Transient Expression Assays

Images were taken of the injected embryos for closer observation. The fluorescence expression patterns for the four plasmids can be seen in Figure 8. Figure 8 shows images of 24 hour old zebrafish embryos, each of the embryos were injected with one of the four plasmids. The embryos injected with the wild-type *sp5* and *sp5l* reporter plasmids showed high levels of expression in the nervous system, predominantly in the spinal cord region, but with significant expression in the midbrain. The expression for *sp5*mut was low, which made it difficult to analyze. In contrast, *sp5l*mut showed a high level of GFP expression throughout the nervous system, predominantly in the spinal cord region, but also in the brain.

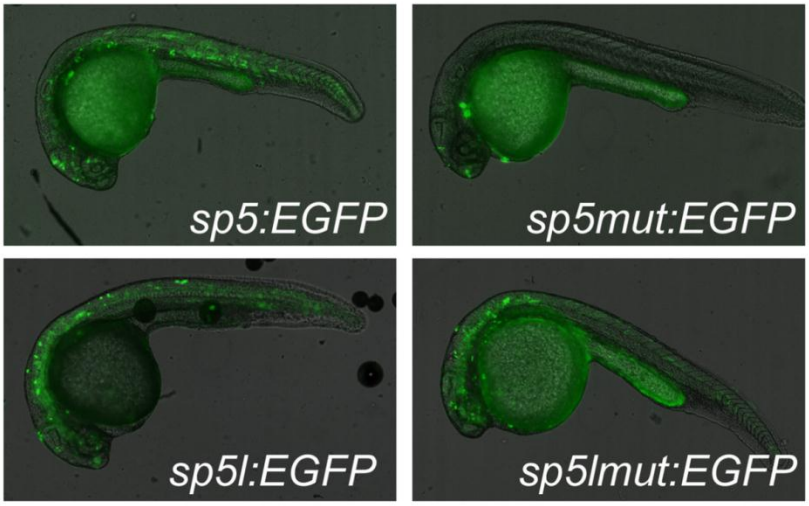

Figure 8. GFP expression in 24 hour old injected embryos. Note that expression from the mutant sp5 promoter is very low, while expression from the mutant sp5l promoter is relatively high.

Low level GFP could be caused by the mutations to the Lef/Tcf sites, or due to injection problems, i.e. too little DNA. To confirm this result, we made 2 additional plasmids that have an additional reporter gene that is expressed in the heart independently of Wnt signaling.

New Reporter Constructs and Their Expression

The *sp5*mut and *sp5l*mut promoters were ligated into a new plasmid containing the *cmlc2* promoter fused to the coding region for monomeric red fluorescent protein (mRFP). The template plasmid used was pT2GRC, which contained the *cmlc2*-mRFP reporter, and EGFP. We used restriction enzymes to excise EGFP from this template and replaced it with *sp5*mut:EGFP.

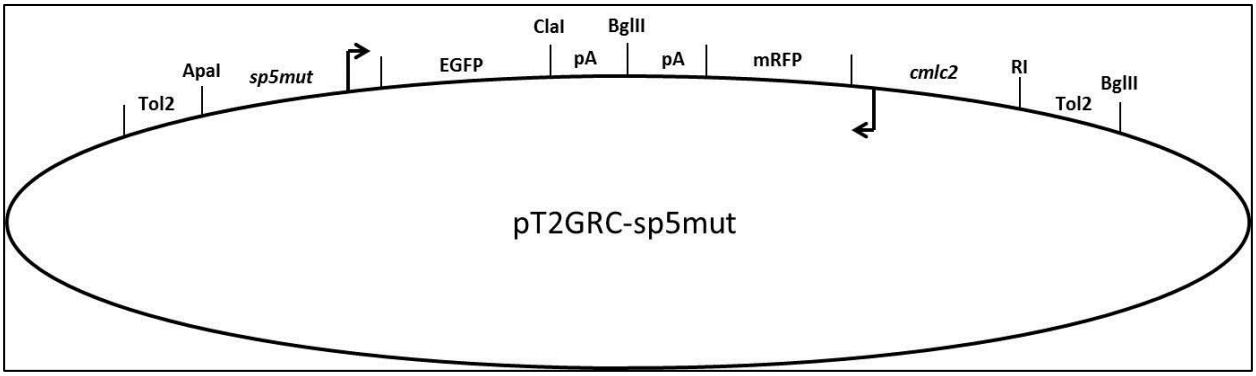

Figure 9. The new plasmid containing both *sp5*mut and *cmlc2* genes. When *sp5* is expressed, EGFP will also express. And when *cmlc2* is expressed, mRFP will also express. However, expression of one gene will not affect the expression of the other.

This was done for *sp5l*mut as well. The schematic diagram of the new plasmid can be seen in Figure 9. The *cmlc2* gene is expressed in the heart by ~30 hours post fertilization. Unlike *sp5* and *sp5l*, this gene is not regulated by Wnt signaling, which means that the expression of this gene should not be affected by Wnt signaling as *sp5* and *sp5l* genes may, and thus expression of mRFP in the heart can serve as a reporter for successful DNA injection and transgenesis irrespective of the expression levels of EGFP.

The new constructs were injected into zebrafish embryos following the method mentioned previously. The expression pattern for *cmlc2*, which was visualized through red fluorescence, was not visible as early as the EGFP expression driven by the *sp5* and *sp5l* promoters. The embryos were screened at approximately 36 hours post fertilization (hfp) and the expression patterns for the genes were as predicted. Figure 10 shows images taken after injection of pT2GRC-*sp5*mut. As expected, *cmlc2*-mRFP expression was observed in the heart, and *sp5*mut expression was located throughout the nervous system at a low level, confirming our prior observations.

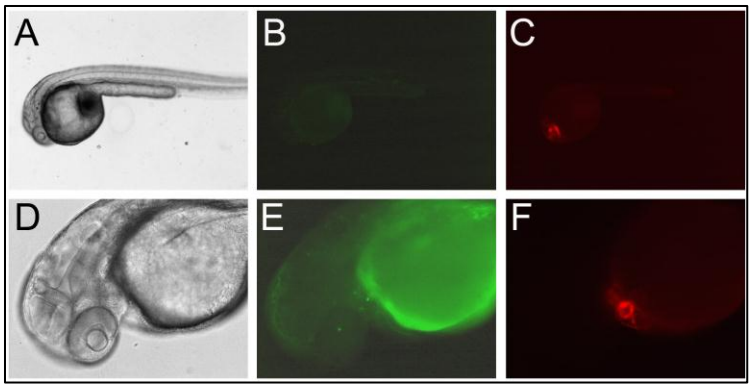

Figure 10. Reporter expression from pT2GRC-sp5mut. (A-C) Low magnification view of the whole embryo. (D-F) High magnification view of the head. (A,D) Bright field. (B,E) EGFP expression driven by the sp5mut promoter. (C,F) mRFP expression driven by the *cmlc2* promoter.

During the screening process, an interesting observation was made. Table 1 shows data collected during the embryo screening in this experiment for the numbers of embryos showing either GFP or RFP fluorescence, or both. Because the GFP and RFP genes are carried on the same plasmid, it was expected that all fluorescent embryos should express both GFP and RFP. However, the observed data did not match this expectation. For pT2GRC-*sp5*mut, a majority of the injected embryos were

cmlc2:sp5mut				
	GFP+:RFP+	GFP+:RFP-	No expression	Total
Number of Embryos	24	44	12	80
cmlc2:sp5mut				
	GFP+:RFP+	GFP+:RFP-	No expression	Total
Number of Embryos	60	15	1	76

Table 1. Number of fluorescent embryos. This data was collected from embryos that were 36 hours old.

GFP-positive and RFP-negative. In contrast, a majority of pT2GRC-sp5lmut injected embryos were both GFP-positive and RFP-positive. These results suggest that GFP-positive embryos that were RFP-negative may not have started expressing *cmlc2* at the time that the embryos were observed.

CHAPTER IV

CONCLUSIONS

The goal of this study was to complete a set of four reporter plasmids designed to determine whether *sp5* and *sp5l* are controlled by Wnt signaling in the zebrafish neural plate. Efforts are currently underway to establish stable transgenic lines for all four reporter constructs. It was possible to make observations of the transient expression produced by the different reporters, which led to the observation that, while both *sp5* and *sp5l* promoters are active in driving reporter expression in the nervous system, Lef/Tcf site mutations in each promoter led to different effects. The *sp5* promoter appears to be more dependent on Lef/Tcf dependent regulation than the *sp5l* promoter, as the *sp5mut* promoter was far less active in injected embryos that the *sp5lmut* promoter. This result suggests that sp5 and sp5l may be regulated through different mechanisms in the zebrafish neural plate, and that they may be responsive to Wnt signaling in different ways. While the expression pattern can be observed in the injected embryos, the stability of the gene expression in these transient assays is not reliable enough to draw any firm conclusions. Therefore, these results must be confirmed in stable transgenic lines.

Wnt signaling-dependent *sp5* and *sp5l* expression is hypothesized to mediate the posteriorization of the nervous system, but the mechanism as to how this is achieved has not been determined. By studying the effect of Wnt on *sp5* and *sp5l* regulation, we can determine how cells can respond to different levels of Wnt signaling during posteriorization of the nervous system, and determine the importance of these two genes during nervous system development.

In this project, GFP fluorescence was used to determine the location of reporter expression during zebrafish development. An important caveat is that EGFP fluorescence cannot be used exclusively to determine the pattern of expression during development because green fluorescent proteins are stable in cells, so they may remain in the cell even after the expression of *sp5* or *sp5l* has terminated. In order to overcome this limitation, *in situ* hybridization experiments will need to be carried out once the stable transgenic lines for the four strains are acquired. With *in situ* hybridization, the localization and pattern of mRNA transcription can be obtained, which is a more direct measurement of gene activation and will help determine the effect of Wnt signaling on its target genes, *sp5* and *sp5l*, and nervous system development.

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