PURDUE UNIVERSITY
GRADUATE SCHOOL
Thesis/Dissertation Acceptance

This is to certify that the thesis/dissertation prepared

By Courtney Lee Curtis

Entitled
WNT SIGNALING IN ZEBRAFISH FIN REGENERATION:
CHEMICAL BIOLOGY USING A GSK3β INHIBITOR

For the degree of   Master of Science

Is approved by the final examining committee:

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            Head of the Graduate Program   Date

WNT SIGNALING IN ZEBRAFISH FIN REGENERATION:

CHEMICAL BIOLOGY USING A GSK3β INHIBITOR

A Thesis

Submitted to the Faculty

of

Purdue University

by

Courtney L Curtis

In Partial Fulfillment of the

Requirements for the Degree

of

Master of Science

August 2013

Purdue University

Indianapolis, Indiana

ACKNOWLEDGEMENTS

First, I wish to thank Dr. James A. Marrs for welcoming me into his lab and providing mentoring and guidance throughout my master's thesis. I also want to thank the members of my committee, Dr. Jiliang Li and Dr. David Skalnik, for their valuable input and the hours they have dedicated to the progress of my graduate work. I would like to express my gratitude to our collaborators at Eli Lilly and Company; Masahiko Sato, Shaoyou Chu, and Manuel Sanchez-Felix for their support throughout this project.

I would especially like to thank my fellow Marrs' lab members, Dr. Swapnalee Sarmah and Pooja Muralidharan, for their assistance and willingness to teach me new techniques. Without their help, completion of this thesis would not have been possible.

Finally, thank you to my wife, Sarah, for her support and understanding over the last two years as I have worked towards the completion of this thesis.

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LIST OF ABBREVIATIONS

APC	Adenomatous Polyposis Coli
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone Morphogenetic Protein
°C	Degrees Celsius
DMSO	Dimethyl Sulfoxide
Dkk	Dickoppf
dpa	Days Post Amputation
Dsh	Dishevelled
FGF	Fibroblast Growth Factor
FGFR1	Fibroblast Growth Factor Receptor 1
Fz	Frizzled
GFP	Green Fluorescent Protein
GSK3β	Glycogen Synthase Kinase 3β
HAT	Histone Acetylases
HDAC	Histone Deacetylases
hpa	Hours Post Amputation
hpf	Hours Post Fertilization

Krm	Kremen
LEF	Lymphoid Enhancer Factor
LRP	Low Density Lipoprotein Receptor-Related Proteins
mm	Millimeter
nM	Nanomolar
NTMT	Alkaline Phosphatase Buffer
PBS	Phosphate- Buffered Saline
PBT	Phosphate- Buffered Saline -0.1% Tween 20
PCR	Polymerase Chain Reaction
PH3	Phospho-Histone 3
PPARγ	Peroxisome Proliferator-Activated Receptor γ
PTH	Parathyroid Hormone
RFP	Red Fluorescent Protein
SFRP	Secreted Frizzled-Related Protein
SHH	Sonic Hedgehog
SOST	Sclerostin
TCF	T-cell Factor
μM	Micromolar
YFP	Yellow Fluorescent Protein

ABSTRACT

Curtis, Courtney L. M.S., Purdue University, August 2013. Wnt Signaling in Zebrafish Fin Regeneration: Chemical Biology Using a GSK3β Inhibitor. Major Professor: James A. Marrs.

Bone growth can be impaired due to disease, such as osteoporosis. Currently, intermittent parathyroid hormone (PTH) treatment is the only approved therapy in the United States for anabolic bone growth in osteoporosis patients. The anabolic effects of PTH treatment are due, at least in part, to modulation of the Wnt/β-catenin pathway. Activation of the Wnt/ β-catenin pathway using a small molecule inhibitor of GSK3β was previously shown to increase markers of bone formation in vitro. Our study utilized a zebrafish model system to study Wnt activated fin regeneration and bone growth. Wnt signaling is the first genetically identified step in fin regeneration, and bony rays are the main structure in zebrafish fins. Thus, zebrafish fin regeneration may be a useful model to study Wnt signaling mediated bone growth. Fin regeneration experiments were conducted using various concentrations of a GSK3β inhibitor compound, LSN 2105786, for different treatment periods and regenerative outgrowth was measured at 4 and 7 days post amputation. Experiments revealed continuous low concentration (4-5 nM) treatment to be most effective at increasing regeneration. Higher concentrations inhibited fin growth, perhaps by excessive stimulation of differentiation programs. *In situ*

hybridization experiments were performed to examine effects of GSK3β inhibitor on Wnt responsive gene expression. Experiments showed temporal and spatial changes on individual gene markers following GSK3β inhibitor treatment. Additionally, confocal microscopy and immunofluorescence labeling data indicated that the Wnt signaling intracellular signal transducer, β-catenin, accumulates throughout GSK3β inhibitor treated tissues. Finally, experiments revealed increased cell proliferation in fin regenerates following LSN 2105786 treatment. Together, these data indicate that bone growth in zebrafish fin regeneration is improved by activating Wnt signaling. Zebrafish Wnt signaling experiments provide a good model to study bone growth and bone repair mechanisms, and may provide an efficient drug discovery platform.

CHAPTER 1: INTRODUCTION

Zebrafish Fin Regeneration

Whereas mammals are limited in their ability to regenerate damaged tissues and are

unable to repair bone fractures beyond a critical size, zebrafish possess the ability to

completely regenerate complex structures, such as the bony rays of the fin. While

zebrafish are able to regenerate all fin types, the accessibility of the caudal fin for surgery

and measurement has made it an important model for use in regeneration studies. The

zebrafish caudal fin is comprised primarily of lepidotrichia, segmented bony rays which

consist of two concave hemirays that surround fibroblast like cells, nerves and blood

vessels, and are covered by a single layer of osteoblasts on both the inner and outer

surface (Figure 1) (Poss, Keating et al. 2003). When subjected to injury, such as

amputation, the zebrafish fin regenerates rapidly. If half of the caudal fin is amputated,

the fin is restored to its original length by approximately 3 weeks post amputation. The

new bones of the regenerate are formed without a cartilage intermediate in a process that

is analogous to intramembranous bone formation (Knopf, Hammond et al. 2011). This

remarkable ability makes zebrafish an ideal model to study the molecular mechanisms of

bone growth and repair.

Zebrafish fin regeneration occurs through three general stages: (1) wound healing, (2) formation of a blastema consisting of mesenchymal progenitors, and (3) regenerative outgrowth and patterning of bony ray structures (Figure 2). The initial stage, wound healing, begins within 1 to 3 hours post amputation (hpa). This process occurs without cell proliferation and is achieved by migration of epidermal cells to form a thin layer covering the wound by 12 hpa (Poleo, Brown et al. 2001). As wound healing progresses, additional layers are added to the wound epidermis and it begins to express genes that play a role in growth and patterning. Over the course of the next 24-36 hours, a blastema forms at the distal tip of each amputated fin ray (Akimenko, Marí-Beffa et al. 2003). The blastema is comprised of proliferative mesenchymal progenitor cells which will eventually develop into the new structures of the regenerated fin. Although the source of the cells which give rise to the blastema is not entirely clear, it is formed, at least in part, by mature osteoblasts and fibroblasts which dedifferentiate, proliferate, and migrate distally (Poleo, Brown et al. 2001; Knopf, Hammond et al. 2011). Beginning at approximately 48 hpa, fin regeneration enters the outgrowth and patterning stage. During this stage, the blastema can be divided into two domains, the distal blastema and the proximal blastema. The distal blastema proliferates extremely slow and is thought to play a role in directing outgrowth (Nechiporuk and Keating 2002). In contrast, the proximal blastema is intensively proliferative. As the cells in proximal blastema proliferate, they migrate to the area immediately proximal and begin to differentiate into osteoblasts which secrete the matrix to form the new bone segment (Poss, Keating et al. 2003). The fin continues to rapidly regenerate until the original length is restored, at which point growth slows via a process that is not well characterized.

Wnt/β-catenin Signaling Pathway

Wnt ligands are cysteine-rich, secreted glycoproteins with crucial roles in embryonic

development and regulation of cell growth, differentiation, and apoptosis (Moon,

Bowerman et al. 2002). Wnt signaling can be divided into two general pathways, the

canonical Wnt/β-catenin signaling pathway and the non-canonical β-catenin independent

Wnt signaling pathway. The canonical Wnt signaling pathway is activated when the Wnt

ligand binds to a transmembrane Frizzled (Fz) receptor and the co-receptors low density

lipoprotein receptor-related proteins – 5/6 (LRP 5/6). Receptor engagement activates

Dishevelled family proteins (Dsh), which in turn, inhibit the cytoplasmic "destruction

complex" consisting of Axin, adenomatous polyposis coli (APC) and glycogen synthase

kinase 3β (GSK3β). Inactivation of the "destruction complex" prevents phosphorylation

of β-catenin by GSK3β, a modification that marks it for degradation (Figure 3). As a

result, unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the

nucleus where it displaces histone deacetylase (HDAC) corepressors from T-cell factor/

Lymphoid enhancer factor (TCF/LEF), recruits histone acetylases (HAT), and activates

Wnt target genes. In the absence of the Wnt ligand, β-catenin is phosphorylated by

GSK3β and subsequently degraded (Agholme and Aspenberg 2011).

There are several proteins that are known to play an antagonistic role in the Wnt/β-

catenin signaling pathway. Most notable are the Dickkopfs (Dkks), sclerostin (SOST),

and secreted Frizzled-related proteins (SFRPs). Dkks bind to the LRP-5/6 co-receptor

blocking the formation of the Wnt-Fz-LRP 5/6 complex, which is necessary for the

activation of Dsh. In addition, Dkks interact with Kremens (Krm) which form a Dkk-

Krm-LRP 5/6 complex that is thought to be internalized and degraded in the lysosome (Westendorf, Kahler et al. 2004). Similar to Dkks, SOST inhibits Wnt signaling by binding the LRP 5/6 co-receptor (Semënov, Tamai et al. 2005). SFRPs antagonize Wnt signaling by directly binding the Wnt ligand (Semënov, Tamai et al. 2005).

<div align="center">Wnt/β-catenin Signaling in Zebrafish Fin Regeneration</div>

Wnt/β-catenin signaling is the earliest genetically identified step in fin regeneration and is required during all stages of regeneration for normal outgrowth to occur (Stoick-Cooper, Weidinger et al. 2007). The Wnt ligand, Wnt-10a, which signals through the canonical Wnt pathway, is upregulated during the first few hours following amputation (Stoick-Cooper, Weidinger et al. 2007). At the same time, β-catenin expression is detectable in the cells of the wound epidermis, where it is maintained throughout regeneration. Additionally, the Wnt/β-catenin signaling target Lef1 is expressed in the wound epidermis as early as 12 hpa (Poss, Shen et al. 2000). Previous studies have shown that inhibition of fibroblast growth factor (FGF) signaling does not affect expression of *lef1*, but inhibition of Wnt/β-catenin signaling is sufficient to repress expression of FGF target genes (Kawakami, Esteban et al. 2006). Taken together, these findings indicate that Wnt/β-catenin signaling is upstream of FGF signaling and later differentiation pathways, such as Shh and Bmp2b signaling, which direct blastema cells to differentiate into osteoblasts (Kulkarni, Halladay et al. 2005; Stoick-Cooper, Moon et al. 2007).

In addition to a role in the immediate regenerative response, Wnt/β-catenin signaling is also important in establishment of the blastema. In studies using a transgenic zebrafish

line, the Wnt/β-catenin signaling transcriptional reporter, TOPdGFP, is detectable in the blastema by 48 hpa. Furthermore, expression levels of several direct Wnt/β-catenin target genes, such as *axin2* and *sp8,* are upregulated in the regenerating tissue during blastema formation (Stoick-Cooper, Weidinger et al. 2007). During this stage of regeneration, interactions between Wnt/β-catenin signaling and FGF signaling are critical for establishing the blastema. Disruption of the Wnt target, FGF receptor 1 (FGFR1), prevents expression of cellular proliferation markers and completely blocks blastema formation (Poss, Shen et al. 2000).

Experiments which utilized a heat shock inducible Dkk transgenic to inhibit Wnt/β-catenin signaling at various times post amputation revealed the necessity of Wnt/β-catenin signaling during each stage of regeneration. Inhibition of Wnt/β-catenin signaling at 0 or 1 dpa does not affect epidermal wound healing, but prevents blastema formation and subsequent regenerative outgrowth. When Wnt/β-catenin signaling is disrupted after the blastema has already formed, fins display only partial regeneration, indicating that Wnt/β-catenin signaling is also involved in blastema maintenance and regulation of outgrowth. Conversely, over activation of Wnt/β-catenin signaling results in enhanced fin regeneration rate. Fish with a mutation in one copy of the Wnt/β-catenin pathway inhibitor, *axin1*, display increased regenerative outgrowth at 7 dpa (Stoick-Cooper, Weidinger et al. 2007).

Interestingly, disruption of β-catenin independent Wnt signaling affects fin regeneration as well. Inhibition of the β-catenin independent Wnt signaling pathway results in

increased regenerative outgrowth, whereas over activation of β-catenin independent Wnt signaling completely blocks fin regeneration (Stoick-Cooper, Weidinger et al. 2007). These findings suggest a model in which β-catenin independent Wnt signaling acts in a negative-feedback loop to antagonize Wnt/β-catenin signaling and regulate regeneration.

<u>Wnt/β-catenin Signaling in Mammalian Bone Formation and Fracture Repair</u>

In recent years, the Wnt signaling pathway has been shown to be an important player in bone formation, homeostasis, and repair. Early indications that canonical Wnt signaling may play a role in bone formation were revealed by finding natural mutations in the LRP 5/6 co-receptor. Loss of function mutations in the LRP 5/6 co-receptor were shown to cause osteoporosis-pseudoglioma syndrome, a disorder in which patients have significantly lower bone mass and an increased risk of fracture (Gong, Slee et al. 2001). Conversely, activating mutations which prevent binding of the Wnt antagonist Dkk to the LRP 5/6 co-receptor result in a high bone mass phenotype with no other obvious defects (Boyden, Mao et al. 2002). These observations laid the foundation for studying the role of Wnt signaling in bone formation and led to examination of the role other key components of the Wnt signaling pathway play in bone formation.

Wnt/β-catenin signaling plays a crucial role in bone formation by regulating osteoblast differentiation and suppressing osteoclast activity (Holmen, Zylstra et al. 2005; Spencer, Utting et al. 2006). Several studies have demonstrated that β-catenin is necessary for mesenchymal progenitors to ultimately differentiate to osteoblasts. A study using mouse embryos with a conditional knock out of β-catenin, specifically in the mesenchymal

progenitors of chondrocytes and osteoblasts, revealed that E 18.5 mutant embryos display a skeleton in which cartilage is present but bone is entirely lacking (Hu, Hilton et al. 2005). A similar study confirmed these results and presented evidence that knocking out β-catenin in osteoblast precursors arrests differentiation and causes the osteoblasts to transdifferentiate into chondrocytes (Hill, Später et al. 2005). While inactivating β-catenin has an inhibitory effect on osteogenesis, ectopic expression of Wnt-14, a canonical Wnt signaling pathway ligand, in early mouse embryos produced enhanced osteogenesis and expansion of osteoblast markers such as Runx2, Osterix, and Osteocalcin (Day, Guo et al. 2005). Wnt-10b, another ligand of the canonical Wnt signaling pathway, plays an important role in stimulating osteoblastogenesis and inhibiting adipogenesis. In mesenchymal progenitors Wnt-10b induces expression of Runx2 and Osterix while at the same time inhibiting expression of adipogenic factors such as peroxisome proliferator-activated receptor γ (PPARγ), thus promoting osteoblastogenesis in favor of adipogenesis (Bennett, Ouyang et al. 2007).

In addition to a role in skeletal development, canonical Wnt signaling was also implicated in the fracture healing process. Data from RT-PCR and gene microarrays have shown that multiple Wnt ligands, along with several components of the Wnt/β-catenin pathway were upregulated during the early stages of fracture repair. During these early stages, β-catenin levels were increased and remained elevated throughout fracture repair before ultimately declining to basal levels as healing completes (Zhong, Gersch et al. 2006). The importance of canonical Wnt signaling in fracture repair was further shown by studies evaluating the effects of Dkk1 on fracture repair, which showed that inhibition of

the Wnt/β-catenin signaling pathway during the first week immediately following injury blocks healing, leaving undifferentiated mesenchymal tissue in place of a callus at the site of injury (Chen, Whetstone et al. 2007).

<u>Wnt/β-catenin Pathway as a Therapeutic Target</u>

The majority of bone fractures heal with minimal intervention, however, a small percentage of fractures fail to heal properly (Secreto, Hoeppner et al. 2009). Therapies designed to induce bone formation could be the key to a shorter, more effective healing process for these patients. Moreover, these same therapies could be applied to osteoporosis patients who are at especially high risk of fractures due to low bone mass and poor bone quality.

Currently, intermittent parathyroid hormone (PTH) treatment is the only approved therapy in the United States for anabolic bone growth in osteoporosis patients (Secreto, Hoeppner et al. 2009). Recent studies have revealed that the anabolic effects of PTH treatment are due, at least in part, to modulation of the Wnt/β-catenin pathway (Kulkarni, Halladay et al. 2005). While parathyroid hormone treatment is effective in increasing bone mass, direct modulation of the Wnt/ β-catenin pathway may be a safer and more efficient way to stimulate bone repair and regeneration. Activation of the Wnt/ β-catenin pathway using a small molecule inhibitor of GSK3β was previously shown to increase markers of bone formation in vitro as well as increase bone mass in vivo. GSK3β inhibitor treatment increased markers of osteoblast differentiation and was sufficient to increase both bone mineral density (BMD) and bone mineral content (BMC) in

osteopenic rats (Kulkarni, Onyia et al. 2006). Collectively, this data points to modulation of the Wnt/ β-catenin pathway as an important area of future research for bone repair and regeneration.

<div style="text-align: center;"><u>Research Goals</u></div>

The goal of this research was to evaluate a zebrafish fin regeneration research model for Wnt mediated bone growth. Wnt signaling is the first genetically identified step in fin regeneration, and bony rays are the main differentiated cell type in fins. Thus, zebrafish fin regeneration may be a useful model to study Wnt signaling mediated bone growth. Our hypothesis was that zebrafish Wnt mediated signaling experiments will model human bone growth and repair, and provide a versatile drug discovery platform. Specifically, we wanted to analyze the effects of activating Wnt/β-catenin signaling, via GSK3β inhibition, on the regenerative response to injury in the zebrafish fin. GSK3β is a well-established drug target for regulating Wnt signaling, and zebrafish GSK3β is very similar to human GSK3β. Human GSK3β contains 420 amino acids versus 421 in zebrafish GSK3β. A 408 amino acid core in the protein is 95% identical between human and zebrafish sequences.

Our hypothesis was tested by analyzing the effects of various GSKβ inhibitor treatment regimens on zebrafish caudal fin regeneration. More specifically, we aimed to measure the effects of GSK3β inhibitor treatment on fin regeneration rate and dissect the cellular and molecular mechanisms of bone growth responses. We examined the rate of regeneration in response to Wnt/β-catenin signaling activation by measuring regenerative

outgrowth at 4 and 7 dpa. Additionally, we evaluated the spatial and temporal effects of

GSKβ inhibitor treatment on Wnt responsive gene expression using *in situ* hybridization.

Using immunostaining, the effects of GSK3β inhibitor treatment on cell proliferation and

β-catenin accumulation were assessed.

CHAPTER 2: MATERIALS AND METHODS

Adult Fin Amputation and GSK3β Inhibitor Treatment

Zebrafish, 6-12 months of age, were obtained from EKKWill Waterlife Resources (Ruskin, FL) and used in fin regeneration experiments. Fish were anesthetized in tricaine (Ethyl 3-aminobenzoate methanesulfonate) and 50% of the caudal fin was amputated using a razor blade. Fish were placed in 2 liters of water with various concentrations of GSK3β inhibitor compound (LSN 2105786) or DMSO vehicle control and kept at 31°C to promote rapid regeneration. Tank water was replaced, and fresh compound was added daily. Tanks and fish were rinsed between treatments to remove any residual compound.

Embryo GSK3β Inhibitor Treatment

Embryos were exposed to LSN 2105786 by incubation in embryo medium containing various concentrations of LSN 2105786 from 6-28 hours post fertilization (hpf), in Petri dishes wrapped with parafilm and maintained at 28.5°C. Images were collected using using a Leica MZ12 microscope equipped with Leica DFC290 camera (Leica Microsystems Inc., Buffalo Grove, IL USA).

Fin Regeneration Measurements

At 4 and 7 dpa, fish were anesthetized and images of regenerating fins were collected using a Leica MZ12 microscope equipped with Leica DFC290 camera (Leica Microsystems Inc., Buffalo Grove, IL USA). The length of the regenerate (from the amputation plane to the distal tip of the fin) at the third, fourth and fifth dorsal and ventral fin rays were measured using IMAGE J software (NIH, http://rsb.info.nih.gov/ij/) and the average length of the regenerate calculated for each fish.

Whole-Mount *In Situ* Hybridization

Fins were collected at various time points post amputation and fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS). Fins were briefly washed two times in PBS, then dehydrated in methanol and stored at 20°C overnight. Fins were rehydrated stepwise in methanol in PBS– 0.1% Tween 20 (PBT), followed by 30 minute incubation with 10 μg/ml proteinase K in PBT. Then, fins were washed two times in PBT and refixed in 4% paraformaldehyde in PBS for 20 minutes. Fins were washed five times in PBT, and then prehybridized for 2 hours at 70°C in hybridization buffer (50% formamide, 5x SSC, 0.1% Tween 20, 50 μg/ml heparin, and 500 μg/ml yeast RNA). Following prehybridization, fins were hybridized overnight in hybridization buffer including 0.5 μg/m l digoxigenin-labeled RNA probe at 70°C. Then, fins were washed at 70°C for 10 minutes each in 75% hybridization buffer/25% 2x SSC, 50% hybridization buffer/50% 2x SSC, 25% hybridization buffer/75% 2x SSC, and 2x SSC. Next, fins were washed two times in 0.05x SSC for 30 minutes each at 70°C, followed by washes at room temperature for 5 minutes each in 75% 0.05x SSC/25% PBT, 50% 0.05x SSC/50% PBT,

25% 0.05x SSC/75% PBT, and PBT. After 2 hour incubation at room temperature in

block buffer (2 mg/ml bovine serum albumin in PBT), fins were incubated overnight in a

1:5,000 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase in block

buffer. The following day, fins were washed with PBT 5 times for 15 minutes each. Fins

were then washed with alkaline phosphatase buffer (NTMT) 3 times for 15 minutes each,

followed by incubation with NBT/BCIP substrate in NTMT to allow color development.

The development of the staining reaction was monitored carefully, and treated and

control fins were stopped at the same time to allow accurate comparison. Following

color development, fins were washed 5 times in PBT and stored in 80% glycerol in PBT.

Digoxigenin-labeled riboprobes for *shh*, *lef1*, *bmp2b*, *bmp4*, and *sox9b* were synthesized

using DIG RNA Labeling Kit (Roche, Indianapolis, IN USA) according to

manufacturer's recommendations. Images were collected using a Leica MZ12

microscope equipped with Leica DFC290 camera.

Whole-Mount Immunostaining

Fins were collected at various time points post amputation and fixed overnight at 4°C in

4% paraformaldehyde in phosphate-buffered saline (PBS). Following fixation, fins were

briefly washed several times with PBS. Next, fins were incubated overnight at room

temperature in block solution. Next, fins were incubated with primary antibody in block

solution for 1-3 days. Primary antibodies used were: mouse anti-Zns 5 (Zebrafish

International Resource Center, Eugene, OR, USA) at 1:200; mouse anti-β-catenin

(Sigma-Aldrich) at 1:300; rabbit anti-phospho-histone H3 (Chemicon International, Inc.)

at 1:500. After incubation in primary antibody, fins were washed with PBT 6 times for

10 minutes each, and then incubated overnight with secondary antibody in block solution. Secondary antibodies used were: goat anti-mouse Alexa 488 (Invitrogen); goat anti-mouse Texas Red (Invitrogen); and goat anti-rabbit Alexa 488 (Invitrogen). Fins were then washed with PBS 4 times for 10 minutes each, and incubated with 1 μM TO-PRO-3 Iodide for 1 hour at room temperature to visualize nuclei. Confocal images were acquired using a Zeiss Observer Z1 LSM 700 confocal microscope (40X 1.1 NA W or 20X 0.8 NA objectives; Carl Zeiss Microscopy, Thornwood, NY USA).

Cell Proliferation Analysis

Fin regenerates were collected at 1 and 2 dpa and stained using anti-phospho-histone H3 antibody, as described above. Phospho-histone 3 (PH3)-positive cells were counted from at least 3 fins per group. The number of PH3-positive cells per unit area was calculated for each fin.

β-catenin Staining Analysis

Fin regenerates were collected at 3 dpa and stained using anti-β-catenin antibody, as described above. Intensity measurements in the regenerating fin rays were made with ImageJ Software along rectangular (ray matched) regions of interest in the tissue immediately distal to the amputation plane.

<u>Statistical Analysis</u>

Analyses on fin regenerate length, fluorescence intensity, and cell proliferation were performed using unpaired two-tailed Student's t-test (GraphPad Software, La Jolla, CA USA).

CHAPTER 3: RESULTS

GSK3β Inhibition Increases Fin Regeneration Rate

To test the effects of enhanced Wnt/β-catenin signaling on the rate of zebrafish fin

regeneration, we treated fish with a small molecule GSK3β inhibitor (LSN 2105786).

Fish were treated for different treatment periods (1-24 hours per day) at various

compound concentrations following caudal fin amputation. Half of the caudal fin was

amputated and fish were kept in tanks at 31°C with or without compound present during

the 7 days immediately following amputation. To determine an optimal dosing regimen

for maximal regenerative outgrowth at 4 and 7 dpa, fish were treated continuously with

low concentrations (1-30 nM) or intermittently with higher concentrations (30-300 nM)

of LSN 2105786. Experiments showed that continuous exposure using low

concentrations (4-5 nM) were most effective at increasing regeneration. In both the 4 nM

and 5 nM continuous treatment groups, fin regenerates were significantly longer than

control fins at 4 and 7 dpa. Control regenerates were an average length of 1.00 mm at 4

dpa compared to 1.18 mm and 1.16 mm for the 4 nM and 5 nM treatment groups,

respectively (Figure 4). This trend continued through 7 dpa, at which point control

regenerates averaged 1.72 mm whereas the 4 nM and 5 nM treatment groups displayed

average lengths of 1.85mm and 1.95 mm, respectively (Figure 2).

Surprisingly, we did not observe any consistent effect on fin regenerate length with intermittent treatment (1 or 6 hours per day at various concentrations). Interestingly, continuous treatment with higher concentrations of LSN 2105786 inhibited fin growth.

LSN 2105786 Treatment Recapitulates Ectopic Wnt Signaling Phenotype

To confirm that LSN 2105786 was acting upon the target enzyme, GSK3β, in zebrafish, we treated embryos with various concentrations (300 nM – 5 μM) of LSN 2105786 from 6-28 hours post-fertilization (hpf) and analyzed the resulting phenotypes. It was previously shown that ectopic Wnt signaling produces a phenotype in which embryos are eyeless or the size of the eye is dramatically reduced (van de Water, van de Wetering et al. 2001). Treatment with LSN 2105786 was able to recapitulate these phenotypes, producing eye defects in a dose dependent manner, with the highest concentrations resulting in 100% eyeless embryos. The lower concentrations used resulted in variable defects, ranging from eyes that were nearly normal in appearance to eyes that were reduced in size, or in some cases completely absent (Figure 5).

GSK3β Inhibition Augments Expression of Wnt Target Genes

To determine the spatial and temporal effects of GSK3β inhibition on Wnt responsive gene expression in fin regenerates, we utilized *in situ* hybridization to examine the expression of the direct Wnt/β-catenin target gene, *lef1*, and several downstream genes: *shh*, *bmp2b*, and *bmp4*. Since continuous LSN 2105786 treatment at a concentration of 5 nM produced consistent increases in regenerative outgrowth, this concentration was used for *in situ* hybridization experiments.

We examined the expression of *lef1* at 1, 2, and 3 dpa in control and GSK3β inhibitor treated tissue. Previous studies demonstrated that *lef1* is expressed in the basal epidermal layer surrounding the blastema at 1 dpa and is thought to be involved in blastema formation. During the regenerative outgrowth phase (48 hpa and beyond), *lef1*is expressed in the distal blastema and two sets of cells on each side of the fin ray (Poss, Shen et al. 2000). In our experiments, *lef1* transcripts appeared to be more strongly expressed, and the expression domain expanded in the treated group at both 1 and 2 dpa (Figure 4). At 1 dpa, *lef1* is restricted to the area immediately surrounding the blastema in control fins; whereas, LSN 2105786 treated fins showed an expansion of *lef1* expression in to the wound epidermis and interray tissue. Interestingly, at 3 dpa, differences in staining intensity were less pronounced. However, *lef1* was detected proximal to the blastema, in the newly formed bone ray, in the treated group but undetectable in the same region of control fins (Figure 6).

We then tested the effects of LSN 2105786 treatment on expression of *shh*, a downstream target involved in bone differentiation pathways (Quint, Smith et al. 2002). Similar to *lef1*, *shh* expression is typically restricted to two sets of cells on each side of the distal tip of the fin ray during the regenerative outgrowth phase. In the LSN 2105786 treated group, an expansion of the *shh* expression domain was observed at 3 dpa, with *shh* transcripts being detectable in the tissue proximal to the blastema and extending to near the amputation plane (Figure 7).

The bone morphogenetic protein (BMP) family members, Bmp4 and Bmp2b, were previously shown to regulate skeletal development and patterning (Wan and Cao 2005). These signaling molecules play a role in osteoblast differentiation and maturation during bone formation (Canalis 2003). Previous studies revealed that both *bmp2b* and *bmp4* are expressed in the regenerating zebrafish caudal fin and disruption of BMP signaling results in decreased bone formation (Laforest, Brown et al. 1998; Murciano, Fernández et al. 2002; Smith, Avaron et al. 2006). During regeneration, *bmp4* is expressed in the distal blastema where it likely plays a role in controlling proliferation and regenerative outgrowth (Murciano, Fernández et al. 2002). In contrast, *bmp2b* is expressed in newly differentiating osteoblasts in the area of the proximal blastema, indicating that it contributes to bone formation (Laforest, Brown et al. 1998).

Our results showed faint expression of *bmp4* in control tissues at 3 dpa, whereas treated tissues displayed robust expression and a slightly expanded expression domain (Figure 6). At 4 dpa, expression of *bmp4* remained higher in treated fins as compared to controls at the same time point. Additionally, in treated fins *bmp4* was expressed in the distal most epidermis; whereas, it was undetectable in the same region of control fins (Figure 8).

Expression of *bmp2b* was not dramatically altered in response to LSN 2105786 treatment at the time points analyzed. At 3 dpa, expression in treated fins was nearly indistinguishable from that of controls (Figure 9).

GSK3β Inhibition Effects on Cell Proliferation

We next investigated the effects of GSK3β inhibition, using 5 nM LSN 2105786 continuously, on cell proliferation in the regenerating fin tissue. Anti-phosphorylated histone H3 (PH3) antibody staining, a marker for chromosome condensation during mitosis, was used to determine the number of proliferating cells in fin regenerates at 1 and 2 dpa. PH3 positive cells were counted in the regenerating tissue of both control and treated fins, and a "nuclei per unit area" value was calculated for each fin. At 1 dpa, the number of proliferating cells counted in treated and control fins was nearly identical (Figure 10). However, on the 2nd day following amputation, treated fins displayed a markedly higher number of proliferating cells than controls (Figure 11). There was an approximately 3 fold increase in the number of PH3-positive cells in treated fins relative to control at this time point (Figure 10).

GSK3β Inhibition Alters β-catenin Accumulation in Regenerating Tissue

The end point of Wnt/β-catenin signaling is stabilization and accumulation of β-catenin. To test the effects of LSN 2105786 treatment on β-catenin accumulation in regenerating fin tissue, fins were stained using a monoclonal anti-β-catenin antibody. Fish were treated continuously with either DMSO vehicle or LSN 2105786 at the following concentrations; 1 nM, 3 nM, 6 nM, 10 nM, or 15 nM. Immunofluorescence intensity was measured at 3 dpa in an identically sized region, beginning at the amputation plane and extending distally, in the 2nd and 3rd lateral rays of each fish.

LSN 2105786 treatment produced a dose dependent effect on immunofluorescence intensity (Figure 10). Immunofluorescence intensity was highest in the 6 nM treated group, which exhibited a 37% increase in average intensity over the DMSO vehicle treated control group (Figure 12). Interestingly, the highest concentration analyzed, 15 nM, produced a decrease in β-catenin expression. However, variation was high among all treatment groups and statistical analysis did not yield statistically significant differences.

CHAPTER 4: DISCUSSION

This research has demonstrated that zebrafish fin regeneration is a useful model for studying Wnt mediated bone growth and repair, and may provide an efficient drug discovery platform. Although it has been used extensively in developmental and genetic studies, zebrafish has only recently been widely utilized as a model in pharmacological studies. The small size, ease of care, and relatively low cost of zebrafish give this model the potential to be adapted for large scale bone therapeutic compound screening. Fin regeneration studies allow the generation of large amounts of data in a relatively short period of time compared to other bone repair models.

Our research shows that zebrafish fin regeneration is responsive to Wnt/β-catenin signaling modulation via GSK3β inhibition. In agreement with previous studies by Stoick-Cooper et al, our findings showed that enhanced Wnt/β-catenin signaling increases regenerative outgrowth in the zebrafish caudal fin (Stoick-Cooper, Weidinger et al. 2007). LSN 2105786 treated fish displayed as much as a 13% increase in regenerate length at 7 dpa as compared to DMSO vehicle treated controls. Similarly, it was previously shown that over activation of Wnt/β-catenin signaling in *axin1* heterozygous mutant fish produced a 10.5% increase in regenerative outgrowth compared to wild type fish at the same time point (Stoick-Cooper, Weidinger et al. 2007). Interestingly, our

results using intermittent LSN 2105786 treatment are in agreement with previously published data as well. It was previously shown that pulsed activation of a *wnt8* transgene was sufficient to increase cell proliferation, but did not affect the length of fin regenerates (Stoick-Cooper, Weidinger et al. 2007).

The inhibitory effects on regeneration observed following continuous LSN 2105786 treatment at higher concentrations invite speculation that extreme over activation of the Wnt/β-catenin pathway may excessively stimulate differentiation programs, thus depleting the progenitor cell population and preventing outgrowth. Further studies aimed at determining the threshold at which Wnt/β-catenin signaling over activation transitions from a stimulatory to an inhibitory factor of regeneration, and dissecting the molecular mechanisms regulating that process, could provide valuable insight to the role of Wnt signaling in the regenerative process.

Since zebrafish can regenerate fins normally, without intervention, the extent to which regenerative outgrowth can be augmented in healthy fish is limited. Fin regenerate length may not be sensitive enough to detect subtle variations of compound efficacy in normal, healthy fish. Fish that are defective in the regenerative process, or display abnormal bone phenotypes, perhaps due to aging, chronic alcohol, or chronic nicotine, may show a more dramatic response to Wnt/β-catenin signaling modulation, and thus provide more insight into bone repair mechanisms.

The ability of LSN 2105786 treatment to recapitulate previously characterized ectopic Wnt signaling phenotypes is consistent with LSN 2105786 acting upon the target enzyme, GSK3β, in zebrafish. Additionally, upregulation of the direct Wnt target, *lef1*, and accumulation of β-catenin in regenerating tissue provide strong evidence that LSN 2105786 treatment activates Wnt/β-catenin signaling during fin regeneration. Our attempts to directly measure GSK3β inhibitor effects using a Wnt/β-catenin signaling transcriptional reporter, TOPdGFP, zebrafish transgenic line were unsuccessful. LSN 2105786 was fluorescent and detected at the same wavelength as GFP, thus preventing quantification of GFP expression changes in response to LSN 2105786 treatment. Future studies using an alternative Wnt/β-catenin signaling transcriptional reporter, such as RFP or YFP, could be utilized to directly measure these effects.

LSN 2105786 treatment was sufficient to increase cell proliferation and augment expression of important downstream genes. One important observation from our *in situ* hybridization experiments was the upregulation of *bmp4* in response to LSN 2105786 treatment. Given the role *bmp4* plays in controlling blastema cell proliferation and outgrowth, it is likely that upregulation of *bmp4* expression plays a role in the increased cell proliferation and subsequent increased regenerative outgrowth observed following continuous LSN 2105786 treatment.

Additionally, expression of the bone differentiation pathway member, *shh*, was expanded at 3 dpa. *shh* is widely purported to be involved in the proliferation and alignment of osteoblasts in the regenerating fin. Ectopic expression of *shh* in the interray tissue was

previously shown to induce bone formation and lead to fusion of adjacent rays (Quint, Smith et al. 2002). Furthermore, it was previously shown that down regulation of *shh* using cyclopamine, a known inhibitor of *shh* signaling, drastically reduced regenerative outgrowth (Quint, Smith et al. 2002).

The *in situ* hybridization experiments performed during this research provide semi-quantitative data regarding gene expression changes following LSN 2105786 treatment; future quantitative PCR assays could be utilized to evaluate these responses with more precision. Quantitative PCR assays for additional genes involved in osteoblast differentiation, such as *runx2*, *osterix*, and *osteocalcin* would provide further details regarding the time course and extent of osteoblast differentiation events in response to GSK3β inhibition.

In summary, our analysis of the effects of GSK3β inhibition on zebrafish fin regeneration showed that enhanced Wnt/β-catenin signaling increased cell proliferation; increased β-catenin accumulation; augmented expression of Wnt target genes; and increased the length of fin regenerates at 4 and 7 dpa. Taken together, these results indicate that zebrafish fin regeneration is a useful model for studying Wnt mediated bone growth and repair, and may have clinical implications for the development of new anabolic bone growth therapies.

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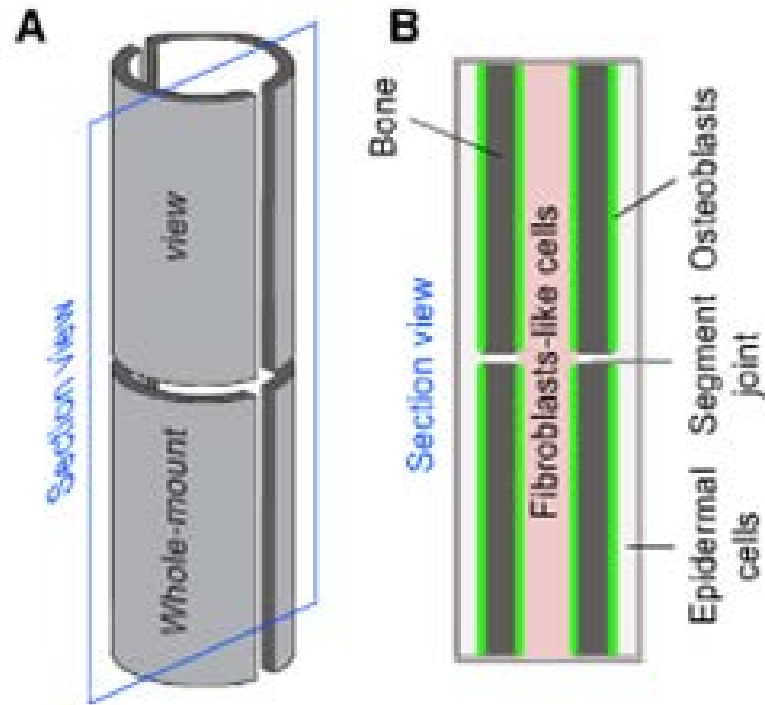
FIGURES

Figure 1: Fin Ray Structure. A: Whole-mount view of bony ray consisting of segmented concave hemirays. **B:** Longitudinal section of fin ray showing fibroblast-like cells surround by concaving facing hemirays, which are covered by a single layer of osteoblasts.

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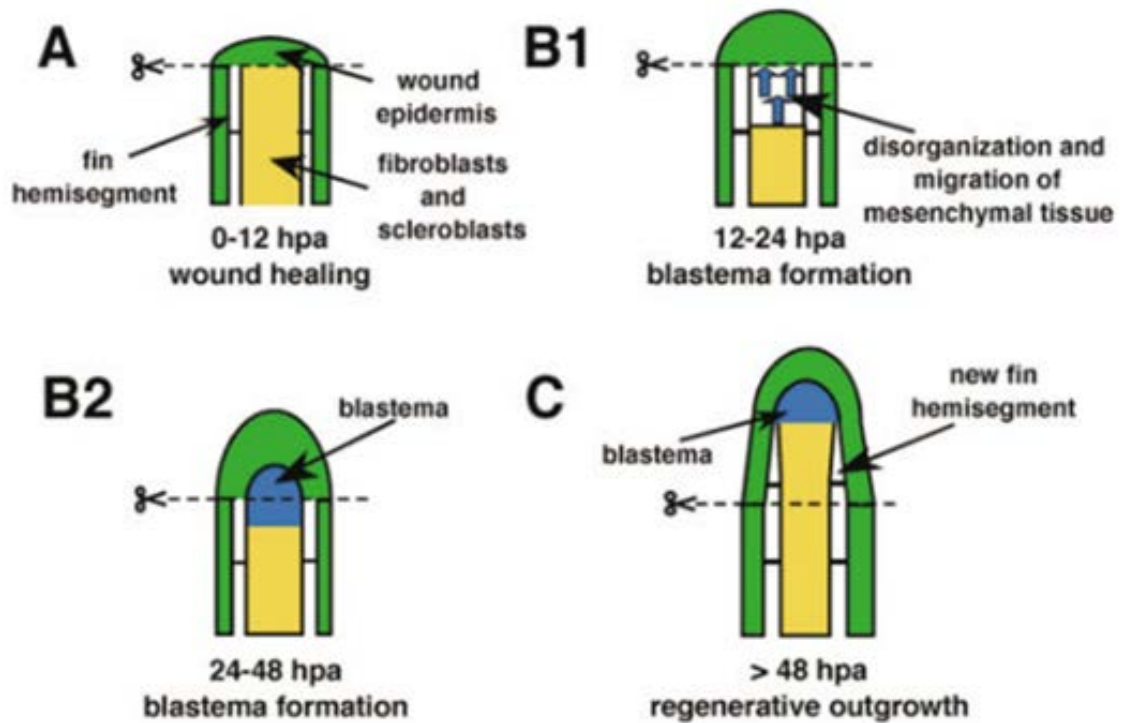
Figure 2: Phases of Regeneration. A: By 12 hpa, epithelial cells migrate to cover the wound and form the wound epidermis. **B1:** Mesenchymal tissues disorganize and migrate distally to contribute to blastema formation by 24 hpa. **B2:** A blastema forms at the distal tip of each fin ray within 24-48 hpa. **C:** At 48 hpa and beyond, regenerative outgrowth occurs as cells of the blastema proliferate, differentiate, and migrate proximally to form the new structures of the fin.

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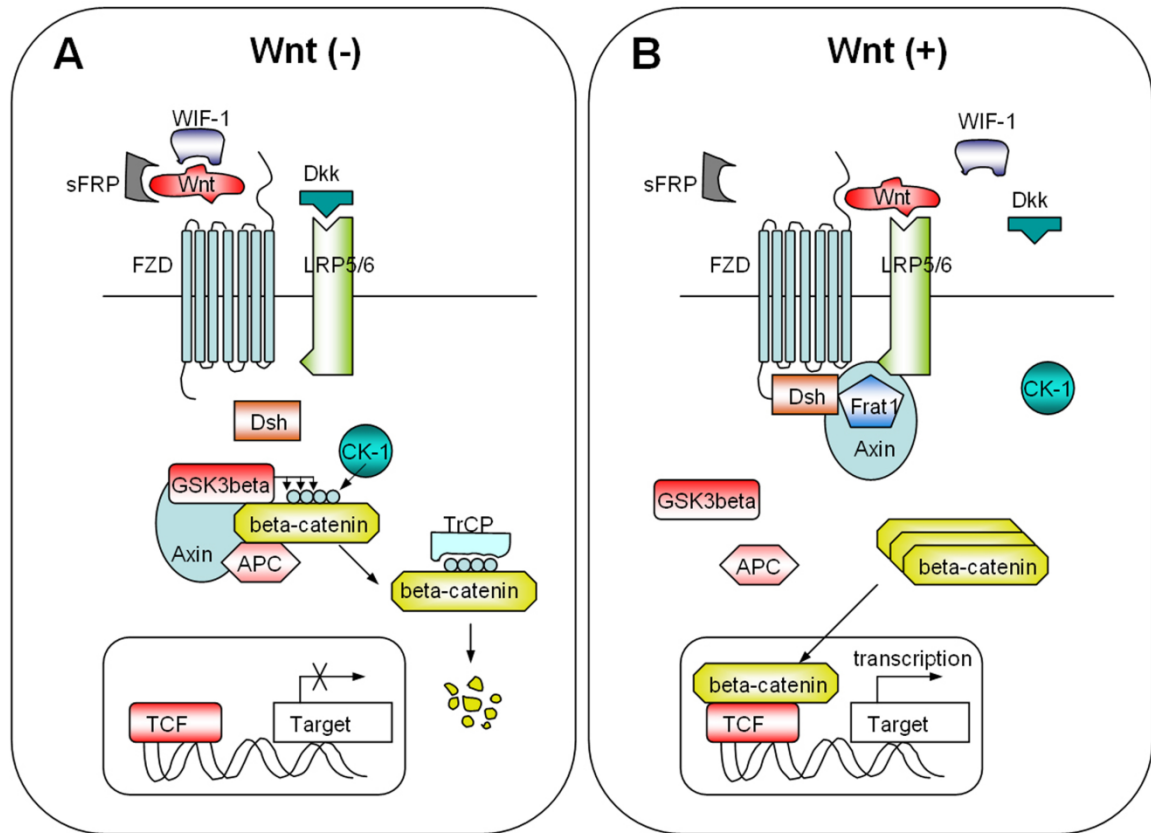
Figure 3: Wnt/β-catenin Signaling Pathway. **A:** In the absence of the Wnt ligand, β-catenin is phosphorylated by GSK3β and subsequently degraded. **B:** Binding of the Wnt ligand to the transmembrane Fz receptor and co-receptors LRP 5/6 activates Dsh which in turn inhibits the cytoplasmic "destruction complex" consisting of Axin, APC and GSK3β, leading to β-catenin accumulation and activation of Wnt target genes.

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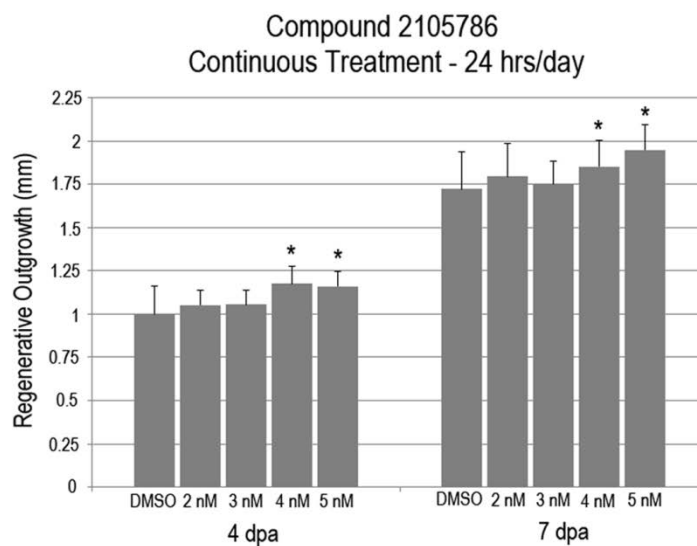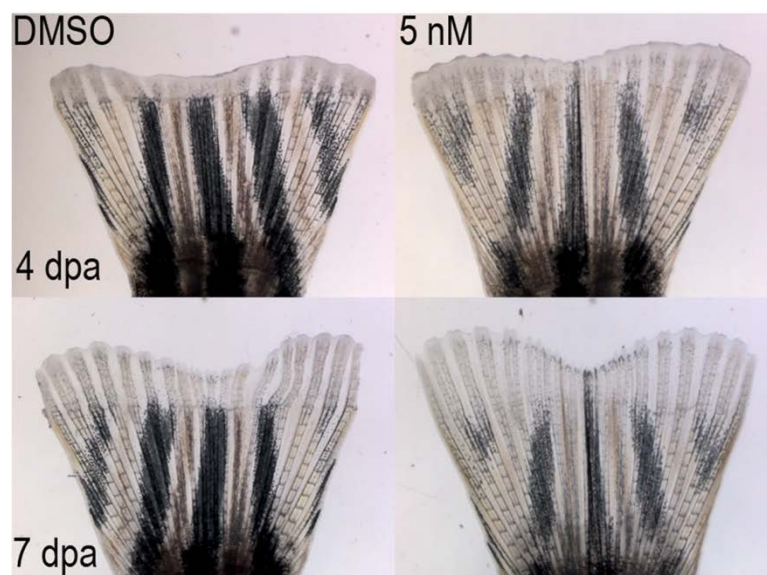
Figure 4: LSN 2105786 Accelerates Fin Regeneration. Continuous treatment with 4 nM and 5 nM LSN 2105786 produced increased regenerate lengths at 4 and 7 dpa compared to control fish. To determine the length of regenerates for each fish, the third, fourth and fifth dorsal and ventral fin rays were measured and the average length of the regenerate calculated for each fish. *, $P < 0.05$.
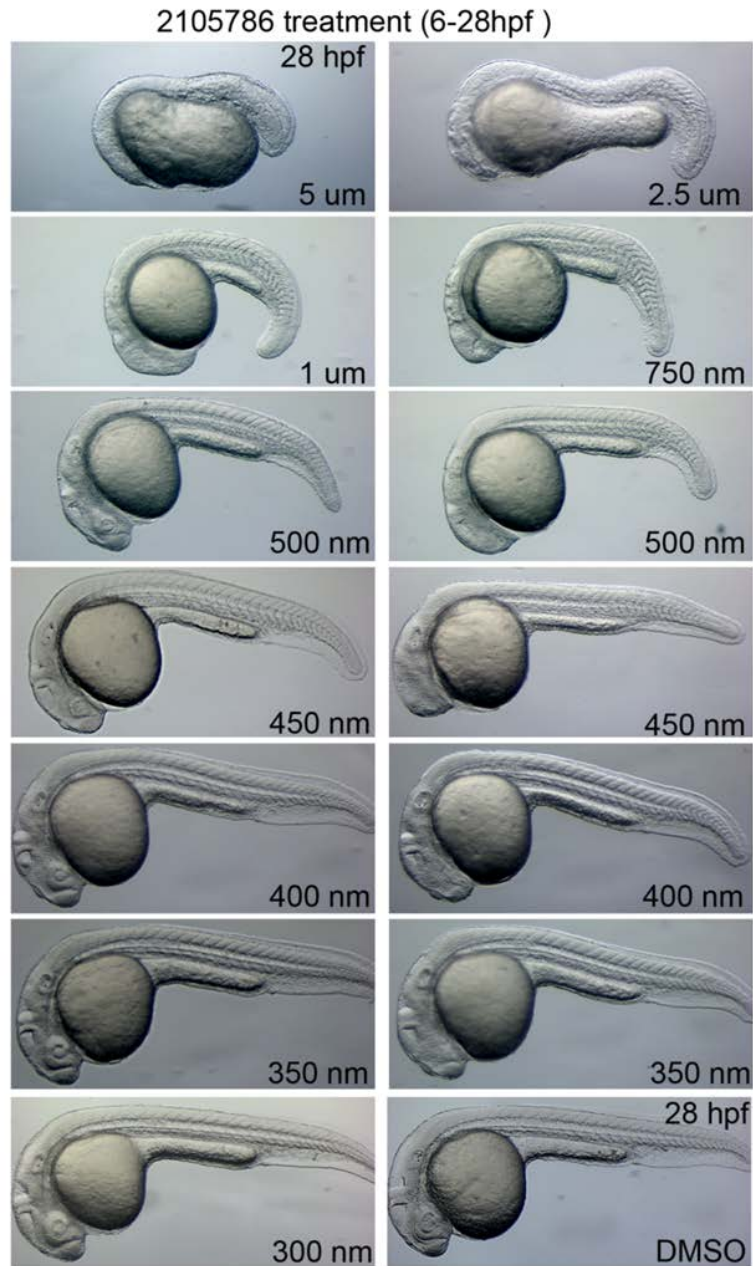
Figure 5: LSN 2105786 Recapitulates Ectopic Wnt Signaling Phenotype. Embryos treated with LSN 2105786 displayed the characteristic small or absent eye phenotype observed as a result of ectopic Wnt signaling. Eye defects varied in severity in a dose dependent manner.
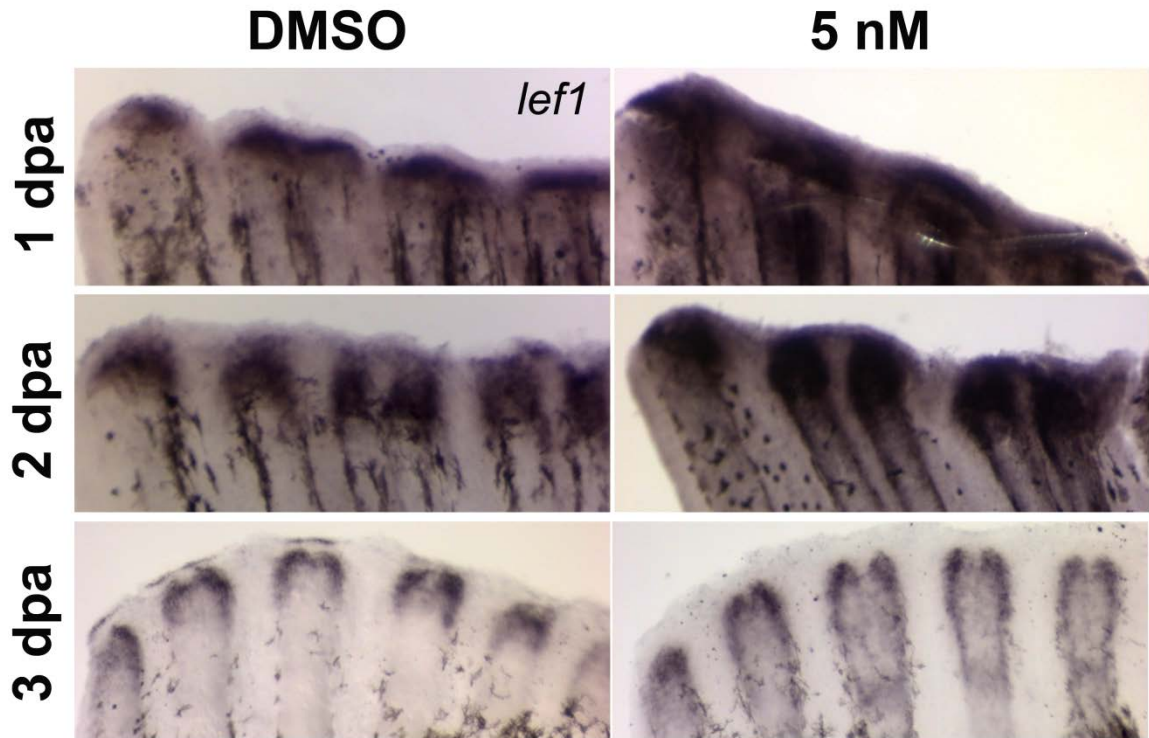
Figure 6: *In Situ* **Hybridization of *lef1*.** At 1 and 2 dpa, *lef1* was restricted to the area immediately surrounding the blastema in control fins; whereas, 5 nM LSN 2105786 treated fins showed an expansion of the expression domain as well as intensified staining. Staining intensity at 3 dpa was not significantly altered in treated fins compared to control fins; however, the expression area was expanded distally in treated fins.
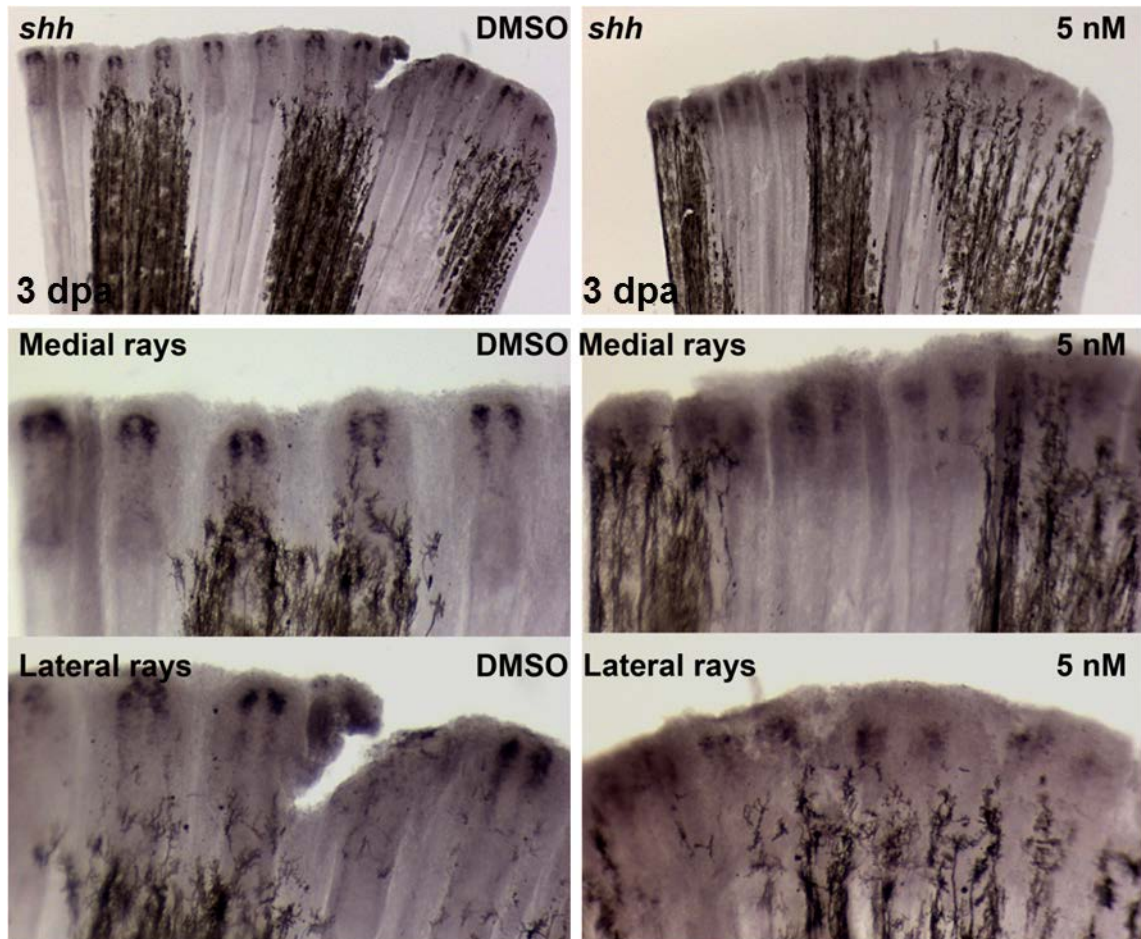
Figure 7: *In Situ* **Hybridization of** *shh***.** *shh* is expressed in two distinct subsets of cells at the distal tip of control fins at 3 dpa. Fins treated continuously with 5 nM LSN 2105786 showed a more diffuse expression pattern with *shh* transcripts being detected in the tissues proximal to the blastema.
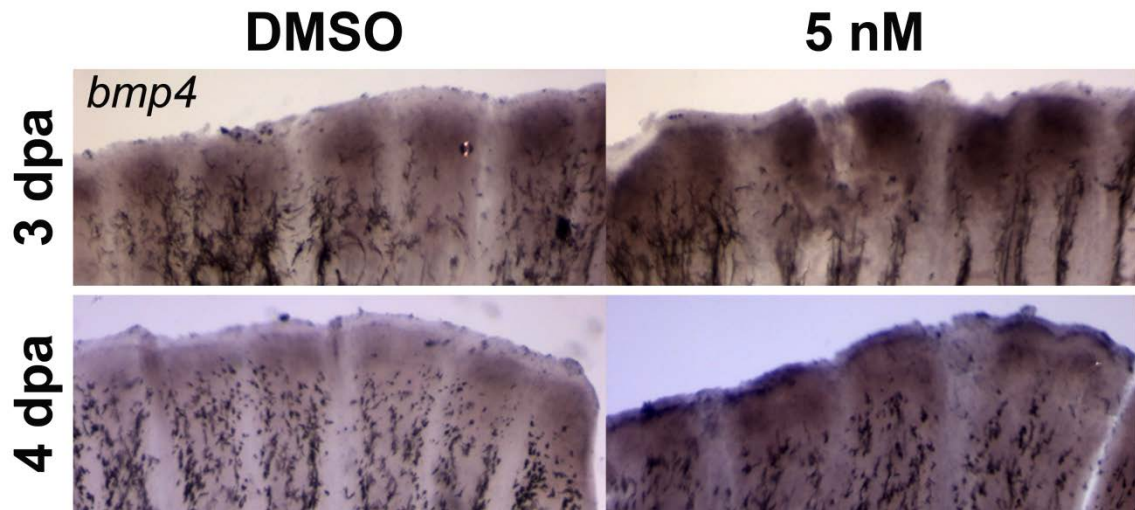
Figure 8: *In Situ* **Hybridization of *bmp4*.** *bmp4* expression was dramatically increased in the 5 nM LSN 2105786 treated fins at 3 dpa. At 4 dpa, *bmp4* was robustly expressed in the distal most epidermis of treated fins but was not detected in the same region of control fins.
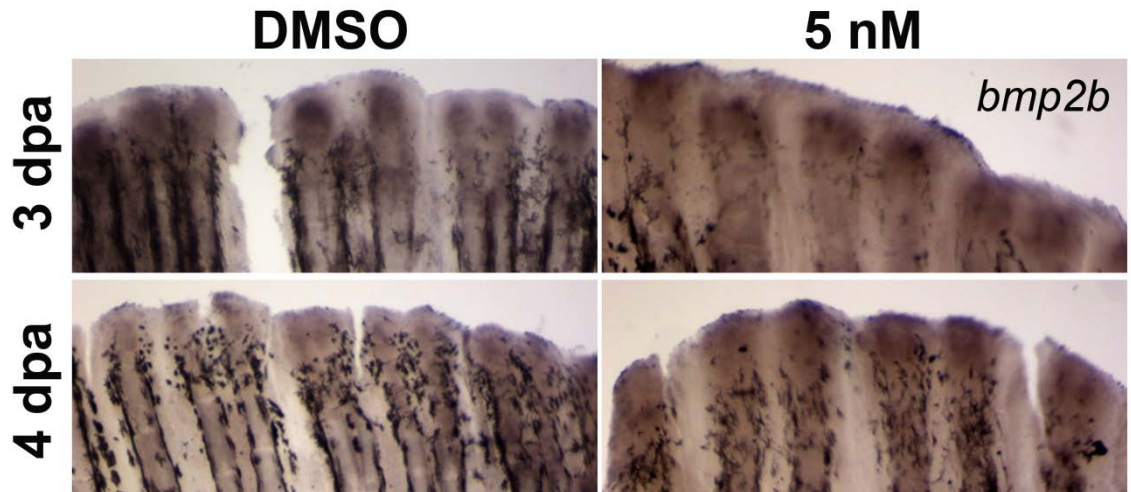
Figure 9: *In Situ* **Hybridization of *bmp2b*.** Expression of *bmp2b* was not dramatically altered in response to 5 nM LSN 2105786 treatment at 3 and 4 dpa.
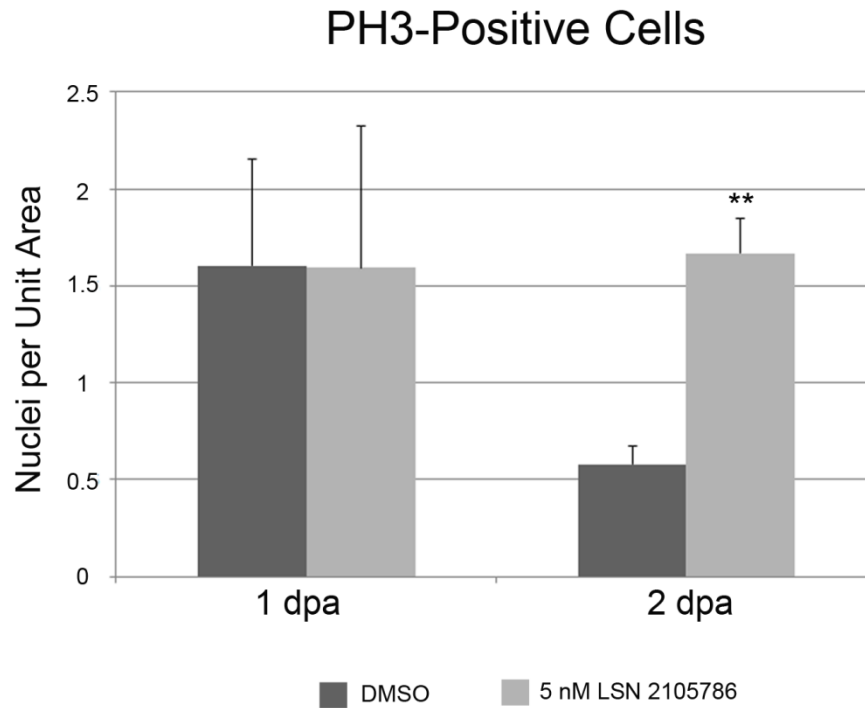
Figure 10: Cell Proliferation Analysis. Cell proliferation was analyzed using anti-phosphorylated histone H3 (PH3) antibody staining. PH3 positive cells were counted in the regenerating tissue of both control and treated fins, and a "nuclei per unit area" value was calculated for each fin. At 1 dpa, the number of PH3 positive cells was not dramatically altered in 5 nM LSN2105786 treated fins. On the 2nd dpa, the number of PH3 positive cells was drastically increased in treated fins. **, $P < 0.001$.
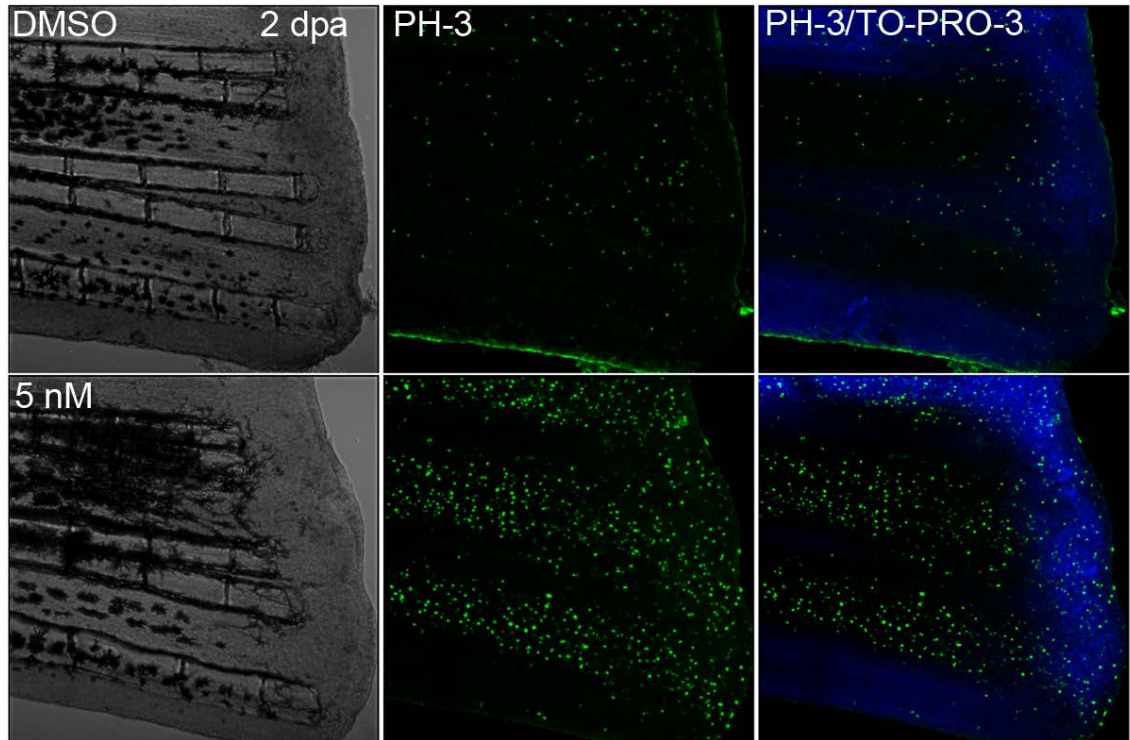
Figure 11: Anti-PH3 Staining at 2 dpa. 5 nM LSN 2105786 treatment produced increased proliferation throughout the fin at 2 dpa. Increased anti-PH3 staining was observed in both the regenerating tissue as well as the tissues proximal to the amputation plane.
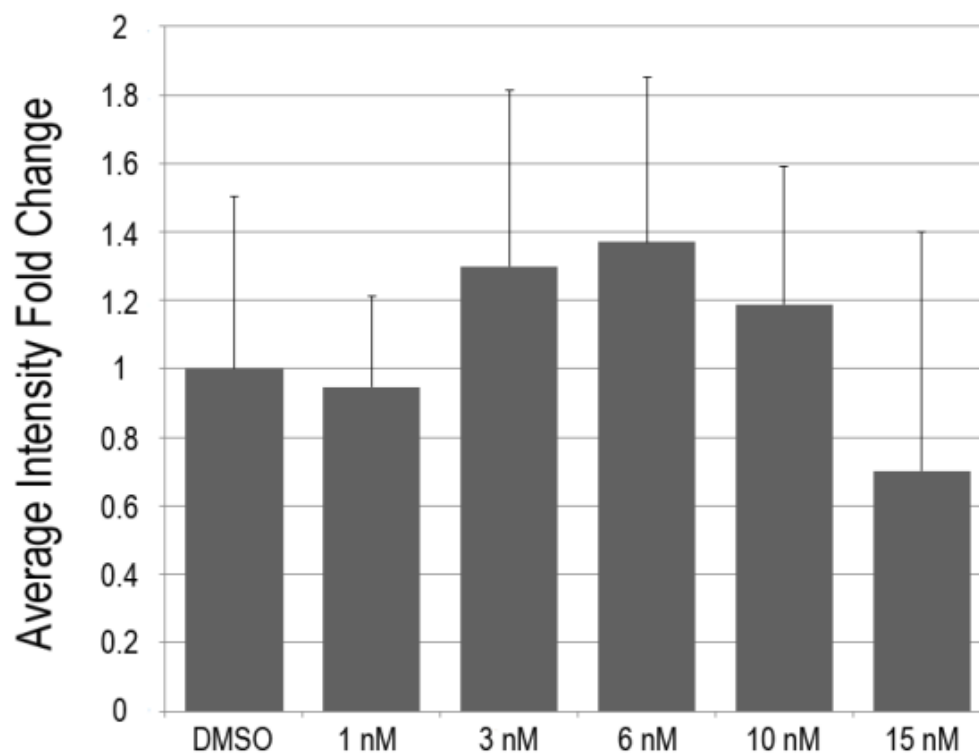
Figure 12: Immunofluorescence Intensity of Anti-β-catenin Staining. LSN 2105786
treatment produced a dose dependent effect on immunofluorescence intensity.
Immunofluorescence intensity was increased following 6 nM treatment, and decreased
following 15 nM treatment. However, statistical analysis did not yield statistically
significant differences among treatment groups.