Wnt/β-catenin Signaling Regulates Regeneration in Diverse Tissues of the Zebrafish

Nicholas Stockton Strand

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Randall Moon, Chair

Neil Nathanson

Ronald Kwon

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Abstract

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Nicholas Stockton Strand

Chair of the Supervisory Committee:

Professor Randall T Moon

Department of Pharmacology

The ability to regenerate tissue after injury is limited by species, tissue type, and age of the organism. Understanding the mechanisms of endogenous regeneration provides greater insight into this remarkable biological process while also offering up potential therapeutic targets for promoting regeneration in humans. The Wnt/β-catenin signaling pathway has been implicated in zebrafish regeneration, including the fin and nervous system. The body of work presented here expands upon the role of Wnt/β-catenin signaling in regeneration, characterizing roles for Wnt/β-catenin signaling in multiple tissues. We show that cholinergic signaling is required for blastema formation and Wnt/β-catenin signaling initiation in the caudal fin, and that overexpression of Wnt/β-catenin ligand is sufficient to rescue blastema formation in fins lacking cholinergic activity. Next, we characterized the glial response to Wnt/β-catenin signaling after spinal cord injury, demonstrating that Wnt/β-catenin signaling is necessary for recovery of

motor function and the formation of bipolar glia after spinal cord injury. Lastly, we defined a role for Wnt/β-catenin signaling in heart regeneration, showing that cardiomyocyte proliferation is regulated by Wnt/β-catenin signaling. These data demonstrate a conserved role for Wnt/β-catenin signaling in promoting regeneration across multiple tissue types in the zebrafish, and further show conservation of some of these functions in human tissue.

Table of Contents

List of Figures

List of Tables

Glossary

BtxB – Botulinum Toxin B

DPA – Days Post Amputation

DPT – Days Post Transection

GRP - Glial-Restricted Precursor

hESC - human Embryonic Stem Cell

NPC - Neural Progenitor Cell

PCR - Polymerase Chain Reaction

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1 – Introduction

1.1 – Regeneration

1.1.1 Historical Study

The loss of tissue, due to injury or disease, is a debilitating and potentially lethal problem for animals. However, some animals are capable of regenerating new cells to replace lost tissue, preserving function and life. Some of the earliest writings on the phenomena of tissue regeneration can be traced to Aristotle, who referred to lizard tail regeneration in his writings (Aristotle et al. 1965). The formalized study of regeneration is also considered to be the birth of experimental biology, advancing upon the observational cataloguing of previous generations. Abraham Trembley published the first report on experimental regenerative biology, documenting the regenerative ability of hydra, a basal freshwater cnidarian capable of regenerating its entire body after amputation (Lenhoff, Lenhoff, and Trembley 1986). This work was followed up by Charles Bonnet, who observed similar traits in annelid worms (Bonnet 1745). Lazzaro Spallanzani published the first work on tadpole and salamander regeneration, studying limb, digit, and tail regeneration (Spallanzani 1768). These foundational works helped develop the study of biology generally and regeneration specifically, and led to questions still grappled with today, including why are some animals and tissues capable of regeneration and not others, and what are the mechanisms that govern regeneration.

1.1.2 Phylogenetic Observations

The ability to regenerate tissues is observed throughout phyla, though this capacity is diminished in less basal animals. Numerous invertebrate species are capable of extensive regeneration after injury. The hydra, for example, is capable of regenerating its entire body after amputation, leading to 2 viable hydra after injury. Planaria, invertebrate flatworms, are also capable of regenerating their entire body from a small portion of their body. This ability to regenerate an entire organism is not observed in vertebrates.

Vertebrate regeneration can be observed in several classes of animals, including fish (zebrafish), amphibians (salamanders), and mammals (mice). Adult zebrafish possess the ability to regenerate several tissues after injury, including caudal fin, heart, spinal cord, retina, and brain (Stoick-Cooper, Moon, and Weidinger 2007). This ability to regenerate numerous tissue types, coupled with its genetic tractability, make the zebrafish a popular model for studying the molecular signals necessary for regeneration. Similarly, salamanders are used to study the regeneration of the tail, hind and forelimb, and spinal cord after injury (Todd 1823). In contrast to the remarkable regenerative capacity of zebrafish and salamanders, mammals possess limited regenerative capabilities. Adult mice are capable of regenerating their digit tips, liver, and skin after injury (Takeo et al. 2013). Furthermore, neonatal mice are able to regenerate their hearts after injury (Porrello et al. 2011). The species, age, and tissue-specific nature of regeneration allows for utilizing different models for comparing and contrasting the mechanisms of regeneration, including attempts at rescuing regeneration in regeneration-deficient tissues.

1.2 – Mechanisms of Regeneration

1.2.1 Whole Organism

Annelids, hydra, and planarians are capable of full body regeneration injury, and the planaria *Schmidtea mediterranea* is one of the most highly studied models of whole organism regeneration. Planaria possess a large number of totipotent stem cells, referred to as clonogenic neoblasts. These neoblasts exist as a slowly proliferating stem cell population that responds to injury by increasing proliferation and producing the cell types necessary to regenerate the entire body, including germ cells (Wagner, Wang, and Reddien 2011; Y. Wang et al. 2007). Planaria are also amenable to gene knockdown through RNAi treatment, which has been used to discover regulators of stem cell proliferation and differentiation. One such regulator of regeneration is the hedgehog signaling pathway (Rink et al. 2009). Decreasing hedgehog signaling leads to the loss of tail regeneration, while increasing it leads to defects in head regeneration, including the formation of a tail in place of the head, demonstrating a role for hedgehog signaling in posterior specification during planarian regeneration. Experiments like this demonstrate the robust regenerative potential of planarians and how they can be leveraged to better understand stem cell biology and tissue regeneration.

1.2.2 Appendage

Several vertebrates are capable of regenerating their appendages in full, a trait exemplified by the salamander limb and the zebrafish caudal fin, which both undergo a process known as epimorphic regeneration. After injury, a layer of epidermal cells covers the amputation plane,

referred to as the wound epidermis or wound epithelium. The wound epithelium later becomes the apical epithelial cap (AEC), a morphologically and genetically distinct structure necessary for regeneration (Han et al. 2005). The AEC is thought to produce the signals necessary to form the blastema, a highly organized mass of lineage-restricted progenitor cells necessary for outgrowth (Tsonis 1996). Differentiated cells are able to dedifferentiate after injury and migrate to the injury site before undergoing proliferation and subsequent outgrowth of the injured appendage (Jeremy P Brockes and Kumar 2002). During outgrowth, proliferating cells differentiate into the tissues necessary for reforming the appendage, including bone, blood vessels, and epidermis.

The cellular and molecular responses that facilitate epimorphic regeneration have been the focus of much attention in the zebrafish, including looking at the formation of the blastema. The blastema is where the majority of proliferation and differentiation occur in the regenerating appendage. Work has been focused on the origin of the cells in the blastema, the molecular cues necessary for blastema formation, and the organization of cells in the blastema. Lineage tracing experiments have demonstrated that some cells in the blastema are formed via dedifferentiation of other cell types, including osteoblasts and fibroblasts, but not epidermal cells (Stewart and Stankunas 2012; Knopf et al. 2011) . Dedifferentiated osteoblasts are lineage restricted, producing new bone but no other tissues (Knopf et al. 2011). However, depletion of osteoblasts does not alter regeneration, suggesting that osteoblasts may also arise through de novo differentiation (Singh, Holdway, and Poss 2012). This suggests that dedifferentiation is not the only source of blastemal cells and that different compartments of the blastema likely arise from different cell types. While the mechanism of dedifferentiation has yet to be elucidated, it

has been shown that the proliferation of pre-osteoblasts is dependent on Fgf signaling (Knopf et al. 2011).

Fgf signaling has multiple roles in the regenerating zebrafish caudal fin, including patterning the wound epidermis (Y. Lee et al. 2009). Fgf signaling is necessary for the induction of shh and wnt5b, which establish the different compartments of the wound epithelium. While the wound epithelium has not been experimentally tested to contribute to zebrafish blastema formation, work in the salamander has shown that AEC formation is necessary for blastemal proliferation (Tsonis 1996). Indeed, perturbation of Fgf signaling inhibits formation of the blastema in the zebrafish caudal fin (K D Poss et al. 2000), but has not yet been directly linked with the wound epithelium through tissue-specific perturbations.

Recent data show that the blastema is also organized into compartments with different roles in regeneration (Wehner et al. 2014). After blastema formation, actively proliferating cells are observed primarily in the proximal blastema, while the distal blastema shows no active proliferation. Distal to the proliferative region of the blastema is a region characterized by differentiation. These regions are also defined by signaling pathway activation, including the notch signaling pathway. Active notch signaling, defined by expression of target gene her4.3, is observed only in the proliferative region of the blastema: inhibition of notch signaling results in defects in proliferation, while overexpression of notch inhibits outgrowth by inhibiting differentiation (Grotek, Wehner, and Weidinger 2013; Münch, González-Rajal, and de la Pompa 2013). These data demonstrate that both the epithelium and blastema are highly organized, and that perturbation of that organization is detrimental to regeneration.

While zebrafish and salamanders are able to regenerate entire appendages, mammals possess limited appendage regeneration. It has been shown that mammals can regenerate digit tips when the amputation spares part of the nail (Borgens 1982; Neufeld and Zhao 1993). This process is dependent in part on BMP signaling (Yu et al. 2010). BMP regulates expression of SDF-1α, which in turn is necessary for recruitment of cells to the blastema (J. Lee et al. 2013). BMP signaling is not required for the formation of the blastema in zebrafish, but is required for regeneration of tissues after blastema formation (Thorimbert et al. 2015), indicating differences in molecular signals governing regeneration of mammalian digit tips and zebrafish fins.

1.2.3 Spinal Cord

The spinal cord possesses differing regenerative capacity across different vertebrates: zebrafish and salamanders are capable of full locomotor recovery after spinal cord injury, but mammals show limited functional recovery after injury. Much work has been done to uncover the mechanisms of zebrafish spinal cord regeneration, focusing on both the generation of new neurons and the extension of axons through the injury site (T. Becker et al. 1997; Reimer et al. 2008). Axon regeneration through the injury site is necessary for functional recovery, as disruption of this process leads to deficits in swimming (C. G. Becker et al. 2004; Goldshmit et al. 2012). Axon extension through the injury site is dependent on the formation of bipolar glia, which bridge the injury site and act as a scaffold for axons to cross the injury site (Goldshmit et al. 2012). Inhibiting motor neuron regeneration has no effect on functional recovery: inhibiting shh signaling and upregulating notch signaling both severely reduce motor neuron generation

but have no observable effect on swimming ability, suggesting that motor neuron regeneration is dispensable for locomotor recovery (Reimer et al. 2009; Dias et al. 2012).

In contrast to zebrafish, mammals exhibit little axonal or neuronal regeneration. Following injury, glial hypertrophy is observed, forming a glial scar that physically and chemically impairs axonal regeneration in mice, rats, and humans. Reactive astrocytes, a major component of the glial scar, express chondroitin sulfate proteoglycans (CSPGs), a group of proteins that actively inhibit axon regeneration (Bradbury and Carter 2011; Silver and Miller 2004). Treatment with chondroitinase ABC (ChABC) dissolves the glycosaminoglycan sidechains of CSPGs, which enhances neurite outgrowth in rats with spinal cord injury and enhances functional recovery (Bradbury et al. 2002), demonstrating the inhibitory role of reactive astrocytes after spinal cord injury. Interestingly, ablation of reactive astrocytes leads to an increase in injury size and a decrease in functional recovery (Faulkner et al. 2004; Anderson et al. 2016). These data suggest that reactive astrocytes are necessary for limiting tissue damage after injury, but their role in forming the glial scar may prevent subsequent regeneration through the injury site.

1.2.4 Heart

Mechanisms of endogenous heart regeneration have been studied extensively in zebrafish and mouse models. Both the zebrafish and neonatal mice are capable of generating new cardiomyocytes after heart injury, while adult mice respond to heart injury by forming a large, collagenous scar, similar to humans. Studies on the cellular and molecular mechanisms of heart regeneration in neonatal mice and zebrafish have led to insights on developing therapies to generate new cardiomyocytes after injury.

In both zebrafish and neonatal mice, differentiated cardiomyocytes re-enter the cell cycle and proliferate to form the regenerated myocardium (Porrello et al. 2011; Kikuchi et al. 2010; Jopling et al. 2010). In the zebrafish, the epicardium upregulates genes involved in heart development, which leads to proliferation of the epicardium (Chablais et al. 2011; Schnabel et al. 2011; J. Wang et al. 2011; González-rosa et al. 2011; Lepilina et al. 2006). Interestingly, ablation of the epicardium inhibits myocardium regeneration, but epicardial cells do not differentiate into new cardiomyocytes (J. Wang et al. 2015; Kikuchi, Gupta, et al. 2011). Instead, lineage tracing experiments show that the new myocardium is formed from pre-existing cardiomyocytes (Kikuchi et al. 2010; Jopling et al. 2010). Cardiomyocytes that express *gata4* have specifically been shown to produce many of the new cardiomyocytes in the zebrafish heart (Kikuchi et al. 2010; Gupta et al. 2013). These data suggest that cardiomyocytes undergo either dedifferentiation or transdifferentiation in the zebrafish heart after injury to generate new cardiomyocytes. This is contrasted by adult mammalian cardiomyocytes, which show very limited proliferation after injury, instead exhibiting signs of cardiac hypertrophy (Soonpaa and Field 1997; Senyo et al. 2014). However, when cell cycle regulators or regulators of signaling pathways are manipulated in cardiomyocytes, some of the cardiomyocytes re-enter the cell cycle and actively proliferate (Campa et al. 2008; Bersell et al. 2009; Engel et al. 2006; Kuhn et al. 2007; Pasumarthi et al. 2005; Chaudhry et al. 2004). When some of these targets are activated after myocardial infarction, increases in heart function and cardiac repair are observed (Bersell et al. 2009; Kuhn et al. 2007; Cheng et al. 2007). These data suggest that adult mammalian cardiomyocytes retain the ability to re-enter the cell cycle, similar to the zebrafish response after injury.

1.3 Wnt/β-catenin Signaling

1.3.1 Canonical Wnt Signaling Pathway

Since the discovery of Wnt proteins, much work has been done to elucidate the mechanism of Wnt signaling. Wnt signaling has several potential signaling pathways, but a majority of the work has been done on the Wnt/β-catenin signaling pathway, also referred to as the canonical Wnt signaling pathway (unless otherwise indicated, Wnt signaling will be used to refer to Wnt/β-catenin signaling). In the absence of ligand, β-catenin is phosphorylated by GSK3B and CK1a in complex with disheveled, axin and APC, referred to as the destruction complex. Phosphorylated β-catenin is ubiquitinated by BTrCP and degraded by the proteasome, preventing signaling from occurring (Aberle et al. 1997; Kitagawa et al. 1999). When Wnt ligand binds to its coreceptors, LRP6 and a Fzd isoform, BTrCP separates from the destruction complex and the destruction complex is recruited to the membrane and blocked from degrading β-catenin (Li et al. 2012). β-catenin is then able to accumulate and translocate into the nucleus, where it binds with TCF and Lef proteins to drive transcription. This process also has numerous points to control Wnt signaling. Secreted antagonists of Wnt/coreceptor binding, such as Dkk and sFRP, are able to prevent signal transduction into the cytosol, inhibiting Wnt signaling. Other signaling pathways are also able to act upon canonical Wnt signaling to inhibit the pathway, including non-canonical Wnt signaling and retinoic acid signaling. Furthermore, Wnt signaling can lead to the transcription of Wnt inhibitors, including naked and axin, a part of the destruction complex. These facets make Wnt signaling an interesting pathway to study, both alone and in conjunction with other phenomena.

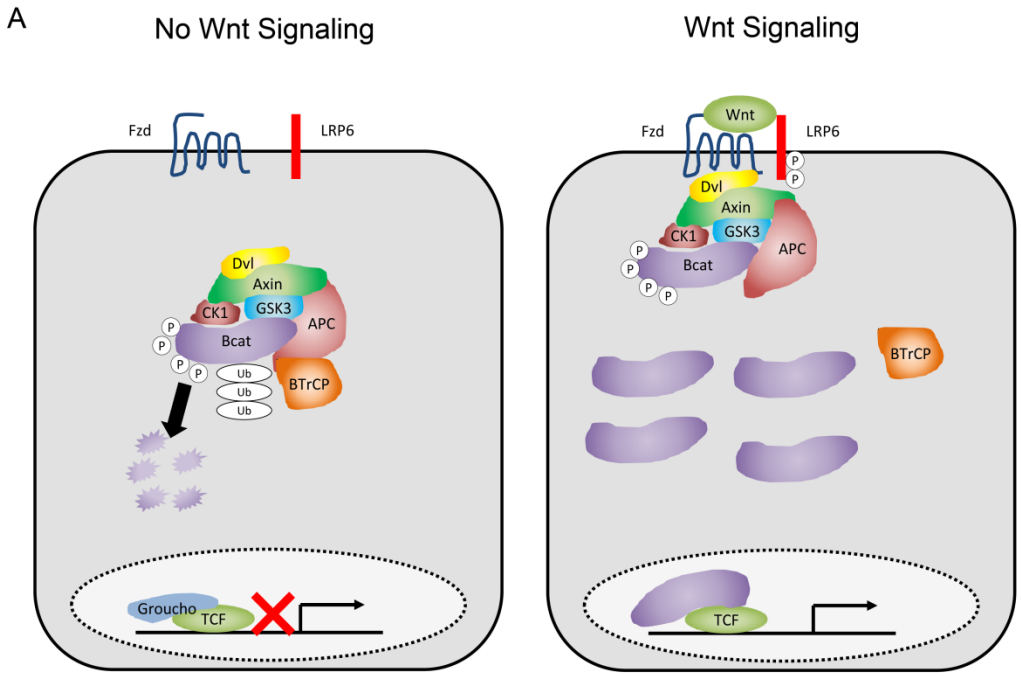

Figure 1.1: Canonical Wnt signaling schematic. (A) In the absence of Wnt ligand, β*-catenin is phosphorylated and subsequently degraded by the destruction complex (APC, Axin, GSK3, CK1, Dvl, and BTrCP). When Wnt ligands bind coreceptors Fzd and LRP6, the destruction complex separates from ubiquitin ligase BTrCP and relocates to the receptors, preventing* β*-catenin degradation.* β*-catenin subsequently accumulates in the cytosol and translocates to the nucleus, where it binds TCF proteins to promote transcription.*

1.3.2 Functions in Regeneration

Wnt signaling has been highly implicated in tissue regeneration across numerous phyla and tissue types. RNA interference in planaria demonstrate that β-catenin is necessary for tail determination in planaria (Petersen and Reddien 2008; Gurley, Rink, and Sanchez Alvorado 2008). Reduction of β-catenin or disheveled inhibits β-catenin signaling and leads to head

formation in lieu of the tail, and conversely reduction of APC activates β-catenin signaling and leads to the formation of a tail in lieu of the head. Further study of Wnt gene expression demonstrates a complex transcriptional response to amputation in the planaria (Gurley et al. 2010). It has been shown that wnt1 is expressed early after amputation along the length of the amputation plane, which is followed by different wnt11 genes during tail regeneration. sFRP genes are also expressed during head regeneration, offering a mechanism for Wnt/β-catenin inhibition in head regeneration (Gurley et al. 2010).

Wnt signaling has also been studied extensively in zebrafish caudal fin regeneration. Inhibition of Wnt signaling results in the loss of fin regeneration after amputation, and inhibition of Wnt signaling after blastema formation still results in a partial inhibition of regeneration (Kawakami et al. 2006; Stoick-Cooper et al. 2007). An in-depth study of β-catenin responsive cells shows that several different cell types respond to Wnt signaling, including osteoblast precursors, non-proliferating distal blastema cells, and actinotrichia-forming cells (Wehner et al. 2014). In the osteoblast precursors, Wnt inhibition results in a loss of EMT and dedifferentiation of osteoblasts (Stewart et al. 2014). Wnt signaling is also necessary for induction of several other signaling pathways, including FGF, Shh, IGF, and RA signaling (Wehner et al. 2014). These experiments demonstrate the importance of Wnt signaling in zebrafish fin regeneration.

Wnt signaling has also been implicated in the regeneration of other zebrafish tissues, including neural tissues. In the regenerating retina, Ascl1a inhibits dkk expression and stimulates wnt4a expression after injury (Ramachandran, Zhao, and Goldman 2011). Inhibition of Wnt signaling leads to a decrease in Müller glia-derived progenitor cells and proliferating cells, demonstrating

its role in retina regeneration. Wnt signaling has also been studied in the regenerating spinal cord of larval zebrafish, looking specifically at neurogenesis from radial glia. A tamoxifen-inducible cre protein was expressed in GFAP+ cells, which include radial glia in the larval spinal cord. After spinal cord injury, the lineage tracing shows that new neurons are formed from GFAP+ cells after injury, and that process is reliant on Wnt signaling (Briona et al. 2015). These experiments demonstrate a role for Wnt signaling in cell fate determination in the regenerating zebrafish nervous system.

Wnt's role in regeneration is also conserved in mammals. Mammals are able to regenerate digit tips, and this is due to a niche of stem cells in the proximal nail epithelium that are required for the formation of the blastema after amputation (Takeo et al. 2013). These cells also provide localized canonical Wnt ligand release, which is necessary for blastema formation in the digit tip (Takeo et al. 2013). As canonical Wnt signaling is necessary for blastema formation in zebrafish (Kawakami et al. 2006; Stoick-Cooper et al. 2007), the role of Wnt signaling in regenerating tissues appears to be conserved across phyla.

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2 - Cholinergic Activity is Required for Blastema Formation & Wnt/β-catenin Signaling in the Regenerating Zebrafish Caudal Fin

2.1 Introduction

The ability to regenerate appendages after injury varies among species. In mammals, appendage regeneration is limited primarily to the tips of digits. In contrast, more phylogenetically basal animals such as zebrafish and salamanders are capable of regrowing entire appendages, i.e. fins or legs, to the preinjury state through a process known as epimorphic regeneration. Epimorphic regeneration is a highly regulated process involving the dedifferentiation of existing cells (Knopf et al. 2011; Stewart and Stankunas 2012; Sousa et al. 2011), cellular proliferation, and reformation and coordinated patterning of multiple tissue types, including skin, bone, muscle, nerves, and blood vessels. Epimorphic regeneration proceeds by wound healing, followed by formation of a blastema - a group of mesenchymal progenitor cells and the primary site of proliferation for the regenerating limb, and finally, outgrowth, differentiation, and patterning of the new tissue (Stoick-Cooper, Moon, and Weidinger 2007).

Cells in the nervous system interact with the wound epidermis and blastema to help orchestrate appendage regeneration, but the mechanism by which the nervous system influences regeneration is poorly understood. Neuronal innervation of injured tissues is required for regeneration in fish, amphibians, and mammals (Suzuki et al. 2005; Simões et al. 2014; Todd 1823; Rinkevich et al. 2014; Mahmoud et al. 2015; Meda et al. 2015). In the zebrafish pectoral fin, physical denervation impairs wound healing and blastema formation, leading to poor regeneration (Simões et al. 2014). Neural tissues produce trophic factors that promote dedifferentiation and proliferation of the regenerating limbs (Kumar, Gates, and Brockes 2007; Kumar et al. 2007; Fior 2014; J P Brockes and Kintner 1986; L. Wang, Marchionni, and Tassava 2000). For example, both Prod1 and nAG proteins are produced by Schwann cells in the regenerating salamander limb, and denervation of the limb inhibits the increase in nAG after limb resection (Kumar, Gates, and Brockes 2007; Kumar et al. 2007). Furthermore, nAG overexpression is able to rescue regeneration in a denervated and amputated limb, defining an important molecular mechanism of nerve-dependent regeneration (Kumar et al. 2007). Though nerve presence is required for regeneration of appendages, the mechanisms by which neurons influence regeneration are not fully understood. How nerves mediate limb regeneration and what cellular and molecular mechanisms are governed by the nervous system remains to be addressed.

One potential pathway regulated by the nervous system during regeneration is the Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is necessary for regeneration of zebrafish fins and retinae, mouse digit tips, and planarian bodies (Stoick-Cooper et al. 2007; Kawakami et al. 2006; Ramachandran, Zhao, and Goldman 2011; Takeo et al. 2013; Petersen and Reddien

2008). In the zebrafish fin, genetic experiments have demonstrated that Wnt/β-catenin signaling is necessary for the formation of the blastema and subsequent regeneration of tissues (Stoick-Cooper et al. 2007; Wehner et al. 2014; Stewart et al. 2014; Petrie et al. 2014). Wnt/β-catenin signaling promotes the activity of several other signaling pathways necessary for diverse aspects of regeneration, including wound epidermis patterning by Fgf and Igf pathways; maintenance and proliferation of blastemas by Fgf, RA, Notch, and Hh pathways; and bone differentiation and patterning by Bmp and Hh signaling pathways (Wehner et al. 2014; Stewart et al. 2014). Furthermore, it has been postulated that Wnt/β-catenin signaling may be decreased in denervated pectoral fins after amputation (Simões et al. 2014). However, whether neurons regulate Wnt/β-catenin signaling and by what mechanism neurons regulate Wnt/β-catenin signaling has not been studied.

Here, we found that spinal cord transection inhibited blastema formation and subsequent regeneration. This inhibition occurred in the absence of denervation of the fin, suggesting that neuronal activity, rather than physical nerve presence, was required for regeneration. Localized injection of Botulinum Toxin B (BtxB), a drug that inhibits neuronal cholinergic signaling, also inhibited fin regeneration in a similar manner. Both spinal cord transection and injection of BtxB inhibited Wnt/β-catenin signaling and transgenic overexpression of Wnt8a activated Wnt/β-catenin signaling and partially rescued fin regeneration in fish with spinal cord transection. These data suggest that cholinergic activity plays a role in zebrafish blastema formation and regeneration in zebrafish caudal fins and illustrates an example of coupling between neuronal electrical activity and activation of a morphogenetic pathway, which may represent a paradigm in other developing or regenerating tissues.

2.2 Results

Spinal Cord Transection Inhibits Fin Regeneration

To study the role of the nervous system in regulation of caudal fin regeneration in adult zebrafish, we surgically transected the spinal cord, which causes paralysis caudal to the lesion site (T. Becker et al. 1997; Goldshmit et al. 2012). We transected the spinal cord approximately 4mm caudal to the brain stem immediately before amputating the distal half of the caudal fin (Fig. 1A). In addition, we analyzed caudal fin regeneration in fish that received no spinal operation (uninjured fish) and in fish that received sham operations, in which the body wall was opened but the spinal cord was not transected (sham fish) (Fig. 1A). To determine if spinal cord transection affected caudal fin regeneration, we measured the total area of new tissue and the length of individual bone rays after fin amputation. Both the rate of caudal fin regeneration (Fig. 1B) and the average bone length at 14 days post amputation (dpa) (Fig. S1A) were reduced in spinal cord transected fish compared to uninjured or sham fish. Moreover, 40% of the caudal fin rays had less than 25% regrowth at 14 dpa after spinal cord transection (Fig. S1B), showing a robust loss of regeneration. Thus, spinal cord transection inhibits caudal fin regeneration in the zebrafish.

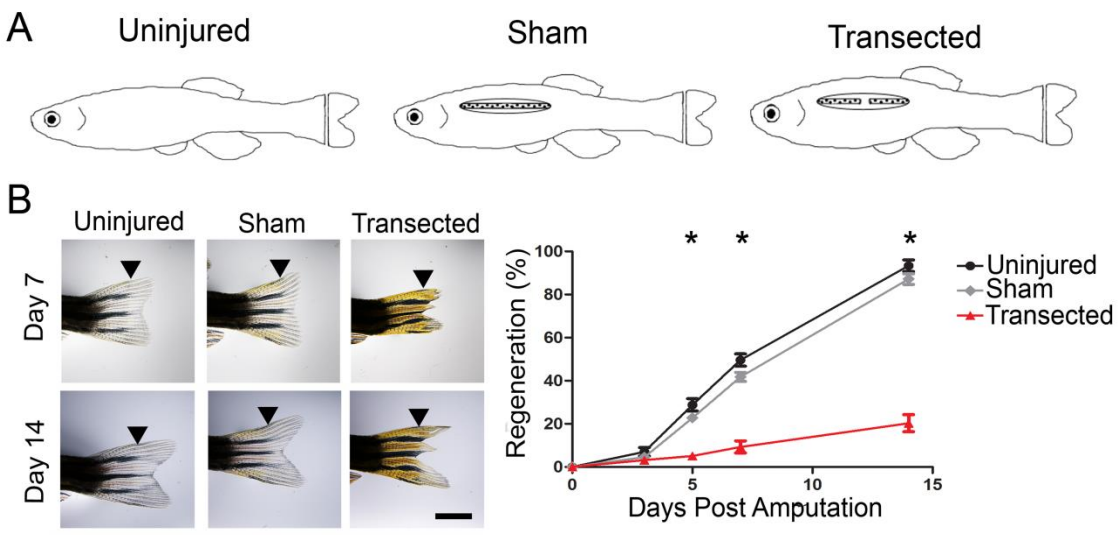

*Figure 2.1: Spinal cord transection impairs caudal fin regeneration. (A) Schematic of surgical groups. (B) Transection of the spinal cord correlates with a severe impairment of caudal fin regeneration, as quantified by total fin area regeneration. *P < 0.05; black triangles indicate amputation plane; scale bars, (B) 5mm; n≥9. Graph shows mean ± SEM.*

Injection of Botulinum Toxin B Inhibits Fin Regeneration

Reduced fin regeneration in spinal cord transected fish could be due to the loss of motor neuron signaling or due to the physical loss of nerve fibers. Physical denervation inhibits regeneration of zebrafish pectoral and caudal fins, as well as salamander limbs (Simões et al. 2014; Todd 1823; Meda et al. 2015). However, we found that labeling for acetylated tubulin, which marks mature axons (Simões et al. 2014), was similar in caudal fins at two days post amputation (dpa) in spinal cord transected fish compared to sham and uninjured control fish (Fig. 2A), demonstrating that spinal cord transection did not cause physical denervation in

regenerating caudal fins. This led us to hypothesize that motor neuron signaling may be necessary for early steps of fin regeneration.

Botulinum Toxin B (BtxB) inhibits the release of acetylcholine, the primary neurotransmitter involved in motor neuron signaling. Recently, we demonstrated that a single low dose (0.1U in 5µL), localized injection of BtxB into the base of the zebrafish caudal fin before amputation reduces the elongation of bones close to the injection site; however, injection of low doses of BtxB did not affect blastema formation in this study (Recidoro et al. 2014; Watson and Kwon 2015). Increasing the dose or injection volume of Botulinum toxins, including BtxB, expands the paralytic region (Kim et al. 2003) and may produce a more complete and wide-spread blockade of cholinergic motor neuron signaling. Thus, we injected a higher dose and volume (0.5U in 20µL) of BtxB into the medial portion of the caudal fin stump before amputation of the caudal fin. These high dose BtxB-injected fish consistently showed reduced growth of new tissue compared to uninjected or vehicle (PBS)-injected fish (Fig. 2B). However, similar to our previous study (Recidoro et al. 2014), the inhibition of regeneration by high-dose BtxB injection appeared localized near the injection site.

Individual fin rays form separate blastemas and regenerate independently of neighboring rays (V. P. Yin and Poss 2008). To formally test if BtxB injection disrupts regeneration in a local manner, we injected BtxB into either the medial or dorsal caudal fin stump, amputated fins, and measured regeneration of individual bone rays. BtxB injections into the medial fin stump resulted in inhibition of regeneration in rays of the medial fin; likewise, dorsal BtxB injections into the fin stump resulted in inhibition of regeneration in rays of the dorsal fin and exhibiting a

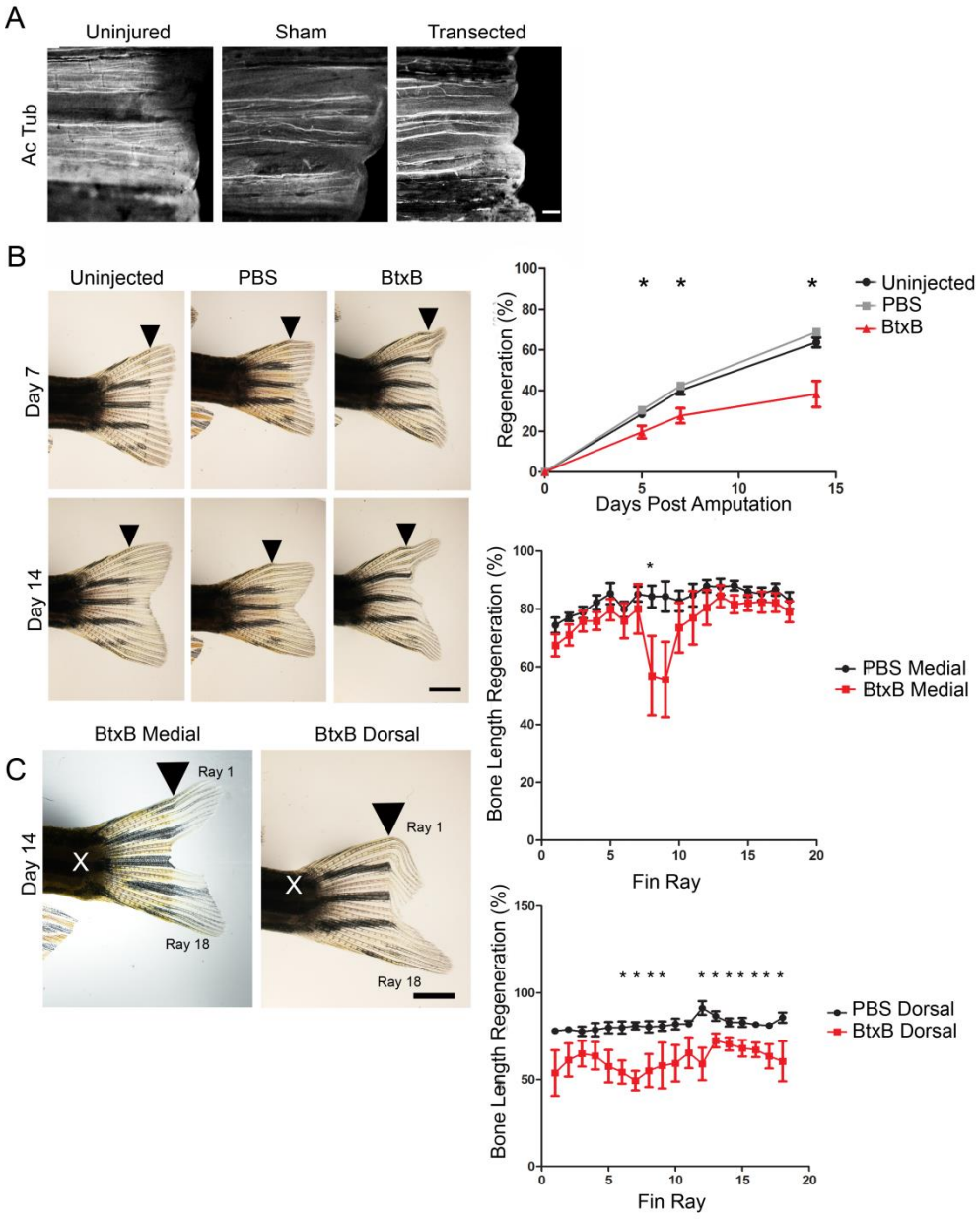

Figure 2.2: BtxB administration inhibits fin ray regeneration localized to the injection site. (A) Spinal cord transection does not cause denervation in the caudal fin at 2 days post amputation. (B) Injection of 0.5U BtxB into the caudal peduncle inhibits caudal fin regeneration, as quantified by total area regeneration. (C) Medial BtxB-injected fish

*display a loss of regeneration in the medial rays of the fin, whereas dorsal BtxB-injected fish display inhibition of fin ray regeneration starting more dorsally. *P < 0.05; black triangles indicate amputation plane, white X indicates injection site; scale bar; (A) 100µm, (B and C) 5mm; n≥5 for (B and C). Graphs show mean ± SEM.*

modest inhibition extending through much of the fin (Fig. 2C). Importantly, focal BtxB injection caused a severe loss of fin ray regeneration localized to the injection site, (Fig. 2C), suggesting that cholinergic signaling is necessary for early regeneration events.

Zebrafish are capable of repairing their spinal cord after injury and regaining motor control of the caudal fin by 14 days after spinal cord transection (Goldshmit et al. 2012). However, a subset of fish do not regain motor control after injury, which may be attributed to either age or misalignment of the spine after injury (T. Becker et al. 1997). We predicted that caudal fins amputated after the spinal cord had regenerated and neuronal activity was restored to the fin would regenerate normally. We transected the spinal cord and amputated the caudal fin on the same day. We measured the regrowth of caudal fins and scored swimming behaviors in fish at 14 dpa and observed no difference in regenerative area between fish that regained motor control of the tail and fish that did not regain motor control (Fig. S2A). Then, we re-amputated the caudal fin at 14dpa and measured the amount of new tissue 28 days after the initial surgery, 14 days after the second amputation. We found that fish that regained tail motor control at 14dpa had more total regenerated area at 28dpa (14 days post second amputation) compared to fish that failed to regain tail motor control or fish that did not have their fin

reamputated (Fig. S2B). These data show that in spinal cord transected fish, regaining muscle movement enables the caudal fin to regenerate.

Spinal Cord Transection Inhibits Blastema Formation, Proliferation, and Gene Transcription in the Amputated Caudal Fin

We also investigated the molecular and cellular mechanisms of caudal fin regeneration regulated by spinal cord transection. To test if early events in fin regeneration are disrupted by spinal cord transection, we compared regenerating fins of spinal cord transected fish and control sham or uninjured fish at 1 and 2 dpa, during wound closure and blastema formation. Blastema formation and wound epithelium thickening were reduced at both 1 and 2 dpa in spinal cord transected fish compared to uninjured fish (Fig. 3A and S3A). We performed in situ hybridization for *lef1*, which is expressed in the wound epithelium at 1 dpa (Kenneth D Poss, Shen, and Keating 2000). *lef1* expression was reduced in spinal cord transected fins at 1 dpa (Fig. S3B), consistent with the loss of wound epithelium thickening. We also labeled sections of regenerating fins for PCNA, a marker of proliferating cells in the fin (Takasaki, Deng, and Tan 1981; Kenneth D Poss et al. 2002). At 2dpa, we observed a decrease in PCNA-positive (PCNA+) cells in fin rays of spinal cord transected fish compared to fin rays in sham and uninjured fish (Fig. 3B). We also performed quantitative PCR on regenerating fins at 1 and 2 dpa from sham and spinal cord transected zebrafish. The abundance of transcripts for *pcna* and *mps1*, which are markers of proliferation, were decreased at both 1 and 2 dpa in fins from spinal cord transected fish compared to sham fish (Fig. 3C), further supporting the hypothesis that spinal

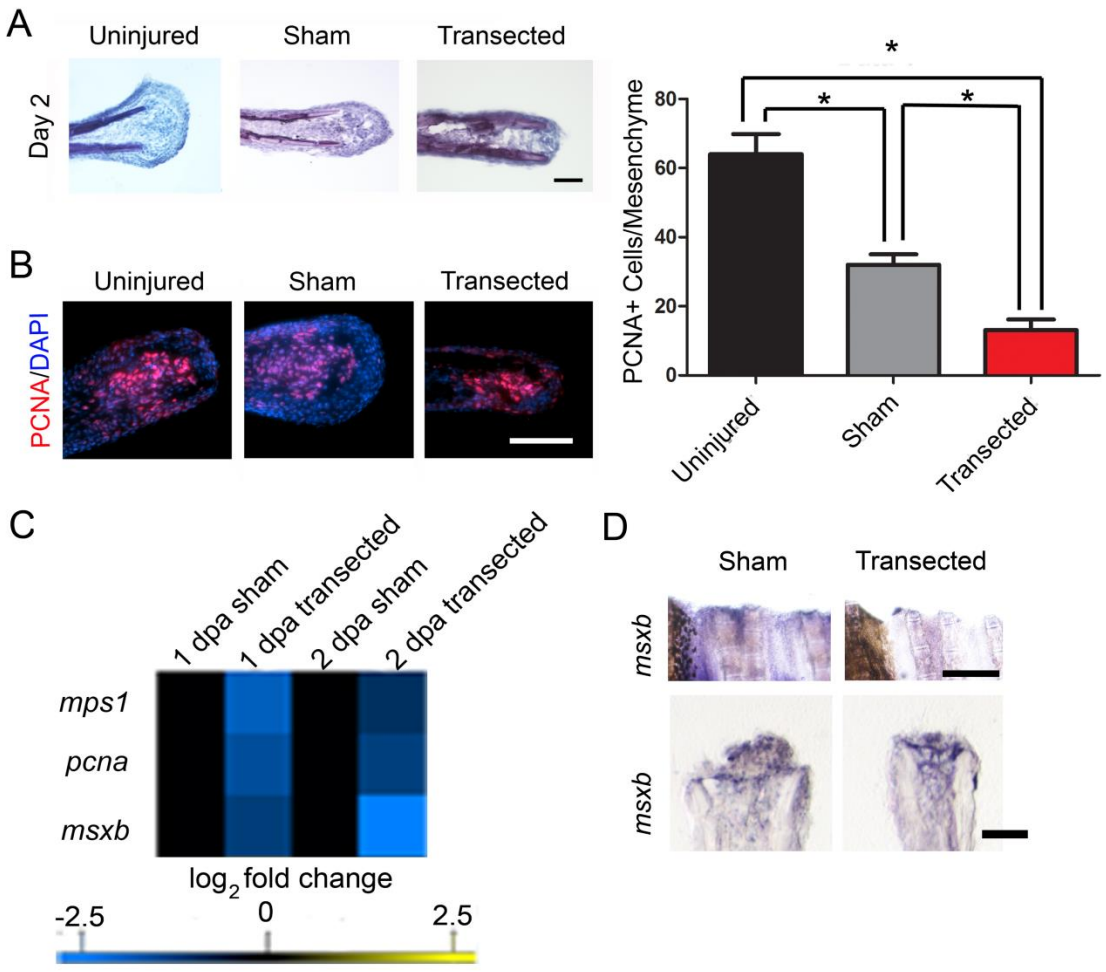

*Figure 2.3: Spinal cord transection inhibits blastema formation and proliferation in the amputated fin. (A) Blastema formation is inhibited in caudal fins after spinal cord transection. (B) PCNA staining is decreased at 2 days post amputation in zebrafish blastemas after caudal spinal cord transection. (C) Gene expression levels of proliferation markers (mps1 and pcna) and a blastema marker (msxb) are decreased in the regenerating fin of spinal cord transected fish compared to sham. (D) in situ hybridization of msxb in the 1 day post amputation fins of sham and spinal cord transected fish.(*P <*

0.05; (A, B, and D section) 100µm, (D whole mount) 1mm; n≥9 for (A and B), n≥3 for (C and D). Graph shows mean ± SEM.

cord transection disrupts proliferation in the amputated fin. Moreover, the abundance of *msxb* transcripts, which are expressed in the blastema, was decreased in fins at 1 and 2 dpa from spinal cord transected fish compared to sham fish (Fig. 3C and 3D), providing additional evidence for the loss of blastema formation in the caudal fin of fish with a spinal cord transection (K D Poss et al. 2000; Smith et al. 2008).

Spinal Cord Transection and BtxB Injection Inhibit Wnt/β-catenin Signaling in the Amputated Fin

We and others have shown that Wnt/β-catenin signaling is required for caudal fin regeneration in zebrafish (Stoick-Cooper et al. 2007; Kawakami et al. 2006). Wnt/β-catenin signaling in the distal compartment of the blastema acts as a signaling organizer for the regenerating tissue by coordinating patterning and proliferative signals (Wehner et al. 2014). Therefore, we tested whether Wnt signaling was impaired in regenerating fins in spinal cord transected fish. The abundance of *wnt10a* transcripts increases during early stages of regeneration in the caudal fin (Stoick-Cooper et al. 2007). However, the expression of *wnt10a* was decreased at 1 and 2 dpa in fins from spinal cord-transected fish compared to sham fish (Fig. 4A and 4B). Moreover, the expression of the Wnt/β-catenin target genes *axin2* and *fgf20a* was decreased at 1 and 2 dpa in fins from spinal cord transected fish compared to sham fish (Fig. 4A). *wnt5a*, which is a feedback inhibitor of Wnt/β-catenin signaling in the regenerating fin (Stoick-Cooper et al. 2007), was not significantly regulated in fins from spinal cord transected fish compared to sham

control fish(Fig. 4A). These data suggest that Wnt/β-catenin signaling in the regenerating caudal fin is inhibited by spinal cord transection.

To determine if Wnt/β-catenin signal transduction in regenerating fins is disrupted by spinal cord transection, we analyzed activation of a heterologous transcriptional reporter of Wnt/β-catenin signaling in Tg(7x*TCF-xla.Siam:nlsmCherry)*^{ia5} transgenic fish [Tg(*TCFsiam:mCherry)* fish] (Moro et al. 2012). We found a decrease in Wnt/β-catenin reporter activity in the distal caudal fin at 3 dpa in spinal-cord transected Tg(*TCFsiam:mCherry)* fish compared to sham-operated and uninjured Tg(*TCFsiam:mCherry)* fish (Fig. 4C). Furthermore, the abundance of *mcherry* transcripts in spinal cord transected fish was reduced compared to sham and uninjured fish at 1 dpa (Fig. S4A), showing that spinal cord transection is inhibiting early Wnt/β-catenin signaling in the amputated caudal fin.

We injected BtxB into the medial part of the caudal fin stump of Tg(*TCFsiam:mCherry)* fish to see if BtxB also inhibited Wnt/β-catenin signaling in the amputated fin. BtxB injection decreased Wnt/β-catenin reporter activity in the medial part of the regenerating fin at 3 dpa compared to a similar injection of PBS (Fig. 4D). Thus, both spinal cord transection and injection of BtxB inhibited the activation of Wnt/β-catenin signaling after caudal fin amputation.

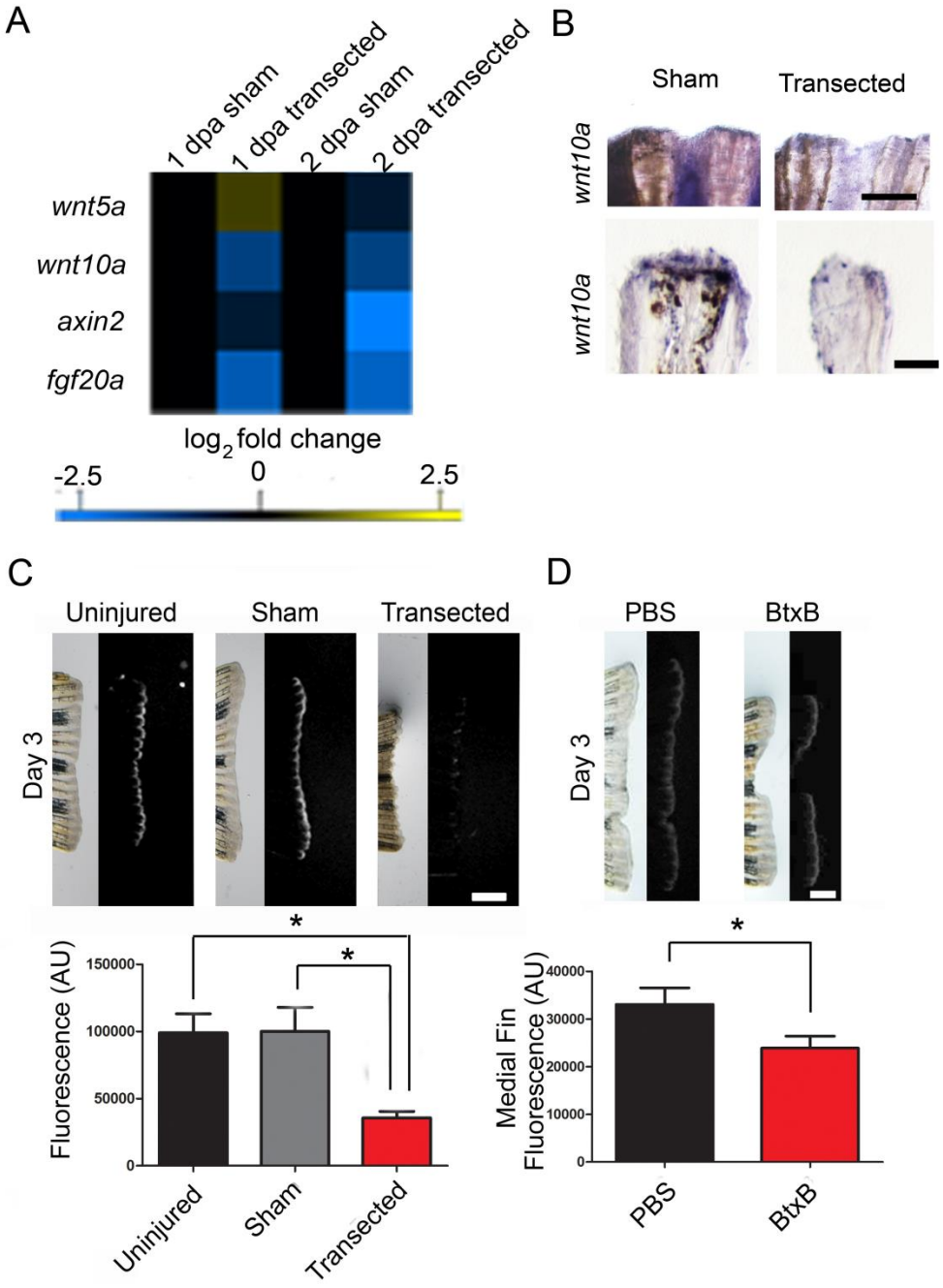

Figure 2.4: Spinal cord transection inhibits Wnt/β-catenin signaling in the amputated fin.

(A) Gene expression levels of Wnt/β-catenin ligands and target genes (wnt10a, axin2, fgf20a) are decreased, while non-canonical Wnt ligand wnt5a is not significantly altered

*in the regenerating fin of spinal cord transected fish compared to sham. (B) in situ hybridization of wnt10a in the 1 day post amputation fins of sham and spinal cord transected fish. (C) mCherry expression in the regenerating fin of β-catenin reporter Tg(TCFsiam:mCherry) fish at 3 days post amputation is decreased after spinal cord transection. (D) mCherry expression in the medial part of the regenerating fin of β-catenin reporter Tg(TCFsiam:mCherry) fish at 3 days post amputation is decreased after injection of 0.5U of BtxB in the medial caudal peduncle. *P < 0.05; (B section) 100µm, (B whole mount, C and D) 1mm; n≥3 for (A and B) n≥4 for (C and D). Graphs show mean ± SEM.*

Elevation of Wnt/β-catenin Signaling Partially Rescues Fin Regeneration in Fish with Spinal Cord Transection

We investigated whether activation of Wnt/β-catenin signaling could enhance regeneration in fish with spinal cord transection. We used Tg(*hsp70l:wnt8a-GFP*)^{w34} fish [Tg(*hsp70l*:*wnt8a-GFP*) fish], which express Wnt8a under the control of a heat-shock-inducible promoter (Weidinger et al. 2005), to activate Wnt/β-catenin signaling in the regenerating caudal fin (Weidinger et al. 2005; Stoick-Cooper et al. 2007). We heat-shocked Tg(*hsp70l*:*wnt8a-GFP*) fish 4 hours prior to fin amputation and spinal cord transection and proceeded to heat shock daily from 2dpa to 14 dpa. Ubiquitous overexpression of Wnt8a increased caudal fin regeneration in spinal cord-transected heat-shocked Tg(*hsp70l*:*wnt8a-GFP*) fish (HsWnt8+) compared to spinal cord transected non-heat shocked Tg(*hsp70l*:*wnt8a-GFP*) fish (No Heat Shock) or spinal cord transected heat-shocked wild-type fish (HsWnt8-) (Fig. 5A). Interestingly, the total area of the

regenerated fin in Wnt8a overexpressing fish with spinal cord transections was similar to that in wild-type sham operated fish (Fig. 5A), though we did observe defects in the interray areas. We verified that Wnt/β-catenin target genes and ligands were increased in the fins of heat-shocked Tg(*hsp70l*:*wnt8a-GFP*) fish at 1 dpa (Figure S5A) Moreover, we observed increases in markers of cell proliferation (*mps1*, *pcna*) and blastema cell populations (*msxb*) (Fig. S5A). In fins at 3 dpa in spinal cord transected fish, reporter fluorescence was increased in heat-shocked Tg(*hsp70l*:*wnt8a-GFP*) and Tg(*TCFsiam:mCherry)* double transgenic fish compared to those with no heat shock or heat-shocked Tg(*TCFsiam:mCherry)* single transgenic fish (Fig. S5B). Moreover, regeneration occurred in all fin rays of wnt8a-overexpressing fish analyzed and the overall bone length regeneration was greater in wnt8a overexpressing fish compared to heat-shocked controls (Figure S5C-D), suggesting Wnt8a may be sufficient to rescue blastema formation in spinal cord transected fish. Consistent with this observation, we found that Wnt8a overexpression in spinal cord transected fish formed blastemas, whereas non-Wnt8a overexpressing fish with spinal cord transection did not by 3dpa (Fig. 5B). Thus, Wnt/β-catenin signaling is likely a mechanism downstream of the defects caused by spinal cord transection that promotes blastema formation and subsequent regeneration of injured caudal fins.

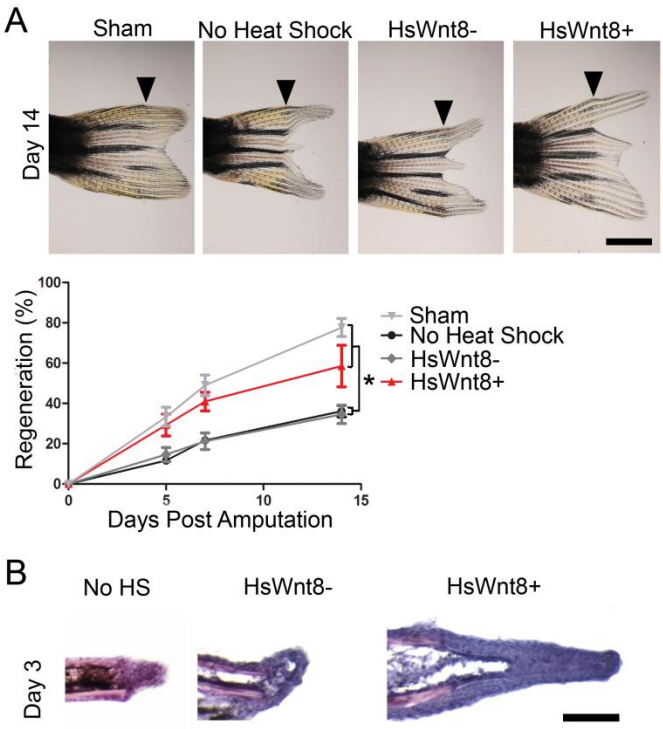

*Figure 2.5: Canonical Wnt8a overexpression rescues loss of regenerating rays after spinal cord transection. (A) Wnt8a overexpression enhances regenerative area after spinal cord transection to non-transected sham surgery levels, compared to non-heat shocked and wild type heat shock controls. (B) Wnt8a overexpression rescues blastema formation in amputated fins at 3 days post amputation after spinal cord transection. *P < 0.05; black triangles indicate amputation plane; scale bars (A), 5mm, (B) 200um; n≥5 for all experiments. Graph shows mean ± SEM*

2.3 Discussion

The mechanism of how nerves influence regeneration has been an open question for several decades. Whereas experiments using physical denervation produce severe defects in regeneration, studies using pharmacological means to inhibit cholinergic activity have shown either modest inhibition or no effect on regeneration. Systemic injection of Botulinum toxin A, which inhibits acetylcholine release by cleaving SNAP-25 and interfering with vesicle fusion, had no effect on adult urodele forelimb regeneration (Drachman and Singer 1971). However, systemic injection of hemicholinium-3, which blocks pre-synaptic choline reuptake and reduces acetylcholine production, reduced elongation of the larval urodele hindlimb after amputation (F. Hui and Smith 1970). These data suggest that cholinergic activity is not an important contributor to salamander appendage regeneration. We show here that spinal cord transection, which causes severe loss of motor function, and BtxB injection, which inhibits cholinergic motor neuron signaling, both inhibit fin regeneration and Wnt/β-catenin signaling. Our data, in conjunction with our previous findings on the role of cholinergic signaling in bone elongation (Recidoro et al. 2014), suggest that cholinergic activity is necessary for appendage regeneration in the zebrafish caudal fin, both in blastema formation and bone elongation, providing an interesting contrast in the two model systems. Although our study implicates cholinergic signaling in fin regeneration, the fin is believed to be innervated primarily by sensory neurons from the dorsal root ganglion (Meda et al. 2015). Thus spinal cord transection and/or BtxB injection could also affect these neurons. Understanding if the activity of sensory neurons is necessary to their function in regeneration would provide an interesting comparison to our study, as inhibition of cholinergic signaling mimics the phenotypes observed in denervation

experiments (Meda et al. 2015; Simões et al. 2014). It is also possible that some of the sensory neurons innervating the caudal fin are cholinergic in nature, but this has not been tested in the zebrafish.

There are several potential mechanisms that may explain the necessity of cholinergic activity for appendage regeneration. Acetylcholine is the primary neurotransmitter released by spinal motor neurons that form synapses with skeletal muscle cells. Our data showed that local injection of BtxB, which inhibits acetylcholine release, inhibits fin regeneration. Thus, it is possible that acetylcholine released by neurons acts as a pro-regenerative, "trophic-like" factor upstream of morphogens, either at the neuromuscular junction or at synapses with other cell types. However, microinfusion of acetylcholine into denervated and amputated urodele limbs did not restore blastema formation (Singer 1960), suggesting that acetylcholine may not be a trophic factor or stimulate the release of trophic factors in neuronal activity-mediated regeneration. These mechanisms have yet to be tested in zebrafish.

An alternative mechanism for neuronal activity-dependent regeneration is the co-release of acetylcholine and Wnt ligands from neurons. We show here that cholinergic activity is necessary for Wnt/β-catenin signaling in the amputated fin. It is possible that neurons in the fin or caudal fin stump secrete Wnt ligands. For example, LTP-inducing tetanic stimulation leads to Wnt3a release from hippocampal neurons in the mouse, an effect that depends on NMDA receptor signaling (Chen, Chang, and Tang 2006). Furthermore, various neuronal sub-populations, including motor neurons, transcribe genes encoding Wnt ligands (Krylova et al. 2002; Agalliu et al. 2009). *wnt10a*, which rapidly increases in transcription after injury, can be inhibited by overexpression of axin1, suggesting that *wnt10a* is also a Wnt/β-catenin target

gene (Wehner et al. 2014). These data suggest that neurons may be a source of Wnt ligand release that stimulates *wnt10a* expression after fin amputation and merits further investigation.

Perturbation of cholinergic signaling may also disrupt the immune response. Previous work has shown that physical denervation of the neonatal mouse heart or pharmacological inhibition of cholinergic signaling in zebrafish inhibits heart regeneration and alters the immune response (Mahmoud et al. 2015). Future studies are necessary to determine if cholinergic signaling may be regulating the immune response, which could be regulating Wnt/β-catenin signaling in the regenerating fin.

Overexpression of Wnt8a partially rescued fin regeneration in spinal cord transected fish, but not defects in the interray tissue of regenerating fins. Thus, cholinergic signaling may also affect signaling pathways in addition to Wnt/β-catenin signaling during regeneration. The sonic hedgehog pathway, which is necessary for bone elongation during regeneration, is perturbed by BtxB injection into the caudal fin stump (Quint et al. 2002; Recidoro et al. 2014). Hedgehog ligands are known to be produced in neurons in the central nervous system, though it has not been studied if their secretion is impacted by synaptic firing (Traiffort et al. 1999; Beug et al. 2011). We also observed a decrease in *fgf20a* mRNA expression, which encodes a ligand in the FGF signaling pathway and is necessary for wound healing and blastemal proliferation and maintenance (K D Poss et al. 2000). There is evidence that acetylcholine receptor activation can increase the abundance of Fgf ligands, consistent with the possibility of post-synaptic regulation of Fgf signaling during regeneration (Moffett, Kratz, and Stachowiak 1998). With the numerous signaling pathways known to regulate fin regeneration, the further study of the molecular

mechanisms governed by cholinergic activity would provide greater detail into the role of nerves in caudal fin regeneration.

Our study demonstrates that zebrafish fin regeneration requires cholinergic activity. We further identified Wnt/β-catenin signaling as being regulated by cholinergic activity and further showed that rescuing Wnt/β-catenin signaling partially rescues regeneration of the caudal fin. Understanding the relationship between cholinergic activity and the morphogenetic pathways involved in regeneration furthers our understanding of how complex tissues are able to reform after injury.

2.4 Materials and Methods

Zebrafish strains and heat shock protocol

We used zebrafish (*Danio rerio*) of either sex from the following lines: Wild-type AB, heterozygous Tg(*7xTCF-Xla.siam:mCherry*)^{ia5} (Moro et al. 2012) to visualize β-catenin responsive cells, and heterozygous Tg(*Hsp70l:Wnt8a-GFP*)^{w34} (Weidinger et al. 2005) to induce canonical Wnt signaling. Wnt8a induction was achieved by exposing Tg(*Hsp70:*Wnt8a-gfp) and age-matched wild type fish to water slowly increased from 28°C to 37°C. Fish remained at 37°C for 60 minutes, and were slowly brought back to normal water temperature. Fish were exposed to this heat shock protocol at least 4 hours prior to surgery and resumed daily heat shocks at 2 days post surgery. All experiments were approved and conducted in accordance with

University of Washington Institutional Animal Care and Use Committee (IACUC) protocol 2057-01 guidelines.

Zebrafish spinal cord transections and fin amputations

Spinal cords of zebrafish were transected as previously described (T. Becker et al. 1997). Briefly, adult zebrafish (>3 months old, either sex) were anesthetized in buffered 0.0168% tricaine methanesulfonate (MS-222) in fish tank water until respiratory movements of the opercula stopped (2-5 minutes). At a point halfway between the operculum and the dorsal fin (at approximately the 8^{th} vertebrae), an incision was made through the muscle layer and the vertebral column was exposed via retracting the muscle. The vertebral column was then cut with microscissors before sealing the wound with a drop of vetbond (3M, St. Paul, MN). Bupivacaine (Hospira, Lake Forest, IL) was administered intramuscularly at a dose no greater than 2mg/kg during surgery to provide additional pain relief during the procedure. Fish were then set on a concave glass slide and the caudal fin was spread out along the slide. A clean razor was used to amputate approximately 50% of the tail fin of the fish. The gills of the fish were flushed in a tank of fish water by puffing water past the gills with a 3mL plastic transfer pipette. Fish resumed breathing within a few seconds and were monitored throughout recovery for signs of pain (e.g. clamped fins, distressed breathing). Fish exhibiting signs of pain were euthanized in an overdose of tricaine methanesulfonate (MS-222) in fish tank water. All fish, including controls, were food deprived for at least 3 days post surgery and housed in the dark to enhance survival. Mean survival rate was 60% over 5 days and greater than 90% of fish regained motor control of the caudal fin by 14 days post surgery.

Zebrafish injections and fin amputations

Zebrafish at least 3 months old of either sex were anesthetized in buffered 0.0168% tricaine methanesulfonate (MS-222) in fish tank water until respiratory movements of the opercula stopped (2-5 minutes). Fish were laid out on a concave glass slide and injected in the caudal peduncle with PBS or 0.5U Botulinum Toxin B (Myobloc, Solstice Neurosciences, Malvern, PA) at a volume of 20µL. Fin amputations were performed the following day as described previously.

Whole mount immunofluorescence staining

Zebrafish, 2 days post amputation, were euthanized in an overdose of buffered tricaine methanesulfonate (MS-222) in fish tank water. The caudal fin and caudal peduncle were amputated and preserved in 90% methanol/10% DMSO overnight at 4°C. Samples were rehydrated via a methanol series and permeabilized with acetone for 20 minutes at -20°C. Mouse acetylated α-tubulin (Sigma Aldrich, T6793; 1:500, St Louis, MO) antibody was added to blocking buffer (0.5% Triton X-100, 5% goat serum, 0.5% BSA in PBS-Tween). After blocking for 1 hour at room temperature, samples were incubated in antibody solution overnight at 4°C. Samples were washed in PBS six times for twenty minutes with gentle shaking, and then stained with goat anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, A11029; 1:1000, Waltham, MA) for 1 hour at room temperature, and washed 6 more times with PBS.

Tissue collection, sectioning, immunofluorescence and hematoxylin staining

Zebrafish, 1-3 days post amputation, were euthanized in an overdose of buffered tricaine methanesulfonate (MS-222) in fish tank water. The caudal fin and caudal peduncle were

amputated and preserved in 4% PFA in ethanol overnight at 4°C. Samples were washed with PBS and sunk in 10% sucrose for 2 hours, followed by 30% sucrose overnight at 4°C. Samples were frozen in O.C.T. (Tissue-Tek, VWR #25608-930, Radnor, PA) at -80°C, and sectioned along the entire dorsoventral span of the fin on a cryostat at 14µM for PCNA staining and 20µm for hematoxylin staining or in situ hybridization and adhered to Superfrost Plus slides (VWR, Radnor, PA) overnight at 40°C. Mouse PCNA antibody (Sigma-Aldrich, P8225; 1:250, St Louis, MO) was added to blocking buffer (0.5% Triton X-100, 5% goat serum, 0.5% BSA in PBS-Tween). After blocking slides for 1 hour at room temperature, samples were incubated in antibody solution overnight at 4°C. Samples were washed in PBS six times for twenty minutes with gentle shaking, and then stained with goat anti-mouse Alexa Fluor 488 secondary antibody (Thermo Fisher Scientific, A11029; 1:1000, Waltham, MA) for 1 hour at room temperature. After 6 more washes with PBS, the slides were sealed with a coverslip with Prolong Gold Antifade Reagent (Thermo Fisher Scientific, Waltham, MA).

For hematoxylin staining, the slides were stained with hematoxylin for 5 minutes, rinsed with water, dipped briefly in acid alcohol, and rinsed with water again. Slides were next soaked in Scott's Blue for 5 minutes, followed by a water rinse and an ethanol soak. Samples were mounted with Permount (Thermo Fisher Scientific, Waltham, MA).

In situ hybridizations

Whole mount in situ hybridizations and cryosectioning of regenerates was performed as described previously (K D Poss et al. 2000; Kenneth D Poss, Shen, and Keating 2000). All in situ

hybridizations were performed on whole mount regenerates at 1 day post amputation and subsequently sectioned. All probes were designed from previously published templates (Moro et al. 2012; K D Poss et al. 2000; Kelly, Lai, and Moon 1993; Dorsky et al. 1999).

Quantitative PCR

Zebrafish, 1 or 2 days post amputation, were anesthetized in buffered 0.0168% tricaine methanesulfonate (MS-222) in fish tank water until respiratory movements of the opercula stopped (2-5 minutes). Fins were amputated 2 fin rays rostral to the initial amputation plane, collecting the regenerating tissue into pools of at least n=4 fins per group. Samples were homogenized and lysed with Trizol to extract RNA. cDNA was synthesized via RevertAid cDNA kit (Thermo Fisher Scientific, Waltham, MO). Quantitative PCR was run on a Roche LightCycler 480. All samples were normalized to β-actin levels and compared to 18s normalization. Primer sequences can be found in table in S1 Table.

Fin area regeneration and fluorescence quantification

Fin regeneration was assessed with a percent regeneration metric. Briefly, full fin images were taken prior to and post amputation, and total pixel area of the fins were measured with ImageJ (NIH). The difference in area was calculated and used as the total area lost. Images were taken at 5, 7, and 14 days post amputation, and the new tissue area (from distal regenerative tip to the amputation plane) was measured and calculated as a percent of amputated tissue, defined as: % regeneration=100 * (new tissue area/amputated tissue area). Total fluorescence was analyzed with ImageJ. To measure fluorescence after transection, the distal part of the fin was

selected from images and the total fluorescence of the selected area was measured via ImageJ and corrected for background fluorescence. The same procedure was used to analyze reporter intensity after BtxB injection, but only rays 7-12 were used to assess localized loss of signal, counting the dorsal-most ray as fin #1.

Bone length regeneration quantification

Bone length regeneration was quantified by measuring pre and post amputation bone lengths and determining the length amputated for each ray of the fin. At 14 days post amputation, the regenerated length was measured per ray, tracing along the bone formation to the distal point of the ray. The data was calculated as a percent, wherein bone length regeneration = 100 * (regenerated bone length/amputated bone length). Rays were sequentially numbered based on position, wherein the dorsal-most ray was designated ray 1.

Wound epithelium thickening quantification

Wound epithelium thickening was quantified on hematoxylin stained slides. Images were analyzed by measuring the epithelial thickness at the thickest point of the distal fin at 1 and 2 days post amputation. The data was calculated as a percent, wherein the average uninjured control was normalized to 100 at both 1 and 2 days post amputation and sham and transected fins were compared to this number.

Image acquisition and processing

Fluorescent images of sections were taken on a Nikon A1R confocal microscope with NIS-elements software. Live imaging and whole fin fluorescent images were taken on the Nikon

SMZ1500. Histological sections were imaged on a Zeiss Axiovert 200. ImageJ was used to merge different channels and figures were assembled using Adobe Photoshop CS3.

Quantifications and statistical analyses

Quantifications were done as indicated in the individual sections. All quantifications were done blinded to the experimental group. All comparisons of 2 variables were analyzed using a T test, while groups of 3 or more were analyzed using a one-way ANOVA with Tukey post-hoc test. All statistics were calculated using Graphpad Prism Version 5.01 for Windows (Graphpad Software, San Diego, CA). Significance was assessed as $p < 0.05$, and is indicated via asterisks. Bar and line graphs all show means with error bars representing standard error of the means (SEM). Number of fish used in each experiment is indicated in the appropriate figure legend.

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2.6 Supplemental Material

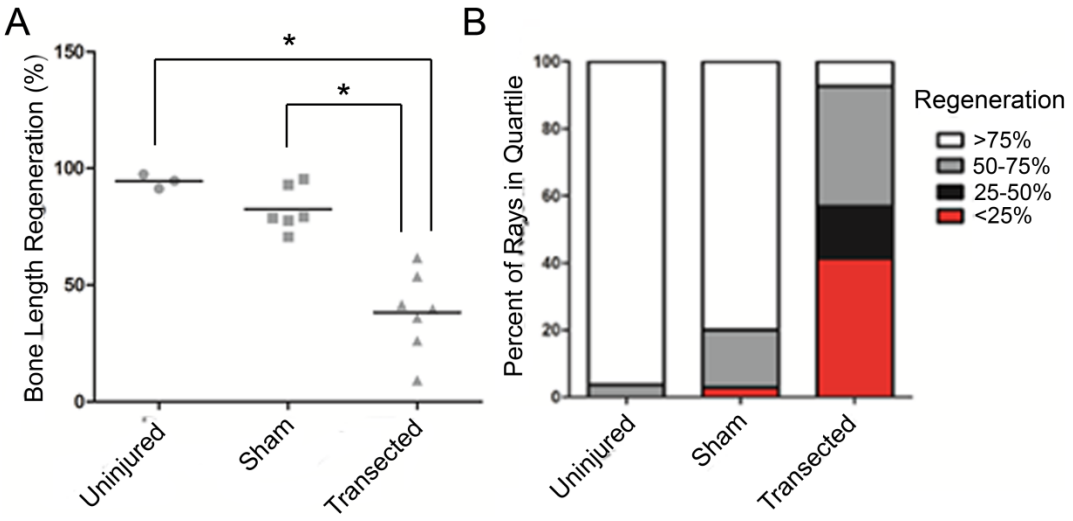

*Figure 2.S1: Spinal cord transection inhibits bone regeneration. (A) Transection of the spinal cord inhibits bone length regeneration of the caudal fin at 14 days post amputation. (B) Spinal cord transection increases percentage of poorly regenerating (<25% regeneration) bone rays. *P < 0.05; (A and B) n≥3. Graph (A) shows mean with each point representing 1 individual fish.*

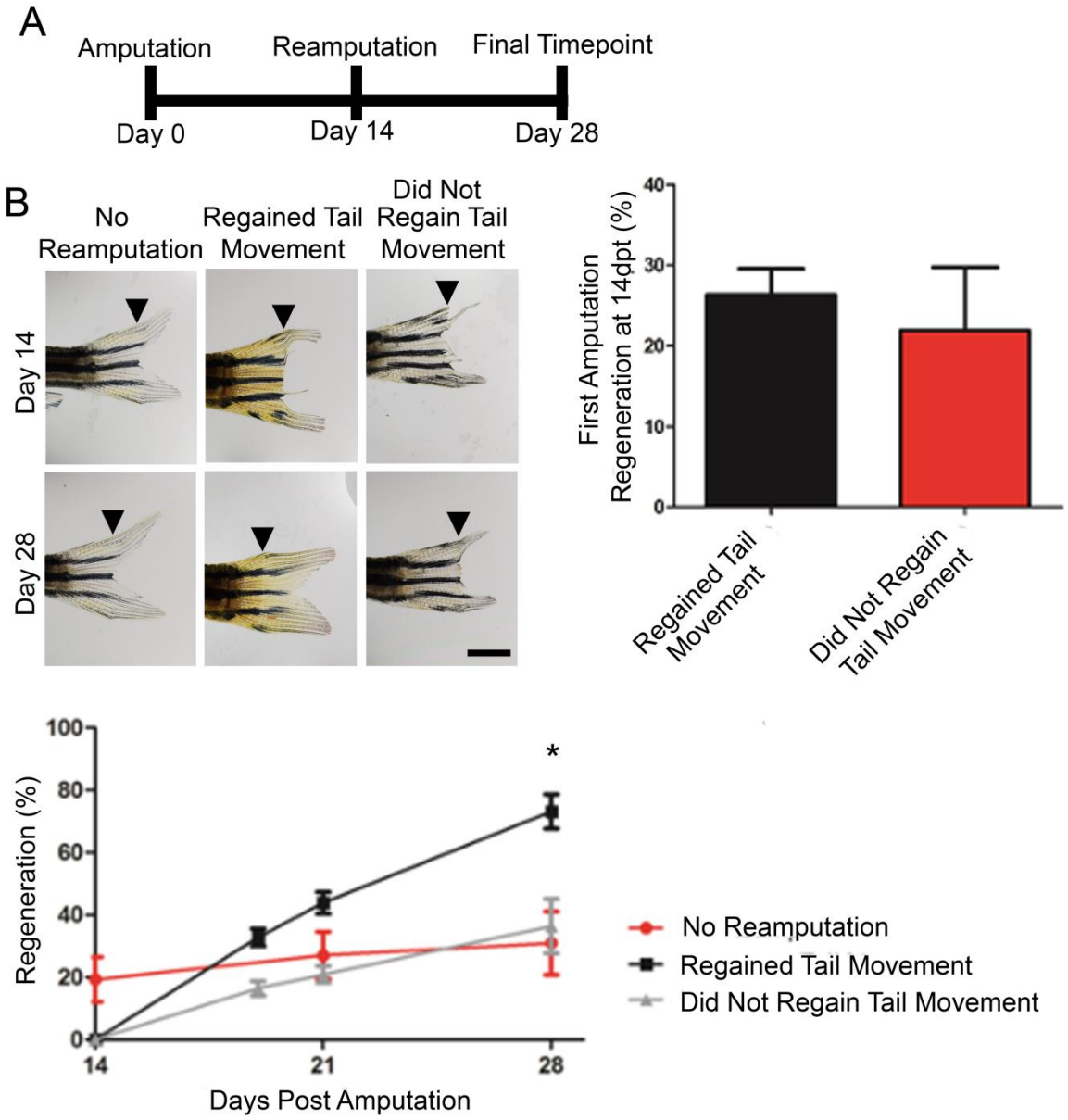

Figure 2.S2: Regaining muscle control in the tail fin rescues regeneration defects after spinal cord transection. (A) Experimental schematic. (B) Fish that regain tail movement after spinal cord transection show improved regeneration after a second fin amputation, compared to controls that fail to regain tail movement or fish that did not receive a

*second amputation. *P < 0.05; black triangles indicate amputation plane; scale bar, (B) 5mm; n≥4 for (B). Graph shows mean ± SEM.*

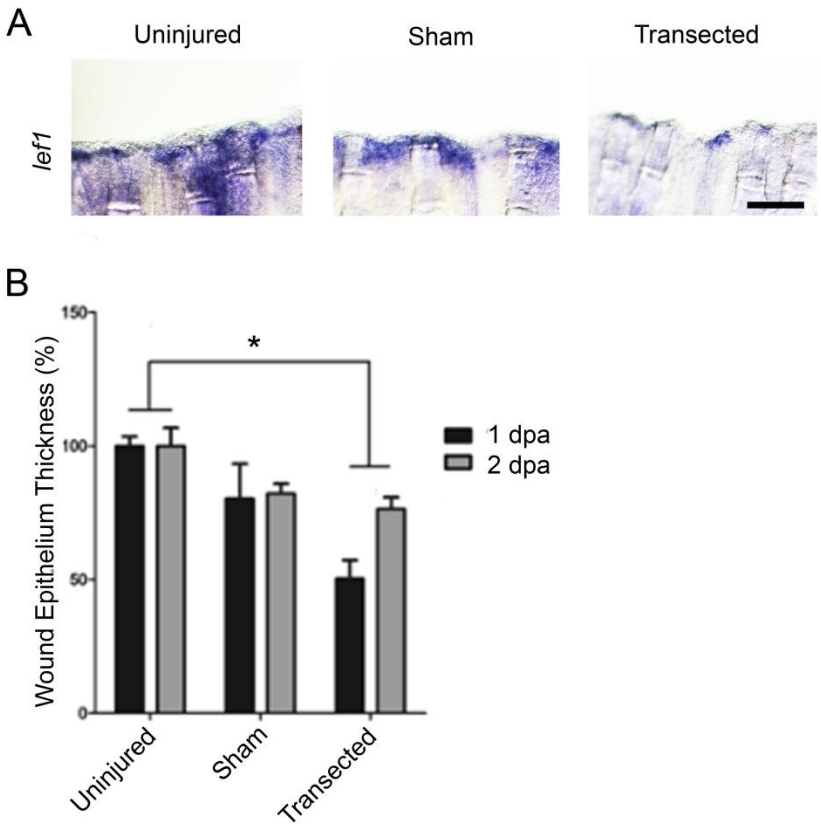

*Figure 2.S3: Wound epithelium thickening is inhibited by spinal cord transection. (A) Wound epithelium thickening is reduced in the caudal fin in spinal transected fish at 1 and 2 days post amputation. (B) In situ hybridization shows a loss of lef1 in 1 day post amputation fins of spinal cord transected fish. *P < 0.05; scale bar, (A) 1mm; (A) n≥9, (B) n≥3. Graph (C) shows mean ± SEM.*

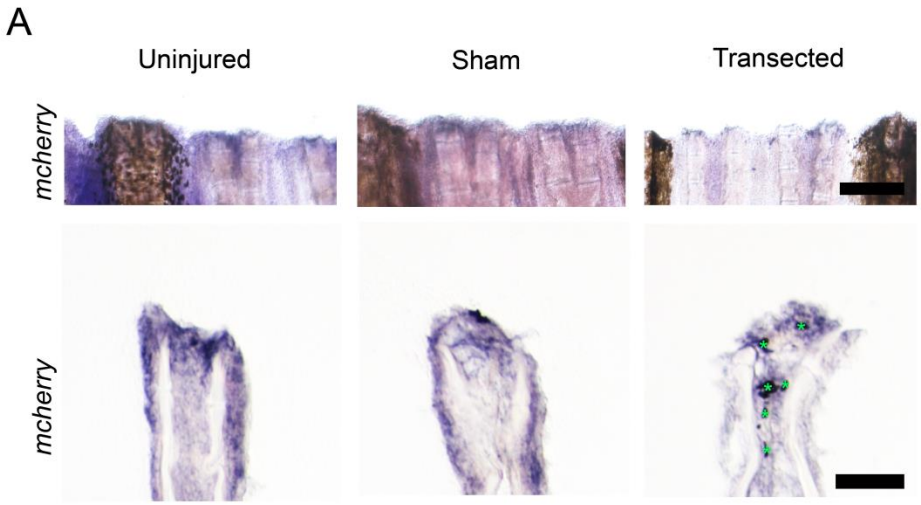

Figure 2.S4: mcherry transcripts are reduced at 1 day post amputation in spinal cord transected fish. (A) In situ hybridization of β-catenin reporter Tg(TCFsiam:mCherry) fish shows a decrease of transcripts in spinal cord transected fish at 1 day post fin amputation. Scale bar, (A whole mount) 1mm, (A section) 100µm; green asterisks indicate pigment cells; n≥3.

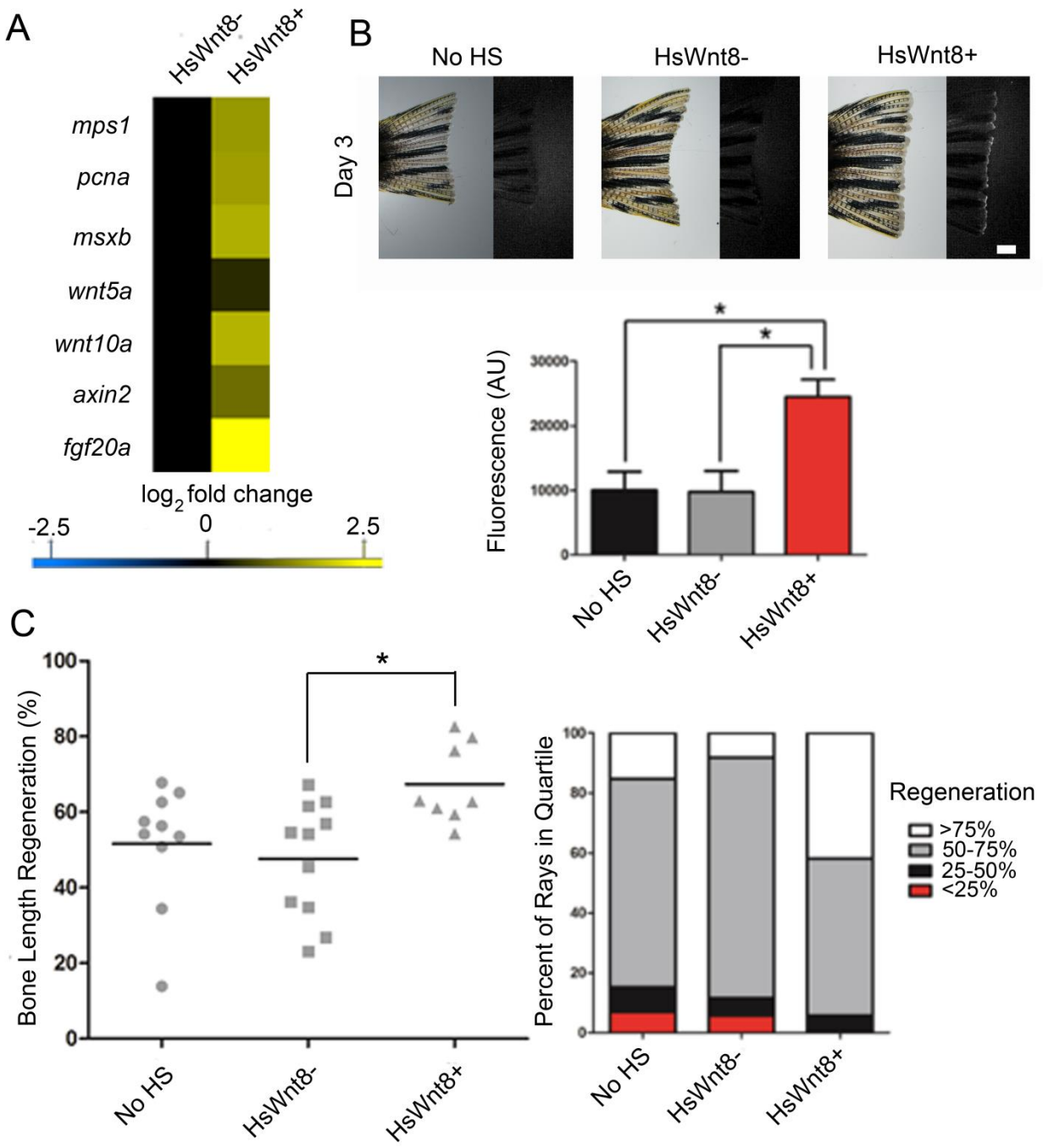

Figure 2.S5: Wnt8a activates Wnt/β-catenin signaling and increases bone length in the caudal fin after spinal cord transection. (A) Overexpression of Wnt8a rescues gene expression of transcripts downregulated in spinal cord transected regenerating fins at 1 dpa. (B) Overexpression of Wnt8a activates the siam:mCherry β-catenin reporter at 3 days post

*amputation in spinal cord transected fish. (C) Wnt8a overexpression increases bone length regeneration and rescues poorly regenerating (<25%) fins in spinal cord transected fish. *P < 0.05; scale bar, (B) 1mm; n≥4 for all experiments. Graph (B) shows mean ± SEM, graph (C) shows mean with each point representing one individual fish.*

Table 2.S1: qPCR primers.

Gene	**Forward Primer**	**Reverse Primer**
bactin	GGTATGGGACAGAAAGACAG	AGAGTCCATCACGATACCAG
18s	CGCTATTGGAGCTGGAATTACC	GAAACGGCTACCACATCCAA
mps1	ACTCGCAGGTCGGAACTCTG	CCACACGTCCCCTTTAGCAC
msxb	GACGACAGTGAAGAACTAAGCG	CCGTTCGGCGATAGAGAGGT
pcna	CCCGATTGTGACCCTCTAAA	TTGGAATGAGCAGTTGGACA
wnt10a	ATTCACTCCAGGATGAGACTTCATA	GTTTCTGTTGTGGGCTTTGATTAG
axin2	ATTCAACATCAAGCCTTCAGAG	TGAAGAAGTATCTGTAGTTGCC
fgf20a	CAGCTTCTCTCACGGCTTGG	AAAGCTCAGGAACTCGCTCTG
wnt5a	GACATCAGTTAGGAGAGTCTTG	TAGAGCTGGCAGAGCTTCTTCT

3 - Wnt/β-catenin Signaling Promotes Glial Bridge Formation and Regeneration After Zebrafish Spinal Cord Injury

3.1 Introduction

Human spinal cord injury can lead to severe disabilities, including loss of motor function caudal to the injury site. This is due in part to the formation of a glial scar - a mass of reactive astrocytes that infiltrates the injury site. The glial scar inhibits axon regeneration through the injury site, limiting recovery of motor function while also reducing inflammatory responses and protecting nerves from secondary insult (Koyama 2014). Various interventions designed to reduce the effects of glial scars can improve axon regeneration and locomotor recovery in rodents (McKeon, Hoke, and Silver 1995; Yick et al. 2004). However, complete ablation of reactive astrocytes exacerbates loss of motor function and further inhibits axonal regrowth due to spinal cord injury (Faulkner et al. 2004; Anderson et al. 2016). This has led to a paradigm in which reactive astrocytes are thought to be necessary for proper wound healing, but may also produce inhibitory molecules and form a physical barrier to inhibit axon elongation.

Unlike mammals, the zebrafish *Danio rerio* possesses a remarkable ability to regain locomotor function after spinal cord injury. Zebrafish are able to generate new motor neurons from

progenitor populations in the spinal cord and also extend axons through the lesion site to form synapses caudal to the injury site (T. Becker et al. 1997; Reimer et al. 2008). Zebrafish produce glia with a bipolar morphology, extending processes across the lesion site. In stark contrast to the mammalian glial scar, axons span this glial bridge to extend through the injury site (Goldshmit et al. 2012). This difference in glial cell response to injury makes the zebrafish an interesting model for studying mechanisms of glial cell biology after spinal cord injury.

Wnt/β-catenin signaling is well known for its roles in regeneration across phyla and tissue types. Wnt/β-catenin signaling is necessary for anterior/posterior determination in planarian regeneration, fin and retina regeneration in zebrafish, and mouse digit tip regeneration, among many others (Petersen and Reddien 2008; Gurley, Rink, and Sanchez Alvorado 2008; Kawakami et al. 2006; Stoick-Cooper et al. 2007; Ramachandran, Zhao, and Goldman 2011; Takeo et al. 2013). Wnt/β-catenin signaling is also necessary for neurogenesis after spinal cord injury in the larval zebrafish (Briona et al. 2015). Furthermore, Wnt receptor Frizzled3a is necessary for the formation of bipolar glial cells in the anterior commissure of developing zebrafish (Hofmeister et al. 2012). Thus, we hypothesized that Wnt/β-catenin signaling promotes spinal cord regeneration in the adult zebrafish by promoting the formation of a glial bridge. We found that activity of a Wnt/β-catenin reporter increased after spinal cord injury in adult zebrafish, and that overexpression of Dkk1, which inhibits Wnt/β-catenin signaling, inhibited functional recovery after injury. We also determined that overexpression of Dkk1 reduced formation of the glial bridge in the injured spinal cord, and that a subset of glia, including bipolar glia, respond to Wnt/β-catenin signaling.

3.2 Results

Wnt/β-catenin signaling is increased after spinal cord transection

To assess whether Wnt/β-catenin signaling is increased after spinal cord injury, we used transgenic zebrafish with a fluorescent reporter of β-catenin-mediated transcription, Tg(*7xTCF-Xla.siam:mCherry*)^{ia5} fish (Moro et al. 2012), heretofore referred to as Siam:mCherry fish. We cut the epidermis and musculature of the body wall and transected the spinal cord at approximately the 8th vertebrae (transected fish). For controls, we used fish that did not undergo operation (uninjured) or those in which only the body wall was opened (sham). We euthanized fish at several times after surgery and quantified the number of cells with Siam:mCherry reporter activation in sections less than 1mm from the lesion site. We found that the number of Siam:mCherry-positive cells increased after injury in sham and transected fish (Fig 1A). However, by 14 days post injury (dpi), there were more Siam:mCherry-positive cells in transected fish compared to sham fish (Fig 1A). By 42dpi, the number of Siam:mCherry-positive cells was not different from uninjured fish in both sham and transected fish.

In order to substantiate the results using the Siam:mCherry reporter, we analyzed the expression of Wnt pathway-related genes in spinal cords of wild-type fish. Increased expression of *wnt4b,* which encodes a Wnt ligand, was found in large scale gene expression analyses of injured zebrafish spinal cords (Guo et al. 2011; S. P. Hui et al. 2014). Similarly, we found that *wnt4b* expression was increased in transected fish compared to sham fish at 3 and 14 dpi (Fig 1B). Dkk is able to inhibit Wnt ligand binding with LRP6, so to confirm that ligand binding is

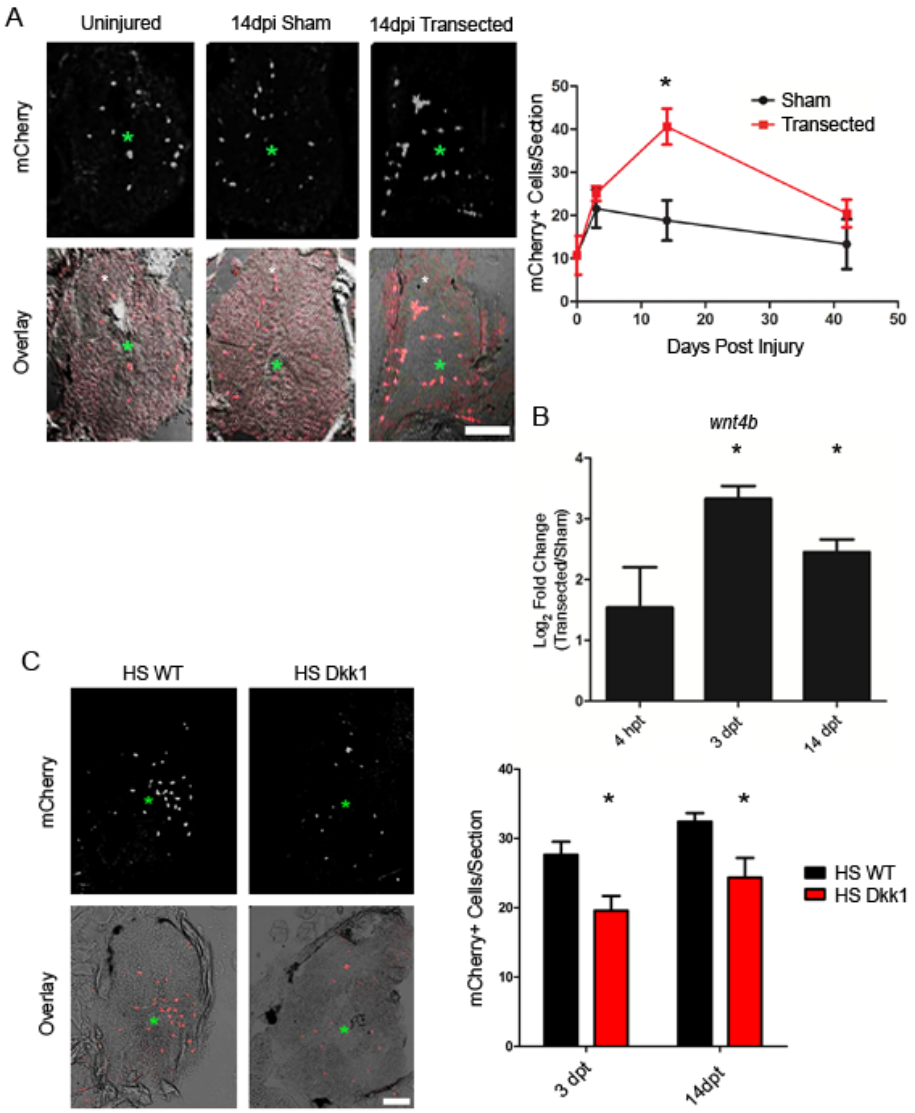

Figure 1: Wnt/β-catenin signaling is increased after spinal cord injury. (A) Transgenic Tg(7xTCFxla.siam:mCherryNLS) fish underwent sham or spinal cord transection and were analyzed for β-catenin responsive cells by counting mCherry+ cells. (B) Quantitative PCR was done at 4 hours post transection, and 3 and 14 days post transection to analyze changes in Wnt ligand wnt4b after spinal cord injury. (C) Overexpression of Dkk via transgenic Tg(Hsp70l:Dkk1-GFP) reduced β-catenin responsive cells at 3 and 14 days post transection, images are

*representative of 3 dpt spinal cords. *P < 0.05; asterisk marks central canal in (A) and (C); scale bar; (A,C) 100µm; n≥5 for (A), n=3 for pooled samples in (B), n≥3 for (C). Graph (A) shows mean ± SEM, graph (B) shows mean ± St Dev.*

required for the β-catenin transcriptional increase we observe, we overexpressed Dkk using a heat-inducible promoter in Tg(*Hsp70l:Dkk1b-GFP*)^{w32} fish (Stoick-Cooper et al. 2007), heretofore referred to as Hsp70l:Dkk fish. Using Hsp70l:Dkk fish, Siam:mCherry double-transgenic fish, we confirmed that inducing Dkk1 overexpression by daily heat-shock starting four hours before spinal cord transection (HS Dkk) inhibited Wnt/β-catenin signaling at 3 and 14 days post transection (dpt) compared to heat shock WT (HS WT) (Fig 1C). Thus, injury leads to a transient increase in Wnt/β-catenin signaling in the spinal cord of adult zebrafish and may be required for regeneration.

Wnt/β-catenin signaling is necessary for spinal cord regeneration

To test if Wnt/β-catenin signaling is necessary for functional recovery after spinal cord injury, we overexpressed Dkk1 the Hsp70l:Dkk fish. We observed both HS WT and HS Dkk fish at 14 and 28 dpt, and assessed them for their total distance of swimming over 5 minutes. We found that HS WT fish showed a significant recovery between 14 and 28 dpt, but HS Dkk fish did not show an increase in free swimming behavior in that same timespan (Fig 2A). Next, we assessed recovery of motor functions using a quantitative analysis of swimming behaviors. We observed impaired recovery after spinal cord transection in HS Dkk1 compared to HS WT or no heat shock (No HS) control fish using a swim score method to semi-quantitatively assess function (Fig 2B). Recovery of normal swimming behaviors in spinal cord transected fish correlates with axonal

regeneration through the lesion site (Vajn et al. 2014). We labeled regenerating axons using an antibody for acetylated tubulin (Briona and Dorsky 2014). We found that daily heat-shock for 21dpt reduced the amount of acetylated tubulin labeling across the lesion site in HS Dkk1 fish compared to No HS and HS WT fish (Fig 2C). These data demonstrate that Wnt/β-catenin signaling is necessary for locomotor recovery after spinal cord injury.

Wnt/β-catenin signaling promotes formation of bipolar glial cells

One of the early events in spinal cord regeneration in zebrafish is proliferation and remodeling of glia near the lesion site. Ultimately, these cells adopt a bipolar morphology that bridges the lesion and serves as a scaffold for regenerating axons. The formation of the glial bridge is necessary for functional recovery after spinal cord injury (Goldshmit et al. 2012). To test if Wnt/β-catenin signaling is required for glial bridge formation, we heat-shocked Hsp70l:Dkk fish daily starting at 4 hours prior to surgery and labeled glia with an antibody targeting glial fibrillary acidic protein (GFAP). We found No HS and HS WT fish had bipolar glia bridging the lesion site at 14dpt (Fig 3A). In HS Dkk1 fish, glia were observed in the lesion site, but did not have a bipolar morphology (Fig 3A). These data suggest that Wnt/β-catenin signaling is necessary for glia to produce a bipolar morphology.

Morphological changes in glia caused by overexpression of Dkk1 could be due to a direct requirement for Wnt/β-catenin signaling in glia or due to secondary effects caused by inhibition of Wnt/β-catenin signaling in other cell types. We found that 20% of mCherry expressing cells in Siam:mCherry fish were also expressing GFAP at 3 and 14 dpt (Fig 3B-C). Moreover, at 7 dpt, we

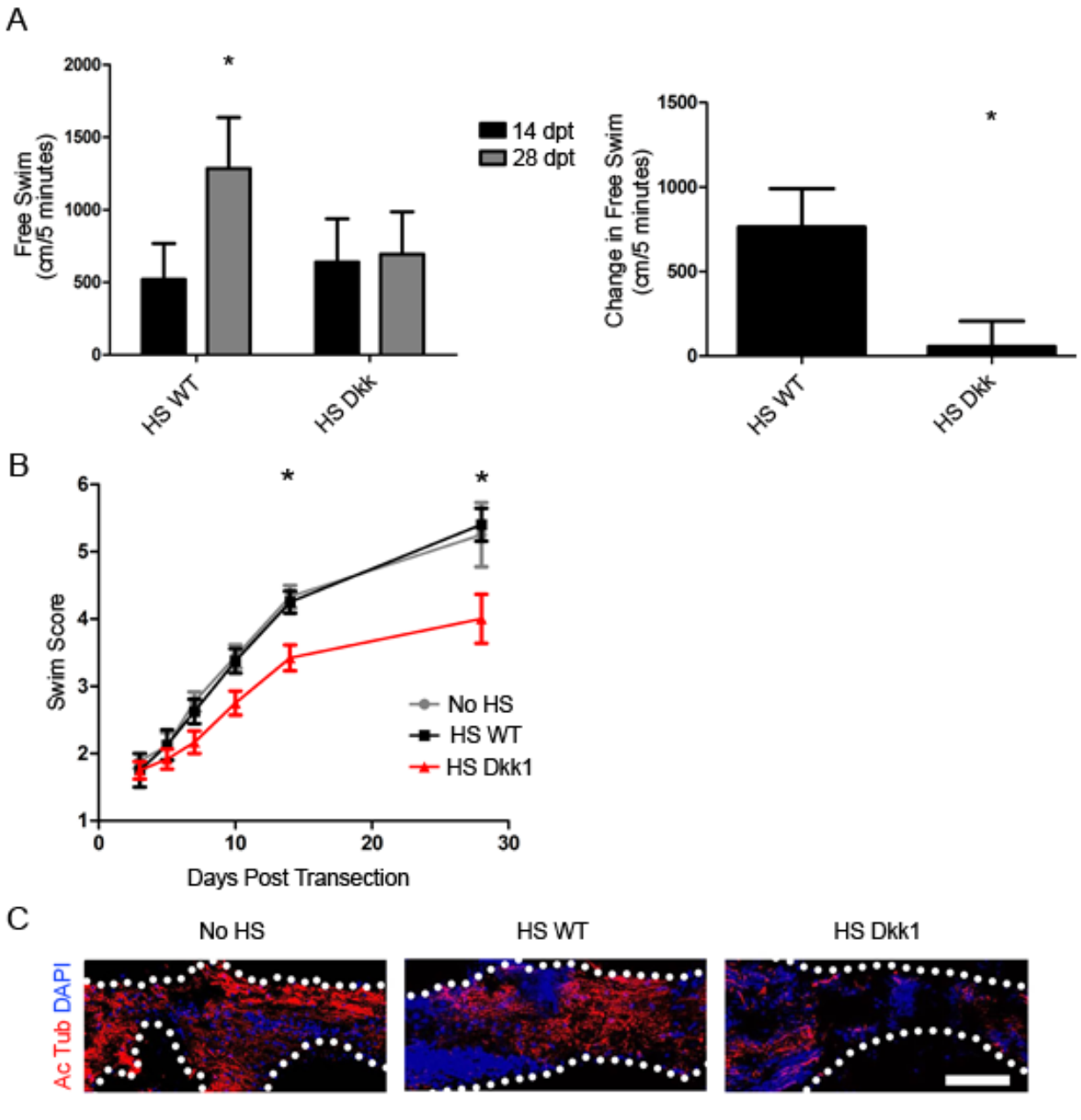
A
Free Swim (cm/5 minutes)
2000
1500
1000
500
0
*
14 dpt
28 dpt
HS WT
HS Dkk
Change in Free Swim (cm/5 minutes)
1500
1000
500
0
*
HS WT
HS Dkk
B
Swim Score
6
5
4
3
2
1
0
10
20
30
*
*
No HS
HS WT
HS Dkk1
Days Post Transection
C
No HS
HS WT
HS Dkk1
Ac Tub DAPI

*Figure 2: Wnt/β-catenin signaling is necessary for locomotor recovery after spinal cord injury. (A) Analysis of free swimming behavior showed that Dkk overexpression prevented recovery of swimming ability compared to HS WT controls. (B) Overexpression of Dkk inhibited locomotor recovery, as measured by swim score, compared to no heat shock and heat shock wild type controls. (C) Acetylated tubulin staining is reduced in fish overexpressing Dkk at 21 days post transection compared to no heat shock and heat shock wild type fish. *P < 0.05; scale bar; (C) 100μm; n≥5 for (A-B), n≥3 for (C). Graphs show mean ± SEM.*

identified double-labeled cells in bipolar glia rostral to the lesion site (Fig 3B). At 14 dpt, we observed double-labeled glia rostral to the lesion site, but very few mCherry+ cells in the lesion site itself, suggesting that either Wnt/β-catenin signaling is not directly influencing bipolar glia in the lesion site or Wnt/β-catenin signaling may be downregulated during this process (Fig 3C). Because we do not know the kinetics of mCherry protein turnover in the spinal cord, we cannot determine at what time Wnt/β-catenin signaling was present in this cell population. Thus, these data suggest that a subset of glia or glia progenitor cells activate Wnt/β-catenin signaling in response to spinal cord injury.

We further looked at how Wnt/β-catenin signaling regulates glial bridge formation by performing quantitative PCR on samples pulsed with Dkk overexpression at 3 dpt. We

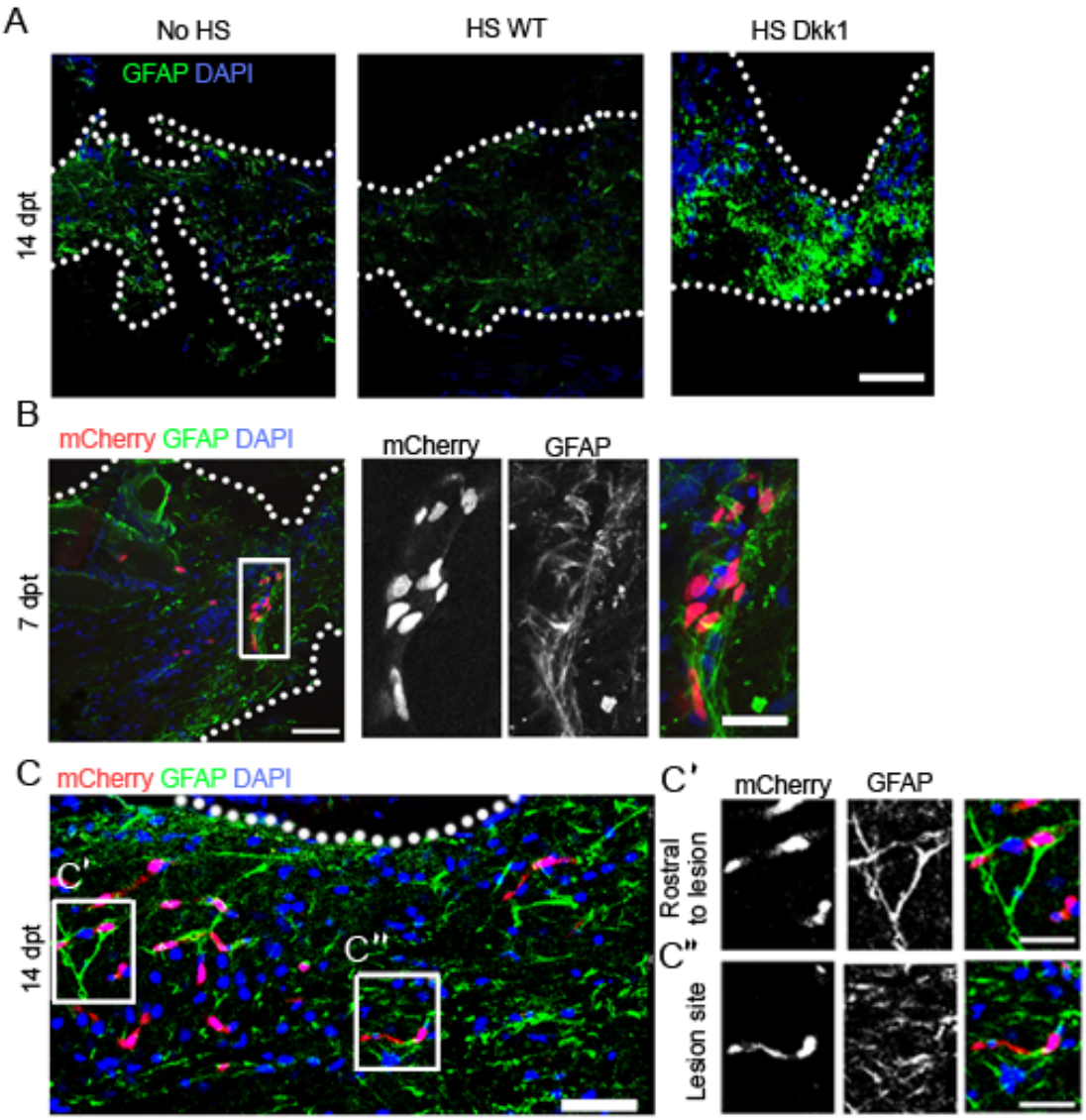

Figure 3: Wnt/β-catenin signaling is necessary for bipolar glial cell formation. (A) Dkk overexpression prevents the formation of bipolar glial cells in the lesion site at 14 days post transection (dpt). (B) GFAP antibody staining colocalizes with mCherry expression in Tg(Siam:mCherry) fish in approximately 20% of mCherry+ cells 7 dpt near the lesion site. (C) GFAP antibody staining colocalizes with mCherry expression rostral to the lesion site, while very few mCherry+ cells are observed in the lesion site at 14 dpt. scale bar; (A) 100µm, (B and C)

50µm and 25µm for the zoom; n≥5 for (A and B), n=3 for(B and C). All sections are laid out with rostral to caudal from left to right.

compared samples of HS Dkk1 overexpressing fish to HS WT controls 6 hours after heat shock and noted decreases in markers of proliferation of proliferation (*ccnd1*, *pcna*), *wnt4b*, and *gfap* (Fig 4A). We also observed an increase in *olig2*, a marker of progenitor cells in the spinal cord (Fig 4A). Interestingly, staining for PCNA at 3dpt revealed no significant change in PCNA+ cells near the central canal, where glial and neuronal precursors are found, in HS Dkk1 fish compared to HS WT controls (Fig 4B). By day 14, we are able to detect a decrease in PCNA+ cells in HS Dkk1 fish compared to HS WT controls (Fig 4B). At day 3, proliferating cells most frequently differentiate into glia, while at day 14, proliferating cells are more likely to become neurons (Goldshmit et al. 2012; Reimer et al. 2008). These data suggest that Wnt/β-catenin signaling does not regulate proliferation of glia after spinal cord injury, but may alter differentiation of glia into a specific bipolar cell.

3.3 Discussion

Our data show that Wnt/β-catenin signaling is dynamically regulated and required for locomotor recovery and the formation of bipolar glial cells after zebrafish spinal cord injury. It was previously shown that activation of the fibroblast growth factor (FGF) pathway is necessary for glial bridge formation in zebrafish, and that activation of FGF in primary astrocytes results in bipolar glia formation in marmosets (4). Furthermore, Fgf2 administration to mice after spinal cord injury increases bipolar glial cells, axonal elongation, and motor function recovery, suggesting that the ability of glia to form a bipolar morphology is conserved in mammals

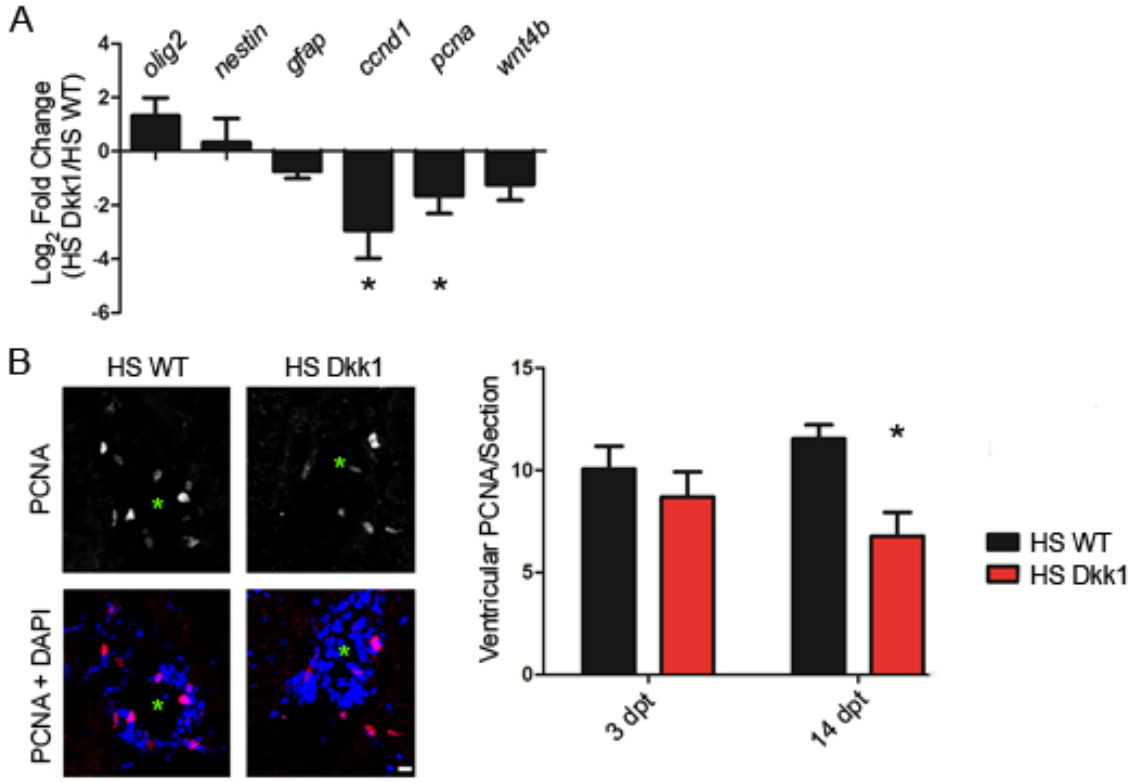

*Figure 3.4: Dkk overexpression does not regulate proliferation of glial progenitors after spinal cord injury. (A) A single pulse of Dkk 3 days post transection inhibits proliferative genes and alters expression of progenitor markers. (B) Dkk overexpression inhibits ventricular proliferation at 14, but not 3, days post transection. *P < 0.05; green asterisks mark central canal; scale bar, (B 10µm; n=3 replicates of pooled samples for (A), n≥4 for (B). Graphs show mean ± SEM.*

(Goldshmit et al. 2014). Glia derivation from neural progenitor cells requires FGF, but these cells form a stereotypic stellate morphology (Davidson et al. 2007; Reubinoff et al. 2001). Our data provide evidence that Wnt/β-catenin signaling is also necessary for bipolar glia formation and may be able to induce a bipolar morphology in human stem cell-derived glia, and is worth a detailed study.

The endogenous activity and use of Wnt/β-catenin signaling as a therapeutic target for spinal cord injury is still a debated issue. Quantitative PCR shows an increase in canonical Wnt ligands after spinal cord contusion in rats, but β-catenin reporter activity is unchanged after spinal cord hemisection in mice (Fernández-Martos et al. 2011; White et al. 2010). Increasing Wnt/β-catenin signaling in rats enhances functional recovery after spinal cord injury, but knockdown of endogenous Wnt/β-catenin signaling in oligodendrocyte precursor cells decreases astrocyte hypertrophy after spinal cord injury in mice (Z.-S. Yin et al. 2008; Suh et al. 2011; Park et al. 2013; Rodriguez et al. 2014). These data suggest that Wnt/β-catenin signaling may have different roles in different species, looking at both pathway activity and downstream effects. Previous work in the zebrafish suggests that Wnt/β-catenin signaling is increased after spinal cord injury in the adult zebrafish, and that Wnt/β-catenin signaling is necessary for neurogenesis in larval zebrafish (S. P. Hui et al. 2014; Briona et al. 2015). Our work extends upon these data, demonstrating that Wnt/β-catenin signaling is necessary for locomotor recovery and the formation of the glial bridge after spinal cord injury in adult zebrafish.

Reactive astrocytes are necessary for spinal cord regeneration, facilitating the healing of the blood-brain barrier, reducing leukocyte infiltration, and promoting neuron survival (Faulkner et

al. 2004; Anderson et al. 2016). With both pro-and anti-regenerative properties, proper modulation of reactive astrocytes could produce enhanced recovery from spinal cord injury. Consistent with this hypothesis, glial-restricted precursors derived via different methods produce astrocytes that are morphologically and molecularly distinct, which lead to different outcomes when transplanted into an injured spinal cord (Davies et al. 2008). Our work shows that inhibition of Wnt/β-catenin signaling by Dkk overexpression leads to the production of glia lacking the bipolar morphology, suggesting that Wnt/β-catenin signaling may be capable of producing pro-regenerative glia after spinal cord injury.

3.4 Materials and Methods

Zebrafish strains and heat shock protocol

We used zebrafish (*Danio rerio*) of either sex from the following lines: Wild-type AB, heterozygous Tg(*7xTCF-Xla.siam:mCherry*)^{ia5} (Moro et al. 2012) to visualize β-catenin responsive cells, heterozygous Tg(*Hsp70l:Dkk1b-GFP*)^{w32} (Weidinger et al. 2005) to induce canonical Wnt signaling, and Tg(*7xTCF-Xla.siam:mCherry*)^{ia5} crossed with Tg(*Hsp70l:Dkk1b-GFP*)^{w32} to monitor β-catenin responsive transcription after Dkk1b overexpression. Dkk1b induction was achieved by exposing Tg(*Hsp70:Dkk1b*-GFP) and age-matched wild type fish to water slowly increased from 28°C to 37°C. Fish remained at 37°C for 60 minutes, and were slowly brought back to normal water temperature. Fish were exposed to three different heat shock protocols: heat shock first induced 4 hours prior to surgery and resumed daily heat shocks at 2 days post surgery, heat shock induced on day 5 for delayed heat shock experiment, and single heat shock

on day 3. All experiments were approved and conducted in accordance with University of Washington Institutional Animal Care and Use Committee (IACUC) protocol 2057-01 guidelines.

Zebrafish Spinal Cord Transection

Spinal cords of zebrafish were transected as previously described (T. Becker et al. 1997). Briefly, adult zebrafish (>3 months old, either sex) were anesthetized in 0.0168% buffered tricaine methanesulfonate (MS-222) in fish tank water until respiratory movements of the opercula stopped (2-5 minutes). At a point halfway between the operculum and the dorsal fin (at approximately the 8^{th} vertebrae), an incision was made through the muscle layer and the vertebral column was exposed via retracting the muscle. The vertebral column was then cut with microscissors before sealing the wound with a drop of vetbond (3M, St. Paul, MN). The gills of the fish were flushed in a tank of fish water by puffing water past the gills with a 3mL plastic transfer pipette. Fish resumed breathing within a few seconds and were monitored throughout recovery for signs of pain (e.g. clamped fins, distressed breathing). Fish exhibiting signs of pain were euthanized in an overdose of tricaine methanesulfonate (MS-222) in fish tank water.

Immunohistochemistry

Zebrafish, 3, 7, 14, or 21 days post transection, were euthanized in an overdose of buffered tricaine methanesulfonate (MS-222) in fish tank water. The spinal cord was removed and preserved in 4% PFA in ethanol overnight at 4°C. Samples were washed with PBS and sunk in 10% sucrose for 2 hours, followed by 30% sucrose overnight at 4°C. Samples were frozen in

O.C.T. (Tissue-Tek, VWR #25608-930, Radnor, PA) at -80°C, and sectioned at 20µM transversely or longitudinally and adhered to Superfrost Plus slides (VWR, Radnor, PA) overnight at 40°C. Rabbit anti-GFAP antibody (Dako, Z0334; 1:500, Carpinteria, CA), and mouse acetylated α-tubulin antibody (Sigma Aldrich, T6793; 1:500, St Louis, MO) were added to blocking buffer (0.5% Triton X-100, 5% goat serum, 0.5% BSA in PBS-Tween). After blocking slides for 1 hour at room temperature, samples were incubated in antibody solution overnight at 4°C. Samples were washed in PBS six times for twenty minutes with gentle shaking, and then stained with goat anti-mouse Alexa Fluor 568, goat anti-mouse 633, goat anti-rabbit 488, secondary antibody (Thermo Fisher Scientific, A11031, A31574, and A11034; 1:1000, Waltham, MA) for 1 hour at room temperature. After 6 more washes with PBS, the slides were sealed with a coverslip with Prolong Gold Antifade Reagent (Thermo Fisher Scientific, Waltham, MA).

Cell Counting

Slides were analyzed for mCherry+ cells and mCherry+/GFAP+ cells. mCherry+ cells were identified by colocalization of mCherry fluorophore and DAPI. mCherry+/GFAP+ cells were defined as being mCherry+ as previously described and exhibiting colocalization of GFAP signal and mCherry signal in a confocal slice. Transverse sections within 1 millimeter of the injury site were used for quantification.

Quantitative PCR

Zebrafish, 4 hours, 3, or 14 days post transection, were euthanized in an overdose of buffered tricaine methanesulfonate (MS-222) in fish tank water. Spinal cords were excised from the fish

1mm rostral and caudal from the transection site, collecting the regenerating tissue into pools of n≥3 fins per group. Samples were homogenized and lysed with Trizol to extract RNA and cDNA was synthesized via RevertAid cDNA kit (Thermo Fisher Scientific, Waltham, MO). Quantitative PCR was run on a Roche LightCycler 480. All samples were normalized to β-actin levels and compared to 18s normalization. Primers used: *bactin* F (GGTATGGGACAGAAAGACAG), *bactin* R (AGAGTCCATCACGATACCAG), *18s* F (CGCTATTGGAGCT GGAATTACC), *18s* R (GAAACGGCTACCACATCCAA), *wnt4b* F (AAGGCTTCAGAGATGGTCATCG), *wnt4b* R (AGAATGCAACGCCGTAGGACAG).

Free Swim Analysis

Swimming capability was quantified at 14 and 28 days post injury by analyzing the total distance swum over a 5 minute timespan. Fish were placed in a brightly illuminated tank filled with tank water (5cm deep, 27°C) and recorded from above. The resulting files were then analyzed by Tracker software (Cabrillo.edu/~dbrown/tracker) to calculate distance traveled in cm.

Locomotor Analysis

Locomotor analysis was performed with a swim score modified from Goldshmit et al, 2012. Fish were scored blindly based on swimming ability between 1 and 6; 1, fish is lying on its side on the bottom of the tank; 2, fish is upright and can swim forward and backward; 3, fish is able to turn by making jerky movements to move tail in line for next set of forward movements; 4, fish regains movement caudal to the injury site, but still swims primarily by head and pectoral fin

movements; 5, fish is swimming by utilizing caudal fin but shows noticeable differences from an uninjured fish; 6, swimming is indistinguishable from uninjured fish.

Image Acquisition and Processing

Fluorescent images of sections were taken on a Nikon A1R confocal microscope with NIS-elements software. ImageJ was used to merge different channels and figures were assembled using Adobe Photoshop CS3.

Statistics

Quantifications were done as indicated in the individual sections. All quantifications were done blinded to the experimental group. All comparisons of 2 variables were analyzed using a T test, while groups of 3 or more were analyzed using a one-way ANOVA with Tukey post-hoc test, except for swim score analysis, which was analyzed with a Kruskal-Wallis test, and free swim analysis, which was analyzed by a two way ANOVA with repeated measures. All statistics were calculated using Graphpad Prism Version 5.01 for Windows (Graphpad Software, San Diego, CA). Significance was assessed as $p < 0.05$, and is indicated via asterisks. Bar and line graphs all show means with error bars representing standard error of the means (SEM). Number of fish used in each experiment is indicated in the appropriate figure legend.

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3.6 Supplemental Material

Table 3.S1: qPCR primers.

Gene	**Forward Primer**	**Reverse Primer**
bactin	GGTATGGGACAGAAAGACAG	AGAGTCCATCACGATACCAG
18s	CGCTATTGGAGCTGGAATTACC	GAAACGGCTACCACATCCAA
wnt4b	AAGGCTTCAGAGATGGTCATCG	AGAATGCAACGCCGTAGGACAG
olig2	TCCAGCAGACCTTCTTCTCC	ACAACTGGACGGATGGAAACC
nestin	CGGCCCTTGATCCGTTATAA	ACCGATGTGGTGTGAGGTTTC
gfap	ACCCGTGACGGAGAGATCAT	CACCTGCTCTGACCACTAGC
ccnd1	GCCAAACTGCCTATACATCAG	TGTCGGTGCTTTTCA
pcna	CCCGATTGTGACCCTCTAAA	TTGGAATGAGCAGTTGGACA

4 – Wnt/β-catenin Signaling Regulates Adult Zebrafish Cardiac Repair and Scar Remodeling

4.1 Introduction

Acute myocardial infarction (MI), caused typically by coronary artery occlusion and ischemic injury, is a leading cause of death in humans. Humans and adult mammals have a reduced capacity to restore functional cardiac tissue after infarction, healing largely by accumulation of a non-functional collagenous scar (Laflamme and Murry 2011; Kikuchi and Poss 2012). This fibrotic scar is not contractile and increases likelihood of uncontrolled cardiac remodeling, additional myocardial infarct events, and heart failure (Ptaszek et al. 2012; Kikuchi and Poss 2012). However, there is evidence that mammals may retain some cardiac regenerative ability, as neonatal mice can completely restore functional cardiac tissue in an infarct model but lose this ability within 7 days of birth (Porrello et al. 2011). In contrast, zebrafish maintain robust cardiac regenerative capacity throughout adulthood, fully regenerating vascularized cardiac tissue after ventricular resection, cardiomyocyte ablation, and cryoinjury (Itou et al. 2012; Chablais et al. 2011; González-rosa et al. 2011; Schnabel et al. 2011; J. Wang et al. 2011; Zhang et al. 2013). Therefore, zebrafish have become a popular vertebrate model to study molecular and cellular aspects of cardiac regeneration (Kenneth D Poss 2007).

After resection of 20% of the ventricle apex, a number of critical events occur that culminates in regeneration of all the resected tissue with cardiac muscle by 1 to 2 months post amputation (Kenneth D Poss, Wilson, and Keating 2002). Within 3 days of injury, the clot sealing the apex

transforms into a complex, fibrin-rich milieu of serum factors and erythrocytes (Kenneth D Poss, Wilson, and Keating 2002). Next, new cardiomyocytes are produced to replace the lost tissue. Cre-based genetic fate mapping studies have demonstrated that resident cardiomyocytes de-differentiate and re-enter the cell cycle in adult zebrafish, rather than differentiation from existing progenitors (Jopling et al. 2010; Kikuchi et al. 2010). Moreover, non-myocardial cells also regenerate and play critical spatiotemporally orchestrated structural and signaling roles in this organ-wide regenerative response. Endocardial cells undergo morphological and gene expression changes that manifest within several days post amputation at the injury site (Kikuchi, Holdway, et al. 2011). Epicardial cells proliferate and migrate toward the amputation plane within 14 days post amputation (Lepilina et al. 2006; Kikuchi, Gupta, et al. 2011). Both of these cell types maintain high expression of *raldh2*, which is vital to myocardial proliferation after injury, demonstrating the contribution of non-cardiomyocytes to cardiomyocyte differentiation (Kikuchi, Holdway, et al. 2011).

Our knowledge of the cellular and molecular basis behind cardiomyocyte proliferation is incomplete. Fgfr2 and Fgfr4 are expressed in injury-activated epicardial cells and inhibition of Fgf signaling by transgenic overexpression of a dominant-negative Fgfr inhibits epicardial epithelial-to-mesenchymal transition, inhibiting neovascularization and halting regeneration (Lepilina et al. 2006). TGFβ signaling is also increased in non-cardiomyocytes and has been implicated in regulating cardiomyocyte proliferation and collagen scar remodeling (Chablais and Jazwinska 2012). The notch signaling pathway also regulates cardiomyocyte proliferation in the zebrafish by upregulating notch ligands in the epicardium and endocardium without

altering the activation of endocardial and epicardial cells, providing further evidence that cardiomyocytes require other cell types to initiate cell cycle re-entry (Zhao et al. 2014).

The Wnt/β-catenin signaling pathway has been implicated in a number of regenerative processes, including caudal fin regeneration, retina, and the lateral line, and is largely conserved through evolution (Kawakami et al. 2006; Stoick-Cooper et al. 2007; Kenneth D Poss, Shen, and Keating 2000; Valdivia et al. 2011; Ramachandran, Zhao, and Goldman 2011). Moreover, Wnt/β-catenin signaling regulates cardiac differentiation in human embryonic stem cells, exerts a bi-phasic effect in zebrafish heart development and cardiac specification, and has been implicated to direct cardiac fibroblast activity in mice (Ueno et al. 2007; Gessert and Kuhl 2010; Duan et al. 2011), making it a potential candidate for participating in cardiac regeneration.

In this study, we explored the effect of Wnt/β-catenin signaling modulation in the adult zebrafish after ventricular resection. We show that Wnt/β-catenin signaling is activated after heart amputation near the injury site, including in a subset of cardiomyocytes. We further show that overexpression of Dkk, a ligand that inhibits Wnt/β-catenin signaling, leads to increased scarring of the amputated heart, misregulated gene expression, and a reduction in proliferating cardiomyocytes.

4.2 Results

Wnt/β-catenin signaling is dynamically regulated after heart amputation

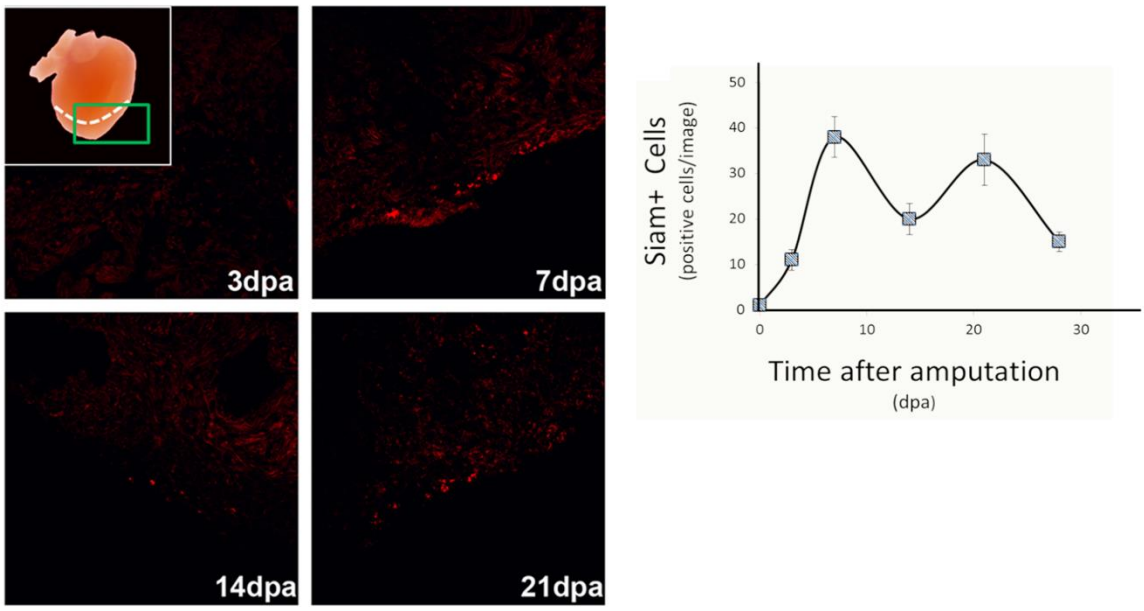

Figure 4.1: Cells undergoing Wnt/β-catenin signaling are present in injury site throughout regeneration. (A) Utilizing the Tg(TCFsiam:mCherry) transgenic fish, cells expressing mCherry were visualized at various timepoints throughout the heart regeneration timeline. (B) Numbers of mCherry+ cells in the bottom third of the heart were quantified from amputated heart sections of Tg(TCFsiam:mCherry) throughout regeneration. Scale bar=100μm; N=3.

To monitor the spatiotemporal molecular and cell dynamics of Wnt/β-catenin signaling after heart amputation, we analyzed activation of a heterologous transcriptional reporter of Wnt/β-catenin signaling in Tg(7x*TCF-xla.Siam:nlsmCherry)*^{ia5} transgenic fish [designated as Tg(*TCFsiam:mCherry)* fish] (Moro et al. 2012). Using the Tg(*TCFsiam*:mCherry) line, we discovered that a small population of cells undergoing Wnt/β-catenin signaling become present from as early as 3 through 28 days post amputation (dpa) in the regenerating heart, confined almost exclusively to the wound boundary initially before penetrating into the wound and

extracellular matrix clot around 7 dpa (Fig. 1A). Quantification of all heart sections of injured fish through 28 dpa revealed that the relative density of these cells peaks at 7 and 21 dpa, although cells were still present in the same localized areas at other timepoints (Fig. 1B). These data show that β-catenin mediated transcription is dynamically regulated during zebrafish heart regeneration.

Dkk overexpression inhibits Wnt/β-catenin signaling and impairs collagen scar remodeling after heart amputation

After amputation of up to 20% of the adult zebrafish heart, the resected cardiac tissue is completely replaced by 42 dpa by new cardiac tissue. This regenerative process eventually fully replaces the collagen-based scar that is observed from 7 dpa onward. The relative level of collagen scarring at 28-30 dpa has been correlated to degree of functional recovery (Kenneth D Poss, Wilson, and Keating 2002) and represents a robust proxy of the extent of heart regeneration. To test if Wnt/β-catenin signaling is regulating collagen scar resolution in the regenerating heart, we quantified total collagen scar area per area in a transgenic line containing heat-shock inducible Dickkopf (*hsDKK1*:GFP), a secreted inhibitor of Wnt/β-catenin signaling (Stoick-Cooper et al. 2007). We first validated that Dkk overexpression inhibited Wnt/β-catenin signaling in the amputated heart by examining hsDKK1:GFP x Tg(TCFsiam:mCherry) transgenic fish. Dkk overexpression reduced the number of mCherry+ cells at 14 dpa by flow cytometry, including a subpopulation of cells that were also expressing Mef2, a cardiomyocyte marker (Fig S1). Dkk was overexpressed daily over 28 days after amputation, and the average total collagen area was assessed across all sections of the heart sample. To

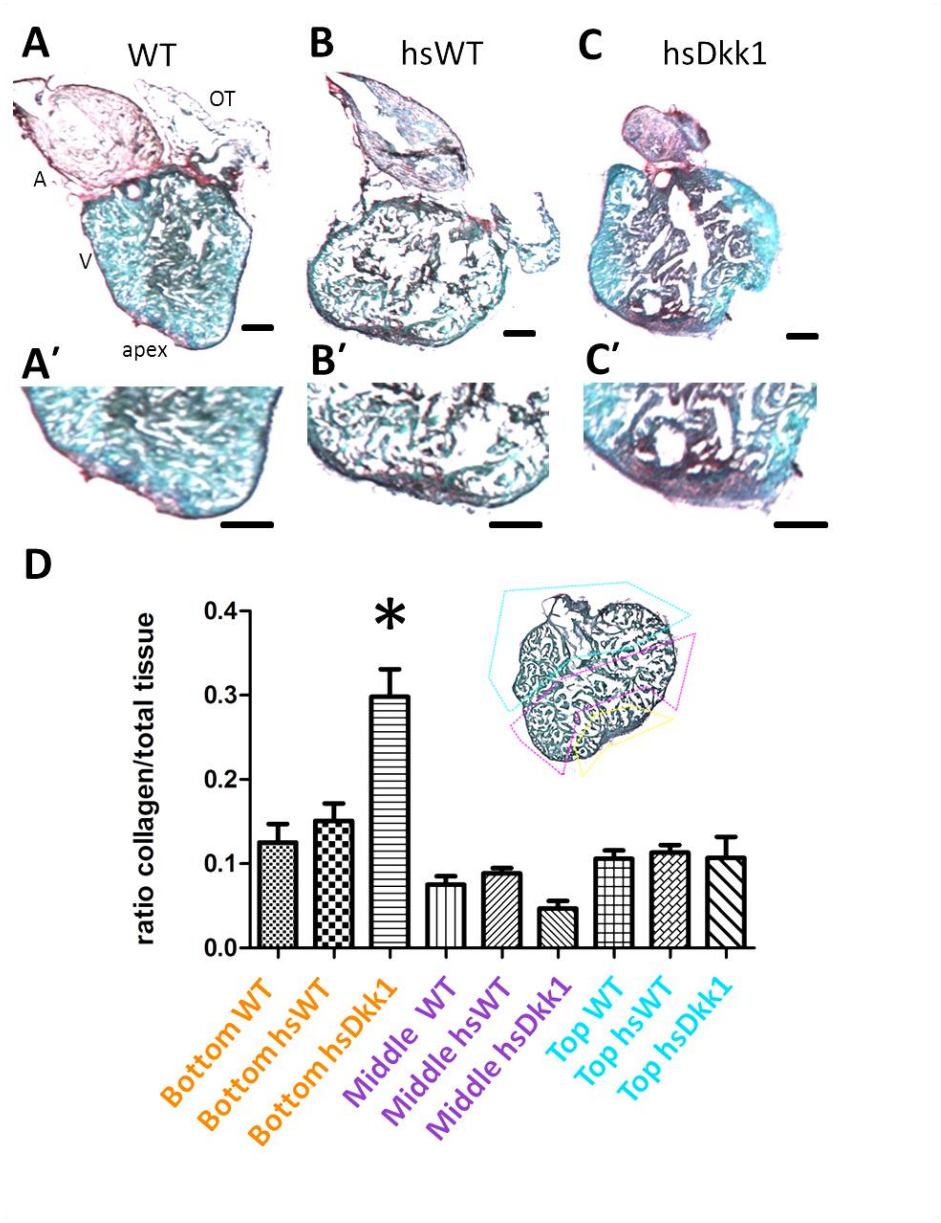

Figure 4.2: Dkk overexpression modulates extent of collagen scar formation after cardiac amputation. (A-C) Dkk overexpression leads to increases in collagen stained area (red) at 28 days post heart amputation compared to controls Scale bar = 300 µm. Conditions evaluated were (A,A') WT (no heat shock), (B,B') hsWT (WT heat shocked daily), and (C,C') hsDkk1 (Tg(hsDKK1:GFP)) heat shocked daily. (D) Collagen scarring was increased by Dkk overexpression only in the apex of the heart (bottom) at 28 days post heart amputation.

**P<0.00; scale bar (A-C) = 300 µm. N= WT (N=27), hsWT (N=27), and hsDkk1 (N=13), total quantification is cumulative from 3 separate experiments of N fish number for each condition as stated. Graph shows mean ± SEM.*

further illustrate and magnify spatial differences in scar presence, we employed a blinded and unbiased computer algorithm to sub-divide scarring into either top, middle, or bottom (incorporating the original resection plane) thirds of the heart (Fig. 2), forming a simplified quantification of scar presence. Dkk1-overexpressing fish displayed greater visible levels of collagen staining at 28 dpa in the bottom third of the heart at the amputation site compared to no heat shock and heat-shock wild type controls (Fig. 2A-C). Moreover, quantification revealed almost 2 times the level of collagen scar area in the injured area in DKK1-overexpressing fish compared to controls (Fig. 2D). Importantly, this difference in scar presence was restricted to the amputation area, and no differences were observed in other areas of the heart (Fig. 2D). Taken together, these data indicate that Dkk overexpression modulates the extent of long-term scarring during regeneration of amputated zebrafish hearts.

Dkk overexpression directly modulates Wnt/β-catenin signaling targets and regenerative cardiac gene expression

We next assessed the role of Dkk overexpression on regulating gene expression in the regenerating heart at 14 dpa by using heatshock to induce a 6 hr pulse Dkk prior to sample collection (Fig. 3). This particular timepoint was chosen since it has been correlated with the peak of cardiomyocyte activation and re-differentiation, epicardial migration, and advent of scar remodeling (Kikuchi and Poss 2012). In agreement with previous studies, several wound healing

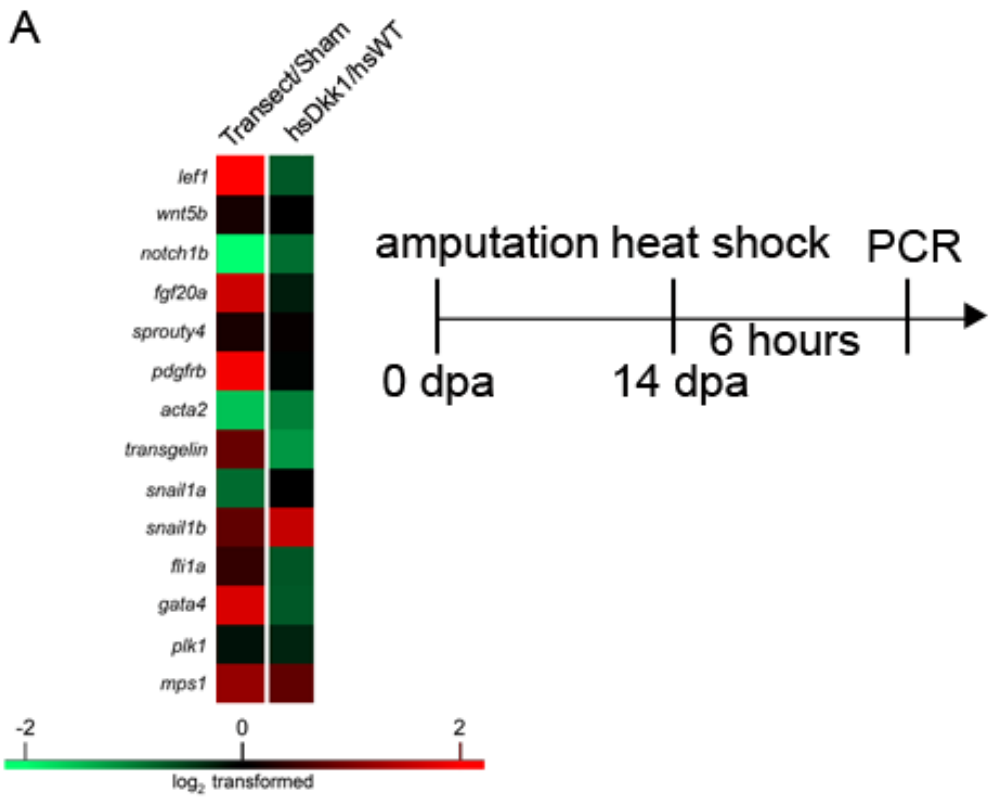

Figure 4.3: Dkk overexpression directly affects tissue expression profile of cardiac injury, healing, and regeneration genes. (A) Gene expression analysis was conducted on amputated cardiac tissue in 7 dpa, 14 dpa, and (after one 6 hr heat shock) 14.25dpa heat-shock inducible Tg(hsDKK1:GFP) fish. mRNA expression was expressed as fold over uncut. N=3.

(*pdgfrb*), regenerative (*fgf20a, mps1, gata4*), and re-vascularization (*fli1*) genes were upregulated in cardiac tissue at 14 dpa, while several genes (*acta, snaila*) associated with epithelial–to-mesenchyme transition were downregulated at 14 dpa (Fig. 3). DKK1-pulsed fish displayed reduced levels of known Wnt/β-catenin signaling target genes after just 6 hrs (*lef1*, *fgf20a*), confirming rapid signaling knockdown. Importantly, Dkk overexpression was sufficient

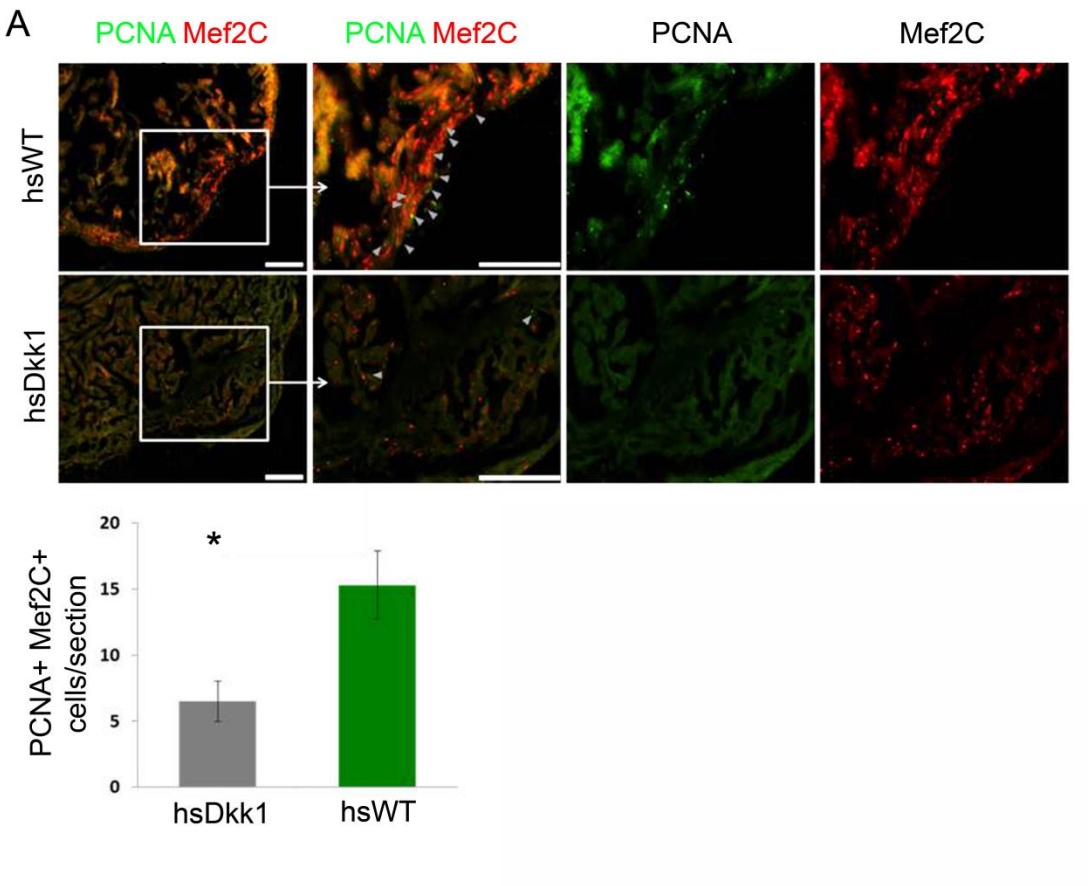

*Figure 4.4: Dkk overexpression reduces activity of renewed cardiomyocytes in wound area. (A) Proliferating cardiomyocytes, defined as PCNA and Mef2C double positive cells, are decreased at 14dpa in Wnt-inhibited Tg(hsDKK1:GFP) fish subjected to daily heat shock compared to heat shock wild type controls. *P<0.05; Scale bar =300μm; N=6. Graph shows mean ± SEM.*

to significantly reduce many assayed regeneration and re-vascularization genes over heat-shock WT controls. Notably, Dkk overexpression reduced expression levels of *gata4* (Fig. 3), which is necessary for cardiomyocyte de-differentiation and proliferation, suggesting that Wnt/β-catenin signaling may be regulating cardiomyocyte proliferation.

Dkk overexpression inhibits cardiomyocyte proliferation

In several zebrafish heart injury models, a critical stage during regeneration is cardiomyocyte de-differentiation, proliferation, and re-differentiation into the wound from 7-14 dpa (Kikuchi, Holdway, et al. 2011). Diminished proliferative and migratory activity of cardiomyocytes during this stage has been linked to reduced heart function (Kikuchi, Holdway, et al. 2011). We assessed the effect of Wnt inhibition on the proliferative activity of cardiomyocytes near the injury site. To examine this, we co-stained regenerating heart sections at 14 dpa for a marker of proliferation, PCNA, indicative of active DNA synthesis, and Mef2c, a marker of cardiomyocytes. Proliferating cardiomyocytes were active mainly in the wound, as well as near the original amputation plane. DKK1-overxpressing fish displayed diminished numbers of proliferating cardiomyocytes, especially deep in the wound and collagen scar (Fig. 4). Taken together, these data reveal a role for Wnt/β-catenin signaling in modulating the proliferation of cardiomyocytes in the wound site at peak activity periods.

Wnt/β-catenin signaling promotes the proliferation of hESC-derived Cardiomyocytes

Work in both rat and human induced pluripotent stem cell-derived cardiomyocytes indicate that inhibition of GSK3B by small molecules increases the number of proliferating cardiomyocytes (Tseng, Engel, and Keating 2006; Titmarsh et al 2016). GSK3B inhibition enhances Wnt/β-catenin signaling, amongst altering other pathways in the cell. Based on the role of Wnt/β-catenin signaling in promoting cardiomyocyte proliferation after zebrafish heart resection, we predicted that Wnt/β-catenin signaling enhanced the proliferation of mammalian, terminally differentiated cardiomyocytes. To test this, we stimulated human embryonic stem cell (hESC)-derived cardiomyocytes for 5 days with recombinant Wnt3a or GSK3B inhibitor Chir-99021,

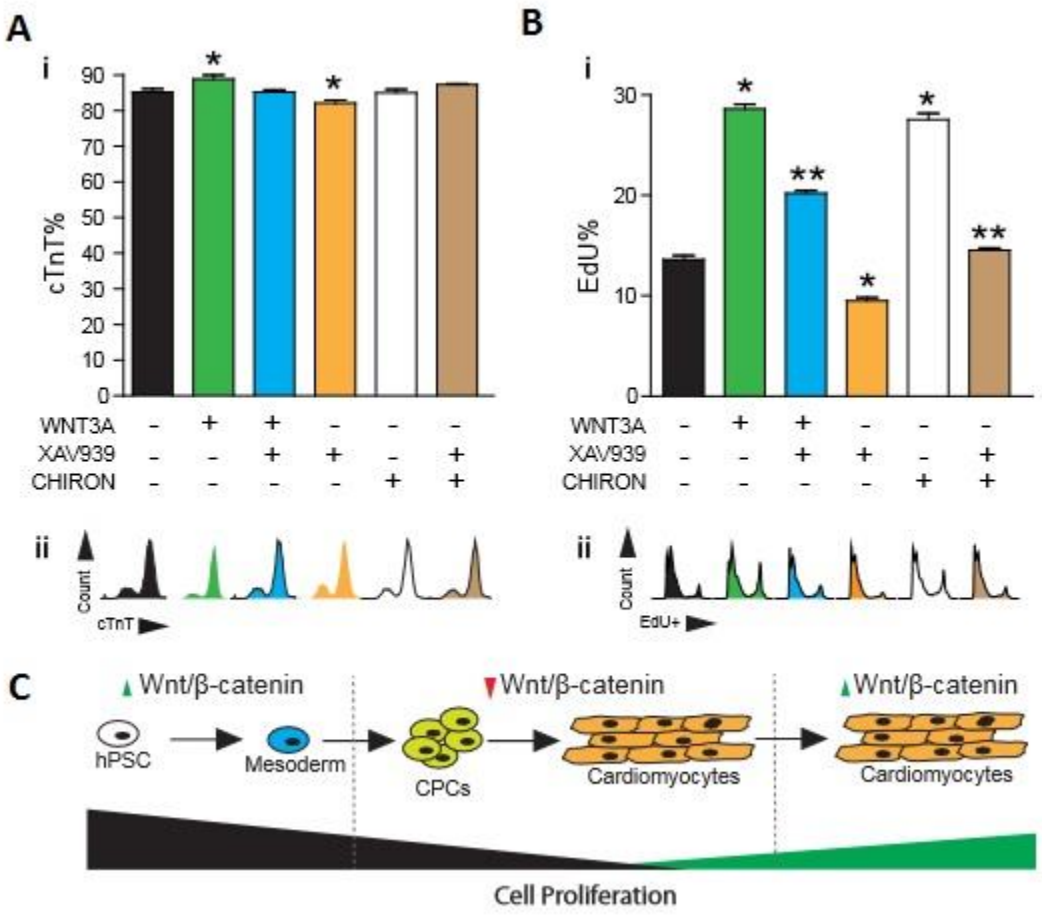

Figure 4.5: Wnt/β-catenin signaling promotes cell proliferation in human embryonic stem cell-derived cardiomyocytes. (A) Cardiomyocyte purity following exposure to Wnt3A, XAV-939, CHIR-99021 (Chiron) alone or in combination as illustrated. Shown are total percentage of cTnT+ cells (i) and representative histograms (ii) with cell count on the y-axis and cTnT on the x-axis. (B) Total percent EdU positive cells (i) and representative histograms following exposure to WNT3A, XAV939, or Chiron alone or in combination as illustrated. (C) Illustration of Wnt/β-catenin signaling during cardiac differentiation and re-entry into the cell cycle. Wnt/β-catenin signaling is induced for pluripotent stem cells to differentiate towards mesoderm which also corresponds with high levels of cell proliferation. The pathway is required to be subsequently inhibited for transition towards to cardiac progenitor cells and definitive cardiomyocytes, resulting in a reduction in cell proliferation. Lastly, re-activation of Wnt/β-catenin signaling by Wnt ligand exposure and GSK3β inhibition induces cell proliferation in terminally differentiated

*cardiomyocytes. *P<0.05, **P<0.05 compared to Wnt3A or Chir-99021 treatment; N=6. Graphs show mean ± SEM.*

either wth DMSO or tankyrase inhibitor XAV-939, which leads to the stabilization of axin to prevent Wnt/β-catenin signal transduction (Huang et al 2009). We found that stimulation of cardiomyocytes with Wnt3a led to an increase in the percent of cells that were cardiac troponin T positive (cTnT+), which was blocked by the addition of XAV-939 (Fig 5A). We also observed that XAV-939 stimulation decreased the percent of cTnT+ cells (Fig 5A). We exposed cells to EdU 24 hours and collected samples to analyze for proliferating cardiomyocytes, defined as being both cTnT and EdU positive. We found that stimulation of cardiocmyocytes by either Wnt3a or Chir-99021 induced significant increases in cardiomyocyte proliferation, and that the addition of XAV-939 was able to inhibit this increase in proliferating cardiomyocytes (Fig 5B). These data suggest that Wnt/β-catenin signaling is able to mediate cardiomyocyte proliferation in terminally differentiated cardiomyocytes derived from human embryonic stem cells (Fig 5C).

4.3 Discussion

Understanding the molecular and signaling events that dictate the cardiac regenerative process in adult zebrafish might reveal molecular or cellular differences from the less robust regenerative responses of injured mammalian hearts. In this study, we utilized a combination of cell tracking and inducible inhibition approaches to evaluate the activity and function of the Wnt/β-catenin signaling pathway after ventricular resection. We show that Wnt/β-catenin signaling is increased after ventricular resection and that Wnt/β-catenin signaling is necessary

for collagen scar remodeling. We also observe that Wnt/β-catenin signaling is necessary for regulating cardiomyocyte proliferation.

Upregulation of Wnt/β-catenin signaling after injury has been observed after injuries in multiple phyla and tissue types (Kawakami et al. 2006; Stoick-Cooper et al. 2007; Ramachandran, Zhao, and Goldman 2011; Petersen and Reddien 2008; Gurley, Rink, and Sanchez Alvorado 2008; Takeo et al. 2013; Briona et al. 2015). In the regenerating heart, we observe active β-catenin mediated transcription in cardiomyocytes and other undefined populations of cells, showing that multiple cell types respond to Wnt/β-catenin signaling, similar to the regenerating fin (Wehner et al. 2014). This suggests that Wnt/β-catenin signaling may be influencing regeneration through cell-specific mechanisms. To that end, stimulation of cultured rat cardiomyocytes with BIO, which enhances β-catenin mediated transcription, or viral overexpression of β-catenin, is sufficient to induce proliferation (Tseng, Engel, and Keating 2006; Hahn et al. 2006). Furthermore, Titmarsh et al has shown that Chir-99021, which also enhances Wnt/β-catenin signaling, is also able to induce proliferation in human induced pluripotent stem cell-derived cardiomocytes. Our work demonstrates that Chir-99021 also induces cardiomyocyte proliferation in hESC-derived cells, but further shows that downstream inhibition of Wnt/β-catenin signaling is able to inhibit the effect of Chir-99021. We also show that stimulation with recombinant Wnt3A is also able to induce cardiomyocyte proliferation, which again can be inhibited by downstream inhibition of signal transduction. This work demonstrates that Wnt/β-catenin signaling is capable of driving terminally differentiated cardiomyocytes to proliferate in the regenerating zebrafish heart and in cultured hESC-derived cardiomyocytes

Although initial transient scar deposition after wound healing is advantageous for restoring contractile function in heart regeneration in zebrafish, scar remodeling by 28 dpa in areas of new cardiomyocyte propagation is essential to restore full myocardial muscle tissue and long-term cardiac function (Chablais and Jazwinska 2012). Our finding that Wnt/β-catenin signaling inhibition increases scar presence and disturbs cardiomyocyte proliferation after heart resection mirrors the similar roles of FGF and TGFβ signaling in positively modulating scar remodeling and cardiomyocyte proliferation (Lepilina et al. 2006; Chablais and Jazwinska 2012). Mammals also initially develop a collagenous scar but, unlike zebrafish, do not eventually remodel it into functional myocardium. Interestingly, overexpression of the Wnt inhibitor FrzA after myocardial infarction in mice leads to improved cardiac function and increased collagen deposition (Barandon et al. 2003). Furthermore, recent work demonstrates that neonatal mouse heart injury induces Wnt/β-catenin signaling in epicardial cells that undergo EMT to become fibroblasts (Mizutani, Wu, and Nusse 2016). Our study suggests that the Wnt/β-catenin pathway may show some conserved functions between zebrafish and mammals, but may also show some differences downstream of Wnt/β-catenin signaling (i.e. scar resolution) and merits further investigation.

Here, we have identified a role for Wnt/β-catenin signaling in zebrafish heart regeneration. We show that Wnt/β-catenin signaling is increased after injury in multiple cell types, and that disruption of Wnt/β-catenin signaling inhibits scar remodeling and cardiomyocyte proliferation. These findings show both conserved and divergent roles of Wnt/β-catenin signaling after heart injury in zebrafish compared to mammals. Gaining a deeper understanding of how individual

cell types are responding to Wnt/β-catenin signaling in the zebrafish heart could provide the mechanistic insights into why these differences are occurring.

4.4 Materials and Methods

Zebrafish strains and heat shock protocol

We used zebrafish (*Danio rerio*) of either sex from the following lines: Wild-type AB, heterozygous Tg(*7xTCF-Xla.siam:mCherry*)^{ia5} [Moro et al., 2012] to visualize β-catenin responsive cells, and heterozygous Tg(*Hsp70l:DKK1-GFP*)^{w34} to inhibit canonical Wnt signaling. Dkk induction was achieved by exposing Tg(*Hsp70:*Dkk1-GFP) and age-matched wild type fish to water slowly increased from 28°C to 37°C. Fish remained at 37°C for 60 minutes, and were slowly brought back to normal water temperature. Fish were exposed to this heat shock protocol at least 4 hours prior to surgery and resumed daily heat shocks at 2 days post amputation. All experiments were approved and conducted in accordance with University of Washington Institutional Animal Care and Use Committee (IACUC) protocol 2057-01 guidelines.

Adult zebrafish ventricular amputation surgeries

Zebrafish of ~6-12 months of age were used for all studies. Heart amputation surgeries were performed as previously described (Kenneth D Poss, Wilson, and Keating 2002). Briefly, Zebrafish were anaesthetized in in buffered 0.0168% tricaine methanesulfonate (MS-222), immobilized in a sponge, and skin posterior to heart cavity pulled open with tweezers, exposing the apex of the ventricle. Using microscissors, 10-20% of the heart at the ventricular apex was amputated. The zebrafish was allowed to clot for 10 seconds and returned to a solitary recovery tank, where it was revived by gentle squirting of oxygenated water over the gills.

Water was changed every day for 2 days while injured zebrafish were kept in solitary confinement, and returned to the general population 2 days post amputation.

Collagen staining and analysis

Whole zebrafish hearts were harvested and then fixed with 4% paraformaldehyde for 45 min and prepared for OCT sectioning. The samples were sectioned with cryostat (CM3050S, Leica) at the thickness of 14 mm. To distinguish the collagen scar tissues histologically from normal tissue, a Fast Green stain was used. Cytosol (i.e., normal tissue) should stain green, and collagen deposition (i.e., scar tissue) should appear reddish in the sections. For image quantification, at least 7 sections with complete morphologies at the slides of each heart sample were chosen to represent each sample. These sections were unbiasedly analyzed by automated computer software to quantify total collagen scar area and total normal cardiac tissue area in each sample. Each section was labeled into bottom (encompassing regenerative tissue), middle, and top (closer to atrium). For each sample, the measure of scarring was the total scar area of each section/total area of tissue averaged among all sections of the same sample.

Flow cytometry and sorting of zebrafish hearts

Flow cytometry and flow-sorting was used to isolate mcherry+ cells from zebrafish hearts. This was performed beginning with isolation of the injured area heart tissue. Once isolated, this tissue was immediately placed in a tissue disassociation solution of 2 mg/ml Collagenase (Sigma-Aldrich) and 0.3 mg/ml Protease (Type XIV, Sigma-Aldrich) in Hanks® solution. The solution was moderately shaken at 30^{o}C for 1 hr with gentle trituration performed every 10 minutes with an 18 gauge needle. After 1 hr, the solution was incubated for 5 minutes in 0.05% trypsin in PBS. Before flow cytometry, cells were stained with rabbit anti-Mef2c (Santa Cruz, C-

313, 1:50, Dallas, TX) and goat anti-rabbit secondary antibody AlexaFluor® 488 (Thermo Fisher Scientific, A11034, 1:1000, Waltham, MA). Disassociated cells were washed in 2% FBS in cell disassociation solution. Disassociated cells from wild type fish at an identical timepoint were used to set up the lower limit (background) of fluorescence expression in each experiment. DAPI (1:600) was used prior to the last wash before flow reading to gauge live/dead cells.

Immunostaining

Whole adult hearts (encompassing ventricle, atrium, and outflow tract) were harvested, washed in PBS for 10 minutes and allowed to bleed out, and fixed in 4% formaldehyde in PBS for 45 minutes at 4^{o}C. Tissue was then washed for 30 min at room temperature with 5% sucrose in PBS, followed by 2 more washes for 1 hour each in 5% sucrose in PBS, and an overnight wash in 30% sucrose in PBS at 4^{o}C. After another overnight wash in a 1:1 ratio of 30% sucrose:100% OCT at 4^{o}C, the tissue was embedded directly in 100% OCT in embedding wells and stored at -80^{o}C before sectioning. Embedded tissue was sectioned in a cryostat, cut into 14 µm transverse sections, and subsequently adhered to VWR Superfrost Plus slides overnight at 40^{o}C.

Rabbit anti-DsRed (Clontech, 632496, 1:200,Mountain View, CA), rabbit anti-Mef2C (Santa Cruz, SC-313, 1:50, Dallas, TX) (1:50), or mouse anti-PCNA (Sigma-Aldrich, P8225; 1:250, St Louis, MO) were added in antibody solution (0.5 %Triton© X-100, 5% goat serum, 0.2% BSA in PBS) overnight at 4°C. Slides were washed 6 times for 15 minutes in PBS with gentle shaking, and goat anti-rabbit secondary antibody AlexaFluor® 488 or 647 or goat anti-mouse 488 (Thermo

Fisher Scientific, A11034, A21245, A11029; 1:1000, Waltham, MA) was added for 2 hr at room temperature in the dark. After 6 more washes in antibody solution the slides were sealed with a coverslip with Prolong® gold antifade reagent (Thermo Fisher Scientific, Waltham, MA). PCNA staining was subjected to antigen retrieval prior to staining by heating slides at 85°C in sodium citrate buffer for 30 minutes.

Quantitative RT-PCR

Zebrafish, 14 days post amputation, were euthanized in an overdose of buffered tricaine methanesulfonate (MS-222) in fish tank water. 50% of the heart, including the clot, were amputated and collected into pools of at least n=5 hearts per group. Samples were homogenized and lysed with Trizol to extract RNA. cDNA was synthesized via RevertAid cDNA kit (Thermo Fisher Scientific, Waltham, MO). Quantitative PCR was run on a Roche LightCycler 480. All samples were normalized to β-actin levels and compared to 18s normalization and analyzed by comparing expression levels of amputated hearts to sham surgery groups, or HsDkk+ fish to HsDkk- fish. Primer sequences can be found in table in S1 Table.

Cell culture and cardiomyocyte directed differentiation

Human embryonic stem cells (RUES2, Rockefeller University; hESC-09-0013) were maintained as previously described (Hofsteen et al 2016). Briefly, hESCs were plated on Matrigel (BD) coated tissue culture plates and maintained with irradiated mouse embryonic fibroblast conditioned media containing 5 ng/mL human bFGF (Peprotech, 100-18B). High density hESC directed differentiation towards cardiomyocyte was conducted as previously described (Palpant et al 2015). Cardiomyocytes were harvested on day 25 of the protocol, pooled and randomly re-plated in a 24-well tissue culture plates at a seeding density of 200k cells/well in 1 mL of RMPI

(Invitrogen) containing B27 supplement. Cardiomyocytes were allowed to recover for 4 days until they were exposed to WNT3A (100 ng/mL, R&D Systems), CHIR-99021 (2 µM, Cayman chemical) and XAV-939 (5 µM, Tocris) as shown in Figure 5 for 5 days prior to collection for flow cytometry.

Cardiomyocyte purity assay

Cells were labeled for flow cytometry using cardiac troponin T (Thermo Scientific) or an IgG corresponding isotype control. Cells were analyzed using a BD FACSCANTO II (Beckton Dickinson, San Jose, CA) with FACSDiva software (BD Biosciences). Instrument settings were adjusted to avoid spectral overlap. Data analysis was performed using FlowJo (Tree Star, Ashland, Oregon).

EDU (5-ethynyl-2'-deoxyuridine) assay

Cardiomyocytes were exposed to 10 µM EdU (DMSO) for 24 h one day prior to collection for flow cytometry. Samples were trypsinized to obtain single cells and fixed for 10 min with 4% paraformaldehyde. Staining procedures for EdU incorporation were performed using the Click-iT ® EdU Flow Cytometry Cell Proliferation Assay kit using an Alexa Fluor 647 antibody (ThermoFisher).

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4.6 Supplemental Material

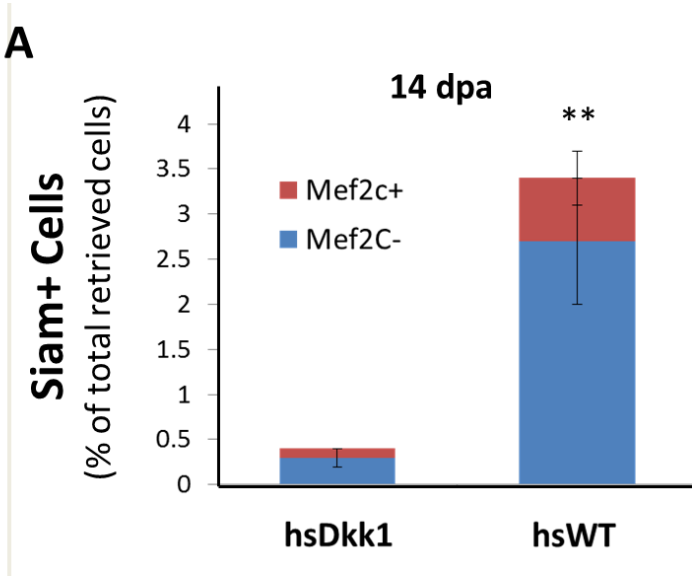

Figure 4.S1: mCherry+ cells are reduced in fish overexpressing Dkk after heart amputation. (A) Pooled hearts 14 days post amputation were disassociated and flow-sorted for cells expressing mCherry and cardiomyocyte marker Mef2c in Tg(*TCFsiam:mCherry)* fish*. **P < 0.05; N=5, Graph shows mean ± SEM.*

Table 4.S1:

Gene	Forward Primer	Reverse Primer
bactin	GGTATGGGACAGAAAGACAG	AGAGTCCATCACGATACCAG
18s	CGCTATTGGAGCTGGAATTACC	GAAACGGCTACCACATCCAA
lef1	GAGGGAAAAGATCCAGGAAC	AGGTTGAGAAGTCTAGCAGG
notch1b	TCACTTCTGCCAACAAGGTG	TCAAAGGGGCTACACTGACC
fgf20a	CAGCTTCTCTCACGGCTTGG	AAAGCTCAGGAACTCGCTCTG
sprouty4	CGGATAGACGTCCGCTTTTA	GTGAGGAACCCTTGACTCCA
pdgfrb	CACACAAACGTCTTCCCAGA	GCATTACAGCAGCCTGAACA
acta2	AAAGCAAGAGGGGAATCCTG	TCTCCCTGTTGGCTTTAGGA
transgelin	CGGTGAAGAAGATCCAGAGC	AAAGCTCCGTCCTCTTTGGT
snail1a	GCAAGAGGCCAAACTACAGC	GTCTGACGTCCGTCCTTCAT
snail1b	ATGCCACGCTCATTTCTTGT	TCATCCTCCTCTCCACTGCT
fli1a	CCAAACATGACGACCAATGA	AGAGCAGGACCTCGGTGTTA
gata4	TTACCTGTGCAATGCCTGTG	TGCAGACTGGCTCTCCTTCT
plk1	GTTGGAAAGCCTCCATTTGA	GAACCTGGGAGGAACAGTGA
mps1	ACTCGCAGGTCGGAACTCTG	CCACACGTCCCCTTTAGCAC

5 - Discussion and Future Directions

5.1 Summary

The work presented in this thesis interrogates the mechanisms of regeneration in 3 highly organized tissues. In the regenerating fin, we establish a role for cholinergic signaling in blastema formation and further demonstrate the necessity of cholinergic activity for Wnt signaling initiation, which has previously been shown to be necessary for blastema formation in the regenerating fin (Stoick-Cooper et al. 2007). Similarly, we found that Wnt signaling is also necessary for functional recovery and glial bridge formation after spinal cord injury. We also demonstrated that inhibition of Wnt signaling prevents scar tissue resolution and cardiomyocyte proliferation after heart resection in the adult zebrafish. These projects illustrate an example of a signaling pathway that is able to regulate multiple regenerating tissues of the zebrafish.

5.2 Future Work

5.2.1 Appendage Regeneration

There are several areas of research to follow up our finding that cholinergic activity is necessary for caudal fin regeneration and for Wnt signaling. While we have shown that cholinergic activity is necessary for Wnt signaling, the mechanism of how neuronal activity modulates Wnt

signaling is not yet understood. It is possible that neurons secrete Wnt ligands, as discussed in chapter 2, and this is now a testable hypothesis, utilizing CRISPR-Cas9. CRISPR-Cas9 technology repurposes a bacterial defense mechanism used to acquire resistance to pathogens (Horvath and Barrangou 2010). This process requires the expression of Cas9 protein and a guide RNA (gRNA) specific to a target sequence that is to be mutated. This process has been leveraged to create mutations in specific genes in organisms and cell lines, including the zebrafish. The generation of a transgenic line featuring heat shock-inducible Cas9 allows for the temporal control of CRISPR mutations (L. Yin et al. 2015). These fish could be crossed with transgenic fish expressing the appropriate gRNA under a tissue specific promoter (e.g. elavl4 for neurons) to knock out Wnt ligands or Wntless, a protein necessary for Wnt secretion, in specific tissues (Fig 1). These double transgenic fish could be heat shocked as adults to produce mutations after development to avoid potential defects in development.

Our work focused specifically on cholinergic signaling, but non-cholinergic sensory neurons are highly present in the zebrafish caudal fin. Determining a potential role for non-cholinergic nerves would provide an interesting comparison to the newt, where it is known that nerve presence is not specific to nerve type, but rather abundance of nerves (Drachman and Singer 1971; Sidman and Singer 1951). Testing this hypothesis would require the generation of new transgenic fish that allow for the inhibition of signaling in sensory neurons. Mutation of Sorbs3 inhibits dorsal root ganglion development (Malmquist et al. 2013), but also affects vasculature development (Vanhollebeke et al. 2015), making a whole organism mutant inappropriate for this experiment, but presents a potential target for cell-specific mutations.

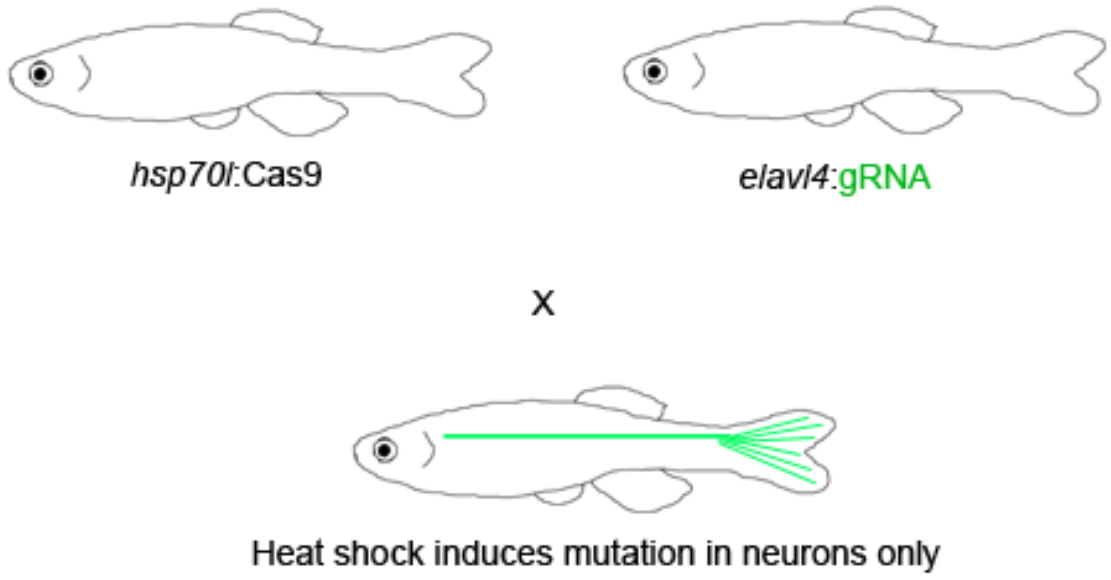

Figure 5.1: Tissue specific, inducible CRISPR/Cas9 Experimental Design. (A) Transgenic fish with a heat shock-inducible Cas9 protein can be crossed with transgenic fish expressing gRNA under the control of a tissue-specific promoter (e.g. elavl4 for neurons), producing fish that can mutate genes with spatial and temporal regulation.

5.2.2 Spinal Cord Regeneration

The work presented here, along with other publications, shows that multiple cell types respond to Wnt/β-catenin signaling in the spinal cord (Briona et al. 2015). The work done in these experiments utilized systemic inhibition of Wnt/β-catenin signaling, but investigation of cell-specific responses, including interactions between Wnt-responsive cells and non-responsive cells, is not easily done in this system. However, an inducible and cell-specific inhibition of Wnt signaling would allow for the interrogation of the mechanisms behind these Wnt-mediated responses. There are several options for producing inducible, tissue specific knockdown of

Wnt/β-catenin signaling, utilizing an intracellular inhibitor of Wnt/β-catenin signaling. One option is to use a tissue specific promoter (e.g. gfap for glia) driving an inducible Cre (CreERT2). This fish could be crossed with a line expressing either a constitutively active promoter (bactin) or an inducible promoter (hsp70l) with a lox-stop-lox before expression of Wnt/β-catenin signaling inhibitor axin1 (Fig. 2A-B). In these lines, tamoxifen treatment would allow for the Cre to excise the lox-stop-lox in only the glia, in this example. Choice of promoter for the axin1 construct allows for either constitutively active or to be induced by heat shock, which allows for more temporal control. Alternatively, the TetON system allows for tissue specific and temporal control through treatment with doxycycline. In this system, the gfap promoter drives expression of TetA, and this fish would be crossed with a transgenic line driving axin1 expression with a Tet responsive element (TetRE) (Fig. 2C). Treatment with doxycycline would allow for expression of axin1 only in glia, again providing a method for tissue specific, inducible inhibition of Wnt/β-catenin signaling.

Translating Wnt signaling's role in bipolar glia formation to mammalian models could be done through enhancing Wnt signaling after spinal cord injury and analyzing glia for a bipolar morphology. It has been shown that Wnt3a-secreting fibroblasts enhance functional recovery in rats with spinal cord injuries (Suh et al. 2011). It has also been shown that Fgf2 treatment can cause glia in the mouse spinal cord to form a bipolar morphology and enhance functional recovery, suggesting that mammalian glia are capable of forming a bipolar morphology *in vivo* (Goldshmit et al. 2014). It is possible that the Wnt3a-secreting fibroblasts are producing glia with a bipolar morphology, but this has not been formally tested.

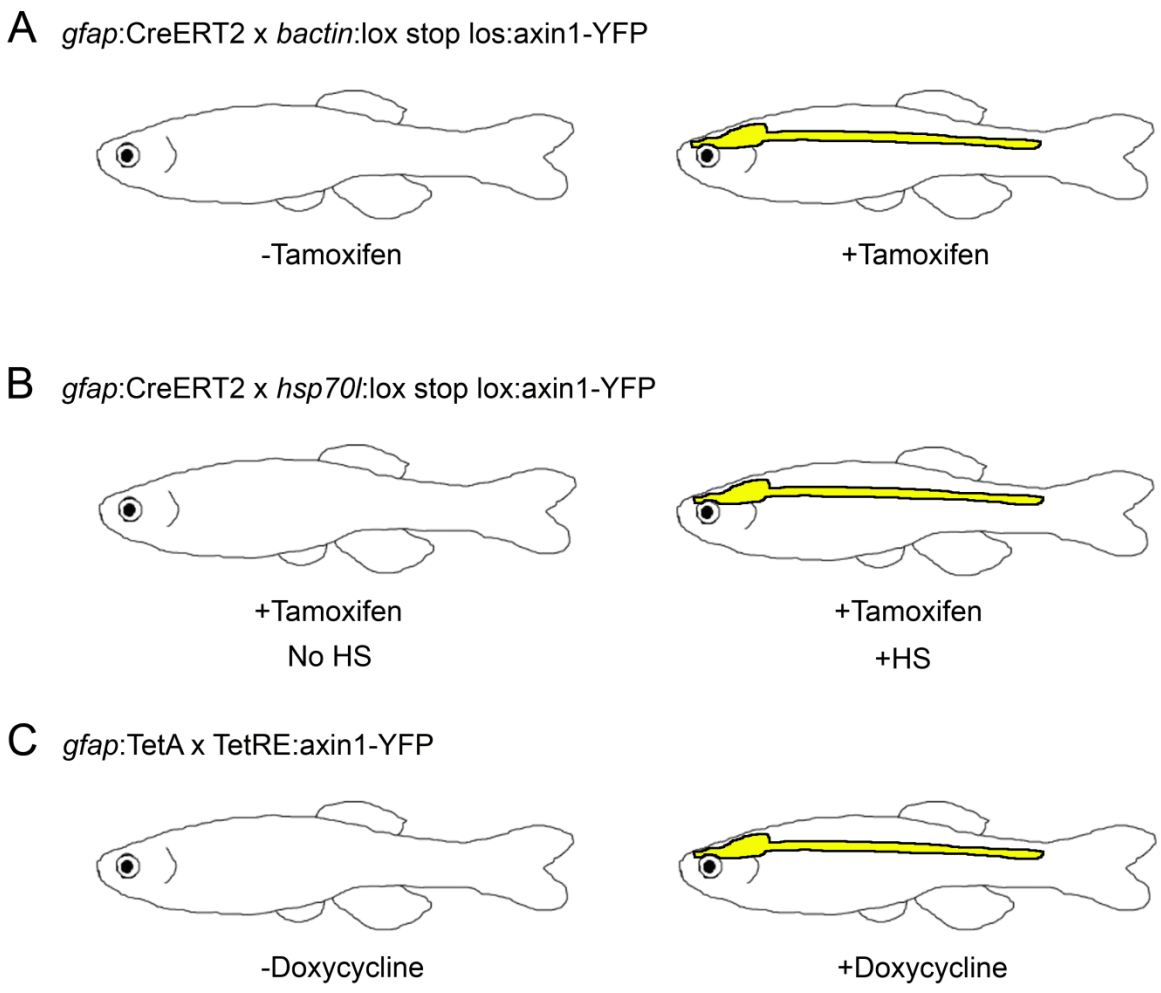

Figure 5.2: Strategies for tissue specific, inducible inhibition of Wnt/β-catenin signaling. (A) A Tg(gfap:CreERT2) x Tg(bactin:lox stop lox:axin1-yfp) transgenic cross would allow for constitutive inhibition of Wnt/β-catenin signaling in glia by treating the fish with tamoxifen. (B) A Tg(gfap:CreERT2) x Tg(hsp70l:lox stop lox:axin1-yfp) transgenic cross allows for temporal control of Wnt/β-catenin signaling inhibition in glia with tamoxifen administration followed by heat shock. (C) A Tg(gfap:TetA) x Tg(TetRE:axin1-yfp) transgenic cross allows for temporal control of Wnt/β-catenin signaling inhibition in glia with doxycycline administration.

Another therapeutic avenue involves injecting glial-restricted precursors (GRPs) or neural progenitor cells (NPCs) into the injury site and using Wnt ligands to induce a bipolar morphology in glial cells. It has been shown deriving GRPs via different methods results in morphologically and molecularly different astrocytes, which in turn have different effects on functional recovery (Davies et al. 2008). Treating GRPs with Wnt ligand may be able to induce these cells into bipolar glia *in vitro*, which would make it possible to inject GRPs or NPCs into the injury site and treat with molecules to produce the bipolar glia. Engraftment of NPCs is able to enhance recovery after mild or moderate spinal cord injuries without directing differentiation (Yokota et al. 2015), but adding small molecule stimulation to the cells may be able to further enhance this recovery.

Understanding the mechanism of bipolar glia formation is also of great interest, specifically looking at the molecular mechanisms downstream of Wnt signaling. Our data indicates that Wnt signaling is acting through β-catenin-mediated transcription, making RNA Sequencing (RNA-Seq) a viable method for exploring the mechanism of Wnt-mediated bipolar glia formation. This could be approached through performing flow cytometry to sort out glia from the regenerating spinal cord and performing single cell RNA-Seq in regenerating WT, heat shock WT, and heat shock Dkk1 fish at different timepoints. This method could identify downstream targets of Wnt/β-catenin signaling, which could be tested for bipolar glia formation using CRISPR/Cas9 as detailed above.

5.2.3 Heart Regeneration

Similar to the spinal cord, the heart increases Wnt/β-catenin signaling in multiple cell types. Understanding the specific role of Wnt/β-catenin signaling in cardiomyocytes compared to other cell types (e.g. epicardial cells) would clarify if Wnt/β-catenin signaling in cardiomyocytes is sufficient for cardiomyocyte proliferation, or if non-cardiomyocyte Wnt/β-catenin signaling is necessary for cardiomyocyte proliferation. The strategies outlined in figure 5.2 would also be applied to answering these questions.

It is interesting to note that only a subset of cardiomyocytes respond to Wnt/β-catenin signaling to reenter the cell cycle and proliferate. Understanding why some cells respond and others do not could allow for a better understanding of how cardiomyocytes proliferate. Single cell RNA sequencing could potentially identify different subsets of cardiomyocytes prior to stimulation to induce proliferation. Identifying and labeling cardiomyocytes displaying a different transcriptome would provide the opportunity to find potential molecular cues that prime cardiomyocytes for proliferation and could be used in concert with small molecules to stimulate proliferation (e.g. Chir99021) to increase cardiomyocyte production after injury in mammals.

5.3 Conclusions

Throughout this work, Wnt/β-catenin signaling is implicated in the regeneration of numerous tissue types. Wnt/β-catenin signaling is also highly studied in development, cancer biology, and adult neurogenesis. All of these processes involve stem cells or putative stem cells, which suggests Wnt/β-catenin signaling may be a general regulator of stem cell biology. Studying Wnt/β-catenin signaling in different contexts of stem cell biology and comparing how different stem cell types (e.g. naïve/prime, embryonic/induced pluripotent stem cells, adult stem

cells/cancer stem cells) respond could uncover novel mechanisms of stem cell regulation and potential therapeutic targets.

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