Molecular Mechanisms Regulating the Pro-Regenerative Glial Cell Response to Spinal Cord Injury in Axolotl

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Dedication

I dedicate this thesis to my family John, Patty and Julia Sabin. Without their constant support and encouragement I would not be who I am or where I am today.

Abstract

Axolotl salamanders have the remarkable ability to functionally regenerate after spinal cord injury. In response to injury, glial cells adjacent to the lesion undergo a pro-regenerative response, proliferate and migrate to reconnect the spinal cord and create a permissive environment for axon regeneration. This is in stark contrast to the mammalian response to spinal cord injury. Damaged astrocytes undergo reactive gliosis and contribute to a glial and fibrotic scar by secreting axon growth inhibitory molecules like chondroitin sulfate proteoglycans and collagens. This ultimately results in failed axon regeneration and a loss of sensory and motor function below the lesion. Why the pro-regenerative glial cell response in axolotl is so different from mammalian astrocytes and the identities of pro-regenerative downstream molecular pathways were not well known. To this end, we identified a dynamic change in glial cell membrane potential that was necessary for the pro-regenerative glial cell response to injury. Disruption of glial cell depolarization by either genetic or pharmacologic approaches inhibited the pro-regenerative glial cell response to injury and blocked spinal cord regeneration. Transcriptional profiling and biochemical approaches identified the ERK/c-Fos signaling pathway as key effector molecules downstream of glial cell depolarization.

Investigations into the identity of the c-Fos binding partner revealed that JunB, not the canonical c-Jun, is the c-Fos binding partner in axolotl glial cells. While reactive astrocytes in mammals express AP-1$^{cFos/cJun}$ which functions to promote reactive gliosis, glial scar formation, and inhibit spinal cord regeneration, AP-1$^{cFos/JunB}$ represses expression of reactive gliosis associated genes. Therefore, we hypothesized that differential composition of AP-1 could regulate the different cellular responses to injury. Consistent with our hypothesis, the ectopic overexpression of AP-1$^{cFos/cJun}$ in axolotl glial cells leads to defects in axon regeneration, similar to mammals.

To determine how glial cells repress *c-Jun* expression, we identified a miR-200a binding site in the 3' untranslated region of axolotl *c-Jun* transcript. Using in vivo and in vitro approaches, we showed that axolotl *c-Jun* is a direct target of miR-200a. Additionally, inhibition of miR-200a leads to axon regeneration defects reminiscent of the AP-1$^{cFos/cJun}$ overexpression phenotype. Finally, transcriptomic profiling of miR-200a inhibitor-electroporated spinal cords revealed differential expression of a subset of genes involved with reactive gliosis, the glial scar, extracellular matrix remodeling, inflammation, migration, and axon guidance compared to control spinal cords. Collectively these results reveal that miR-200a inhibits signaling networks involved with reactive gliosis, the glial scar, and other processes necessary for spinal cord regeneration.

Further examination of the RNA sequencing data revealed that miR-200a inhibition led to the expression of the mesoderm transcription factor Brachyury and down-regulation of a host of neural genes, including *Sox2*. This expression profile is reminiscent of a more developmentally primitive spinal cord progenitor population called neuromesodermal progenitors. This suggests that miR-200a inhibition led to the loss of the neural identity and acquisition of a more neuromesodermal progenitor-like state. Subsequent analysis revealed miR-200a inhibition indirectly promotes Brachyury expression, specifically in axolotl glial cells, perhaps via modulation of FGF and Wnt signaling molecules. Whether modulation of Brachyury expression is sufficient to induce glial cells to fully dedifferentiate into NMPs and contribute to mesoderm-derived tissues (muscle/cartilage) during regeneration is not clear.

In summary, my thesis research identified an injury-induced change in glial cell membrane potential that was necessary for the pro-regenerative glial cell response to spinal cord injury. The injury-induced change in glial cell membrane potential is up-stream of ERK signaling and c-Fos expression. In damaged mammalian astrocytes, c-Fos heterodimerizes with c-Jun to promote reactive gliosis and glial scar formation. However, a majority of axolotl glial cells do not express c-Jun and instead c-Fos heterodimerizes with JunB to form AP-

$1^{cFos/JunB}$. AP-$1^{cFos/JunB}$ functions to inhibit reactive gliosis, glial scar formation, and promote the pro-regenerative glial cell response. Ectopic overexpression of AP-$1^{cFos/cJun}$ in axolotl glial cells inhibits spinal cord regeneration. Axolotl glial cells express the microRNA miR-200a, which functions to repress c-Jun expression. Inhibition of miR-200a blocks spinal cord regeneration, leading to differential expression of genes involved with reactive gliosis and glial scar formation. Finally, miR-200a may play an additional role in stabilizing the neural identity of neural progenitor cells during axolotl spinal cord regeneration. Inhibition of miR-200a could result in dedifferentiation of glial cells to a more neuromesodermal progenitor-like identity, perhaps by modulating Wnt and FGF signaling.

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Chapter 1: Introduction

An Introduction to Regeneration

The ability of certain organisms to regenerate missing or damaged body parts has long fascinated scientists. Since the earliest observations of Aristotle and T.H. Morgan, regenerative biologists have been seeking answers to a unified set of questions: What is the cellular source of the regenerate and from where do these cells come from? What are the signals that turn on the regenerative program and what signals terminate this process? Does regeneration recapitulate embryonic development or are there regeneration-specific differences? Unfortunately, while regeneration is a common phenomenon found across metazoa, most detailed regeneration studies have been confined to a handful of model organisms with a proven track record of thriving in a laboratory environment (Russell et al., 2017; Sanchez Alvarado, 2018). Additionally, many model organisms that have a robust genetic tool kit, like mice, *Drosophila*, and *C. elegans*, do not display robust regenerative characteristics. However, with the technological advances that have occurred within the last 30-40 years, including more efficient transgenesis, transient gene knock down or over expression, genetic knockouts, Cre-mediate DNA recombination, CRISPR/Cas9 genome editing, live cell imaging, and RNA sequencing, we are poised to answer these questions at unprecedented cellular and molecular resolution and across more organisms than ever before.

While the ability of organisms to regenerate missing body parts is wide spread throughout metazoa the degree to which an organism is capable of regeneration is highly variable **(Fig.1-1)** (Poss, 2010; Sanchez Alvarado, 2000; Sanchez Alvarado and Tsonis, 2006; Slack, 2017; Tanaka and Ferretti, 2009; Tanaka and Reddien, 2011). The ability to regenerate has been subdivide into 5 broad biological levels: whole body regeneration, like planarian flatworms that can regenerate their entire body from a small fragment of tissue (Reddien, 2013; Zhu and Pearson, 2016), structural regeneration, like amphibian limb or tail regeneration (Brockes and Kumar, 2005; Slack et al., 2004), internal organ

regeneration, like zebrafish heart (Gemberling et al., 2013) or mammalian liver regeneration (Widmann and Fahimi, 1975), tissue regeneration, such as scar free wound healing in axolotl (Erickson et al., 2016; Seifert et al., 2012b) and the African spiny mouse (Seifert et al., 2012a) and finally cellular regeneration, such as axon regeneration in *C. elegans* (Byrne and Hammarlund, 2017; Tang and Chisholm, 2016) or mammalian muscle regeneration (Hernandez-Hernandez et al., 2017).

The mechanisms that organisms use to regenerate after injury are equally variable but can be subdivided into two broad categories: Morphallaxis and Epimorphic Regeneration. Morphallaxis occurs in the absence of cell proliferation and results from the remodeling of pre-existing tissues to replace the damaged or missing part. This type of regeneration is observed after injury in the fresh water cnidaria *Hydra* (Holstein et al., 1991; Park et al., 1970). Interestingly, a closely related species, *Nematostella vectensis*, does not regenerate in the same manner. Instead, *Nematostella* undergo epimorphic regeneration, which is dependent on cell proliferation (Amiel et al., 2015; Passamaneck and Martindale, 2012). Epimorphic regeneration results in the formation of the regeneration blastema, which is a transient structure comprised of proliferating progenitor cells that will differentiate into the tissues of the regenerate. Formation of a blastema is unique to organisms that have the natural ability to regenerate and in organisms that fail to regenerate, like mammals, a blastema is not formed and a scar is formed instead. Scar formation allows for structural support of the injured tissue but at the expense of functional regeneration of the damaged tissue.

Mammals have a very limited regenerative capacity, which further declines with age. In response to most injuries, mammals form scars and are unable to functionally regenerate missing or degenerated tissues. This is very obvious after neurological trauma or in response to neurodegenerative diseases. Following spinal cord injury or traumatic brain injury, local cells respond by forming a glial and fibrotic scar that inhibits axon and neuronal regeneration (Rolls et al., 2009; Silver and Miller, 2004; Sofroniew, 2009). Additionally, degeneration of

dopaminergic neurons leads to Parkinson's disease and patients with Parkinson's fail to naturally replenish those lost neurons (Di Santo and Widmer, 2018). Human studies and mammalian disease models have provided a lot of valuable information about disease progression and etiology. However, these models have provided significantly less information about how to mount a robust pro-regenerative or pro-restorative response from endogenous stem or progenitor cells. To address these questions, researchers harness the amazing regenerative potential of invertebrate and non-mammalian vertebrate species that are able to regenerate after nervous system injury (Tanaka and Ferretti, 2009).

The following sections will provide an introduction to what is known about nervous system regeneration. We will first discuss what is known about this process in model organisms that have the natural ability to regenerate. Next, we will discuss the mammalian response to spinal cord injury, the formation of the glial scar, and how the glial scar functions to inhibit axon regeneration and functional recovery. We will then specifically focus on the developmental origin and regenerative potential of neural progenitor cells in regenerative and non-regenerative model systems. Finally, we will consider how bioelectric and microRNA signaling can regulate the regenerative response to nervous system injury.

Brain Regeneration in Planaria and Salamanders

Planarian Brain Regeneration

Despite being evolutionarily more primitive than humans, planarian flatworms display a similar organization and cellular diversity in their nervous system (Ross et al., 2017). At their anterior pole there is a concentration of neurons that make up the cephalic ganglia, which functions as a primitive "brain" (Sarnat and Netsky, 1985). Within the cephalic ganglia are neurons that secrete a diverse range of neurotransmitters including dopamine, acetylcholine, GABA,

serotonin, and various neuropeptides (Nishimura et al., 2007a; Nishimura et al., 2007b; Nishimura et al., 2008a; Nishimura et al., 2010; Nishimura et al., 2008b). These classes of neurons are also found in mammals, which suggests an ancient and evolutionarily conserved role for these classes of neurons. Recently, glial cells were identified in the planarian brain, which can be identified by expression of a neurofilament-associated gene *Smed-if-1* and express known astrocyte markers such as glutamine synthetase and excitatory amino acid transporter (Roberts-Galbraith et al., 2016; Wang et al., 2016). These putative glial cells are found within the cephalic ganglia and send cytoplasmic projections to regions of the ganglia and nerve chord with a high density of synapses (Wang et al., 2016). Due to those observations, it was proposed that planarian glial cells could play a role in synapse formation or stabilization similar to mammalian astrocytes. Neurons within the cephalic ganglia send projections to the ventral nerve cords and each nerve cord is connected via transverse commissures (Okamoto et al., 2005). While many of the classes of neurons and the rough anatomical organization of the planarian central nervous system is similar to mammals, the respective response to injury is drastically different. In humans, traumatic brain injury results in scar formation and cognitive or behavioral deficits, however planarians are able to fully regenerate their brain after decapitation (Cebria et al., 2002b; Inoue et al., 2004).

Decapitated planaria regenerate their heads, including the cephalic ganglia, within 7 days (Cebria et al., 2002b; Inoue et al., 2004). Following decapitation, resident stem cells called neoblasts proliferate and migrate to the wound site and contribute to the blastema (Reddien, 2013). Neoblasts are a heterogeneous population of stem cells which appear to be comprised of both pluripotent and multipotent progenitors (Reddien, 2018). Pluripotent neoblasts are sufficient to rescue lethally irradiated planarians after transplantation and can be prospectively identified via the expression of the transmembrane protein TSPAN1 (Wagner et al., 2011; Zeng et al., 2018). Multipotent, neural committed neoblasts have been identified using in silico approaches on published single cell

RNA sequencing data sets as well as comprehensive in situ gene expression profiling approaches (Cowles et al., 2013; Molinaro and Pearson, 2016). Furthermore, subsets of neoblasts express SoxB1 orthologous, similar to vertebrate neural stem cells (Roberts-Galbraith et al., 2016; Ross et al., 2018). Genetic loss of function analysis of two different SoxB1 genes in planaria showed important roles for nervous system maintenance and regeneration (Roberts-Galbraith et al., 2016; Ross et al., 2018). These results suggest that planarians harbor a committed neural progenitor neoblast compartment whose function is dependent on SoxB1 genes.

The first clusters of neurons appear within the blastema between 24 and 48 hours post decapitation (Cebria et al., 2002b; Nishimura et al., 2007a; Umesono et al., 1997, 1999). Post mitotic, newly specified neurons begin to express neurotransmitter and region specific genes as early as 36 hours after decapitation (Nishimura et al., 2007a; Umesono et al., 1997, 1999). Rudimental brain morphology begins to form between 2-3 days after decapitation, including the reappearance of the anterior commissure that connects the cephalic ganglia (Nishimura et al., 2007a; Umesono et al., 1997). Additionally, the optic chiasm and projections between the new brain and ventral nerve cords are formed. Brain morphology similar to uninjured brain is restored by 7 days after decapitation and is coincident with complete behavioral recovery (Inoue et al., 2004; Inoue et al., 2014; Reddien et al., 2005).

Reverse genetic screens using RNA interference have begun to shed light on the molecular mechanisms regulating whole brain regeneration in planaria. RNAi against the epidermal growth factor receptor (EGFR) *Smed-egfr-3* impairs blastema formation and head regeneration, partially due to defects in asymmetric division of neoblasts (Fraguas et al., 2011; Lei et al., 2016). Furthermore, knockdown *Smed-egr-4*, an EGFR target gene, specifically inhibits neural specification and formation of the brain primordium during head regeneration (Fraguas et al., 2014). Interestingly, *Smed-egr-4* RNAi does not affect reestablishment of anterior identity on the regenerating tissue but specifically

affects brain regeneration (Fraguas et al., 2014). This suggests multiple pathways act in parallel to ensure the proper identity of (1) the regenerating head and (2) the regeneration of discrete tissues within the head. Further molecular characterization identified a neuregulin homologue, *Smed-nrg-7*, which functions as a putative ligand for EGFR during planarian head regeneration (Lei et al., 2016). Knockdown of *Smed-nrg-7* affects neoblast division and neural specification, similar to *Smed-egfr-3* knockdown, suggesting these molecules function in the same pathway (Lei et al., 2016).

An additional RNAi screen identified a fibroblast growth factor (FGF)-like receptor called *nou-darake*, which resulted in ectopic neurogenesis and posterior expansion of nervous tissue (Cebria et al., 2002a). The precise signaling cascade down stream of *nou-darake* is unclear, however knockdown results in a significant increase in the number of neural progenitor cells immediately posterior to the cephalic ganglia (Cowles et al., 2013). The increase of neural progenitors within the posterior boundary of the cephalic ganglia could explain the dramatic posterior expansion of nervous tissue after *nou-darake* knockdown. To date, there has not been an FGF-like ligand identified in the planarian genome. Collectively, these data have led to the speculation that *nou-darake* could function to inhibit the posterior diffusion of a neural specifying factor to precisely regulate nervous tissue size (Cebria et al., 2002a; Ross et al., 2017).

In addition to growth factor signaling, Hedgehog signaling also regulates neoblast behavior during brain regeneration (Rink et al., 2009; Wang et al., 2016; Yazawa et al., 2009). The planarian hedgehog ligand, *Smed-hh*, is expressed by cholinergic, GABAergic, and octopaminergic neurons in the cephalic ganglia (Currie et al., 2016). Neoblasts located between the two cephalic lobes express genes for the Hedgehog receptor and various down stream signaling components (Currie et al., 2016). Knockdown of *Smed-hh* results in decreased expression of glial cell genes, a decrease in the number of neural progenitor cells, and a decrease in the number of cholinergic neurons (Currie et al., 2016; Wang et al., 2016). Collectively, these results suggest an important role for

Smed-hh in regulating various aspects of nervous tissue homeostasis and regeneration in planaria.

Apart from whole brain regeneration, planarians can regenerate discrete portions of their nervous systems following dissection. In response to removal of their eyes, planarians are able to fully and functionally regenerate those tissues (Lapan and Reddien, 2011, 2012; LoCascio et al., 2017). The planarian eyes are located dorsal to the cephalic ganglia, consist of 25 photoreceptors each and are necessary for responding to light (Carpenter et al., 1974). Transcriptomic analysis of isolated planarian eyes identified 600 genes that are specifically enriched in the eyes compared to non-eye tissue (Lapan and Reddien, 2012). Further analysis of those genes revealed that many of them have human orthologous that are also eye enriched, including TRP channels, R-opsins and β-arrestin (Lapan and Reddien, 2012). Functional validation of a subset of eye-enriched genes identified the transcription factor *Smed-ovo* as a specific regulator of eye regeneration (Lapan and Reddien, 2012). *Smed-ovo* is specifically expressed in cells of the planarian eye and eye progenitors (Lapan and Reddien, 2012). Genetic loss of function analysis revealed *Smed-ovo* knockdown resulted in a failure to regenerate the eyes after decapitation (Lapan and Reddien, 2012). Furthermore, *Smed-ovo* is necessary for eye maintenance in uninjured animals (Lapan and Reddien, 2012).

While *Smed-ovo* is specifically required for maintenance and regeneration of the planarian eye, another gene *Smed-pbx* plays dual roles in regulating tissue regeneration and eye regeneration. Knockdown of *Smed-pbx* results in a failure to induce expression of positional genes during head or tail regeneration, which ultimately inhibits head or tail regeneration (Chen et al., 2013). Additionally, *Smed-pbx* knockdown inhibits eye regeneration after eye resection compared to controls (Chen et al., 2013). Similar to *Smed-ovo*, knockdown of *Smed-pbx* leads to a loss of eye cells in uninjured animals, suggesting that *Smed-pbx* plays an important role during homeostatic tissue turnover in the eyes (Chen et al., 2013).

Decades of research and recent advances in experimental technologies have allowed for exquisite analysis of nervous system regeneration in planaria. While the ability of this fresh water flatworm to regenerate its nervous system after injury might seem superhuman, there are examples of vertebrates with comparable abilities. Next, we will discuss the ability of an evolutionarily closer relative to humans, the salamander, which is miraculously able regenerate its brain after mechanical injury and chemical ablation of neuronal subtypes.

Salamander brain regeneration

Similar to planarians, salamanders display an almost superhuman ability to regenerate after traumatic brain injury. Thymidine analogue pulse chase experiments have revealed neurogenic hotspots in the telencephalon of adult newts and axolotl (Berg et al., 2010; Maden et al., 2013). Following mechanical injury, cells within normally quiescent regions of the salamander brain respond to injury and become highly proliferative (Maden et al., 2013). The cells that respond to injury are located adjacent to the ventricles and express the intermediate filament protein glial fibrillary acidic protein (GFAP) (Maden et al., 2013). These cells function as neural stem cells during brain and spinal cord regeneration, which will be discussed in more detail in the following section (Echeverri and Tanaka, 2002; Fei et al., 2014; Maden et al., 2013). After mechanical injury, the telencephalon is morphologically regenerated around 6 weeks post transection (Maden et al., 2013). However, it was not clear if the identity or number of the regenerated neurons was the same as in the uninjured brain.

Initial characterization of neuronal identity and organization in the axolotl brain first looked for expression of region specific markers, such as *FezF2* and *CTIP2*, which are expressed in layer 5 neurons of the mammalian cortex. Both *FezF2* and *CTIP2* are expressed in a subset of neurons located in the medial and dorsal pallium of the axolotl telencephalon (Amamoto et al., 2016). While the salamander brain does not have a laminar organization like the mammalian

cortex, neuronal subtypes are clustered in nuclei-like structures within the mantel zone of the dorsal, lateral, and medial pallium (Amamoto et al., 2016). Detailed analysis of neuronal regeneration after stab injury revealed that the number and identities of neurons in the axolotl brain are precisely regenerated. The total number of mature $NeuN^+$ neurons was the same in the uninjured and regenerated dorsal pallium (Amamoto et al., 2016). Additionally, neuronal subtypes were precisely regenerated. For example, the number of $CTIP2^+$ neurons, $Calbinden2^+$ interneurons and $Satb2^+$ neurons was not different between uninjured and regenerated brains (Amamoto et al., 2016). However, the anatomical organization of the regenerated tissue was disorganized. While neuronal subtypes are organized in nuclei-like structures in the uninjured brain, their distribution in the regenerated tissue was not confined to discrete anatomical regions (Amamoto et al., 2016). However, electrophysiological approaches confirmed that even though the regenerated neurons were disorganized they were functional and fully integrated into the preexisting brain circuitry (Amamoto et al., 2016). These experiments confirmed that the salamander brain precisely regenerates after traumatic injury.

In addition to regenerating after tissue resection, salamanders are able to regenerate specific neuronal subpopulations after chemical ablation. Injection of the neurotoxin AF64A into the ventricle of adult newts results in the specific ablation of cholinergic neurons in the newt telencephalon (Kirkham et al., 2014). The number of cholinergic neurons is completely restored by 25 days post ablation (Kirkham et al., 2014). Following ablation of cholinergic neurons, $Sox2^+$ glial cells proliferate and differentiate into cholinergic neurons (Kirkham et al., 2014). Interestingly, inhibition of the Notch signaling pathway with the γ-secretase inhibitor DAPT did not significantly affect the glial cell response to neuronal ablation (Kirkham et al., 2014). This result suggests that Notch signaling does not regulate the injury-induced response of glial cells to cholinergic neuron ablation.

Apart from regenerating cholinergic neurons newts also regenerate dopaminergic neurons. Injection of 6-hydroxydopamine (6-OHDA) into the ventricle of adult newts results in apoptosis of TH^+ dopaminergic neurons (Berg et al., 2010; Parish et al., 2007). Coincident with dopaminergic neuron cell death, the newts displayed locomotor defects reminiscent of the tremors associated with Parkinson's disease (Parish et al., 2007). The locomotor defects gradually become less and less severe over the next 30 days, which coincided with the regeneration of dopaminergic neurons up to pre-lesion levels (Parish et al., 2007). Similar to regeneration of cholinergic neurons, the regeneration of dopaminergic neurons is dependent on the proliferation and differentiation of $Sox2^+$ glial cells (Berg et al., 2010; Berg et al., 2011). Microarray based transcriptional profiling approaches identified a significant change in expression of sonic hedgehog target genes during regeneration of dopaminergic neurons (Berg et al., 2010). Pharmacologic inhibition of sonic hedgehog signaling with cyclopamine resulted in fewer regenerated dopaminergic neurons compared to controls (Berg et al., 2010). This result suggests that regeneration of dopaminergic neurons is depended on sonic hedgehog signaling.

Further investigation into the signals that activate the glial cell response to ablation of dopaminergic neurons identified dopamine itself as an essential signal (Berg et al., 2011). Glial cells express the D2R dopamine receptor and respond to experimental manipulation of dopamine signaling. Injection of a D2R agonist blocks glial cell proliferation and differentiation after ablation of dopaminergic neurons. Conversely, injection of a D2R antagonist results in increased glial cell proliferation and increased number of dopaminergic neurons in the uninjured brain (Berg et al., 2011). Collectively, these results suggest a model where glial cells sense the concentration of dopamine in the microenvironment to increase or decrease the rate of dopaminergic neuron differentiation accordingly.

Apart from brain regeneration, salamanders are able to functionally regenerate after spinal cord injury. Indeed, the ability of salamanders to regenerate the nervous system has been most commonly studied in the context

of tail amputation or complete spinal cord transection. The ability of salamanders to regenerate after spinal cord injury is drastically different than their mammalian counterparts. Next, we will discuss the current state of the field regarding the ability of salamanders and other model organisms to regenerate after spinal cord injury. For the context of this introduction, I will focus on discussing what is known about the glial cell response to spinal cord injury, axon regeneration, and the developmental origin and regenerative potential of spinal cord neural progenitor cells.

Spinal Cord Regeneration in Naturally Regenerating Organisms

While mammals are unable to functionally regenerate their spinal cord after injury, other vertebrates have retained this remarkable ability. The organisms most extensively used to study natural spinal cord regeneration are fish, frogs, salamanders, and lamprey (Tanaka and Ferretti, 2009). While fish, salamanders and lamprey can regenerate their spinal cord throughout their life frogs lose this ability after metamorphosis (Beattie et al., 1990). A unifying theme throughout examples of natural spinal cord regeneration is the absence of reactive glial cells or a glial scar. In naturally regenerating organisms, glial cells adjacent to the lesion proliferate and migrate to repair the damaged spinal cord and create a permissive environment for axon regeneration (Butler and Ward, 1965, 1967; Goldshmit et al., 2012; Zukor et al., 2011). Due to advances in biochemical, molecular, and transcriptomic technologies, it is becoming clear that glial cells express neural stem cell markers, such as SoxB1 genes (Sox1-3), and function as bona fide neural progenitor cells in the uninjured and regenerating spinal cord. In the following sections I will discuss what is known about the glial cell response to injury and their role in functioning as neural progenitor cells to promote spinal cord regeneration.

Glial Cell Response to Spinal Cord Injury

After spinal cord injury in zebrafish and lamprey, $GFAP^+$ glial cells adjacent to the injury adopt a bipolar, elongated morphology and form a glial bridge connecting the rostral and caudal stumps (Goldshmit et al., 2012; Mokalled et al., 2016). Formation of the glial bridge is dependent on FGF and connective tissue growth factor (CTGF) signaling, and overexpression of CTGF is sufficient to speed up bridge formation and functional recovery (Goldshmit et al., 2012; Mokalled et al., 2016). Regenerating axons use the glial bridge as a substrate to migrate across the lesion and restore motor function. Whether these same signaling mechanisms regulate glial bridge formation in lamprey is not clear.

In contrast to zebrafish and lamprey, $GFAP^+$ glial cells in the salamander spinal cord do not form a glial bridge. Early reports show that after spinal cord transection, glial cells seal over the rostral and caudal stumps and migrate towards each other (Butler and Ward, 1965, 1967; Sabin et al., 2015; Zukor et al., 2011). A detailed characterization of spinal cord regeneration in newts suggests that glial cells and regenerating axons are intimately associated with each other throughout the regeneration process (Zukor et al., 2011). This is slightly different than in zebrafish where the glial bridge forms first and then axons regenerate over the bridge and through the lesion. Regardless of the mechanism, the overall conclusion remains similar: glial cells adjacent to the lesion undergo a pro-regenerative response to injury, function to reconnect the spinal cord, and create a permissive/instructive environment for axon regeneration.

In addition to FGF signaling, many other developmental signaling pathways regulate the pro-regenerative glial cell response to injury. Genetic epistasis experiments in Xenopus identified that FGF and Wnt signaling downstream of BMP signaling are necessary for tadpole tail regeneration, including the regeneration of the spinal cord (Beck et al., 2003; Lin and Slack, 2008; Sugiura et al., 2009). Similar to tadpoles, newts also require FGF signaling to respond to tail amputation. Glial cells in the newt spinal cord up-regulate FGF2

expression after tail amputation, which promotes the proliferative outgrowth of the regenerating spinal cord (Zhang et al., 2000). In a later study, FGFR1 was shown to have a similar expression profile to FGF2 in glial cells after tail amputation, and FGFR1 remained highly expressed for 4 weeks after tail amputation (Zhang et al., 2002). Another FGF receptor, FGFR4, was specifically up-regulated in glial cells by 3 weeks after amputation (Zhang et al., 2002). Signaling through FGFR4 promotes neuronal differentiation of neural stem cells (Hongo et al., 1999; Zhang et al., 2002) and new neurons appear in the regenerating spinal cord around 3 weeks post amputation (Zhang et al., 2002). These observations led the authors to hypothesize that differential expression of FGF receptors could function as a switch in glial cells to balance proliferation versus differentiation.

Additional studies in Xenopus identified an important role for Notch signaling during spinal cord regeneration after tail amputation. Pharmacologic and genetic perturbations of Notch signaling during tail regeneration affected spinal cord regeneration but not muscle regeneration or overall tail outgrowth (Beck et al., 2003). This is in contrast to tail bud development where Notch signaling is required for tail outgrowth (Beck and Slack, 2002). These studies show that Notch signaling regulates slightly different processes during development and regeneration. This observation supports the hypothesis that regeneration is not simply a recapitulation of the developmental program but utilizes unique gene regulatory pathways.

Axon Regeneration and Functional Recovery

In addition to the pro-regenerative glial cell response to spinal cord injury, there is substantial axon regeneration through the lesion. Spinal cord circuits are comprised of axons from locally acting interneurons or long tract axons from spinal cord projecting brain regions. Retrograde tracing approaches have identified specific brain regions that project axons to the spinal cord in fish and salamanders. Interestingly, every spinal cord projecting brain region regenerates

axons through the lesion. However the path the axons take during regeneration is different from the pre-injury trajectory (Becker et al., 1998; Becker et al., 1997; Clarke et al., 1988). During regeneration, axons preferentially migrate through the spinal cord grey matter (Becker and Becker, 2001) and functional recovery of swimming activity is positively correlated with the percent of axons that regenerate through the lesion (Becker et al., 1997). Investigation of TH^+ (dopaminergic) and 5-HT^+ (serotonergic) axons revealed that the organization of axonal tracts in the spinal cord is drastically different in uninjured compared to regenerated white matter (Kuscha et al., 2012). Similar to previous findings, it was found that functional recovery positively correlated with the percent of regenerated TH^+ and 5-HT^+ axons (Kuscha et al., 2012). Interestingly, dopamine released from supraspinal TH^+ axons promotes motor neuron development and regeneration in the zebrafish spinal cord (Reimer et al., 2013). Pharmacologic or genetic activation of dopamine signaling during development leads to increased numbers of spinal cord motor neurons. Similarly, activation of dopamine signaling promotes regeneration of motor neurons after spinal cord transection (Reimer et al., 2013).

Collectively, these studies suggest that the best predictor of functional recovery after spinal cord injury is the percent of axons that regenerate through the lesion rather than the organization of regenerated axons within the spinal cord. These observations support the high degree of plasticity within spinal cord neural networks. Even though the regenerated networks were not identical to the uninjured circuitry, it was sufficient to restore motor function. Whether there is a minimum requirement of synapses that need to regenerate to restore motor function is not clear. Additionally, how regenerating axons form synapses with the correct postsynaptic neuron is not known.

Response to Spinal Cord Injury in Mammalian CNS

Spinal cord injury (SCI) in mammals results in neuronal cell death, axon degeneration, and formation of a glial and fibrotic scar that collectively lead to the loss of sensory and motor function below the lesion. There are many factors that contribute to regenerative failure in mammals, including the inability of neurons to initiate new axon growth (Ferguson and Son, 2011; Kadoya et al., 2009; Liu et al., 2011; Mar et al., 2014), as well as a lesion microenvironment that inhibits axon regeneration (Diaz Quiroz and Echeverri, 2013; Rolls et al., 2009; Silver and Miller, 2004; Sofroniew, 2009). Mechanical injury to axons results in an influx of calcium that activates cell signaling pathways. This results in degeneration of the severed axons by a process called Wallerian degeneration (**Fig. 1-2**) (Bradke et al., 2012). Axons that do not undergo Wallerian degeneration form dystrophic end bulbs and do not regenerate across the lesion.

Dystrophic end bulbs are unable to naturally regenerate across the growth inhibitory microenvironment of the lesion. However, if given a permissive substrate (i.e. peripheral nerve graft) dystrophic end bulbs are able to migrate into and over the graft. Transplantation experiments have shown that dystrophic end bulbs retain this ability 4 weeks (Houle, 1991), 13 weeks (Li and Raisman, 1995), and even up to 1 year after initial injury (Kadoya et al., 2009; Kobayashi et al., 1997). It is important to note that to stimulate dystrophic end bulbs to migrate a year after injury, the graft needs to be supplemented with the neurotrophins BDNF and/or NT-3 (Kadoya et al., 2009; Kobayashi et al., 1997). Prior to these experiments it was thought that severed axons with dystrophic end bulbs were unable to regenerate. These results suggest that some axons retain the ability to migrate into a permissive environment up to a year after injury. This further highlights the growth inhibitory environment of the lesion as a major obstacle to stimulating regeneration.

The Fibrotic and Glial Scar

There are two distinct compartments found within and around the lesion after SCI. There is a fibrotic scar that is comprised of meningeal and

perivascular-derived fibroblasts, pericytes and inflammatory cells, as well as growth inhibitory extracellular matrix (ECM) (**Fig. 1-2**) (Adams and Gallo, 2018; Diaz Quiroz and Echeverri, 2013; Rolls et al., 2009; Silver and Miller, 2004; Sofroniew, 2009). Surrounding the fibrotic scar is a barrier comprised of reactive glial cells collectively referred to as the glial scar (Adams and Gallo, 2018; Faulkner et al., 2004; Sofroniew, 2009; Voskuhl et al., 2009). Cells found within the glial scar express axon growth inhibitory molecules such as chondroitin sulfate proteoglycans (CSPGs), semaphorins and ephrins (Rolls et al., 2009; Silver and Miller, 2004). Apart from growth inhibitory ECM components, cells contributing to the fibrotic core also express axon growth inhibitory molecules collectively creating an inhospitable environment for axon regrowth (Bundesen et al., 2003; De Winter et al., 2002; Pasterkamp et al., 1999; Silver and Miller, 2004).

Reactive astrocytes are the major glial cell type contributing to the glial scar. Oligodendrocytes and specific myelin products derived from degenerated oligodendrocytes also contribute to the glial scar, but these factors will be discussed later. Following injury, astrocytes adjacent to the injury site undergo reactive gliosis, up-regulate expression of the intermediate filament proteins glial fibrillary acidic protein (GFAP) (Bignami and Dahl, 1974; Eng, 1985; Yang and Wang, 2015) and vimentin (Yang et al., 1994), and migrate towards the lesion. The origin of reactive astrocytes within the glial scar is still under investigation, but the most convincing lines of evidence show that pre-existing astrocytes re-enter the cell cycle, proliferate, and migrate towards the lesion (Buffo et al., 2008; Bush et al., 1999; Gadea et al., 2008). More recent lineage tracing experiments suggest ependymal cells, a putative spinal cord neural stem cell population, could give rise to astrocytes after injury, which then undergo reactive gliosis and contribute to the glial scar (Barnabe-Heider et al., 2010; Meletis et al., 2008). However, a recent report from the Sofroniew lab using a similar lineage tracing approach contradicts these findings (Ren et al., 2017). Additionally, NG2 expressing cells in the injured spinal cord can differentiate into astrocytes that

similarly contribute to the glial scar (Hackett et al., 2018; Huang et al., 2018). The contribution of endogenous spinal cord neural stem cells and other cell types to the mammalian response to SCI will be discussed in more detail later.

While reactive astrocytes do secrete growth inhibitory molecules, recent reports using sophisticated cell ablation and conditional gene knockout approaches suggest a protective role for the glial scar. Initial studies used the conditional expression of herpes simplex virus thymidine kinase (TK) gene under the transcriptional control of an astrocyte specific promoter (GFAP) to ablate proliferative, reactive astrocytes, which contribute to the glial scar (Bush et al., 1999). Viral TK phosphorylates the inert pro-drug Ganciclovir converting it to a competitive deoxyguanosine analogue that is preferentially incorporated into the genome of dividing cells. However, cellular DNA polymerase is unable to read the analogue leading to failed genome duplication and the cell ultimately undergoes apoptosis. This approach is dependent on genome duplication and cell division and therefore ablates only dividing cells. Thymidine kinase mediated ablation of proliferating, glial scar associated astrocytes led to increased axonal degeneration, a failure to repair the blood brain barrier and decreased functional recovery compared to wild type mice (Bush et al., 1999). The increased axonal degeneration was attributed to a massive influx of inflammatory cells that are normally restricted to the fibrotic scar at the lesion center (Bush et al., 1999). However, ablation of reactive astrocytes led to a breakdown of the glial barrier separating the fibrotic lesion core from surrounding spinal cord tissue (Bush et al., 1999), allowing the aberrant infiltration of inflammatory cells into the spinal cord parenchyma. Further analysis identified the transcription factor STAT3 as being necessary for induction of reactive gliosis in spinal cord astrocytes after injury (Herrmann et al., 2008). Specific knockout of STAT3 from astrocytes using a GFAP-Cre phenocopied the cell ablation experiments, further highlighting a potentially protective role of reactive astrocytes after injury (Herrmann et al., 2008).

While it is clear that reactive astrocytes do play a beneficial role by sealing off the injury and mitigating the inflammatory response, the fact remains that they express growth inhibitory molecules that block axon regeneration. Stimulation of robust axon regeneration after spinal cord injury will likely require the specific targeting of the growth inhibitory properties of reactive astrocytes, while maintaining the protective immune modulatory function. How these two functions are balanced or related within reactive astrocytes remains unclear.

Chondroitin Sulfate Proteoglycans

The major axon growth inhibitory molecules secreted by reactive astrocytes are chondroitin sulfate proteoglycans or CSPGs (Jones et al., 2003; McKeon et al., 1999; Rolls et al., 2009; Sandvig et al., 2004; Silver and Miller, 2004; Thon et al., 2000). CSPGs are composed of a proteoglycan protein core which is modified by the addition of sulfated sugar groups (Kwok et al., 2012). In vitro experiments showed that various CSPG family members are capable of inhibiting axon growth and neurite extension (Hynds and Snow, 1999; Snow et al., 2003). While CSPGs do not cause growth cone collapse, they act as a repulsive stimulus and cause the retraction of filopodia (Hynds and Snow, 1999; Snow et al., 1990; Snow et al., 2003). Furthermore, CSPGs are capable of blocking the otherwise growth promoting properties of fibronectin or the cell adhesion molecule L1CAM (Dou and Levine, 1994; Snow et al., 1996). Further biochemical characterization revealed that the sulfated sugar groups added to the proteoglycan core are specifically involved in the repulsive nature of CSPGs. Treating CSPGs with the sugar cleaving enzyme chondrotinase reduces their repulsive behavior in vitro (McKeon et al., 1995) and in vivo, leading to improved functional outcomes (Bradbury et al., 2002; Caggiano et al., 2005).

Similarly, in vivo studies that inhibited the CSPG receptor, PTPσ, stimulated significant axon regeneration through the lesion and an increase in locomotor recovery compared to control mice (Lang et al., 2015). These results support an overall growth inhibitory role for CSGPs during spinal cord

regeneration in mammals. Furthermore, injection of mice with a neutralizing antibody to the specific CSPG NG2 resulted in increased functional recovery after SCI (Tan et al., 2006). Oligodendrocyte progenitor cells, microglia, macrophages, pericytes and Schwann cells all express NG2 and are known to participate in the fibrotic or glial scar (Adams and Gallo, 2018; McTigue et al., 2001). Paradoxically, NG2 knockout mice had more axonal retraction and less functional recovery after SCI compared to wild type mice (de Castro et al., 2005). Interestingly, severed axons were shown to closely interact with NG2 and form synapse-like associations with NG2 expressing cells (Filous et al., 2014; McTigue et al., 2006). While these close, synapse-like associations prevent excessive axonal retraction after SCI, they also prevented axon regrowth through the lesion (Filous et al., 2014). It is not clear why the neutralizing antibody promoted functional recovery after SCI compared to controls but there was no difference in the NG2 knockout mice. Additionally, the mice were global NG2 knockout, so it would be interesting to use a cell type specific Cre to determine which $NG2^{+}$ cell population is responsible.

Myelin Components

As mentioned above oligodendrocytes and myelin components also contribute to the growth repulsive nature of the spinal cord lesion. Oligodendrocytes produce myelin sheaths, which wrap around axons and are indispensible for fast, efficient conduction of action potentials. However, after injury, damaged oligodendrocytes degenerate leaving behind myelin components such as the neurite outgrowth inhibitor (Nogo) proteins, myelin associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp), which function to promote growth cone collapse and inhibit axon regeneration.

Nogo ligands (Nogo-A, -B and –C) signal through their cognate receptor NgR and stimulate growth cone collapse (Chen et al., 2000b; Schwab, 2004). Biochemical studies identified a conserved domain at the C-terminal of all 3 Nogo isoforms that was sufficient to induce growth cone collapse independent of the

rest of the protein (GrandPre et al., 2000). This domain was called Nogo-66 and injection of mice with a peptide antagonist of Nogo-66, which blocked receptor binding, or a Nogo-A neutralizing antibody lead to significant functional improvement after spinal cord injury compared to controls (GrandPre et al., 2002; Merkler et al., 2001). These results were consistent with genetic knockouts of Nogo-A/B, which led to increased axon regeneration through the glial scar and improved functional recovery (Kim et al., 2003). However, other reports contradict these findings by showing that neither a double Nogo-A/B nor triple Nogo-A/B/C knockout led to significant axon regeneration or functional recovery (Zheng et al., 2003). Interestingly, the genetic background of the knockout mice was different between the two reports (Cafferty et al., 2007) and it was previously shown that C57BL/6 mice and Sv129 mice reacted differently to Nogo inhibition after spinal cord injury (Dimou et al., 2006). These findings suggest that other genomic loci could dictate the sensitivity of regenerating spinal cord axons to Nogo-mediated repulsion, thus explaining the contradictory findings.

Apart from Nogo, MAG, and OMgp, there are other myelin-derived components that contribute to the glial scar (Sandvig et al., 2004). Similar to Nogo, both MAG and OMgp induce growth cone collapse and inhibit neurite outgrowth of neurons in vitro (Li et al., 1996; Wang et al., 2002). Whether these factors play a similar role in vivo is less clear. MAG-null animals did not exhibit robust axon regeneration or a significant functional improvement after spinal cord injury compared to controls (Bartsch et al., 1995). OMgp-null animals in the C57BL/6 background similarly did not show increased axon regeneration or functional recovery compared to controls (Ji et al., 2008). However, when researchers out bred the C57BL/6 to Sv129, resulting in a more heterogeneous genetic background, OMgp knockout led to significant improvement of motor function and axon regeneration through the lesion (Ji et al., 2008). The functional differences observed between the various genetic backgrounds of either Nogo or OMgp knockout further suggests that additional, unidentified genetic interactions contribute to axon sensitivity to myelin components. Finally, triple knockout of

Nogo/MAG/OMgp did lead to significant improvement of motor function and regeneration of sensory and motor axons through the glial scar after spinal cord injury (Cafferty et al., 2010). These experiments support a model in which various myelin-derived components could cooperate to inhibit robust axon regeneration and functional recovery. These results further highlight the need to target multiple aspects of the growth inhibitory glial scar to stimulate functional regeneration.

Spinal Cord Neural Progenitor Cells: Developmental Origins and Regenerative Potential

Committed neural progenitor cells (NPCs) are largely responsible for nervous system development and production of the neuronal diversity found within an organism. Cells with neural potential in vertebrates can be defined by expression of SoxB1 genes (Sox1-3) (Bylund et al., 2003; Graham et al., 2003), and even in anemones and corals, the nervous system is derived from SoxB expressing cells (Flici et al., 2017; Richards and Rentzsch, 2014, 2015). Resolution of the exact identity of vertebrate SoxB genes compared to anemone and coral SoxB genes has not been trivial (Jager et al., 2011; Magie et al., 2005; Shinzato et al., 2008), but functional data support the hypothesis that regulation of the NPC state is an ancient function of SoxB genes throughout metazoa. While SoxB expressing NPCs contribute to the developing nervous system across phyla, the NPC response to injury varies greatly across species. For example, $Sox2^+$ glial cells have been identified in the spinal cords of Xenopus, zebrafish, and axolotl, and are necessary for spinal cord regeneration (Fei et al., 2014; Hui et al., 2015; Munoz et al., 2015). Sox2 expression decreases as tadpoles undergo metamorphosis which is one hypothesized reason why adult frogs are unable to regenerate after spinal cord injury (Munoz et al., 2015). However, $Sox2^+$ cells are also found in the mammalian spinal cord but respond to injury by differentiating into scar-associated glial cells without undergoing neurogenic differentiations (Barnabe-Heider et al., 2010; Meletis et al., 2008).

The precise reasons for the drastically different response to injury across species are not clear and remain an active area of investigation in regenerative biology.

Developmental Origins of Spinal Cord Neural Progenitor Cells

Early fate mapping experiments labeled anterior epiblast stem cells and found that prior to E7.5 labeled cells were competent to contribute to anterior (forebrain, midbrain, hindbrain, anterior spinal cord) and posterior (spinal cord) central nervous system (Lawson and Pedersen, 1992). In contrast, after E7.5 anterior epiblast stem cells only contribute to anterior CNS structures and not to the developing spinal cord (Forlani et al., 2003). However, vertebrate embryos undergo extensive elongation during later stages of development (post-E7.5), resulting in an extensive expansion of both the posterior mesoderm and developing spinal cord. These observations suggest that either committed mesoderm and spinal cord progenitors are solely responsible for contributing to axis extension or, as has been recently hypothesized, an additional bipotent progenitor contributes to both mesoderm and spinal cord to accommodate embryo elongation (Wilson et al., 2009). These models are not mutually exclusive and early experiments using various lineage tracing and fate mapping approaches support the existence of a bipotent axial progenitor cell.

Labeling of single cells in the node streak border (NSB) of chick embryos revealed labeled progeny in paraxial mesoderm and spinal cord, paraxial mesoderm and notochord, or just notochord at later developmental time points (Selleck and Stern, 1991). This is consistent with later findings that grafted transgenic GFP^+ NSB tissue or caudal lateral epiblast (CLE) tissue into wild type embryos (Cambray and Wilson, 2007). While GFP^+ NSB grafts contributed to paraxial mesoderm, notochord, or spinal cord, GFP^+ grafts from the CLE only contributed to paraxial mesoderm or spinal cord but not notochord (Cambray and Wilson, 2007). Collectively, these results suggest that a population of progenitors in the post-gastrula embryo within the NSB or CLE have the ability to contribute to either spinal cord or mesoderm (paraxial mesoderm/notochord). Additionally,

the cells in these regions displayed true stem cell-like characteristics as they could be serially transplanted into new embryos and continue to contribute labeled progeny to either paraxial mesoderm or neural tissue (Cambray and Wilson, 2002). Taken together, these observations support the existence of a self-renewing, bipotent axial progenitor cell. However, vital dye labeling or tissue grafting can be messy and imprecise, resulting in the accidental co-labeling or transplantation of a committed neural or mesoderm progenitor that could bias the interpretation. Therefore, it was necessary to genetically and irreversibly label single cells and their progeny to indisputably determine the existence of a bipotent axial progenitor cell.

Single cell labeling during mouse embryonic development can be performed by the random recombination of a non-functional LacZ allele (LaacZ) to the functional LacZ form (Bonnerot and Nicolas, 1993; Tzouanacou et al., 2009). This recombination event is extremely rare and would not occur in multiple cells independently. By employing this technique, multiple patterns of $LacZ^{+}$ clones were found. As would be expected, labeled clones that were restricted to neural, non-neural ectoderm, mesoderm or endoderm were represented. However, another distribution of clones was observed, which contained $LacZ^{+}$ cells in both paraxial mesoderm and spinal cord tissue (Tzouanacou et al., 2009). These results suggest that a proportion of cells within paraxial mesoderm and spinal cord are derived from a common progenitor. Taken together, embryological transplantation experiments and fate mapping approaches (vital dye and genetic labeling) confirmed the existence of a self-renewing, bipotent axial progenitor subsequently named neuromesodermal progenitor (NMPs).

Due to advances in transgenesis, transcriptional profiling, and in vitro cell culture techniques, we now know that NMPs are found within the tail bud and CLE of vertebrate embryos (**Fig. 1-3**) (Gouti et al., 2015; Henrique et al., 2015; Wilson et al., 2009). This population of bipotent progenitors is molecularly defined by the co-expression of the NPC marker Sox2 and the early mesoderm marker Brachyury (Martin and Kimelman, 2012; Olivera-Martinez et al., 2012;

Tsakiridis et al., 2014; Wymeersch et al., 2016). FGF and Wnt signaling are necessary to induce Sox2 and Brachyury expression within NMPs. However, prolonged Wnt signaling activity is sufficient to induce NMP differentiation into committed mesodermal progenitors (Martin, 2016; Rivera-Perez and Magnuson, 2005; Takemoto et al., 2006). Conversely, environments with high levels of retinoic acid signaling function to oppose FGF and Wnt signaling and lead to NMP differentiation into committed NPCs (Diez del Corral et al., 2003; Gouti et al., 2014; Shum et al., 1999). Neuromesodermal progenitor cells can be derived from embryonic stem cells in vitro and possess similar characteristics to their in vivo counterparts (Gouti et al., 2014; Turner et al., 2014; Verrier et al., 2018). In vitro derived NMPs co-express Sox2 and Brachyury and will differentiate into mesoderm derivatives in the presence of prolonged Wnt activity (Gouti et al., 2014; Turner et al., 2014; Verrier et al., 2018). While addition of retinoic acid to the cultures results in loss of Brachyury expression and differentiation of NMPs to neural progenitors that are capable of becoming spinal cord neuron subtypes (Gouti et al., 2014; Verrier et al., 2018). The in vitro experiments were carried out using mouse embryonic stem cell lines, but recently these approaches have been duplicated using human embryonic stem cell lines (Verrier et al., 2018). Additionally, a population of $Sox2^{+}$/$Brachyury^{+}$ cells exists in human embryos (Olivera-Martinez et al., 2012), collectively supporting the existence of an analogous bipotent progenitor population in humans.

Initial studies aimed at determining the contribution of NMPs to embryo elongation during mouse development utilized conditional, tamoxifen inducible Cre recombinase under the Brachyury promoter (Anderson et al., 2013; Imuta et al., 2013). Interestingly, linage tracing experiments with the Brachyury: CreERT line crossed to a Rosa 26 LoxP STOP LoxP LacZ reporter revealed massive contribution of $LacZ^{+}$ cells to the trunk spinal cord (Chalamalasetty et al., 2014; Garriock et al., 2015). Tamoxifen was injected at E7.5, a time when Brachyury is only expressed in committed mesoderm and NMPs; therefore, any $LacZ^{+}$ cells in the spinal cord would have been derived from NMPs. This analysis revealed that

up to 65% of NPCs in the posterior, trunk spinal cord were derived from NMPs (Chalamalasetty et al., 2014; Garriock et al., 2015; Gouti et al., 2015).

Taken together these sets of experiments provide strong evidence for the existence of a self-renewing, bipotent axial progenitor that co-expresses Sox2 and Brachyury and contributes to both paraxial mesoderm and spinal cord neural progenitor cells. The exact importance of this population to proper spinal cord development is still not clear. In the future, it would be interesting to devise a scheme to specifically ablate NMPs ($Sox2^+$/$Brachyury^+$ cells) and determine the effect that has on spinal cord or mesoderm development.

Mammalian Neural Progenitor Cell Response to Injury

While NPCs have been found in the adult central nervous systems in mammals (Gage, 2000; Horner et al., 2000; Johansson et al., 1999; Weiss et al., 1996), their ability to productively contribute to CNS regeneration after injury is lacking. Three populations of putative NPCs have been proposed: ependymal cells, astrocytes, and cells of the oligodendroglial lineage (Barnabe-Heider et al., 2010; Meletis et al., 2008; Stenudd et al., 2015). Previous cell sorting experiments suggested that ependymal cells were the only cells from the adult spinal cord capable of generating neurospheres in vitro (Meletis et al., 2008). Isolated ependymal cells expressed the NPC markers Sox2/3, Nestin, and PDGFRα and did not express the oligodendrocyte progenitor cell (OPC) marker Olig2 (Meletis et al., 2008). Collectively, these results suggest that ependymal cells function as NPCs in the adult mammalian spinal cord. However, in vitro culture conditions are drastically different than the in vivo environment, potentially leading to an artificial selection of cell behaviors due to those conditions. Therefore, it is important to follow the dynamics of each of these populations in vivo in uninjured and damaged spinal cords.

Each population of putative NPC can be discretely marked with a specific conditional Cre. Ependymal cells, which line the central canal of the adult spinal cord, are efficiently labeled using a FoxJ1: CreERT (Meletis et al., 2008).

Astrocytes throughout the adult spinal cord can be labeled with a connexin 30 (Cx30): CreERT, and cells of the oligodendroglial lineage can be labeled with an Olig2: CreERT. BrdU pulse chase experiments revealed that Olig2: CreERT cells, representing $NG2^+$ oligodendrocyte progenitors (OPCs) and their progeny, are the most proliferative cell type within the uninjured spinal cord, accounting for ~80% of $BrdU^+$ cells (Barnabe-Heider et al., 2010). In stark contrast, Foxj1: CreERT ependymal cells and Cx30: CreERT astrocytes represent 4% and <1% of $BrdU^+$ cells respectively, with non-neural spinal cord cell types making up the difference (i.e. microglia, vascular associated cells) (Barnabe-Heider et al., 2010).

In response to spinal cord injury, all three populations of labeled cells drastically increased proliferation and were found within the glial scar 2 weeks after injury (Barnabe-Heider et al., 2010). Ependymal cells displayed the highest degree of multipotency by differentiating into astrocytes and oligodendrocytes after injury (Barnabe-Heider et al., 2010). Ependymal-derived cells were found in the glial and fibrotic scar, displayed astrocyte-like morphology, and expressed the astrocyte markers Sox9 and vimentin. Interestingly, these progeny expressed little to no GFAP protein (Barnabe-Heider et al., 2010). Additionally, a small proportion of ependymal-derived cells expressed the oligodendrocyte marker Sox10, suggesting that ependymal cells are competent to differentiate into oligodendrocytes after injury.

Oligodendrocyte progenitor cells marked with the Olig2: CreERT did not display any degree of multipotency and only gave rise to $Sox10^+$ oligodendrocytes. As mentioned before, OPCs also express NG2 and recent genetic fate mapping experiments used an NG2: CreERT to follow the cell fate during development and after injury. These experiments showed that, during development, cells that express NG2 only differentiate into $Olig2^+$ OPCs or $Sox10^+$ oligodendrocytes (Hackett et al., 2018; Huang et al., 2018). However, after spinal cord injury, $NG2^+$ cells differentiated into astrocytes that underwent reactive gliosis and contributed to glial scar formation (Hackett et al., 2018;

Huang et al., 2018). This suggests that $NG2^+$ OPCs have the potential to differentiate into astrocytes, but only after injury.

Finally, genetically labeled astrocytes were found to proliferate the most after spinal cord injury, giving rise to 58% of labeled cells at the lesion 2 weeks after injury (Barnabe-Heider et al., 2010). Consistent with other reports, these newly generated astrocytes were restricted to the periphery of the lesion and not found within the fibrotic core (Faulkner et al., 2004; Voskuhl et al., 2009). Importantly, it was found that genetically labeled astrocytes only gave rise to new astrocytes and did not differentiate into any other neural cell type (Barnabe-Heider et al., 2010).

Collectively, these analyses confirm that ependymal cells, OPCs, and astrocytes all proliferate in response to spinal cord injury. Furthermore, ependymal cell progeny localize to the glial and fibrotic scar while OPCs, oligodendrocytes, and reactive astrocytes were only found in the glial scar or surrounding parenchyma. Additionally, only ependymal cell progeny express markers of either astrocytes (Sox9, S100β, vimentin, GFAP) or oligodendrocytes (Sox10), further supporting the role of ependymal cells as NPCs in vivo. Interestingly, no newborn $BrdU^+$ neurons were observed at either 2 or 4 weeks post injury (Barnabe-Heider et al., 2010). While neurospheres formed from ependymal cells can differentiate into neurons in vitro, this clearly does not happen naturally in vivo. This suggests that the local environment of the uninjured and damaged spinal cord is not conducive to neurogenesis and might preferentially promote glial differentiation. This is consistent with reports from the rat spinal cord where pro-neural homeobox and basic helix loop helix transcription factors are expressed after injury, but no new neurogenesis was observed (Yamamoto et al., 2001). This could be due to an increase in Notch expression after spinal cord injury, which is known to inhibit neuronal differentiation, but needs to be explored in more detail (Yamamoto et al., 2001).

Axolotl, Zebrafish and Frog Neural Progenitor Response to Injury

Work from salamanders and fish have shown that, even in the adult spinal cord, glial cells express dorsoventral patterning molecules. During neural tube development the antagonistic relationship between BMP and Shh signaling sets up various progenitor domains defined by expression of specific transcription factors (Briscoe and Small, 2015). While these embryonic expression domains are lost post development in mammals, they remain or are re-established after injury in fish and salamanders (Dias et al., 2012; McHedlishvili et al., 2007; Reimer et al., 2009; Schnapp et al., 2005). Specifically, dorsal Pax7, lateral Pax6, and ventral Shh domains are present in the adult axolotl spinal cord and inhibition of Shh signaling leads to expansion of the dorsal Pax7 domain and blocks spinal cord regeneration after tail amputation (Schnapp et al., 2005). Similarly, after spinal cord transection in zebrafish, Shh is up-regulated by glial cells in the ventral spinal cord which co-express Nkx6.1, another ventral marker in the neural tube (Reimer et al., 2009). Shh inhibition was found to specifically affect the differentiation of glial cells within the pMN domain of the spinal cord into motor neurons (Reimer et al., 2009). Whether Shh inhibition affects motor neuron differentiation during spinal cord regeneration in axolotl was not addressed. While it is likely that the proportion and identity of new neurons generated during regeneration was affected by Shh inhibition, there was also a gross inhibition of cell proliferation after amputation. It is likely that perturbed glial cell and blastemal cell proliferation was responsible for failed spinal cord and tail outgrowth after amputation (Schnapp et al., 2005). In the future, it will be necessary to perturb Shh signaling in a discrete, genetically defined population of cells within the axolotl spinal cord to delineate between a general proliferative defect and effects on neuronal differentiation.

While injury induced the expression and expansion of embryonic progenitor cell domains within the zebrafish spinal cord, axolotl glial cells immediately adjacent to the injury lose expression of these markers. Previous experiments established that all the cellular material required for spinal cord regeneration after tail amputation comes from a region 500μm rostral to the injury

(McHedlishvili et al., 2007). This is similar to reports in tadpoles during spinal cord regeneration after tail amputation (Lin et al., 2007; Slack et al., 2008). Closer examination found that dorsal Pax7 and lateral Pax6 expression was lost in glial cells within the 500μm zone (McHedlishvili et al., 2007). While these results suggest that these cells lost their dorsoventral identity, how this specifically affects their response to injury or their potential is not clear. Recent work using gene expression profiling suggests these cells actually dedifferentiate into a more neuroepithelial-like identity based on differential expression of particular neuroepithelial junction proteins like ZO-1, occludin, and claudin-5 (Rodrigo Albors et al., 2015). However, this study only profiled the change in expression of 100 genes correlating to axolotl homologues of chick genes that are differentially expressed in the tail bud, preneural tube, and neural tube of developing chick embryos (Olivera-Martinez et al., 2014). Due to the small set of genes analyzed, this approach could have missed a more complex transcriptional state assumed by these cells during tail regeneration. An unbiased, genome wide approach would allow for higher resolution of transcriptional states that may have been missed.

Neural Progenitor Identity during Axolotl Tail Regeneration

Apart from the limb, salamanders can also regenerate their spinal cord after tail amputation or spinal cord transection, which will be discussed in more detail later. However, lineage-tracing experiments sought to determine the potential of spinal cord neural progenitor cells after tail amputation. Glial cells in the axolotl spinal cord can be prospectively labeled by electroporation of a plasmid where the GFAP promoter drives GFP expression (Echeverri and Tanaka, 2002). Labeled cells were observed using live in vivo fluorescent imaging for the duration of tail regeneration. Unsurprisingly, a majority of GFP^+ glial cells differentiated into neural subtypes: neurons, new glia or neural crest derivatives. However, a small population of labeled glial cells unexpectedly left

the spinal cord and differentiated into myosin-heavy chain $(Myh)^+$ muscle fibers or contributed to cartilage within the regenerate (Echeverri and Tanaka, 2002).

While controversial, these observations support the hypothesis that, during tail regeneration, spinal cord neural progenitors are competent to contribute to mesoderm-derived tissues (muscle/cartilage) as well as neuroectoderm-derived spinal cord. This is reminiscent of the NMP identity discussed earlier in this section. Interestingly, similar lineage tracing experiments after spinal cord transection did not observe this same potential (Sabin et al., 2015). This suggests that after spinal cord transection, specific molecular pathways could function to stabilize or maintain the neural progenitor identity or inhibit differentiation toward a mesoderm fate. The identity of these pathways and how they relate to spinal cord regeneration after transection compared to tail amputation are not clear, but are partially addressed by this dissertation.

Bioelectricity and Tissue Regeneration

Much progress has been made in elucidating the cell signaling pathways and transcription factor networks necessary for tissue regeneration. However, identification of early injury pathways that lead to the initial changes in gene expression that stimulate the cellular/tissue response to injury is still lacking. Recent work has identified changes in the cellular membrane potential as a potent stimulator of cellular proliferation, migration, survival, and tissue patterning (**Fig. 1-4**).

Membrane Potential

Cellular membrane potential refers to the separation of charge across the plasma membrane. The inside of the cell is more negative than the outside of the cell. This electrical gradient is established and maintained by the function of hundreds of ion channels, pumps, and solute transporters and can be propagated though a tissue via gap junctions (Levin, 2009a; McLaughlin and

Levin, 2018). Membrane potential has been largely studied in excitable cell types, like neurons and muscle fibers, which respond to and generate action potentials. However, every cell establishes a membrane potential, and changes in membrane potential of non-excitable cells leads to measurable changes in their physiology. Initial observations revealed that quiescent cells, which rarely or never undergo cell division, have a much more negative membrane potential compared to highly dividing cells (**Fig. 1-5**) (McLaughlin and Levin, 2018). Cancer cells often over express electrogenic channels, pumps or transporters that lead to a more positive membrane potential (Becchetti et al., 2017; Funk, 2015; Pollak et al., 2017). Pharmacologic or genetic inhibition of these factors decreases cell proliferation and inhibits migration/metastasis (Matusik, 2017; Persson and Bondke Persson, 2016).

Treatment of developing Xenopus embryos with the drug ivermectin led to the depolarization (more positive membrane potential) of a subgroup of cells within the embryo resulting in excessive melanocyte development (Blackiston et al., 2011). Ivermectin did not directly affect the melanocyte neural crest progenitors, but instead affected a different group of cells, which led to melanocyte precursor hyperplasia. The hyperplastic melanocytes were highly invasive and colonized organ systems normally devoid of melanocytes (Blackiston et al., 2011). Genetic perturbation of membrane potential by injection of mRNA encoding for various ion channels into Xenopus blastomeres resulted in defects in neurodevelopment (Pai et al., 2015). Overexpression of an inward rectifying potassium channel Kir5.1 led to hyperpolarization (more negative membrane potential) of neuroepithelial cells, decreased proliferation, and smaller and mis-patterned brains (Pai et al., 2015). Similarly, injection of single blastomeres with mRNA encoding for a chloride channel and rearing embryos in an environment leading to chloride influx (leading to hyperpolarization) phenocopied the brain defects observed Kir5.1 overexpressing embryos. Collectively, two independent approaches show that hyperpolarization leads to defects in neurodevelopment.

Membrane Potential and Regeneration

Apart from effects on development, it is clear that changes in membrane potential are important during tissue regeneration as well. As mentioned previously, planarian flatworms are able to perfectly regenerate both their head and tail following amputation. Interestingly, using the anionic fluorescent dye DiBAC it was determined that blastemas at an anterior cut, which regenerate a head, are much more depolarized compared to posterior blastemas, which regenerate a tail (Beane et al., 2011). Artificially depolarizing cells in the posterior blastema with ivermectin led to regeneration of a head in place of a tail (Beane et al., 2011). Conversely, hyperpolarization of the anterior blastema with a hydrogen/potassium ATPase inhibitor (SCH-28080) lead to regeneration of a tail in place of a head (Beane et al., 2011). The genetic underpinnings of head versus tail regeneration have been well defined in planaria. Inhibition of canonical Wnt signaling by β-catenin RNAi leads to posterior blastemas regenerating a head instead of a tail (Gurley et al., 2008; Petersen and Reddien, 2008). Remarkably, co-treatment of planarians with β-catenin RNAi and the hydrogen/potassium ATPase inhibitor blocked ectopic head regeneration and instead allowed for proper tail regeneration (Beane et al., 2011). This suggests the bioelectric state of the posterior blastema cells is downstream of Wnt signaling and is sufficient to instruct a tail identity even in the absence of β-catenin transcriptional activity.

Apart from planaria, membrane potential plays a critical role in regulating Xenopus tadpole tail regeneration and zebrafish fin regeneration (Adams et al., 2007; Monteiro et al., 2014; Tseng et al., 2010). Both studies identified various subunits of the vesicular hydrogen ATPase pump (V-ATPase) as being differentially expressed as early as 6 hours after amputation. Pharmacologic inhibition and genetic knockout of essential subunits of the V-ATPase pump blocked tail and fin regeneration, respectively. Interestingly, key patterning genes such as Msx1, Notch, and Mkp3 are downstream of V-ATPase activity (Adams et

al., 2007; Monteiro et al., 2014). Additionally, inhibition of V-ATPase signaling blocked proper innervation of the regenerated fin and inhibited spinal cord regeneration in the Xenopus tail (Adams et al., 2007; Monteiro et al., 2014).

During Xenopus tail regeneration, V-ATPase signaling at 6 hours post injury is necessary to induce expression of the voltage gated sodium channel $Na_v1.2$ by 24 hours post amputation (Tseng et al., 2010). The sequential action of these pumps function to first depolarize blastema cells early (V-ATPase at 6 hours post amputation) and then repolarize the cells at later time points ($Na_v1.2$ by 24 hours post amputation) (Tseng et al., 2010). Expression of $Na_v1.2$ leads to activation of the salt inducible kinase (SIK), which is necessary for expression of key morphogenetic factors mentioned above (Msx1 and Notch) (Tseng et al., 2010). Finally, overexpression of either V-ATPase or $Na_v1.2$ is able to induce tadpole tail regeneration during a refractory period in which tadpoles are temporarily unable to regenerate (Adams et al., 2007; Tseng et al., 2010). Collectively, these studies highlight the important role that dynamic changes in blastema cell membrane potential have on tissue regeneration.

MicroRNAs and Spinal Cord Regeneration

(Adapted from Sabin, K and Echeverri, K. "MicroRNA signaling during regeneration." *Regenerative Engineering and Developmental Biology: Principles and Applications.* Ed. David M. Gardiner. CRC Press, 2017. 183-205. Print.)

Biogenesis of microRNAs

MicroRNAs (miRNAs) were first identified in *C. elegans* in 1993 (Lee et al., 1993) and have proven to be potent regulators of post-transcriptional gene expression (**Fig. 1-6**). Most miRNA genes are transcribed by RNA polymerase II to form a primary miRNA that is capped, polyadenylated, and further processed in the nucleus by a multi-enzyme complex, called the microprocessor, to form pre-miRNAs, which are subsequently exported from the nucleus to the cytoplasm

(Denli et al., 2004; Gregory et al., 2004; Lee et al., 2004; Lund et al., 2004; Yi et al., 2003). Once exported into the cytoplasm, the pre-miRNAs are processed further by an RNase III enzyme called DICER to produce a ~22 nucleotide RNA molecule that is incorporated into the miRNA-induced signaling complex, or miRISC (Bernstein et al., 2001; Chendrimada et al., 2005; Park et al., 2011). Once the miRISC is assembled, the guide strand of RNA directs target specificity by binding to complementary seed sequences in the 3' untranslated region of mature mRNAs (Bartel, 2009; Shukla et al., 2011). Mature miRNAs regulate gene expression by annealing to the complementary seed sequence in the 3' untranslated region of target transcripts to promote mRNA degradation and/or inhibition of translation (Filipowicz et al., 2008). Considering that a single miRNA can regulate the expression of hundreds or thousands of individual mRNAs, and that a single mRNA can be efficiently regulated by multiple miRNAs, therapeutic manipulation of a single miRNA could have profound effects on the cellular response to injury. Indeed, recent evidence supports the hypothesis that differential regulation of miRNA expression after injury is critical for promoting functional spinal cord regeneration.

MicroRNAs and Spinal Cord Regeneration

Traumatic spinal cord injury (SCI) in mammals results in extensive neuronal cell death, axonal degeneration, and deposition of scar tissue leading to the loss of sensory and motor function below the injury site. While mammals are unable to regenerate damaged nervous tissue to restore sensory and motor function other vertebrates, like zebrafish and salamanders, are able to functionally regenerate (Becker and Becker, 2015; Diaz Quiroz and Echeverri, 2013; Gemberling et al., 2013; Tanaka and Ferretti, 2009). The molecular mechanisms that promote functional nervous tissue regeneration are still being elucidated, but recent data from our lab and others have firmly established an essential role for miRNAs in regulating faithful nervous system regeneration in organisms with the inherent ability to regenerate. Studies in mammals, which

have limited regenerative capabilities after CNS injury, have identified cohorts of miRNAs that are differentially regulated in response to various injury paradigms (reviewed in (Bhalala et al., 2013; Di et al., 2014; Nieto-Diaz et al., 2014; Nin et al., 2009)). However, for the context of this Introduction, I will focus on a few pertinent examples from model organisms with the natural ability to regenerate.

Following tail amputation in salamanders, the regenerating spinal cord forms a transient structure called the ependymal bulb that protrudes into the blastema and migrates posteriorly throughout regeneration. The adult spinal cord maintains expression of dorsoventral markers including Pax7, Pax6, and Msx1/2, which are down regulated after tail amputation within a 500μm zone anterior to the injury (McHedlishvili et al., 2007; Schnapp et al., 2005). How these DV expression domains are differentially regulated between the adult and regenerating spinal cord is not well understood. A microarray-based profiling approach identified miR-196 as highly up-regulated during axolotl tail regeneration (Sehm et al., 2009). In situ hybridization showed miR-196 is specifically expressed in the glial cells of the regenerating spinal cord. Inhibition of miR-196 resulted in decreased glial cell proliferation, defects in ependymal blub formation, and overall inhibited spinal cord regeneration. Additionally, the dorsal Pax7 expression domain did not diminish but was instead expanded within the 500μm zone. Other dorsal patterning molecules (BMP4, Msx1/2, Meis2) were up-regulated while the ventral patterning molecule Shh was not affected after miR-196 inhibition (Sehm et al., 2009).

Deep sequencing of small RNAs after axolotl tail amputation identified additional miRNA families that were differentially regulated during tail amputation (Gearhart et al., 2015). This study identified members of the miR-1 and let-7 families, as well as miR-21 and miR-206 as being significantly differentially regulated after injury compared to uninjured tissue. This is consistent with other reports suggesting important regulatory roles of these miRNAs during regeneration of various organs (Aguirre et al., 2014; Holman et al., 2012; Lepp and Carlone, 2015; Ramachandran et al., 2010). Of interest was the identification

and functional validation of putative novel miRNAs that didn't share significant homology with any known vertebrate miRNA (Gearhart et al., 2015). This suggests the existence of salamander-specific miRNAs that could represent a species-specific difference in regenerative capabilities compared to mammals.

Similar to axolotl tail regeneration, studies in the newt have identified a critical signaling network between miR-1, miR-133a, and retinoic acid receptor β2 (RARβ2) (Carter et al., 2011; Lepp and Carlone, 2014). RARβ2, miR-1, and miR-133a are all specifically expressed in glial cells of the newt spinal cord and pharmacologic inhibition of RARβ2 blocked spinal cord regeneration (Carter et al., 2011; Lepp and Carlone, 2014). This suggests that RARβ2 signaling is necessary for faithful spinal cord regeneration. Interestingly, RARβ2 is a direct target of both miR-1 and miR-133a and overexpression of miR-133a leads to decreased RARβ2 expression and phenocopies the regenerative defects associated with RARβ2 inhibition (Carter et al., 2011; Lepp and Carlone, 2014). Collectively these results show a complex feedback network aimed at regulating the precise timing and levels of RARβ2 signaling which is necessary for spinal cord regeneration.

Additional work on miR-133 in fish identified that miR-133b was up-regulated in neurons of the medial longitudinal fasciculus (MLF) 7 days after spinal cord transection (Yu et al., 2011b). Neurons within the MLF are known to project to the spinal cord in fish (Becker et al., 1997), suggesting that the injury-induced up-regulation of miR-133b could regulate axon regeneration of supraspinal projecting neurons. Indeed, inhibition of miR-133b function after injury led to defects in axon regeneration. The small GTPase RhoA was found to be a direct target of miR-133b in MLF neurons (Yu et al., 2011b). RhoA blocks axon regeneration in mammals through modulation of actin dynamics, ultimately leading to growth cone collapse (Chiba et al., 2009; Dergham et al., 2002; Holtje et al., 2009). The authors postulate that up-regulation of miR-133b in MLF neurons attenuates RhoA levels leading to a permissive environment for growth cone migration and axon regeneration.

Apart from tail amputation, axolotls also regenerate their spinal cord after transection injury (Butler and Ward, 1965, 1967; Piatt, 1955). This type of injury is widely used in mammals to study spinal cord regeneration and allows direct comparisons of molecular differences in injury response between mammals and axolotl. Microarray-based transcriptional profiling identified miR-125b was down regulated by 1 day post injury in axolotl but was not down regulated until 6 days post injury in rat (Diaz Quiroz et al., 2014). Additionally the overall levels of miR-125b were 6 times higher in axolotl compared to rat. The authors hypothesized that the differential regulation and/or differences in expression level of miR-125b might be one factor regulating the different responses to SCI in axolotl compared to rat. The axon growth inhibitory molecule Semaphorin4D (Sema4D) is a direct target of miR-125b in rats and axolotl, and Sema4D is up-regulated by reactive astrocytes after SCI in mammals (Diaz Quiroz et al., 2014). Down regulation of miR-125b after SCI in axolotls helps create an instructive environment guiding axon regeneration through the lesion. Over expression of miR-125b leads to unorganized axonal sprouting throughout the lesion, leading to failed spinal cord regeneration. Conversely, miR-125b inhibition or Sema4D overexpression completely blocks axon regeneration (Diaz Quiroz et al., 2014). Collectively, these results support a model whereby the precise levels of miR-125b are necessary to modulate the levels of Sema4D to create a permissive and instructive environment for axon regeneration.

The 6-fold lower levels of miR-125b in the rat spinal cord would allow for much higher levels of Sema4D leading to blocked axon regeneration. This is similar to Sema4D overexpression or miR-125b inhibition in axolotl spinal cord. Indeed, surgical implantation of a chemically synthesized miR-125b mimic embedded in a pluronic gel, which facilitates mimic uptake by cells, into the lesion of injured rats led to significantly higher levels of miR-125b, similar to axolotls. Increased miR-125b levels led to decreased expression of genes involved with reactive gliosis, glial scar formation and increased functional recovery compared to controls (Diaz Quiroz et al., 2014). These experiments

provide efficacy for comparative studies in lower vertebrates aimed at elucidating molecular targets that promote regeneration for subsequent manipulation in mammalian regeneration.

Collectively, these studies highlight the diverse cellular responses regulated my miRNA signaling during natural spinal cord regeneration. These processes include patterning, glial cell proliferation, and appropriate axon regeneration through the lesion. While these studies have provided valuable insight into miRNAs and spinal cord regeneration, this is by no means an exhaustive list and there remain many more miRNA families to be described and functionally validated. Indeed, this thesis has identified miR-200a as an important regulator of spinal cord regeneration in axolotl. While miR-200a has an appreciated role in regulating neural development in vertebrates and invertebrates, its function had not previously been extensively characterized during neural regeneration.

Figures

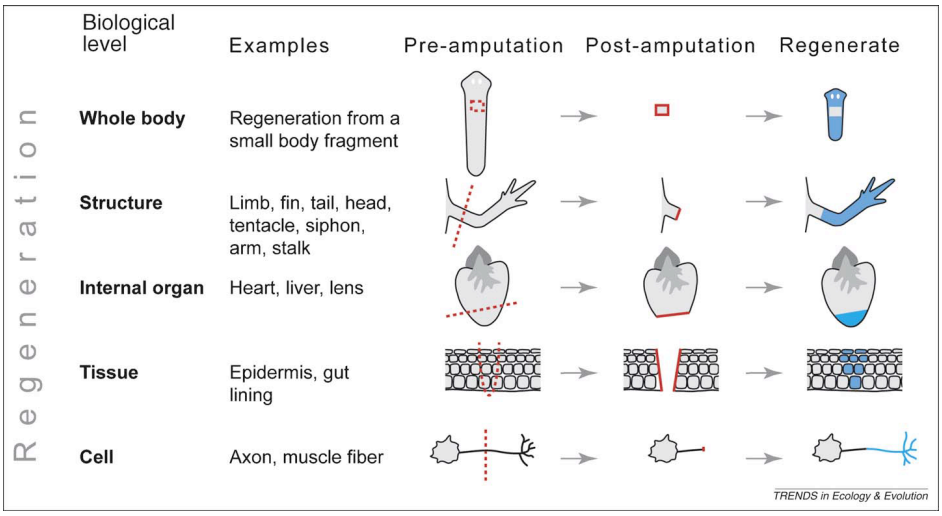

Figure 1-1: The ability to regenerate diverse structures is variable within organisms.

The ability to regenerate is widespread throughout metazoa and comes in many different forms. Planarian flatworms are able to regenerate their whole body from a small piece. Salamanders are able to regenerate complex structures, such as the limb. Zebrafish have the robust ability to undergo organ regeneration following amputation of the apex of the heart. Humans are able to undergo tissue level regeneration following insult to skeletal muscle or liver. Finally, nematodes display the ability to regenerate cellular structures such as axons after axotomy. (Modified from (Bely and Nyberg, 2010))

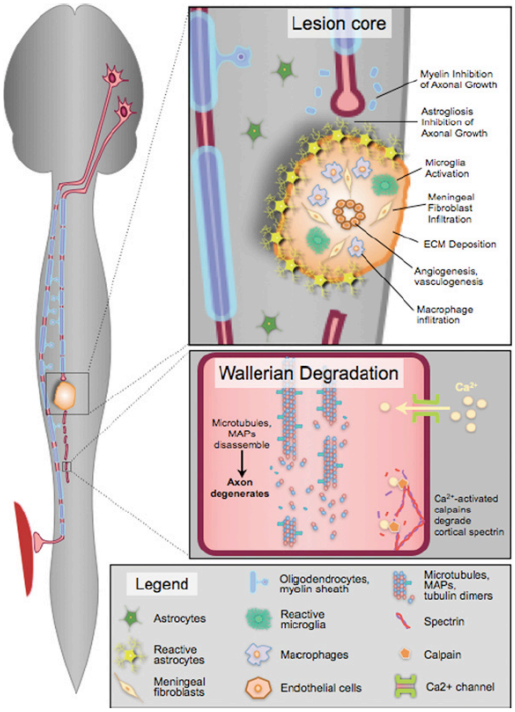

Figure 1-2 Cell types, cellular response and scar formation after spinal cord injury in mammals.

Following spinal cord injury in mammals, a fibrotic scar forms at the lesion core. The fibrotic scar is comprised of fibroblasts, pericytes and infiltrating immune cells as well as axon growth repulsive extracellular matrix components. Surrounding the fibrotic core is a glial scar primarily comprised of reactive astrocytes and myelin-derived growth repulsive molecules from degenerated oligodendrocytes. Molecules expressed on the cell surface of reactive astrocytes and secreted by reactive astrocytes, as well as the myelin debris, collectively function to inhibit axon regeneration through the glial scar. Additionally, mechanical injury and calcium influx into severed axons sets off a series of events leading to cytoskeletal depolymerization and Wallerian degeneration. (Modified from (Diaz Quiroz and Echeverri, 2013))

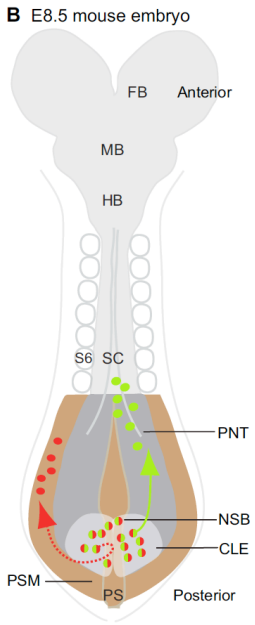

Figure 1-3 Neuromesodermal progenitors reside in the caudal lateral epiblast of elongating embryos.

Neuromesodermal progenitors reside within the caudal lateral epiblast of elongating vertebrate embryos. Neuromesodermal progenitors are competent to contribute to either mesoderm or spinal cord. PS: primitive streak. PSM: presomitic mesoderm. CLE: caudal lateral epiblasat. NSB: node streak border. PNT: preneural tube. SC: spinal cord. S6: somite 6. HB: Hindbrain. MB: midbrain. FB: forebrain. (Modified from (Henrique et al., 2015)).

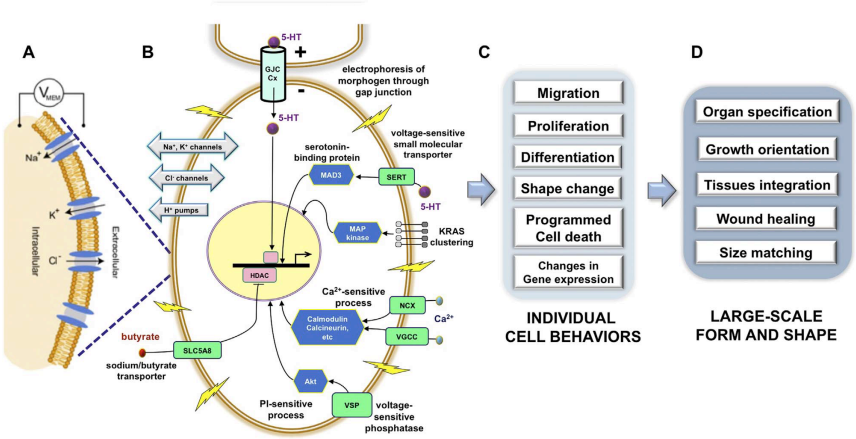

Figure 1-4 Membrane potential regulates diverse cellular processes associated with different biological states.

(A) Cellular membrane potential is established via the coordinated functions of various ion channels, pumps, and transporters located within the cellular membrane. **(B)** Cellular membrane potential, ionic flow, and transporter substrates can regulate the activity of various cell signaling pathways capable of affecting **(C)** multiple cellular processes that have implications for **(D)** tissue and organism level functions. (Modified from (McLaughlin and Levin, 2018))

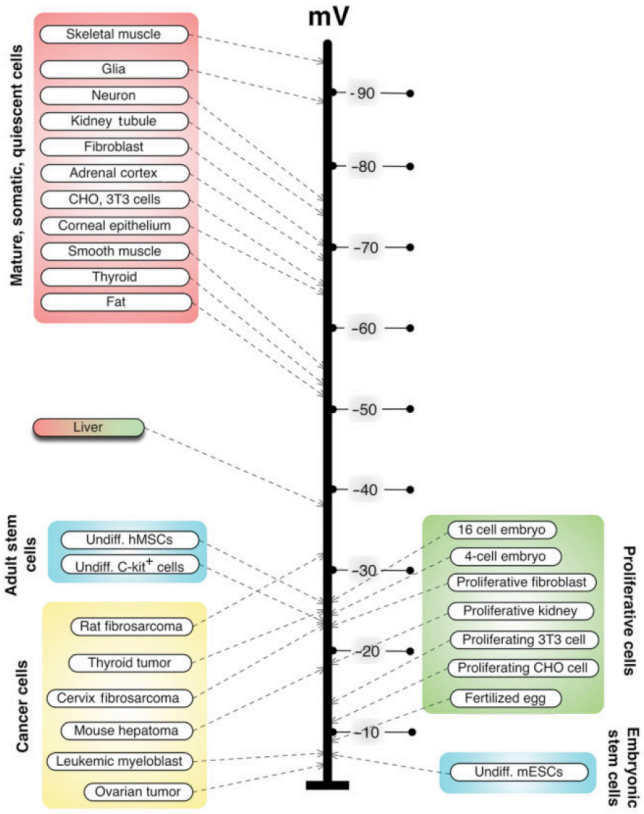

Figure 1-5 The resting membrane potential is different across quiescent and highly proliferative cells.

Cellular membrane potential correlates with the proliferative status of a cell. Highly differentiated cells, like muscle fibers or neurons, have a very negative resting membrane potential. However, highly proliferative cells, like stem cells or cancer cells, have a much more positive membrane potential. (Modified from (Levin, 2012)).

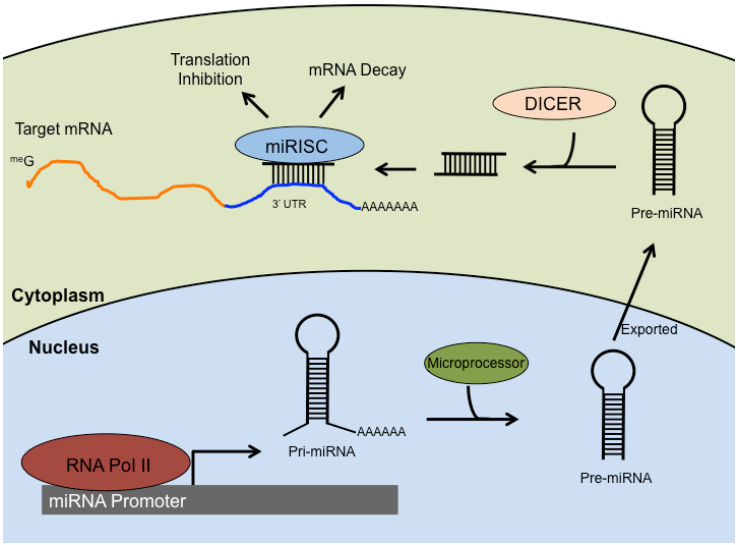

Figure 1-6 Biogenesis of microRNAs.

A microRNA gene is transcribed by RNA polymerase II and polyadenylated. A multi-enzyme complex, called Microprocessor, cleaves the primary miRNA (pri-miRNA) transcript to generate a pre-miRNA, which is subsequently exported to the cytoplasm. After nuclear export, the pre-miRNA is cleaved to ~21 nucleotides by DICER and the guide strand is incorporated into miRISC. Finally, the guide miRNA will anneal to a 6-8 nucleotide seed sequence in the 3' untranslated region (UTR) of target mRNAs to promote transcript degradation or inhibition of translation. (Modified from (Sabin, 2017)).

Chapter 2: Identification of early injury signals that regulate the pro-regenerative glial cell response to spinal cord injury in axolotl

(Adapted from the paper, “Sabin K, Santos-Ferreira T, Essig J, Rudasill S, Echeverri K. “Dynamic membrane depolarization is an early regulator of ependymoglial cell response to spinal cord injury in axolotl.” Dev. Bio. 2015 Dec 1;408(1):14-25. doi: 10.1016/j.ydbio.2015.10.012. Epub 2015 Oct 20. PubMed PMID: 26477559; PubMed Central PMCID: PMC5096653.”)

Introduction

The ability to regenerate lost tissue after injury is a widespread phenomenon across diverse phyla, ranging from amphibian limb regeneration to full body regeneration in planaria flatworms (Alvarado, 2000; Brockes, 1991, 1997; Poss et al., 2003; Tanaka, 2003). Among vertebrates, amphibians, such as the Mexican axolotl, are unique in their ability to regenerate multiple structures after injury including limbs, skin, heart, liver, and the central nervous system (Abate et al., 1993; Diaz Quiroz and Echeverri, 2013; Gardiner et al., 2002; Goss, 1969; Tanaka and Reddien, 2011). In contrast, mammals have a very limited regenerative capacity that declines even more with age. Mammals can regenerate small lesions in skin, muscle, or peripheral nerves and can regenerate part of the liver (Becker and Diez Del Corral, 2015; Borena et al., 2015; Cregg et al., 2014; Heinrich et al., 2015; Lepousez et al., 2015; Silver J and Miller JH, 2004; Yamakawa and Ieda, 2015). Why mammals react differently to injury than lower vertebrates like salamanders or fish represents a fundamental question in regenerative biology.

Strikingly, after spinal cord injury (SCI) axolotls are able to functionally regenerate their spinal cord, ultimately regaining sensory and motor function comparable to pre-lesion levels (Butler and Ward, 1965, 1967; Chernoff et al., 2003; Clarke et al., 1988; Clarke and Ferretti, 1998; Clarke et al., 1986b). In response to SCI, glial cells, which function as resident neural stem cells and line the central canal of the spinal cord, proliferate and migrate to bridge the lesion and provide guidance signals to support and direct subsequent axon regeneration (Butler and Ward, 1965, 1967; Chernoff et al., 2003; Diaz Quiroz et al., 2014; O'Hara et al., 1992; Quiroz and Echeverri, 2012). Despite extensive characterization of the cellular response to SCI, the molecular signals that drive functional regeneration are only now being elucidated.

In recent years we have made extensive progress in understanding some of the signaling molecules that are necessary at the injury site to ensure faithful

regeneration in terms of size and patterning of the lost appendage or damaged tissue (Becker and Diez Del Corral, 2015; Chaar and Tsilfidis, 2006; Chernoff et al., 2003; Ferretti et al., 2003; Frobisch and Shubin, 2011; Gardiner and Bryant, 1996; Gardiner et al., 1999; Kumar and Brockes, 2012). Many of the molecules recently shown to be essential for spinal cord regeneration are also essential for neural tube development. After tail amputation the transcription factor Sox2, a known neural progenitor cell marker, is required for glial cell proliferation and subsequent spinal cord regeneration (Fei et al., 2014). Additionally, sonic hedgehog, which is important in specifying the dorsoventral axis in the developing neural tube, is similarly important for glial cell proliferation and patterning of the regenerated spinal cord after tail amputation (Schnapp E et al., 2005). Many of these gene products are not expressed in uninjured adult tissue or are differentially expressed after injury (Chernoff, 1988, 1996; Chernoff et al., 2000; Chernoff and Robertson, 1990; Chernoff et al., 2002; Chernoff et al., 2003; Diaz Quiroz et al., 2014; Monaghan et al., 2007; Scadding and Maden, 1986; Schnapp E et al., 2005; Sehm et al., 2009). How the expression of these genes is precisely regulated after injury remains a fundamental question in the field.

Recent reports show that regulation of cellular membrane potential, the electrical charge separation across the plasma membrane, plays an integral role in regulating regeneration (Barghouth et al., 2015; Beane et al., 2013; Borgens et al., 1986; Borgens et al., 1977; Levin, 2007, 2009a, b; Stewart et al., 2007; Tseng and Levin, 2013; Tseng and Levin, 2012). After Xenopus tadpole tail amputation the hydrogen (H^+) V-ATPase pump is highly up-regulated in the regeneration blastema within 6 hours after injury (Adams et al., 2007; Tseng et al., 2011; Tseng and Levin, 2008, 2012). The H^+ V-ATPase functions to repolarize the injury site to resting membrane potential by 24 hours post injury. If the expression or function of H^+ V-ATPase is blocked, then cells at the injury site fail to proliferate and tail regeneration does not occur. Furthermore, inhibition of the early electrical response to injury blocks expression of key morphogenetic factors, such as Msx1, Notch and BMP, 48 hours post injury (Tseng et al., 2010).

Recent studies in the axolotl using ion sensitive dyes and *in vivo* imaging shows rapid and dynamic changes in H^+ and Na^+ ion contents and a depolarization of the membrane potential in cells adjacent to the injury site (Ozkucur et al., 2010). However, the functional significance of these biophysical signals in regulating regeneration was not addressed.

Using our *in vivo* spinal cord injury model, we analyzed the role of membrane potential in the glial cells after spinal cord injury. Here we demonstrate that there is a rapid depolarization of glial cells after spinal cord injury and repolarization to resting membrane potential within 24 hours post injury. We show that perturbing this dynamic change in membrane potential after injury inhibits glial cell proliferation and axon regeneration through the lesion. Additionally, we identified c-Fos as an important target gene that is normally up-regulated after injury in glial cells. However, in glial cells whose normal electrical response is perturbed after injury, c-Fos is not up-regulated and regeneration is inhibited. Our results indicate that axolotl glial cells must undergo a dynamic change in membrane potential in the first 24 hours post injury to initiate a pro-regenerative response.

Results

Injury induces rapid changes in glial cell membrane potential

Previous work in the lab established a spinal cord transection model whereby ~500μm of spinal cord is removed using a 26 gauge needle (Diaz Quiroz et al., 2014; Sabin et al., 2015). Lineage tracing analysis during spinal cord regeneration after transection labeled the glial cells using either the GFAP or Nestin promoter to drive GFP expression. This approach revealed that glial cells within a 500μm rostral and 300μm caudal to the lesion proliferate and migrate to reconnect the injured spinal cord by 7 days post injury (Sabin et al., 2015). Labeled glial cells outside of this region did not respond to injury or contribute to regeneration. Specific analysis of glial cell proliferation kinetics within this region using BrdU pulse chase revealed glial cell proliferation peaked at 3 days post injury and returned to homeostatic levels by 7 days post injury (Sabin et al., 2015). Furthermore, axon regeneration through the lesion was also completed by 7 days post injury (Diaz Quiroz et al., 2014; Sabin et al., 2015). Taken together, these results suggest that upon injury glial cells respond to a specific signal that is transduced over a certain distance to stimulate glial cells to participate in regeneration. However, the identity of this injury signal was not clear.

Previous work in planaria, axolotl, zebrafish, and Xenopus has demonstrated dynamic changes in the resting membrane potential occur after injury and this response is essential for faithful regeneration to occur (Beane et al., 2013; Blackiston et al., 2009; Borgens, 1986; Monteiro et al., 2014; Ozkucur et al., 2010). However, it remains unknown if a similar response occurs after SCI. To address this question we used *in vivo* imaging to measure the change in the membrane potential after injury with the fluorescent voltage sensitive dye $DiBAC_4(3)$ (bis-[1,3-dibutylbarbituric acid]trimethine oxonol (**Fig. 2-1A,B**). This method enabled us to identify a significant increase in membrane potential approximately seven hours post injury. However, by twenty-four hours post injury

the membrane potential of the glial cell population adjacent to the injury site had returned to almost uninjured levels (**Fig. 2-1C**). This change in membrane potential was observed in the cells adjacent to the injury site and within approximately 500μm of the injury, but no change was observed in cells further away. To investigate if this change in membrane potential is essential for regeneration to occur we used the commercially available drug ivermectin, which opens glycine gated chloride channels and induces depolarization (Beane et al., 2013; Blackiston et al., 2011). We confirmed that ivermectin treatment caused prolonged depolarization of the glial cells by causing a decrease in intracellular chloride levels by measuring the chloride levels after ivermectin treatment compared to controls (**Fig. 2-2**).

Prolonged depolarization led to an overall decrease in the number of $BrdU^+$ glial cells 3 days post injury compared to vehicle control (**Fig. 2-3 A-C**). Additionally, it was noted that the distance between the rostral and caudal ends of the injured spinal cord did not decrease over time like in the control animals (**Fig. 2-4**). Taken together, these data suggest that prolonged depolarization of glial cells causes a defect in cell division and/or migration in response to injury.

To determine if ivermectin affects specifically the glial cells or other cell types present in the spinal cord we performed immunohistochemistry looking for the target of ivermectin, the glycine gated chloride channel. Only $GFAP^+$ glial cells lining the central canal express the glycine receptor (**Fig. 2-5A-D**). To assess if prolonged depolarization of the glial cells after injury had any effect on functional regeneration we looked at axon regeneration 7 days post injury using whole mount β-III tubulin staining. In vehicle control injected animals, normal axon regeneration across the lesion site was observed (**Fig.2-6A, A'**). However, in animals injected with ivermectin, axons completely failed to grow across the lesion (**Fig. 2-6B, B'**). To further verify that this effect was specific to the ability of ivermectin to activate glycine gated chloride channels we injected spinal cords with the native ligand, glycine, and assayed for axon regeneration 7 days post injury. Axons in glycine injected animals also failed to grow across the lesion,

phenocopying the ivermectin injected animals. (**Fig. 2-6A-C**). To further confirm that dynamic changes in glial cell membrane potential regulates spinal cord regeneration, we altered the membrane potential in a predictable direction by specifically electroporating the glial cells with overexpression constructs of various ion channels. When we overexpressed a constitutively active mutant of the potassium channel Kir2.1, which causes hyperpolarization (Hinard et al., 2008), or the GABA-gated Na^+/Ca^{2+} channel Exp-1, which causes depolarization (Beg and Jorgensen, 2003), in the glial cells adjacent to the injury site, these animals failed to regrow axons across the lesion at 7 days post injury compared to vector control (**Fig. 2-7A-C**). Collectively, these data show that a dynamic electrical response of glial cells is necessary for the subsequent proliferative response to injury and axon regeneration across the lesion.

Molecular pathways downstream of membrane potential

To investigate the molecular pathways necessary for spinal cord regeneration, the lab performed microarray analysis on spinal cord injury samples 1, 3, and 7 days post injury compared to uninjured tissue (Sabin et al., 2015). From this analysis they identified that the transcription factor c-Fos was specifically up-regulated 1 day post injury and down regulated throughout the rest of regeneration (Sabin et al., 2015). Immunohistochemical analysis revealed that c-Fos was specifically expressed in the glial cells 500μm rostral and 300μm caudal to the lesion at 1 day post injury and was absent throughout the rest of regeneration, in agreement with our microarray (Sabin et al., 2015). Interestingly, microarray analysis of uninjured, 1 day post injury control, and 1 day post injury ivermectin injected spinal cords revealed that prolonged glial cell depolarization inhibited c-Fos up-regulation (Sabin et al., 2015). While this data clearly demonstrates that c-Fos expression is downstream of membrane potential after injury the biochemical signal up-stream of c-Fos expression and activation was not known.

Previous work has shown that c-Fos expression and activity are regulated by the MAP kinase ERK (Abate et al., 1993; Vesely et al., 2009; Vial et al., 2003). In addition, c-Fos has been shown to be an essential regulator of cell division (Bakiri et al., 2007; Herdegen and Leah, 1998; van Dam and Castellazzi, 2001). Therefore, we hypothesized that injury induced changes in membrane potential led to ERK activation and subsequent c-Fos expression. To test if c-Fos activation is necessary for glial cell proliferation after injury, we injected the spinal cord with an ERK inhibitor immediately prior to injury and assessed regenerative defects by assaying for (1) glial cell proliferation, and (2) axon regeneration through the lesion. BrdU pulse chase analysis revealed that ERK inhibition inhibited glial cell proliferation (Sabin et al., 2015). Additionally, ERK inhibition blocked axon regeneration through the lesion (Sabin et al., 2015). Collectively, these results show that inhibiting ERK function and the downstream regulation of c-Fos phenocopies the regenerative defects associated with prolonged glial cell depolarization.

Finally, to directly determine if prolonged glial cell depolarization affects ERK activation after injury, we performed Western blot analysis for di-phospo-ERK (dpERK) in uninjured and 1 day post injury control compared to ivermectin-injected spinal cords. In control 1 day post injury spinal cords dpERK is increased compared to the uninjured spinal cord, and prolonged depolarization of glial cells with ivermectin largely blocks ERK activation after injury (**Fig. 2-8**) This data collectively supports our hypothesis that membrane depolarization after injury is upstream of ERK and c-Fos activation, which is necessary for glial cell proliferation and subsequent spinal cord regeneration. Taken together, our data show that rapid depolarization of glial cell membrane potential is essential to activate the pro-regenerative circuitry necessary for spinal cord regeneration.

Discussion

Pro-regenerative glial cell response to injury is dependent upon rapid changes in membrane potential

The present study offers insight, at the cellular and molecular level, into the response of axolotl glial cells to acute injury. Previous work in axolotl has shown that, in response to tail amputation, glial cells will amplify and migrate to regenerate a new ependymal tube (Echeverri and Tanaka, 2002, 2003b; McHedlishvili et al., 2007; McHedlishvili et al., 2012). However, the molecular signals that initiate this response to injury have remained elusive. Our previous observation that cells from defined distances rostral and caudal to the injury site respond to injury suggests to us that upon injury, specific signals are transmitted a certain distance that are responsible for initiating a regenerative response in these cells.

In recent years, several groups have reported that biophysical cues may be the first signals that are transmitted in response to injury to regulate gene expression and elicit a regenerative response. After tail amputation in *Xenopus* tadpoles, the activation of the H^+ V-ATPase is necessary and sufficient to promote tail regeneration (Adams et al., 2007). Recent work in the axolotl established that changes in calcium, sodium, and membrane potential occur in cells at the injury site after tail amputation (Ozkucur et al., 2010). To determine if these early injury-induced signals were essential for spinal cord regeneration in the axolotl, we injected the fluorescent voltage sensitive dye DiBAC into the spinal cord central canal and used in vivo fluorescent imaging to investigate the electrical response of the glial cells to injury. We identified a dynamic change in membrane potential after injury in cells adjacent to the injury site (**Fig. 2-1**). We observed a rapid increase in membrane potential within the first 7 hours that is largely repolarized to resting membrane potential twenty-four hours after injury. To determine if this dynamic change in the membrane potential of glial cells is essential to the regenerative response we microinjected the depolarizing drug

ivermectin, which activates glycine gated chloride channels leading to chloride efflux (**Fig. 2-2**), into the spinal cord central canal or overexpressed various ion channels before injury. We found that prolonged depolarization of glial cells blocked their proliferative response to injury and inhibited migration of the rostral and caudal ends of the lesion (**Fig. 2-3, Fig. 2-4**). Furthermore, perturbation of the endogenous electrical response of the glial cells to injury by overexpression of depolarizing or hyperpolarizing channels inhibited axon regeneration compared to vector control animals (**Fig. 2-5**).

Our observation that prolonged depolarization blocks glial cell proliferation conflicts with a previous study that shows that ivermectin treatment of developing Xenopus embryos leads to a hyperproliferation of melanocyte-producing neural crest cells in a cell non-autonomous manner (Blackiston et al., 2011). Interestingly, other studies report that global perturbation of the electrical response of damaged tissue blocks cell proliferation and subsequent regenerative outgrowth after Xenopus tadpole tail amputation and zebra fish fin amputation (Adams et al 2007, Tseng et al 2010, Monteiro et al 2014). Taken together, these observations suggest that membrane potential-sensitive changes in cell proliferation are cell type and context specific.

In an effort to identify common and cell type specific pathways regulated by changes in membrane potential Pai et al took a comparative approach looking at developing *Xenopus* embryos, axolotl spinal cord regeneration, and human mesenchymal stem cell differentiation. The authors identified several pathways that were similarly activated across species and across cell types in response to prolonged depolarization (Pai et al., 2016). Interestingly, there were also species/cell type specific responses to prolonged depolarization supporting the idea that changes in membrane potential can differentially regulate cellular responses based on developmental context and cell type (Pai et al., 2016). How changes in membrane potential are detected and transduced into diverse biochemical pathways based on cellular identity is still not clear.

Dynamic changes in membrane potential regulate gene expression

To investigate how changes in glial cell membrane potential could lead to a physiologic response to injury, the lab previously performed microarray analysis and compared changes in gene expression in vehicle treated and depolarized samples. This approach identified a group of genes that are up-regulated by 1 day post injury and then rapidly return to homeostatic levels (Sabin et al., 2015). Within this group of genes was the transcription factor c-Fos and subsequent immunohistochemical analysis revealed that c-Fos was specifically expressed in glial cells adjacent to the lesion and its expression is downstream of the rapid change in glial cell membrane potential (Sabin et al., 2015). The transcription factor c-Fos is a well characterized early response gene that has been studied in many model systems and is known to regulate a diversity of signaling pathways and cellular processes including, but not limited to, proliferation, differentiation, and apoptosis (Bakiri et al., 2007; Herdegen and Leah, 1998; Ueyama et al., 1997; van Dam and Castellazzi, 2001; Vial et al., 2003). The upstream biochemical signal regulating c-Fos expression and activity was not clear. However, the MAP kinase ERK is a well-established regulator of c-Fos expression and nuclear translocation. Therefore, we wanted to determine if ERK signaling was downstream of glial cell membrane potential after injury.

Subsequent biochemical analysis revealed that the MAP kinase ERK becomes activated at 1 day post injury and prolonged glial cell depolarization inhibits ERK activation (**Fig. 2-8**). This is consistent with our hypothesis that the rapid and dynamic change in glial cell membrane potential is up stream of ERK and c-Fos signaling. Taken together, these results support a working model where the ERK/c-Fos signaling cascade is necessary for the pro-regenerative glial cell response to injury and subsequent spinal cord regeneration in axolotl.

It is interesting to note that the ERK/c-Fos signaling cascade is a well established pathway associated with activity-dependent synaptic signaling associated with neuronal firing (West and Greenberg, 2011). Our data suggests that ERK/c-Fos signaling represents a conserved voltage-sensitive signaling

network, even in non-excitable cells, such as glial cells. It will be exciting in the future to determine if the voltage-sensitive machinery that leads to ERK activation after injury is the same as during neuronal firing.

Taken together, this data show that upon injury to the spinal cord the glial cells experience a rapid and dynamic change in resting membrane potential that is an essential signal to induce downstream gene expression changes, like activation of c-Fos, to drive the glial cell response to injury to promote a pro-regenerative response. c-Fos is known to heterodimerize with another early response protein, c-Jun, and form the AP-1 transcription factor (Norwitz et al., 2002; van Dam and Castellazzi, 2001). In axolotl we have found no evidence for up-regulation of c-Jun in glial cells, however it is found in the nucleus of neurons close to the injury site. It will be interesting to determine other potential interacting partners of c-Fos in the glial cells. In addition, it will be essential to investigate the other potential signals, like changes in other ions or mechanical transduction, that act in parallel or directly downstream of membrane potential to illicit a pro-regenerative response.

Figures

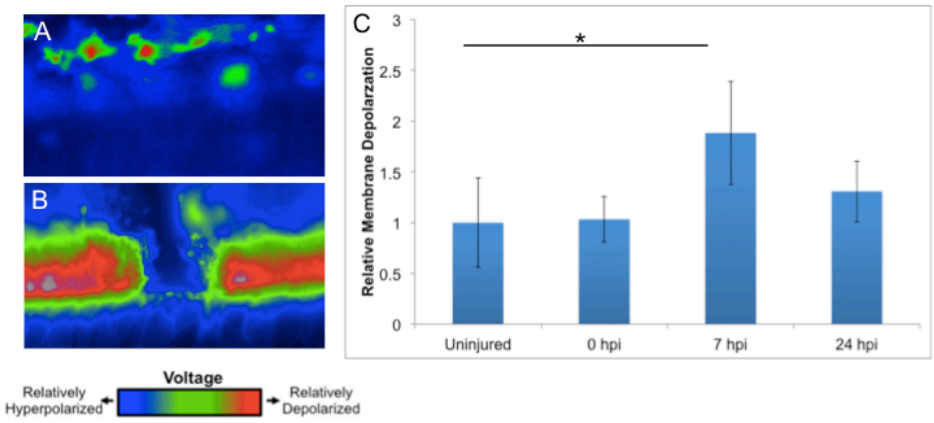

Figure 2-1: Spinal cord injury induces rapid but transient membrane depolarization.

Animals were injected with the membrane potential sensitive dye DiBAC and were imaged under the FITC channel before injury, immediately after injury, 7 hours post injury, and 24 hours post injury. Images were converted into a heat map using Fiji and pixel intensity was measured within a 500μm region rostral and 350μm caudal of the injury. Representative images of the polarization state of (A) uninjured and (B) injured spinal cords shows a drastic change in the polarization state following spinal cord ablation. (C) The spinal cord is significantly depolarized at 7 hours post injury compared to uninjured animals and is largely repolarized by 24 hours post injury. ***P<0.05; n=5.

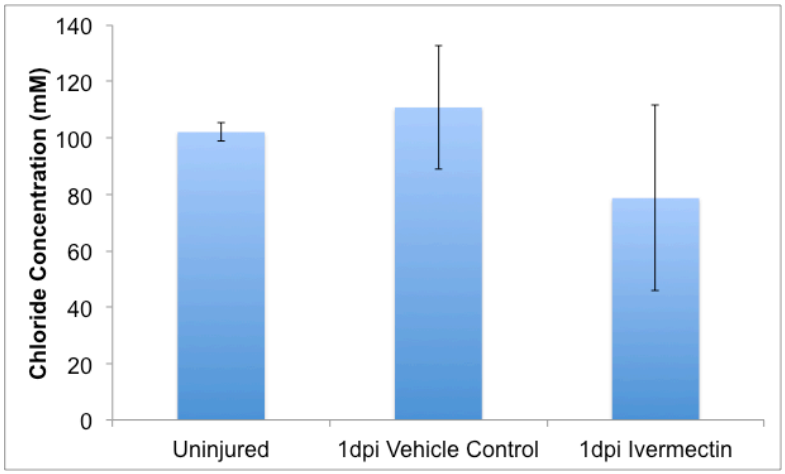

Figure 2-2: Chloride levels are decreased in ivermectin treated animals. Intracellular chloride levels were measured 1 day post injury in control versus ivermectin injected animals, as expected with ivermectin intracellular chloride levels increase. Extracts were made from groups of 6 -10 animals. N= 2.

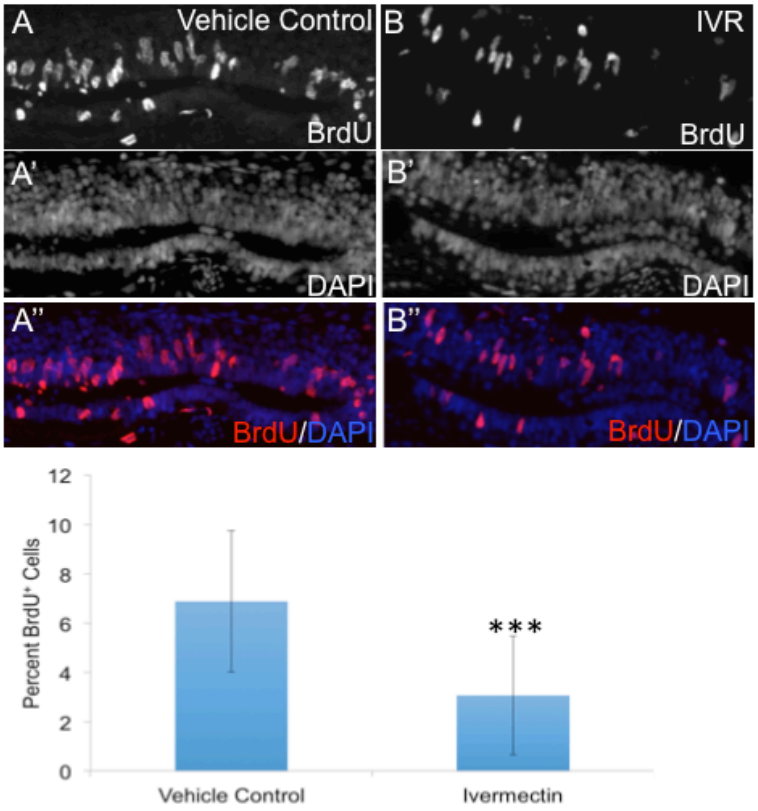

Figure 2-3: Prolonged depolarization inhibits glial cell proliferation in response to spinal cord injury.

Animals were injected with vehicle control of PBS (n=15) (A, A', A'') or ivermectin (IVR) to induce prolonged depolarization of the membrane (n=16) (B, B', B'') prior to spinal cord ablation. One day after injury animals were subjected to an intraperitoneal injection with BrdU and harvested for staining 24 hours later. (A", B") Comparison of the percent of BrdU+ cells in IVR treated and control axolotls shows there are significantly fewer BrdU+ cells in IVR treated animals 48 hours post injury compared to control axolotls (C). ***; P≤0.001. Error bars represent +/- SEM. Scale bar is 75 micrometers.

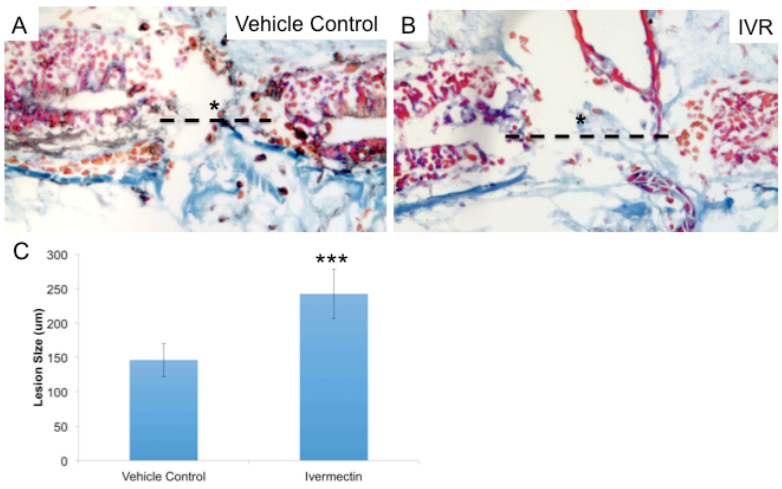

Figure 2-4: Prolonged depolarization of the glial cells adjacent to the injury site inhibits the progression of the rostral and caudal ends of the injured spinal cord during regeneration.

AFOG stains of longitudinal sections of 5 days post injury spinal cords injected with either (A) PBS (n=6) or (B) IVR (n=5) shows that the rostral and caudal ends of IVR injected spinal cords are significantly further from one another compared to PBS injected spinal cords (C). ***P ≤0.001; error bars represent +/- SEM.

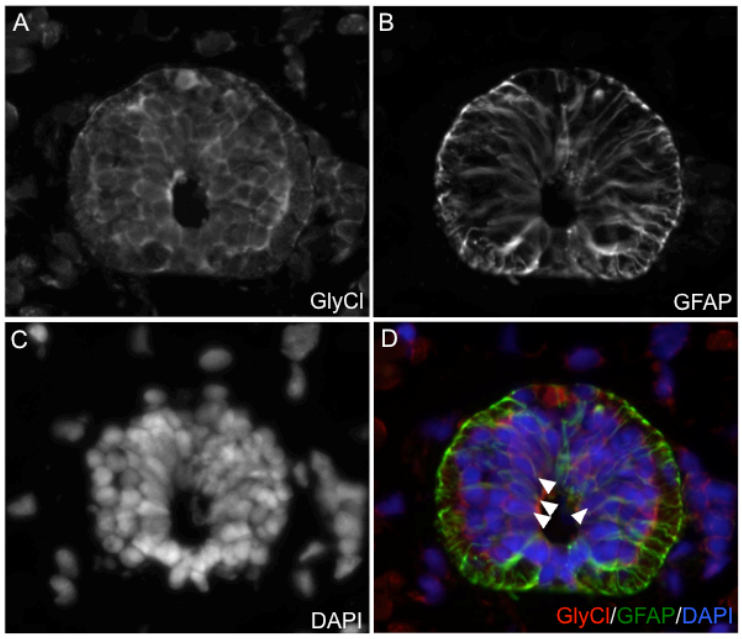

Figure 2-5: GlyCl-R is expressed by glial cells in the axolotl spinal cord. Cross sections of axolotl tails were stained with antibodies against (A) GlyCl-R or (B) the glial cell marker GFAP. (D) Overlay with DAPI (Blue) GlyCl-R (red) is expressed by GFAP+ (green) glial cells (white arrows). Scale bar 50 micrometers.

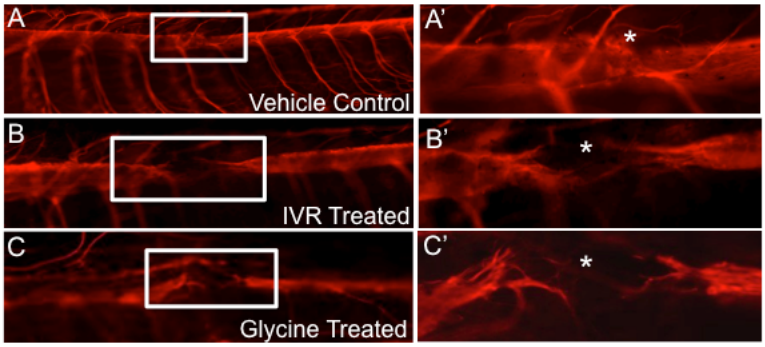

Figure 2-6: Prolonged depolarization of glial cell membrane using ivermectin or glycine inhibits axon regeneration across the injury site. Spinal cords were injected with either vehicle control PBS (A), the GlyCl-R agonists IVR (B), or the native ligand for GlyCl-R glycine (C) immediately before spinal cord ablation. Activation of GlyCl-R causes Cl^- efflux leading to chronic depolarization. In control animals 7days post injury the axons have regrown through the injury site, as visualized using whole mount anti-ß III tubulin staining (A, A'). In animals where the glial cells were kept in a prolonged state of membrane depolarization by injection of ivermectin, axon regeneration was inhibited (B, B'). Injection of the native ligand for glycine gated chloride channels, glycine, phenocopied the drug phenotype (C, C'). * denotes injury site. Control N = 47, Ivermectin N=32, Glycine N=17.

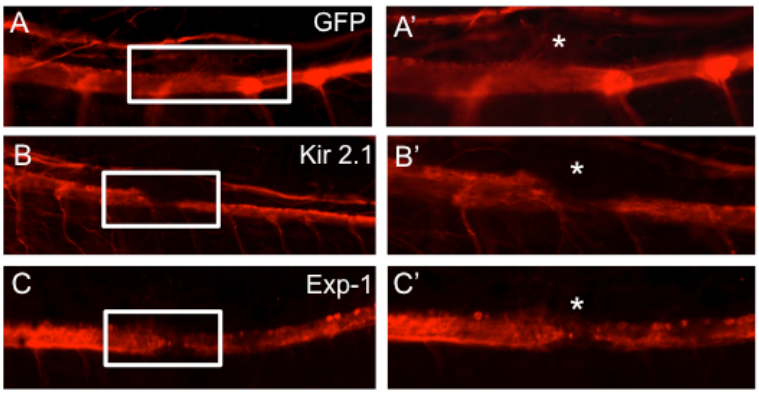

Figure 2-7 Blocking the normal injury-induced change in membrane potential following injury inhibits axon regeneration across the lesion. Spinal cords were electroporated with constructs encoding (A, A') H2B GFP, (B, B') a constitutively active mutant of the potassium channel Kir2.1, which causes hyperpolarization, or (C, C') the GABA-gated Na+/Ca++ channel Exp-1, which causes depolarization. Whole mount anti-beta-III tubulin staining of electroporated spinal cords 6 days post injury show that axons failed to regenerate in (B, B') hyperpolarized or (C, C') depolarized spinal cords compared to (A, A') control axolotls. White box and * denotes original injury site. Scale bar 75 micrometers.

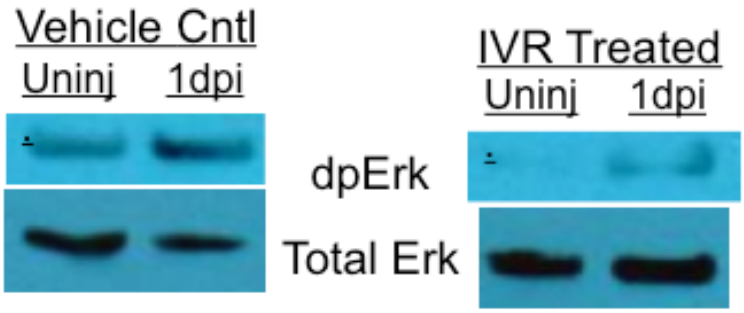

Figure 2-8: Prolonged glial cell depolarization blocks injury induced ERK activation.

Western blot analysis of control versus ivermectin treated animals. In vehicle control injured animals dpERK protein levels increase after spinal cord injury. This normal increase of dpERK is inhibited in ivermectin treated animals. Lysates were made from pools of 7-10 spinal cords per sample. N=3.

Chapter 3: Identification of molecular pathways that regulate the pro-regenerative glial cell response after spinal cord injury in axolotl

(Adapted from the manuscript, “Sabin K, Jiang P, Gearhart M, Stewart R, Echeverri K. “AP-1$^{cFos/JunB}$/miR-200a regulate the pro-regenerative glial cell response during axolotl spinal cord regeneration.” Under Review.”)

Introduction

Salamanders have retained the remarkable ability to functionally regenerate after spinal cord injury (SCI) (Butler and Ward, 1965, 1967; Chernoff et al., 2002; Chernoff et al., 2003; Clarke et al., 1988; Diaz Quiroz and Echeverri, 2013; Egar and Singer, 1972a; Tazaki et al., 2017). In response to SCI, glial fibrillary acidic protein $(GFAP)^+$ glial cells proliferate and migrate through the lesion to create a permissive environment for axon regeneration (Chernoff et al., 2003; Diaz Quiroz et al., 2014; Sabin et al., 2015; Zukor et al., 2011). This is in stark contrast to the mammalian response to SCI where damaged astrocytes undergo reactive gliosis and contribute to the glial and fibrotic scar by secreting axon growth inhibitory proteins like chondroitin sulfate proteoglycans (CSPGs) and collagens (Adams and Gallo, 2018; Cregg et al., 2014; Rolls et al., 2009; Silver and Miller, 2004). In addition to salamanders, many different vertebrate animals have the ability to regenerate a functional spinal cord after injury, including lamprey, Xenopus and zebrafish. Common to all these animals is that regeneration occurs in the absence of reactive gliosis and glial scar formation (Diaz Quiroz et al., 2014; Hui et al., 2013; Sabin et al., 2015; Zukor et al., 2011). The molecular pathways that promote functional spinal cord regeneration in the absence of reactive gliosis and glial scar formation are poorly understood.

Recent advances in molecular genetics and transcriptional profiling techniques are beginning to elucidate the molecular and cellular responses necessary for functional spinal cord regeneration. Lampreys can fully regenerate locomotive function within 12 weeks of a full spinal cord transection. After SCI in lamprey, resident $GFAP^+$ astrocytes elongate and form a glial bridge that facilitates axons to regenerate through the lesion (Cohen et al., 1989; Davis and McClellan, 1994; Davis et al., 1993; Herman et al., 2018; Lurie and Selzer, 1991; Mackler et al., 1986; Rovainen, 1976; Selzer, 1978; Yin et al., 1984). This is reminiscent of the injury-induced glial bridge formed by $GFAP^+$ glial cells in zebrafish spinal cord, which is similarly necessary for axon regeneration

(Goldshmit Y et al., 2012; Hui SP et al., 2010). Xenopus display robust functional spinal cord regeneration in the larval stages by activating the $GFAP^{+}/Sox2^{+}$ glial cells to divide, migrate, and repair the lesion site, which allows axons to regenerate, however their ability to regenerate is lost after metamorphoses in the adult frog (Beattie et al., 1990; Gaete et al., 2012; Gibbs and Szaro, 2006; Lee-Liu et al., 2014; Lin et al., 2012; Munoz et al., 2015). A common theme in these species is the fulfillment of a pro-regenerative glial cell response and the absence of reactive gliosis and glial scar formation.

Previously, we identified a pro-regenerative role for c-Fos and the transcriptional complex AP-1 in glial cells during axolotl spinal cord regeneration (Sabin et al., 2015). AP-1 is commonly formed as a heterodimeric complex of FOS and JUN proteins (Chinenov and Kerppola, 2001; Chiu et al., 1988; Halazonetis et al., 1988) capable of regulating the expression of various genes involved with cell cycle, extracellular matrix remodeling, and cell migration (Chiu et al., 1988; Shaulian and Karin, 2001; Szabowski et al., 2000; Zenz et al., 2008). The identity of AP-1 target genes and the ability of AP-1 to transcriptionally activate or repress target genes is partially dependent on the combination of FOS and JUN proteins that comprise the AP-1 dimer (Hess et al., 2004; Piechaczyk and Farras, 2008; Rodriguez-Martinez et al., 2017; Szabowski et al., 2000). Interestingly, after CNS injury in mammals both c-Fos and c-Jun are up-regulated in reactive astrocytes and function to promote reactive gliosis and glial scar formation (Cao et al., 2015; Di Giovanni et al., 2003; Gadea et al., 2008; Gao et al., 2013). While c-Fos is significantly up-regulated in glial cells after SCI in axolotl, whether c-Jun is up-regulated similar to reactive mammalian astrocytes or a different JUN family member was unknown (Sabin et al., 2015).

Here, we identify that axolotl glial cells express a non-canonical AP-$1^{cFos/JunB}$ after SCI. Axolotl glial cells up-regulate miR-200a expression, which directly represses c-Jun expression during spinal cord regeneration, thereby blocking the formation of AP-1-$^{cFos/cJun}$. Chronic overexpression of AP-$1^{cFos/cJun}$ in axolotl glial cells leads to defects in axon regeneration, similar to mammals.

Specific inhibition of miR-200a in axolotl glial cells partially phenocopied AP-$1^{cFos/cJun}$ overexpression leading to defects in axon regeneration. Transcriptomic profiling of control or miR-200a inhibitor electroporated spinal cords identified differential expression of genes indicative of reactive gliosis, glial scar formation, and other genes known to be necessary for functional spinal cord regeneration. Our data support a role for the non-canonical AP-$1^{cFos/JunB}$ in promoting the pro-regenerative glial cell response necessary for spinal cord regeneration and miR-200a in blocking reactive gliosis and glial scar formation in the axolotl.

Results

Axolotl glial cells up-regulate expression of the non-canonical AP-1$^{cFos/JunB}$ after Injury

Previous research showed that the transcription factor c-Fos is up-regulated in GFAP^{+} glial cells in axolotl after SCI (Sabin et al., 2015). However, the identity of the c-Fos binding partner in axolotl glial cells was unknown (Sabin et al., 2015). To determine whether the canonical AP-1$^{cFos/cJun}$ is expressed in axolotl glial cells, we performed immunohistochemical staining for c-Jun at 1 day post injury in axolotl spinal cords. This analysis revealed that c-Jun is not expressed in GFAP^{+} glial cells but is expressed in NeuN^{+} neurons (**Fig. 3-1A**). Therefore, axolotl glial cells do not up-regulate the canonical AP-1$^{cFos/cJun}$ but instead must up-regulate a different JUN family member.

To discover putative c-Fos binding partners, we mined previously published transcriptional profiling data of axolotl spinal cord regeneration (Sabin et al., 2015). This approach identified JunB as a potential c-Fos binding partner due to its similar transcriptional dynamics after SCI. Subsequent qRT-PCR confirmed that *JunB* is up regulated after SCI, which mirrors the expression dynamics of *c-Fos* (**Fig. 3-1b**). In situ hybridization confirmed that *JunB* is expressed in glial cells, which are adjacent to the central canal, not neurons, in a more dorsolateral domain, adjacent to the lesion at 1 day post injury (**Fig. 3-1C**). Finally, to confirm that axolotl c-Fos and JunB interact biochemically, we utilized the biotin based BioID system (Roux et al., 2012). Axolotl JunB-BioID showed robust c-Fos pull down, confirming their specific biochemical interaction (**Fig. 3-2**). Taken together these results support our hypothesis that after SCI, axolotl glial cells express the non-canonical AP-1$^{cFos/JunB}$.

Differential activity of AP-1$^{cFos/JunB}$ compared to AP-1$^{cFos/cJun}$ at the GFAP promoter

After SCI in mammals, damaged mammalian astrocytes become reactive, up-regulate *GFAP* expression, and contribute to glial scar formation (Rolls et al., 2009; Silver and Miller, 2004). To determine whether axolotl glial cells undergo a similar process we used qRT-PCR to assay *GFAP* expression during axolotl spinal cord regeneration. We found that *GFAP* expression is significantly down regulated during axolotl spinal cord regeneration (**Fig. 3-3A**). This is consistent with previous reports during axolotl spinal cord regeneration after tail amputation (Rodrigo Albors et al., 2015). This result suggests that axolotl glial cells undergo a fundamentally different molecular response to injury than mammalian astrocytes. It is known that AP-1$^{cFos/cJun}$ directly regulates *GFAP* expression during reactive gliosis in mammals (Gao et al., 2013). Therefore, as a proxy for reactive gliosis, we wanted to test whether different compositions of the AP-1 complex could lead to differential GFAP promoter activity.

We subcloned the axolotl GFAP promoter upstream of a luciferase reporter and co-transfected neural cells with axolotl AP-1$^{cFos/cJun}$ or AP-1$^{cFos/JunB}$. Similar to mammals, the canonical AP-1$^{cFos/cJun}$ acts as a strong transcriptional activator of the GFAP promoter (**Fig. 3-3B**). Interestingly, when we transfected the same neural cell line with the axolotl AP-1$^{cFos/JunB}$ sequences this significantly repressed the GFAP promoter compared to the control cells (**Fig. 3-3B**). These results suggest that AP-1$^{cFos/JunB}$ could function to mitigate reactive gliosis in axolotl glial cells and help promote a pro-regenerative glial cell response.

Finally, we wanted to determine whether chronic over expression of AP-1$^{cFos/cJun}$ in axolotl glial cells, similar to what is observed in mammals after SCI, is sufficient to affect axolotl spinal cord regeneration. Electroporation was used to introduce GFP tagged versions of axolotl c-Fos and c-Jun overexpression constructs into the glial cells of the spinal cord (Echeverri and Tanaka, 2002, 2003b). After 24 hours, animals were screened for GFP^{+} glial cells and animals with >70% labeled glial cells were selected for SCI. The animals were harvested 7 days post injury, a time point at which axons have regenerated though the lesion (Diaz Quiroz et al., 2014; Sabin et al., 2015), and the extent of axon

regeneration was examined by whole mount immunohistochemistry for β-III tubulin. In control spinal cords the β-III tubulin^{+} axons have regenerated through the lesion (**Fig. 3-3C**), however when AP-1$^{cFos/cJun}$ was overexpressed in the glial cells this lead to disordered axon regeneration (**Fig. 3-3C**) indicative of an overall growth repulsive environment (Diaz Quiroz et al., 2014) that could be reminiscent of the mammalian glial scar.

miR-200a represses c-Jun expression in axolotl glial cells

Our previous work showed that c-Jun is expressed in NeuN^{+} neurons and in dorsal root ganglia close to the injury site, but not in glial cells, suggesting that c-Jun expression is repressed in glial cells (**Fig. 3-1A**) (Sabin et al., 2015). Work from our lab and many others have shown dynamic regulation of target gene expression by microRNAs (miRNA) is critical for functional tissue regeneration (Aguirre et al., 2014; Beauchemin et al., 2015; Diaz Quiroz et al., 2014; Hodgkinson et al., 2015; Sehm et al., 2009; Thatcher et al., 2008; Witman et al., 2013). Therefore, we wanted to test the hypothesis that axolotl glial cells repress c-Jun expression via a miRNA-mediated mechanism. Analysis of the c-Jun 3' untranslated region (UTR) using TargetScan identified a conserved miR-200 seed sequence across many species, including axolotl. While miR-200 family members are known to play a pivotal role during neurodevelopment (Trumbach and Prakash, 2015), little is known about their function during regeneration.

Time course analysis revealed that miR-200a is expressed at homeostatic levels in the uninjured spinal cord and is significantly up-regulated at 3 days post injury (**Fig. 3-4A**). In situ hybridization confirmed that miR-200a is specifically expressed in glial cells during axolotl spinal cord regeneration (**Fig. 3-4B**). To determine whether miR-200a function is necessary for functional spinal cord regeneration, we injected and electroporated spinal cords with a commercially available chemically synthesized anti-sense miR-200a inhibitor that knocked down miR-200a levels but did not affect a closely related family member, miR-200b (**Fig. 3-4C,D**). Whole mount β-III tubulin staining revealed that miR-200a

inhibition resulted in a complete failure of axons to regenerate through the lesion by 7 days post injury compared to controls (**Fig. 3-4E**). Furthermore, axons were observed sprouting from the spinal cord both rostral and caudal to the lesion, similar to regeneration defects observed with AP-1$^{cFos/cJun}$ over expression (**Fig. 3-3G**). Further histological analysis revealed that by 10 days post injury the two ends of the spinal cord failed to reconnect (**Fig. 3-5**).

To determine whether *c-Jun* transcript abundance is affected by miR-200a inhibition we used qRT-PCR at 3 days post injury, the time point miR-200a is most highly expressed (**Fig. 3-4A**), and found an increase in *c-Jun* transcript compared to control 3 day post injury and uninjured spinal cords (**Fig. 3-6A**). Immunohistochemical analysis revealed a small proportion of c-Jun$^+$/GFAP$^+$ cells in control spinal cords (**Fig. 3-6B,C**), which is different than what we observed at 1 day post injury. This finding suggests that at 3 days post injury a few glial cells do express c-Jun, and miR-200a inhibition leads to a drastic and significant increase in the percentage of GFAP$^+$/c-Jun$^+$ glial cells compared to controls (**Fig. 3-6B,C**). Luciferase reporter experiments confirmed that the axolotl c-Jun 3' UTR is directly targeted by miR-200a (**Fig. 3-6D**) and mutation of the miR-200a seed sequence alleviates that repression (**Fig. 3-6D**). Collectively, these experiments confirm that miR-200a represses c-Jun expression in GFAP$^+$ glial cells.

miR-200a inhibition causes dysregulation of genes involved with reactive gliosis and glial scar formation

MicroRNAs are able to regulate expression of dozens, if not hundreds, of genes in multiple pathways to precisely coordinate various cellular processes (Bartel, 2018). To get a more comprehensive picture of the role of miR-200a in regulating the pro-regenerative glial cell response during axolotl spinal cord regeneration, we performed RNA sequencing analysis. Uninjured and 4 days post injury spinal cords were electroporated with either a control or miR-200a specific inhibitor. We chose 4 days post injury because this is one day after peak

miR-200a up-regulation and would allow us to identify downstream signaling networks normally regulated by miR-200a during regeneration.

Analysis of the RNA sequencing data compared gene expression in control uninjured, 4 days post injury control, and 4 days post injury miR-200a inhibitor spinal cords. From these comparisons, genes that had greater than 2.0-fold and a significant p-value ($p \leq 0.05$) in one or both comparisons were included in further analysis. This approach identified a cohort of genes that were differentially regulated at 4 days post injury after miR-200a inhibition. Among the significantly differentially expressed genes, we identified 462 genes that were significantly up-regulated and 153 genes were significantly down regulated in the miR-200a inhibitor treated samples compared to control samples (**Fig. 3-7**). From research in mammals, a subset of these genes (**Fig. 3-8**) are known to be involved with reactive gliosis and glial scar formation (LGALS1, DCN, BCAN, TLR2, CSPG5) and have functions related to extracellular matrix (COL21A1, CNTN1, FN1) and ECM remodeling (ADAM23, LOXL1, HYAL4, MMP2, SERPINE1), as well as cell migration (ITGBL1, ITGAD, CD151 ITGB1BP1, ITGB3BP), axon migration (CHL1, CLSTN1, NEFM/H), and inflammation (TLR2, CCL3L3, TNFAIP2) (**Fig. 3-8A**) (Babcock et al., 2006; Flevaris and Vaughan, 2017; Li et al., 2015; Mabon et al., 2000; Renault-Mihara et al., 2017; Schafer and Frotscher, 2012; Sirko et al., 2015; Sofroniew, 2009; Wang et al., 2017). These data collectively suggests that miR-200a inhibition is sufficient to disrupt expression of genes involved with multiple cellular processes necessary for regeneration.

We used qRT-PCR analysis to confirm and analyze more carefully a subset of genes well documented to be involved with reactive gliosis and glial scar formation. This confirmed that miR-200a inhibition leads to a highly significant increase in vimentin expression and a small but significant increase in GFAP expression (**Fig. 3-8B**) compared to 4 days post injury control spinal cords. Remarkably, expression of the chondroitin sulfate proteoglycan, CSPG4 (NG2), which can be used to identify a subset of reactive glia in mammals (Fidler

et al., 1999; Levine, 1994; Sandvig et al., 2004), is specifically up-regulated after miR-200a inhibition (**Fig. 3-8B**). This data suggests that inhibition of miR-200a after injury leads to a more reactive phenotype and an overall more axon growth inhibitory microenvironment similar to what is observed in mammalian astrocytes after SCI.

Discussion

The present study has identified an important role for the non-canonical AP-1$^{cFos/JunB}$ and miR-200a in regulating the pro-regenerative glial cell response to SCI in axolotl (**Fig. 3-9**). Our previous work showed a pro-regenerative role for c-Fos in regulating the glial cell response to SCI (Sabin et al., 2015). Unlike JUN proteins, which can homodimerize to form AP-1 (Chinenov and Kerppola, 2001; Chiu et al., 1988; Halazonetis et al., 1988), c-Fos functions as an obligate FOS:JUN heterodimer. However the identify of the c-Fos binding partner in axolotl glial cells was unclear. Immunohistochemical analysis showed that c-Jun is expressed in NeuN^{+} neurons, not GFAP^{+} glial cells at 1 day post injury (**Fig. 3-1A**). Therefore, pro-regenerative glial cells do not express the canonical AP-1$^{cFos/cJun}$ during axolotl spinal cord regeneration. Interestingly, the JUN family member JunB was up-regulated and heterodimerizes with c-Fos in axolotl glial cells (**Fig. 3-1B,C, Fig. 3-2**). Studies from mammalian CNS injury identified that the canonical AP-1$^{cFos/cJun}$ is up-regulated and promotes reactive gliosis and glial scar formation (Cao et al., 2015; Gadea et al., 2008; Gao et al., 2013; Raivich and Behrens, 2006; Yi et al., 2016). Interestingly, mimicking the mammalian situation by ectopically overexpressing AP-1$^{cFos/cJun}$ in axolotl glial cells led to defects in axon regeneration, indicative of an overall growth inhibitory environment, which could be similar to the glial scar in mammals (**Fig. 3-3D**). While electroporation of the spinal cord with overexpression constructs primarily labels glial cells (Echeverri and Tanaka, 2002, 2003a; Rodrigo Albors et al., 2015; Sabin et al., 2015), we cannot rule out the possibility that a small number of local neurons were labeled which partially contributed to regenerative defects.

In other vertebrates that have the ability to regenerate a functional spinal cord, FOS and JUN family members are also differentially regulated after injury. In zebrafish, similar to the axolotl, c-Fos and JunB are up-regulated 1 day post injury (Hui et al., 2014). In addition, lamprey up-regulate c-Fos and JunD within 6 hours of injury while c-Jun is not up-regulated until 3 days post injury (Herman et

al., 2018). Whether JunD in expressed in glial cells in the lamprey spinal cord and functions similarly to JunB is not clear. Furthermore, JunB is necessary for zebrafish fin regeneration (Hasegawa et al., 2017; Ishida et al., 2010). Additionally, during heart regeneration in zebrafish homologues of c-Fos and JunB are up-regulated but c-Jun is not differentially expressed. Interestingly, medaka, which does not regenerate after heart injury, up-regulates a FOS family member but no JUN members (Lai et al., 2017). Furthermore, the starlet sea anemone, *Nematostella vectensis*, differentially expresses several FOS and JUN family members during early stages versus late stages during oral regeneration and after exposure to heave metals (Agron et al., 2017; Warner et al., 2018). While there is limited sequence homology between *Nematostella* Fos1, Fos2, Jun1-3, and vertebrate c-Fos, c-Jun and JunB, it will be interesting to determine whether there are functional similarities at the protein level (Agron et al., 2017). Collectively, these observations support a model where differential expression of FOS and JUN proteins could regulate the regenerative response to tissue injury. In the future, it will be interesting to look across species to identify how this complex has evolved in regeneration competent and incompetent organisms.

AP-1 is capable of regulating expression of many genes involved with cellular proliferation, migration, cell survival, apoptosis, extracellular matrix, and ECM remodeling (Zenz et al., 2008). Some of these processes are mutually exclusive (i.e. cell survival and apoptosis) and the ability of AP-1 to specifically regulate a subset of genes is highly context dependent (Eferl and Wagner, 2003; Rodriguez-Martinez et al., 2017; Zenz et al., 2008). One factor contributing to the ability of AP-1 to regulate target genes is the combination of FOS and JUN proteins that comprise the heterodimer (Hess et al., 2004; Piechaczyk and Farras, 2008; Rodriguez-Martinez et al., 2017; Szabowski et al., 2000). Additionally, the composition of AP-1 can partially affect its ability to act as a transcriptional activator or repressor. For example, AP-1$^{FosB/JunB}$ transcriptionally activates *FoxD5b* during Xenopus neural development while AP-1$^{cFos/cJun}$ acts as a transcriptional repressor at that locus (Yoon et al., 2013). Furthermore, JunB

can antagonize the ability of c-Jun to transform non-malignant cells (Schutte et al., 1989). Indeed, often times JunB and c-Jun function to directly antagonize one another (Liu et al., 2015; Szabowski et al., 2000). These observations are consistent with our findings that the canonical AP-1$^{cFos/cJun}$ is a potent transcriptional activator of the axolotl GFAP promoter while AP-1$^{cFos/JunB}$ acts as a transcriptional repressor (**Fig. 3-3B**). These experiments highlight the possibility that differential combination of AP-1 subunits could induce very different cellular responses to injury. Recent reports indicate that co-occupancy of AP-1 with key pioneering transcription factors (FoxK2 and glucocorticoid receptor) is necessary for efficient recruitment of p300 histone acetyltransferase and chromatin remodeling (Biddie et al., 2011; Ji et al., 2012). This highlights an intriguing possibility that AP-1$^{cFos/JunB}$ could aid in chromatin remodeling to create a more permissive environment to allow for later waves of gene expression that promote the pro-regenerative glial cell response.

Our data support a model where pro-regenerative glial cells up-regulate AP-1$^{cFos/JunB}$ which promotes the pro-regenerative glial cell response. However, the mechanism by which c-Jun expression is repressed in glial cells was not clear. Our lab and others have shown that microRNAs play a critical role in fine-tuning the genetic programs necessary for tissue regeneration (Aguirre et al., 2014; Beauchemin et al., 2015; Diaz Quiroz et al., 2014; Hodgkinson et al., 2015; Sehm et al., 2009; Thatcher et al., 2008; Witman et al., 2013). Therefore, we sought to determine whether glial cells repress c-Jun expression via a miRNA-mediated posttranscriptional mechanism, thus blocking formation of the canonical AP-1$^{cFos/cJun}$. Subsequent analysis confirmed that miR-200a is up-regulated in glial cells at 3 days post injury and miR-200a function is necessary for spinal cord regeneration (**Fig. 3-4**). In vivo and in vitro experiments confirmed that c-Jun is a direct target of miR-200a (**Fig. 3-6**). Unexpectedly, this analysis also revealed a small population of c-Jun^{+}/GFAP^{+} glial cells in control spinal cords (**Fig. 3-6B,C**). This is different than what we observed at 1 day post injury, where c-Jun is only expressed in NeuN^{+} neurons (**Fig. 3-1A**). We predict that up-

regulation of miR-200a in glial cells is necessary to reduce c-Jun levels to allow JunB to outcompete c-Jun for c-Fos binding. Additionally, glial cells can act as neural stem cells in the axolotl spinal cord, and perhaps this population of c-Jun^{+}/GFAP^{+} cells recently underwent or is about to undergo neuronal differentiation. This would be consistent with our observation at 1 day post injury where only neurons express c-Jun. While the functional significance of this minor population of glial cells is presently unclear, it is important to note that a majority of glial cells do not express c-Jun in control spinal cords. Furthermore, miR-200a inhibition leads to a significant increase in the proportion of c-Jun^{+}/GFAP^{+} glial cells compared to controls (**Fig. 3-6B,C**), and ectopic overexpression AP-1$^{cFos/cJun}$ in glial cells leads to defects in spinal cord regeneration (**Fig. 3-3C**). Collectively, these results support our hypothesis that miR-200a directly represses c-Jun expression in axolotl glial cells to promote the pro-regenerative response to spinal cord injury.

While miR-200a inhibition or overexpression of the canonical AP-1$^{cFos/cJun}$ leads to defects in spinal cord regeneration, miR-200a inhibition resulted in a more severe phenotype (**Fig. 3-3C**, **Fig. 3-4E**). The less severe phenotype could be due to the endogenous JunB competing with ectopic c-Jun for c-Fos heterodimerization. If the molar concentration of JunB is higher than c-Jun in a given cell then that cell will behave like a wild type AP-1$^{cFos/JunB}$ expressing glial cell, resulting in a less severe regeneration phenotype. Furthermore, there are likely other downstream targets of miR-200a that are essential for spinal cord regeneration. In the future, it will be important to identify additional miR-200a targets and determine their role in regulating spinal cord regeneration.

We have identified c-Jun a direct miR-200a target, however miRNA's are able to target dozens if not hundreds of genes to orchestrate multiple cellular pathways (Bartel, 2018). Therefore, we performed RNA sequencing of spinal cord tissue from uninjured spinal cords and 4 day post injury spinal cords electroporated with a control or miR-200a inhibitor. We isolated spinal cord tissue by microdissecting spinal cord 500μm rostral and caudal from the lesion. This

region corresponds to the area from which glial cells contribute to regeneration (Sabin et al., 2015). While this method does not specifically isolate the glial cells, it would allow us to identify gene expression changes that could contribute to the resulting growth inhibitory lesion microenvironment after miR-200a inhibition.

Inhibition of miR-200a led to the differential expression of hundreds of genes at 4 days post injury compared to control 4 days post injury spinal cords (**Fig. 3-7**). Further analysis identified a handful of genes known to be involved in reactive gliosis and glial scar formation, as wells as genes known to be important for spinal cord regeneration in fish and salamanders (**Fig. 3-8**) (Becker et al., 1998; Chen et al., 2016; O'Hara et al., 1992; Yu et al., 2011a; Zukor et al., 2011). Collectively, our work identifies several key signaling nodes responsible for the precise regulation of functional spinal cord regeneration in axolotl.

Figures

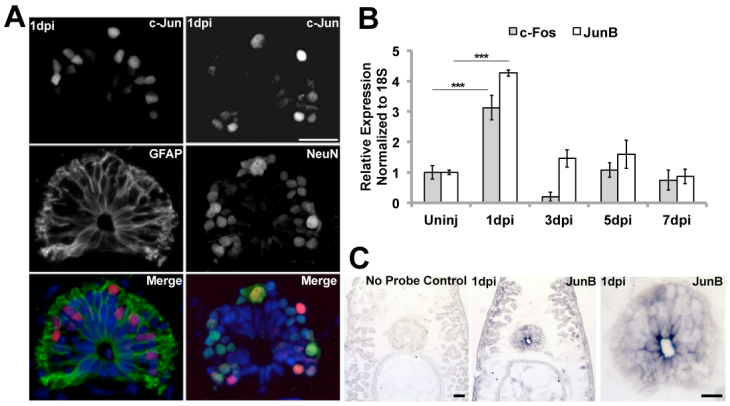

Figure 3-1: Axolotl glial cells express AP-1$^{cFos/JunB}$ after spinal cord injury. (A) c-Jun is only detected at the protein level at 1 day post injury spinal cords using immunohistochemistry in NeuN^{+} neurons but may be detected in other cells using more sensitive methods (n=5) Scale bars= 50μm. (B) qRT-PCR profiling confirms similar expression profile for c-Fos and JunB during axolotl spinal cord regeneration (n=3). (C) In situ hybridization confirms *JunB* expression in glial cells at 1 day post injury (n=5) Scale bars= 50μm. *** p≤0.001. Error bars represent ±S.T.D

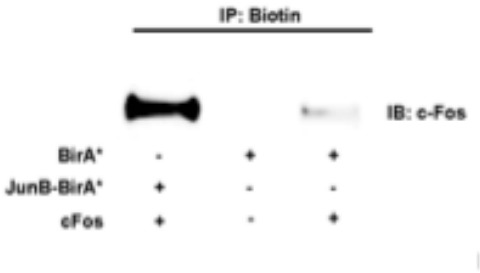

Figure 3-2: Axolotl c-Fos and JunB biochemically interact.

The axolotl JunB open reading frame was subcloned into the BioID vector. HEK293 cells were transfected with JunB-BioID+c-Fos, axolotl c-Fos, or empty BioID. Subsequent Western blot analysis shows there is only robust c-Fos pull down in the presence of JunB-BioID and not empty BioID, confirming specific biochemical interaction (n=2).

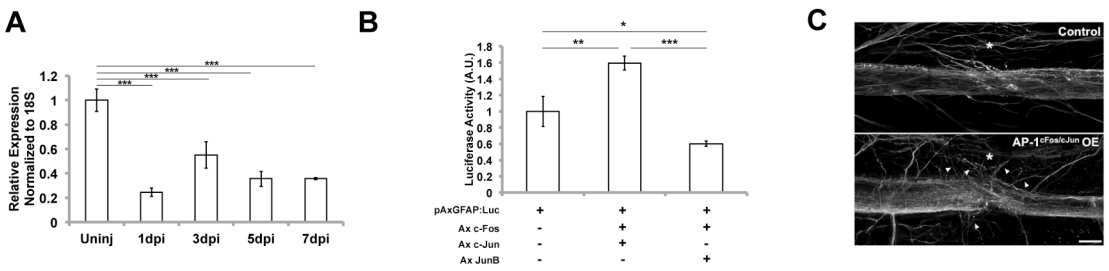

Figure 3-3: AP-1$^{cFos/JunB}$ differentially regulates *GFAP* expression.

(A) qRT-PCR data showing down regulation of *GFAP* throughout the time course of spinal cord regeneration (n=3). (B) Luciferase reporter experiments in the human B35 neuroblastoma cell line reveal different activation levels of the GFAP promoter depending on the AP-1 complex present, (n=3) (C) Whole mount antibody staining of β-III tubulin. Overexpression of axolotl AP-1$^{cFos/cJun}$ inhibits axon regeneration at 7 days post injury compared to control injured animals (control n=11, AP-1$^{cFos/cJun}$ OE n=13) . **p≤0.01, *** p≤0.001. Error bars represent ±S.T.D. In C * denotes injury and arrow heads depict disorganized axons. Scale Bar = 75μm

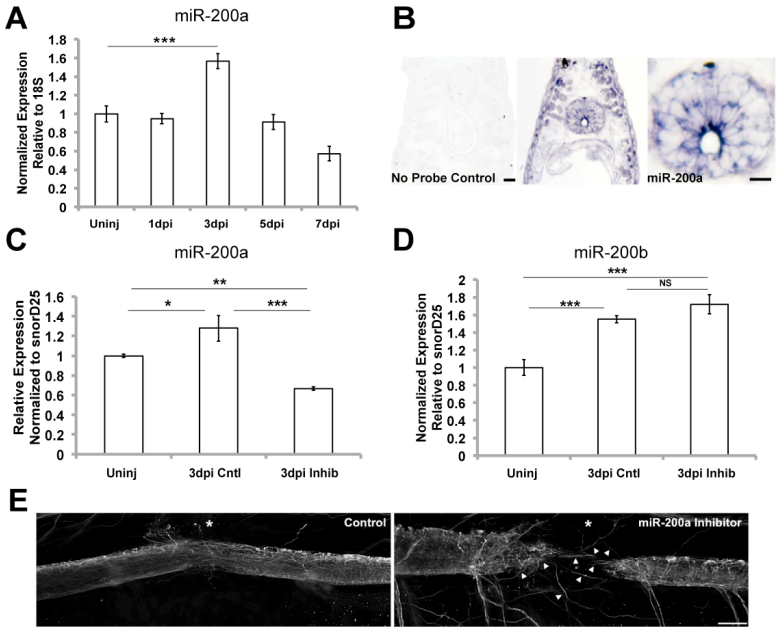

Figure 3-4: Glial cells up-regulate miR-200a after spinal cord injury and this is required for spinal cord regeneration.

(A) qRT-PCR analysis shows that miR-200a is up-regulated at 3 days post injury (n=3). (B) In situ hybridization confirms miR-200a expression specifically in glial cells at 3 days post injury in the regenerating spinal cord (n=6) Scale Bar = 50μm. (C) qRT-PCR analysis confirms knockdown of miR-200a after spinal cords were injected with a miR-200a inhibitor (n=3). (D) miR-200a inhibition does not affect miR-200b levels (n=3). (E) Whole mount β-III tubulin staining at 7days post injury reveals that miR-200a function in glial cells is necessary for proper axon regeneration compared to controls (controls n=33, miR-200a inhibitor n=35). In E * denotes injury and arrowheads depict disorganized axons. Scale Bar = 75μm

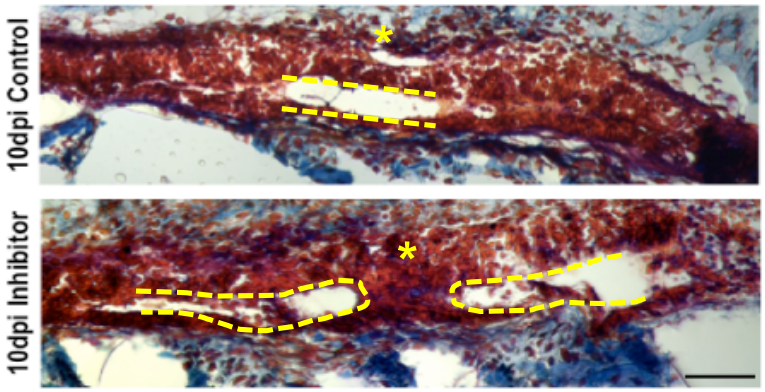

Figure 3-5: miR-200a inhibition results in a failure of spinal cord reconnection by 10 days post injury.

Control and miR-200a inhibitor treated tails were isolated at 10 days post injury and sectioned longitudinally with rostral to the right and caudal to the left. AFOG staining revealed a failure of spinal cord reconnection in miR-200a inhibitor electroporated tails compared to controls (n=8). Dashed lines represent the central canal and the (*) indicates the injury site. In control spinal cords there is a continuous central canal indicating the two ends of the spinal cord have reconnected. In miR-200a inhibitor electroporated spinal cords the two ends of the spinal cord have failed to reconnect.

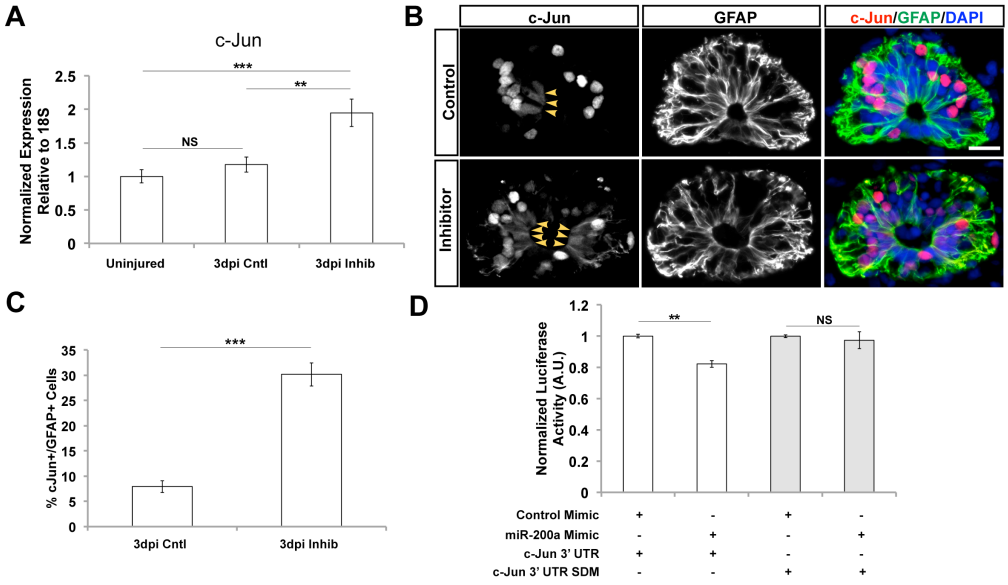

Figure 3-6: miR-200a directly represses c-Jun expression in GFAP$^+$ glial cells during spinal cord regeneration.

(A) qRT-PCR analysis confirms *c-Jun* is not normally differentially expressed at 3 days post spinal cord injury, but miR-200a inhibition leads to significant increase in *c-Jun* expression (n=3). (B) Antibody staining with GFAP and c-Jun. A small population of GFAP$^+$ glial cells express c-Jun in control conditions, but inhibition of miR-200a leads to a significant increase in the number of c-Jun$^+$/GFAP$^+$ glial cells (n=8). Scale Bar = 50μm. (C) Quantification of the percent of c-Jun$^+$/GFAP$^+$ glial cells after inhibition of miR-200a. (D) Co-transfection of a miR-200a mimic and a luciferase reporter containing the axolotl c-Jun 3' UTR confirms that miR-200a actively represses c-Jun, and mutation of the miR-200a seed sequence in the axolotl c-Jun 3' UTR alleviates that repression, (n=3). **p≤0.01, *** p≤0.001. Error bars represent ±S.T.D. In (B) * denotes Jun$^+$/GFAP$^+$ glial cell.

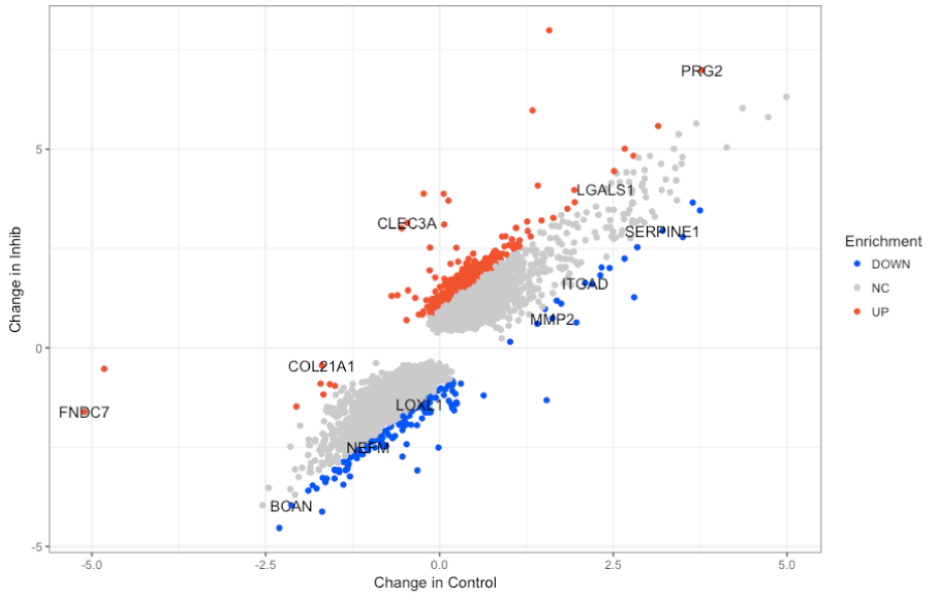

Figure 3-7: miR-200a inhibition leads to differential expression of a cohort of genes.

Scatter plot representing log2 fold changes in gene expression at 4 days post injury in control versus miR-200a inhibitor electroporated spinal cords relative to the uninjured animals. Genes with a mean count greater than 50 and a Benjamini-Hochberg adjusted p-value less than 0.1 in one or both experiments are shown. Genes with expression changes greater than 2-fold (log2 > 1 or log2 < -1) between experiments are colored red or blue for up and down regulated respectively. Labeled points represent a subset of genes involved with reactive gliosis, glial scar formation, ECM, ECM remodeling, and axon migration. Each condition was sequenced in biological triplicate (n=3).

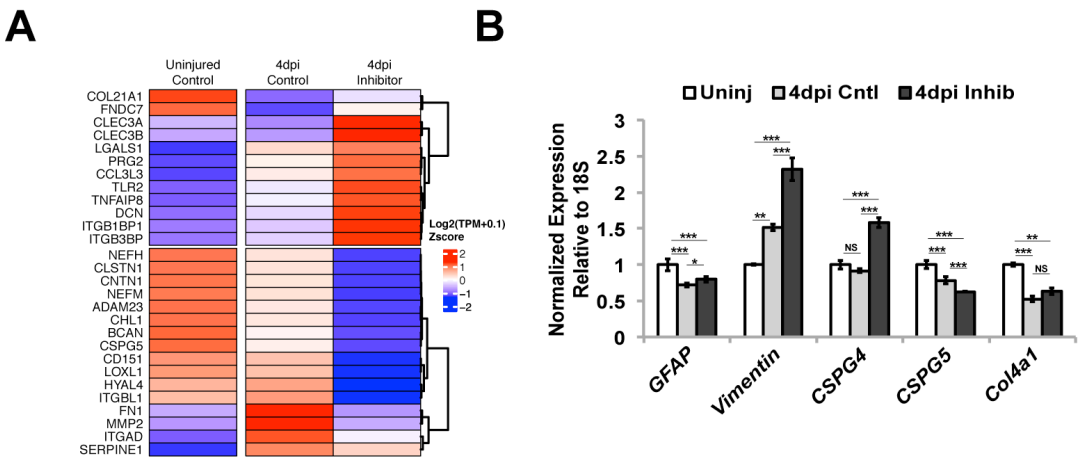

Figure 3-8: miR-200a inhibition leads to differential expression of genes involved with reactive gliosis and glial scar formation.

(A) Heatmap representation of expression values. Transcript per million expression values (TPMs) for uninjured, 4 days post injury Control and 4 days post injury miR-200a inhibitor electroporated samples were padded (+0.1) and log2 transformed. Mean values across replicates were centered and scaled prior to clustering. (B) qRT-PCR analysis confirms differential expression of reactive gliosis and glial scar related genes after miR-200a inhibition. *p≤0.05, **p≤0.01, *** p≤0.001. Error bars represent ±S.T.D

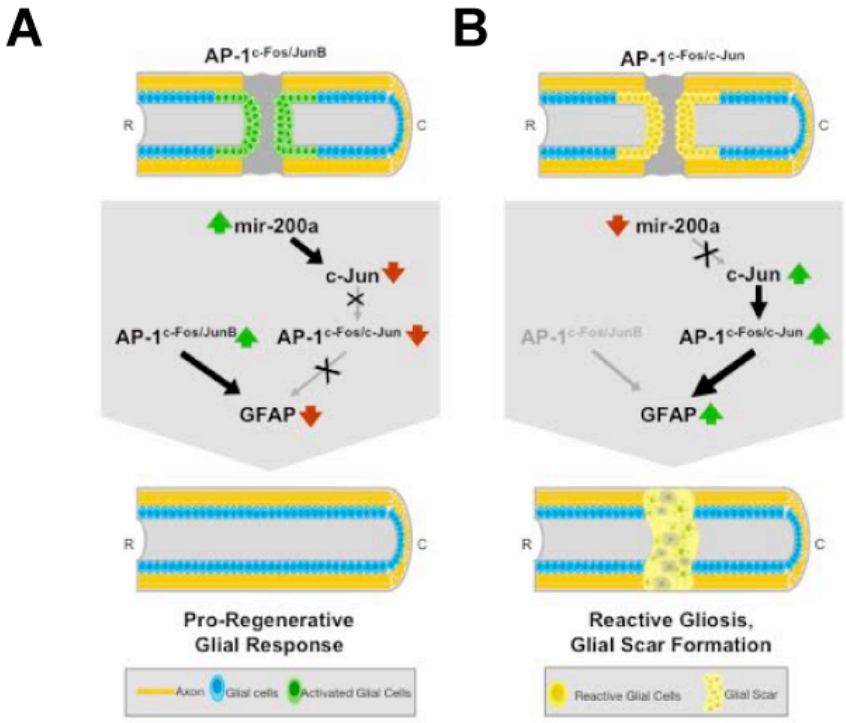

Figure 3-9: AP-1$^{cFos/JunB}$/miR-200a promotes the pro-regenerative glial cell response to spinal cord injury in axolotl.

(A) After SCI, glial cells up-regulate expression of AP-1$^{cFos/JunB}$, which partially functions to repress *GFAP* expression and direct the glial cells to a pro-regenerative response. Additionally, miR-200a is up-regulated which represses c-Jun expression thereby blocking formation of the canonical AP-1$^{cFos/cJun}$. (B) Inhibition of miR-200a during spinal cord regeneration results in higher levels of *GFAP* expression and a more reactive phenotype, expression of genes indicative of a glial scar like environment, and a failure in axon regeneration.

Chapter 4: miR-200a balances mesoderm versus neural identity during axolotl spinal cord regeneration

Introduction

Among vertebrates, the Mexican axolotl salamander has the remarkable ability to faithfully regenerate its spinal cord after injury. This process has been most commonly studied in the context of tail amputation (McHedlishvili et al., 2012; Monaghan et al., 2007; Piatt, 1955; Rodrigo Albors et al., 2015; Zhang et al., 2000; Zhang et al., 2002), but the axolotl spinal cord regenerates after a more targeted transection injury as well (Butler and Ward, 1965, 1967; Clarke et al., 1988; O'Hara et al., 1992; Zukor et al., 2011). These lines of investigation have identified a population of $Sox2^{+}/GFAP^{+}$ glial cell that functions as a *bona fide* neural progenitor cell (NPC) in the axolotl spinal cord (Echeverri and Tanaka, 2002; Fei et al., 2016; Fei et al., 2014; McHedlishvili et al., 2012; Rodrigo Albors et al., 2015). This cell population is interchangeably referred to as glial cells, neural progenitors, or neural stem cells but I will refer to them as glial cells in this text. These $Sox2^{+}/GFAP^{+}$ glial cells proliferate after injury and differentiate into new glia and neurons (Echeverri and Tanaka, 2002; McHedlishvili et al., 2012; Rodrigo Albors et al., 2015). Genetic knockout of Sox2 inhibits spinal cord regeneration after tail amputation, definitively showing the necessity of Sox2 expressing glial cells during regenerative spinal cord outgrowth (Fei et al., 2016; Fei et al., 2014). Other biochemical signaling pathways that regulate the glial cell response to injury are only now being elucidated.

Molecular signals required for the glial cell response to injury have been identified both after tail amputation and spinal cord transection. Sonic hedgehog, Wnt/PCP and FGF signaling are absolutely required for the pro-regenerative glial cell response to tail amputation (Rodrigo Albors et al., 2015; Schnapp et al., 2005; Zhang et al., 2000; Zhang et al., 2002). During spinal cord regeneration after transection, the transcriptional complex $AP\text{-}1^{cFos/JunB}$ and MAP kinase signaling are critical regulators of the glial cell response to injury (Sabin et al., 2015)(**Chapter 3**). Additionally, microRNA (miRNA) signaling is important to fine-tune the glial cell response to injury after both tail amputation and spinal cord

transection (Diaz Quiroz et al., 2014; Gearhart et al., 2015; Lepp and Carlone, 2014; Sehm et al., 2009).

We previously showed that miR-200a is an important regulator of the glial cell response after spinal cord transection (**Chapter 3**). The function of miR-200a has been most extensively studied during neurodevelopment and epithelial-to-mesenchymal transition (EMT) (Trumbach and Prakash, 2015; Zaravinos, 2015). miR-200a functions to inhibit EMT by directly repressing the expression of the transcription factors ZEB1 and β-catenin (Su et al., 2012; Zaravinos, 2015). This leads to maintained epithelial polarity and decreased Wnt signaling, respectively. During neurodevelopment, miR-200 family members regulate many processes: neuronal survival (Karres et al., 2007), neuroepithelial progenitor proliferation, identity and neuroblast transition (Morante et al., 2013), neural progenitor identity and cell cycle dynamics (Peng et al., 2012), as well as fine tunes signaling networks necessary for neurogenesis (Choi et al., 2008; Vallejo et al., 2011) and gliogenesis (Buller et al., 2012). However, the role of miR-200a in regulating neural progenitor identity and response to injury is not well understood.

Early experiments aimed at determining the potential of $GFAP^+/Sox2^+$ glial cells prospectively labeled these cells with the GFAP promoter driving GFP expression and used live *in vivo* fluorescence imaging to follow GFP^+ glial cells after tail amputation. Most GFP^+ glial cells gave rise to new neurons and glia but a small proportion of labeled glial cells left the spinal cord and contributed to muscle and cartilage within the regenerated tail (Echeverri and Tanaka, 2002). This is at odds with similar experiments that followed GFP^+ glial cells after spinal cord transection where GFP^+ cells appeared to only gave rise to neurons and new glia (Sabin et al., 2015). It is not clear why glial cells contribute to mesoderm-derived tissues during tail regeneration but not after spinal cord transection.

Within the last 15 years it has become clear that a population of progenitors, called neuromesodermal progenitors (NMPs), exist during embryonic development within the chondro-neural hinge and caudal lateral

epiblast of amniotes and the tail bud of anamniotes (Henrique et al., 2015; Kimelman, 2016). NMPs are competent to contribute to both mesoderm and spinal cord during embryonic development (Garriock et al., 2015; Henrique et al., 2015; Tzouanacou et al., 2009). Extensive genetic and biochemical analysis determined that NMPs can be defined by the co-expression of the transcription factors Brachyury and Sox2 (Gouti et al., 2017; Koch et al., 2017; Turner et al., 2014; Wymeersch et al., 2016). Additionally, the relative level of FGF and Wnt signaling activity regulate NMP cell fate decisions (i.e. differentiation into mesoderm progenitors or neural progenitors) (Bouldin et al., 2015; Garriock et al., 2015; Goto et al., 2017; Gouti et al., 2017; Gouti et al., 2015; Martin, 2016; Martin and Kimelman, 2008; Turner et al., 2014). Both FGF and Wnt signaling are important regulators of the glial cell response to tail amputation, but the activity of these pathways after spinal cord transection has not been well characterized.

The present study has identified a potential role for miR-200a in stabilizing the neural identity after spinal cord transection in axolotl. Transcriptional profiling revealed that miR-200a inhibition leads to up-regulation of Brachyury and a down regulation of neural genes. Subsequent analysis confirmed specific expression of *Brachyury* in glial cells adjacent to the central canal and down regulation of the neural progenitor cell marker *Sox2*. Brachyury is not a direct target of miR-200a however β-catenin, a known transcriptional co-activator up-stream of *Brachyury* expression, is a direct miR-200a target. Therefore, miR-200a could function to stabilize the neural identity by directly fine tuning β-catenin expression and Wnt signaling activity to prevent glial cells from dedifferentiating into NMP-like identity.

Results

Transcriptional profiling reveals unique and conserved targets in homeostatic compared to regenerating spinal cords

We previously performed RNA sequencing on uninjured and 4 days post injury spinal cord tissue electroporated with a control inhibitor or specific, antisense miR-200a inhibitor. Inhibition of miR-200a in the uninjured spinal cord resulted in 6,235 transcripts greater that 2-fold differentially expressed compared to control uninjured spinal cords. Interestingly, of the 6,235 differentially expressed genes, only 2,760 were up-regulated (**Fig. 4-1A**). This was a surprising result, as one would expect there to be more up-regulated transcripts after inhibiting a miRNA. We used GOrilla analysis to identify gene ontology (GO) terms for the subset of genes that were significantly up regulated after miR-200a inhibition. GO terms involved with translation, RNA metabolism, peptide metabolism, and translation initiation were significantly enriched in this gene set ($p \leq 10^{-24}$) (**Fig. 4-1B**). Interestingly, the 3,475 genes that were significantly down regulated in uninjured spinal cords after miR-200a inhibition were enriched for GO terms involved with organismal development, developmental process, cellular differentiation, and signaling ($p \leq 10^{-22}$) (**Fig. 4-1C**).

Examination of differentially expressed genes at 4 days post injury after miR-200a inhibition identified a total of 1,007 genes that were differentially expressed compared to control samples. This is a much smaller gene set and suggests that there is a higher specificity to genes affected by miR-200a during spinal cord regeneration. A total of 797 genes were up-regulated and 210 genes were down regulated after miR-200a inhibition (**Fig. 4-1D**). Genes that were up-regulated were enriched for GO terms involved with nucleic acid metabolism, specifically RNA metabolism, and protein localization ($p \leq 10^{-6}$) (**Fig. 4-1E**). Interestingly, the top GO terms enriched in down regulated genes were involved with nervous system processes, specifically synaptic signaling and chemical synaptic signaling, as well as nervous system development ($p \leq 10^{-6}$) (**Fig. 4-1F**).

This was interesting because miR-200a has previously been reported to regulate neural development, specifically neural progenitor cell identity and cell fate decisions (Trumbach and Prakash, 2015), and we previously showed that miR-200a is specifically expressed in the glial cell/neural progenitor population in the axolotl spinal cord. Therefore, we were keen to identify which neural related genes and pathways were down regulated during regeneration. Ingenuity Pipeline Analysis revealed the top affected network enriched in down regulated transcripts was Organismal Development, Nervous System Development and Function, Tissue Development. This network revealed genes involved with transcriptional regulation (*HIF-1α, Gsx1/2, NFIX, Six1*), extracellular matrix remodeling (*MMP7, MMP9, Loxl2*), integrin signaling (*ITGAM*), and cell migration (*Myh1, Myh3, Mylk2*) (**Fig. 4-1G**). Importantly, a cohort of transcription factors expressed in neural progenitors (*Nkx6.1*, *Olig1/2*, *Mnx1*, *Neurog1*) is represented within this signaling network. Collectively, these observations support a role for miR-200a in stabilizing or promoting the neural identity and neuronal differentiation (**Fig. 4-1G**).

Finally, we further characterized the unique and overlapping gene sets affected by miR-200a in uninjured compared to 4 days post injury spinal cords. Global comparisons of differentially expressed genes revealed a core of 902 overlapping genes that were similarly affected (757 up-regulated and 137 down regulated genes) by miR-200a independent of injury status (**Fig. 4-1H**). There was a total of 5,334 genes that were only differentially expressed in the uninjured spinal cord, and 106 genes that were only affected after injury (**Fig. 4-1H**).

Taken together, our RNA sequencing analysis identified a subset of genes that are affected by miR-200a in uninjured and regenerating spinal cords. We identified 902 genes that seem to represent a core gene network affected by miR-200a independent of injury status. However, we identified unique gene networks that were affected in an injury dependent manner. Further analysis of these gene sets could help shed light on the context specificity with which miRNA signaling could regulate regeneration and homeostasis.

Regeneration Specific targets of miR-200a could lead to decreased retinoic acid signaling

As discussed above, a total of 106 genes were only differentially expressed at 4 days post injury after miR-200a inhibition. These genes could represent a regeneration specific network regulated by miR-200a and, importantly, could represent pathways independent from its role during spinal cord homeostasis or development. Of the 106 differentially expressed genes, 35 genes were up-regulated, and 71 genes were down regulated (**Fig. 4-1H**). Analysis of GO terms enriched in the regeneration-specific gene set revealed that a subset of these genes is involved in retinol metabolism and retinoic acid signaling (**Fig. 4-1I**). Specifically, *Aldh1a2* (also known as *Raldh2*), an enzyme that catalyzes the conversion of retinaldehyde to biologically active retinoic acid, is down regulated (Molotkov et al., 2005; Sirbu et al., 2005). Additionally, *Cyp1a1*, a cytochrome P450 enzyme capable of oxidizing retinoic acid to a biologically inactive intermediate (Chen et al., 2000a; McSorley and Daly, 2000), is up-regulated. The *Cyp26* gene family is a group of cytochrome P450 genes that are more commonly associated with the inactivation of retinoic acid (Abu-Abed et al., 2001; Uehara et al., 2007; Yashiro et al., 2004). However, these genes are not differentially expressed after miR-200a inhibition. Collectively, these expression profiles suggest an environment with decreased retinoic acid signaling. It is well established that retinoic acid signaling plays an important and complex role in regulating neural progenitor cell identity and neuronal differentiation (Duester, 2008; Maden, 2007; Rhinn and Dolle, 2012). If miR-200a specifically affects retinoic acid signaling in the regenerating spinal cord after miR-200a inhibition, then this could be one reason genes involved with neural progenitor cells and neuronal function are down regulated compared to controls. This remains to be experimentally verified.

miR-200a inhibition leads dedifferentiation of neural progenitors to a neuromesodermal progenitor-like identity

Taking a more targeted gene-level approach, we generated a heat map of the 30 most significantly up-regulated and down regulated genes (**Fig. 4-2A**). Consistent with the GO analysis, genes involved in RNA processing, nucleic acid metabolism, and protein targeting were among the most up-regulated genes in our data set (*Tdrd9*, *Acap1*, *Eme1*, *Zfp324b*). Similarly, genes involved with neurotransmitter transport, neuronal polarization, neurotrophin signaling, and neuronal differentiation were among the most down regulated genes (*Slc6a6*, *Brsk1*, *Slc6a14*, *Arhgap8*, *Neurog1*). Surprisingly, the transcription factor Brachyury (*T*) was among one of the most up-regulated genes at 4 days post injury in response to miR-200a inhibition (**Fig. 4-2A**).

Quantitative RT-PCR confirmed that *Brachyury* is not expressed in uninjured or control 4 days post injury spinal cords but is expressed after miR-200a inhibition in 4 days post injury spinal cords (**Fig 4-2B**). This is an intriguing finding as Brachyury is normally only expressed in mesoderm during embryonic development and is not expressed in the nervous system. However, some spinal cord neural progenitor cells are developmentally derived from $Sox2^{+}$/$Brachyury^{+}$ neuromesodermal progenitors (NMPs) (Garriock et al., 2015; Tzouanacou et al., 2009; Wymeersch et al., 2016). Given that NMPs and axolotl glial cells express Sox2 and that Sox2 is a direct target of miR-200a during mouse brain development (Peng et al., 2012), we assayed *Sox2* transcript abundance. Interestingly, while *Sox2* is normally up-regulated in control 4 days post injury compared to uninjured spinal cords, miR-200a inhibition blocks *Sox2* up-regulation, maintaining transcript abundance near uninjured levels (**Fig. 4-2B**). This suggests that axolotl Sox2 is not a direct target of miR-200a like it is in mammals.

Finally, we wanted to identify the cells that express Brachyury in 4 days post injury spinal cord after miR-200a inhibition. *In situ* hybridization showed that glial cells adjacent to the central canal are $Brachyury^{+}$ after miR-200a inhibition

(**Fig. 4-2C**). This is the same population of cells that express Sox2 (Fei et al., 2016; Fei et al., 2014; Rodrigo Albors et al., 2015). *In situ* hybridization for *Sox2* confirms our qRT-PCR data that miR-200a inhibition leads to down regulation of *Sox2* transcript compared to control 4 days post injury spinal cords (**Fig. 4-2C**).

Collectively, our data suggests that miR-200a inhibition leads $Sox2^+$ glial cells in the axolotl spinal cord to express *Brachyury* and assume a neuromesodermal progenitor-like identity.

miR-200a inhibition creates a permissive environment for neuromesodermal progenitor-like cells

Inhibition of miR-200a leads to expression of *Brachyury* in glial cells in the axolotl spinal cord (**Fig. 4-2**). However, the signaling mechanism leading to *Brachyury* expression was still not clear. We first wanted to test whether miR-200a could directly repress *Brachyury* expression. The axolotl Brachyury 3' untranslated region (UTR) contains 2 miR-200a seed sequences, suggesting miR-200a could directly regulate *Brachyury* expression. However, when we subcloned the axolotl *Brachyury* 3' UTR into a luciferase reporter and co-transfected HEK293 cells with a control mimic or a miR-200a mimic, there was no difference in luciferase activity (**Fig. 4-3**). This suggests that miR-200a does not directly repress *Brachyury* expression in axolotl.

Neuromesodermal progenitor cell fate decisions are exquisitely regulated by the activity of certain cell signaling pathways. Wnt and FGF signaling have been most strongly associated with NMP cell fate decisions (Goto et al., 2017; Gouti et al., 2017; Gouti et al., 2015; Martin, 2016). We first tested whether FGF signaling could be affected by miR-200a inhibition. We assayed for expression of *FGF8* and *FGF10*, which are both associated with NMP development (Goto et al., 2017; Henrique et al., 2015; Kimelman, 2016; Turner et al., 2014). *FGF8* expression is slightly down regulated at 4 days post injury after miR-200a inhibition compared to uninjured spinal cords (**Fig. 4-4A**) and *FGF10* expression is only significantly up-regulated after miR-200a inhibition in 4 days post injury

spinal cords compared to controls (**Fig. 4-4A**). This suggests that miR-200a inhibition could lead to an increase in FGF signaling in regenerating spinal cords. However, given that Wnt signaling directly regulates Brachyury expression (Arnold et al., 2000; Yamaguchi et al., 1999) and NMP cell fate decisions (Bouldin et al., 2015; Garriock et al., 2015; Martin, 2016; Martin and Kimelman, 2008), we wanted to further examine the role of Wnt signaling.

The expression levels of various Wnt ligands involved in NMP development and embryonic axis elongation (*Wnt3a*, *Wnt5a*, *Wnt8a*) were largely unchanged in 4 days post injury spinal cords after miR-200a inhibition compared to controls (**Fig. 4-4B**). Therefore, it is not likely that these Wnt ligands are involved with inducing the NMP-like identity in glial cells after miR-200a inhibition. However, there are many Wnt ligands, therefore Wnt signaling activity could still be affected by miR-200a inhibition. To establish a base line for Wnt signaling activity after spinal cord injury we assayed *Lef1* expression, which is a direct transcriptional target downstream of Wnt signaling (Filali et al., 2002). *Lef1* expression was significantly up-regulated in control 4 days post injury compared to uninjured spinal cords, indicating a potential increase in Wnt signaling activity after injury. Remarkably, *Lef1* expression was significantly up-regulated even further after miR-200a inhibition in 4 days post injury compared to control regenerating spinal cords (**Fig. 4-5A**). Collectively, these data suggest that miR-200a inhibition could result in increased Wnt signaling, potentially independent of changes in Wnt ligand expression.

miR-200a modulates Wnt signaling activity by directly targeting β-catenin

While miR-200a inhibition could lead to increased Wnt signaling, it was not clear how this was occurring. During tumor progression, miR-200a inhibits the epithelial-to -mesenchymal transition subsequently blocking tumor cell metastasis (Su et al., 2012; Zaravinos, 2015). This is partially achieved through the direct repression of β-catenin by miR-200a, resulting in decreased Wnt signaling (Su et al., 2012). We did not observe a significant up-regulation of

specific Wnt ligand expression after miR-200a inhibition (**Fig. 4-4B**). However, as determined by *Lef1* expression, miR-200a inhibition could leads to increased Wnt signaling (**Fig. 4-5A**). Therefore, we hypothesized that miR-200a might regulate Wnt signaling by targeting β-catenin. To test our hypothesis, we first assayed for changes in β-catenin transcript abundance (*Ctnnb1*). qRT-PCR analysis confirmed that after injury in control 4 days post injury spinal cords, there is an increase in *Ctnnb1* abundance compared to uninjured spinal cords, similar to what we observed for *Lef1*. There is a slight increase of *Ctnnb1* transcript levels after miR-200a inhibition compared to control 4 days post injury spinal cords (**Fig. 4-5A**), suggesting β-catenin could be a direct target of miR-200a in axolotl.

To determine whether miR-200a could target axolotl β-catenin, we cloned the *Ctnnb1* 3' UTR and identified 2 miR-200a seed sequences. We subcloned the *Ctnnb1* 3' UTR into a luciferase reporter and co-transfected cells with a control mimic or miR-200a specific mimic. There was decreased luciferase activity in miR-200a mimic transfected cells compared to control, suggesting that miR-200a could regulate β-catenin expression (**Fig. 4-5B**). To confirm that the decrease in luciferase activity is due to direct regulation by miR-200a, we mutated both seed sequences in the *Ctnnb1* 3' UTR and repeated the luciferase experiments. Mutation of the miR-200a seed sequences completely alleviated the repression, confirming that axolotl β-catenin is a direct target of miR-200a, similar to mammals (**Fig. 4-5B**).

Taken together, these data suggest that miR-200a could modulate Wnt signaling through the direct regulation of β-catenin transcript levels. Inhibition of miR-200a leads to increased *Lef1* expression, which is indicative of increased Wnt signaling. Increased levels of Wnt signaling may contribute to induction of *Brachyury* expression and an NMP-like phenotype in axolotl glial cells. Collectively, these experiments have shed light on the context dependent nature of miRNA signaling in uninjured and regenerating spinal cords, and identified new signaling pathways that regulate glial cell identity during axolotl spinal cord regeneration.

Discussion

The current study has identified miR-200a as a putative regulator of glial cell identity during spinal cord regeneration in the axolotl. GO term analysis of genes down regulated in the uninjured and 4 days post injury spinal cord after miR-200a inhibition showed that these genes were involved with nervous system development, organismal development, synaptic signaling and cellular differentiation (**Fig. 4-1C,F,G Fig. 4-2**). Specifically, genes involved with neuronal differentiation (*Neurog1, NeuroD4*) and neuronal processes like synaptic transmission (*CHRNB1*, *GABRA4*) and neurotransmitter uptake (*SLC6A6*, *SLC18A3, SLC6A14*) were down regulated (**Fig. 4-1C,F,G Fig. 4-2A**). This suggests that miR-200a normally functions to promote NPC identity and neuronal differentiation, perhaps via repression of currently unidentified inhibitor(s) of NPC function. This is consistent with multiple reports across various species that inhibition of miR-200a and other miR-200 family members results in the loss of neural progenitor identity and precocious neuronal or glial differentiation (Buller et al., 2012; Choi et al., 2008; Morante et al., 2013; Peng et al., 2012; Trumbach and Prakash, 2015; Vallejo et al., 2011).

In the context of regeneration, the apparent loss of NPC identity and neuronal differentiation could be due to decreased retinoic acid signaling. Our RNA sequencing analysis identified a regeneration specific gene network affected by miR-200a signaling (**Fig. 4-1H**). A subset of those 106 regeneration specific genes are involved with regulating retinoic acid synthesis and degradation (*Aldh1a2*, *Cyp1a1*) and would suggest miR-200a inhibition might result in decreased retinoic acid signaling (**Fig.4-1I**). Defects in retinoic acid signaling affect neural development and regeneration, specifically affecting neural progenitor cells and neurogenesis (Duester, 2008; Maden, 2007; Rhinn and Dolle, 2012). Retinoic acid signaling is active in the developing axolotl spinal cord, in the regenerating spinal cord after tail amputation (Monaghan and Maden, 2012; Nguyen et al., 2017), and is necessary for spinal cord regeneration after

tail amputation in newts (Carter et al., 2011; Lepp and Carlone, 2014). Additionally, increased retinoic acid signaling causes commitment of neuromesodermal progenitors to the neural lineage (Gouti et al., 2017; Kimelman, 2016). Decreases in retinoic acid signaling after miR-200a inhibition could help create a permissive environment for Brachyury expression and acquisition of an NMP-like identity, which is discussed in more detail below. However, it will be important to functionally validate the role of retinoic acid signaling in regulating the glial cell response to spinal cord transection in axolotl and the putative regulatory role miR-200a plays in that process.

If the NPC identity is lost after miR-200a inhibition, then one hypothesis could be that they are reverting or dedifferentiating into a more developmentally primitive progenitor type. The anterior nervous system (brain and anterior spinal cord) derives almost exclusively from neuroectoderm, but the posterior spinal cord is comprised of a mixture of committed neural progenitors and neural progenitors derived from $Sox2^{+}$/$Brachyury^{+}$ NMPs (Garriock et al., 2015; Henrique et al., 2015; Tzouanacou et al., 2009; Wymeersch et al., 2016). Remarkably, miR-200a inhibition in 4 days post injury spinal cords leads to up-regulation of *Brachyury* and blocks *Sox2* up-regulation in glial cells adjacent to the central canal (**Fig. 4-2C**). Without performing double in situ hybridization, we cannot definitively claim that this cell population co-expresses both *Sox2* and *Brachyury*. However, it is very probable that this is the case given previous publications on the location and staining patters of $Sox2^{+}$ cells in the axolotl spinal cord (Fei et al., 2016; Fei et al., 2014; Rodrigo Albors et al., 2015) and the similarity in staining pattern of each single in situ (**Fig. 4-2C**). It will be important in the future to clarify this distinction.

Neuromesodermal progenitor cell commitment to the neural lineage is partially determined by the relative levels of Sox2 compared to Brachyury, given that the two transcription factors function to antagonize one another (Koch et al., 2017). These reports are consistent with our observation that miR-200a inhibition leads to *Brachyury* expression and blocks of *Sox2* up-regulation in glial cells after

injury (**Fig. 4-2B,C**). It is still not clear whether Brachyury directly affects Sox2 levels in the regenerating axolotl spinal cord or whether it is via an indirect mechanism. Collectively, these results support our hypothesis that miR-200a inhibition leads to reversion or dedifferentiation of glial cells to a more developmentally primitive NMP-like identity. However, it will need to be determined whether these cells dedifferentiate into true NMPs, leave the spinal cord, and become competent to contribute to mesoderm-derived tissues during regeneration.

Our results support a model where *Brachyury* is not directly regulated by miR-200a, but instead miR-200a inhibition leads to a more permissive signaling environment for *Brachyury* expression and acquisition of an NMP-like phenotype (**Fig. 4-3, 4-4, 4-5**). Specifically, Wnt and potentially FGF signaling appear to be increased after miR-200a inhibition (**Fig. 4-4**). Canonical Wnt signaling is crucial for radial glial cell proliferation during neural tube development (Shtutman et al., 1999) and spinal cord regeneration in zebrafish (Briona et al., 2015). Therefore, it is not surprising to see a potential increase in Wnt signaling activity after spinal cord injury. However, it is interesting that miR-200a does not affect expression of Wnt ligands, but instead regulates β-catenin levels (**Fig. 4-5**). This is reminiscent to the role of miR-200a in inhibiting EMT by repressing β-catenin and canonical Wnt signaling (Su et al., 2012; Zaravinos, 2015). The increase in *Ctnnb1* levels after miR-200a inhibition is not statistically significant, however it is known that slight changes in transcript abundance can have profound affects on protein levels (Schwanhausser et al., 2011). Therefore, a modest increase in transcript abundance could represent a biologically significant increase in β-catenin protein levels. It will be important in the future to verify Wnt signaling activity and β-catenin protein levels after miR-200a inhibition.

Glial cells with NMP-like characteristics have already been reported during tail regeneration (Echeverri and Tanaka, 2002). However, there is not a functionally analogous glial cell population after spinal cord transection (Sabin et al., 2015). One reason glial cells in the tail have this capability might be because

tail regeneration requires the coordinated activity of several progenitor lineages to accurately reconstruct a complex tissue comprised of muscle, blood vessels, cartilage, skin, and nerves. Therefore, it could be beneficial during tail regeneration to have a bipotent cell population capable of contributing to multiple tissue types. Indeed, some of the spinal cord and mesoderm-derived tissues in the tail would have been developmentally derived from NMPs. It could be that those cells retained a developmental memory of where they came from so that after injury they reassume the less differentiated state to faithfully contribute to tail regeneration. However, after a more targeted spinal cord transection there is not extensive damage to mesoderm-derived tissues and therefore no need for a bipotent progenitor. Indeed, the presence of such a bipotent progenitor could be deleterious to functional spinal cord regeneration, as that could result in the depletion of the neural progenitor pool and subsequent imprecise reconstitution of local neural cell types. In the future it will be interesting to tease apart the cellular and molecular mechanisms regulating the distinction between glial cell responses after spinal cord transection compared to tail amputation.

Figures

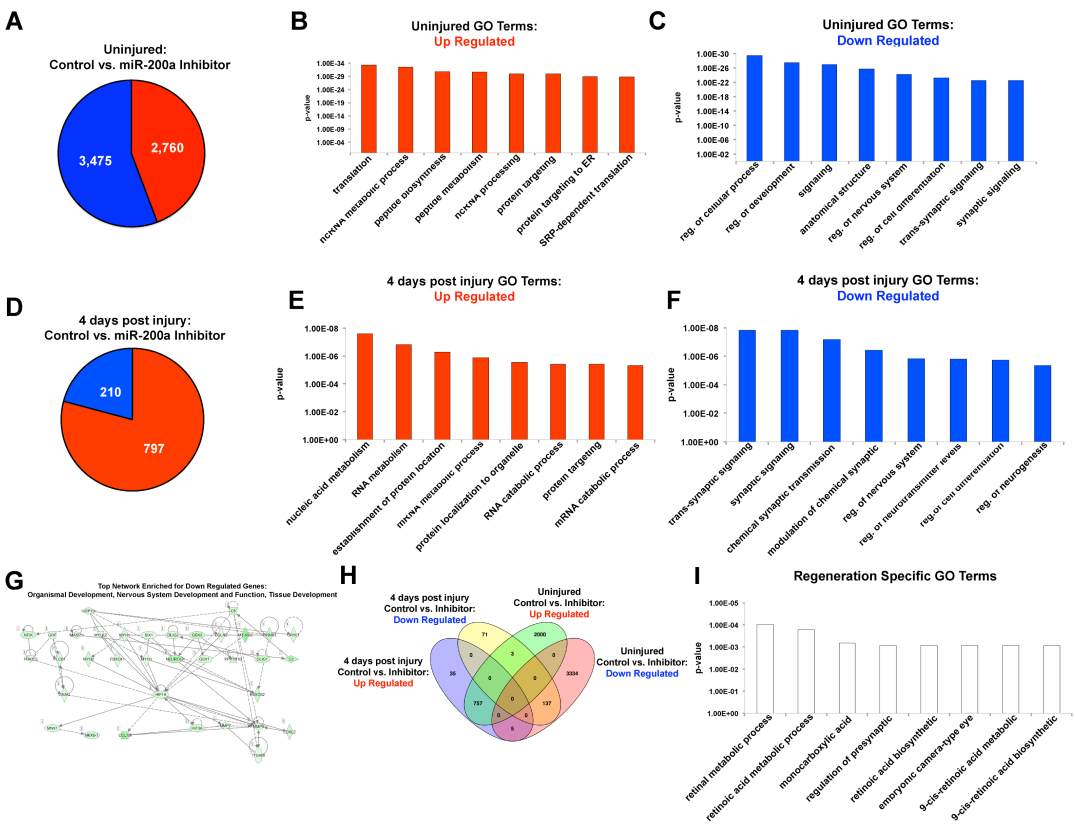

Figure 4-1: miR-200a affects expression of common and unique gene sets in the uninjured and regenerating spinal cord.

(A,D) Pie chart representation of the proportion of genes up-regulated (Red) or down regulated (Blue) in (A) uninjured control compared to uninjured miR-200a inhibitor electroporated spinal cords or (D) 4 days post injury control compared to 4 days post injury miR-200a inhibitor electroporated spinal cords. (B,E) Gene Ontology terms enriched in gene sets up-regulated in (B) uninjured or (E) 4 days post injury spinal cords after miR-200a inhibition. (C, F) Gene Ontology terms enriched in genes sets down regulated in (C) uninjured or (F) 4 days post injury spinal cords. (G) Ingenuity Pipeline Analysis of the top network represented by down regulated transcripts at 4 days post injury after miR-200a inhibition. (H) Pie Chart Representation of the unique and specific genes differentially up- or down-regulated in uninjured compared to 4 days post injury spinal cords. (I) Gene

Ontology terms enriched in the set of genes that are only differentially expressed after miR-200a inhibition at 4 days post-injury and not in uninjured spinal cords.

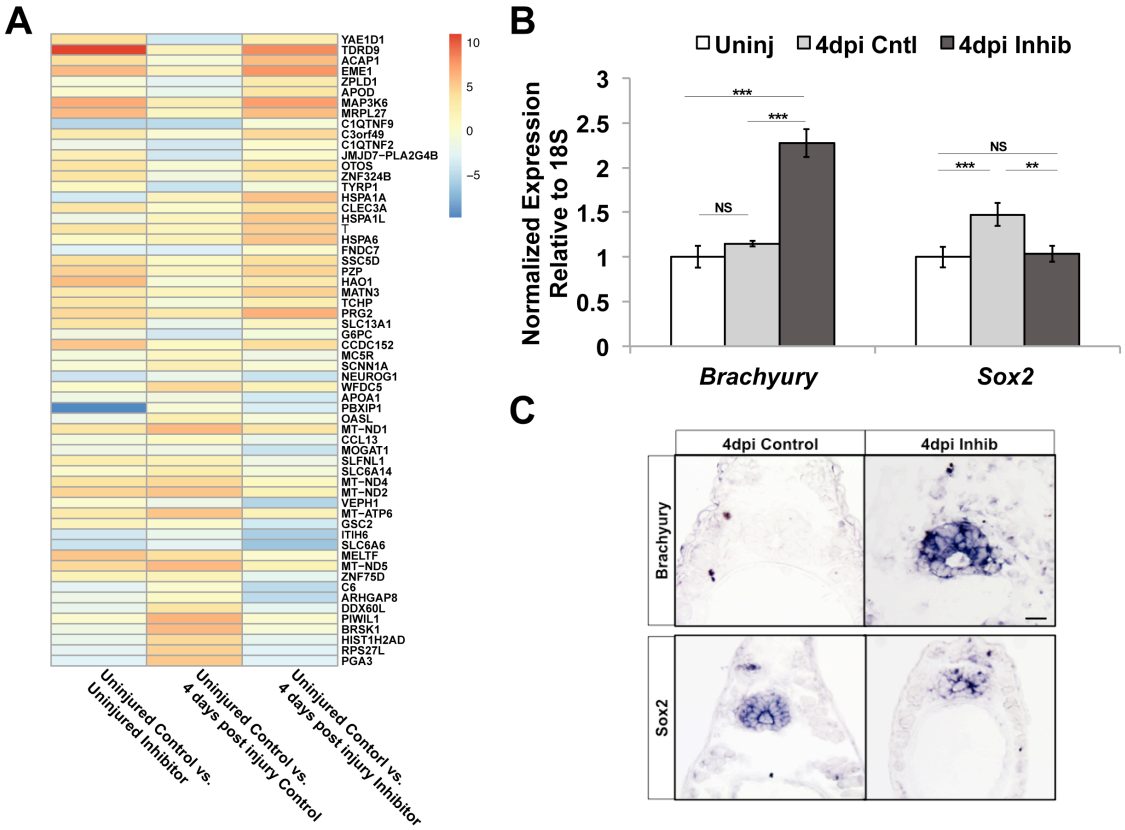

Figure 4-2: miR-200a inhibition leads to Brachyury expression in glial cells. (A) A heat map representation of the 30 most up-regulated and 30 most down-regulated genes after miR-200a inhibition in uninjured and 4 days post injury spinal cords compared to indicated controls (Bottom of heat map). (B) qRT-PCR analysis of the NMP markers Brachyury and Sox2 confirmed that *Brachyury* expression is only up-regulated at 4 days post injury after miR-200a inhibition and *Sox2* expression remains at homeostatic levels after miR-200a inhibition; (n=5). (C) In situ analysis of *Brachyury* (n=5) and *Sox2* (n=3) transcripts corroborates the RNA-seq and qRT-PCR data and reveals the transcripts are specifically expressed in the glial cells adjacent to the injury site. Scale bar = 50μm. Error bars represent STD. ** p≤0.01, ***p≤0.001

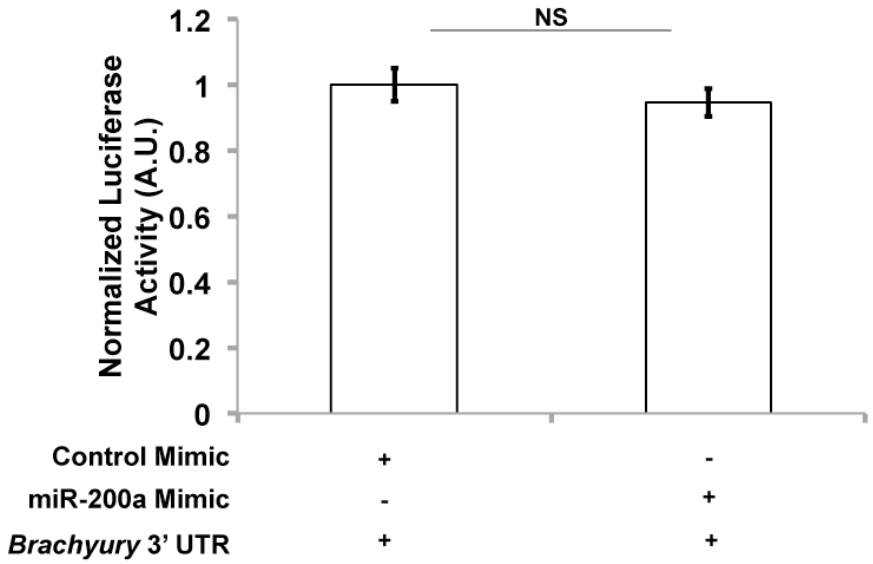

Figure 4-3: Brachyury expression is not regulated by miR-200a.

The axolotl Brachyury 3' untranslated region was cloned from RNA isolated from embryos at various developmental stages and subcloned into a 3' UTR luciferase reporter. HEK293 cells were transfected with the luciferase reporter and either a control mimic or miR-200a mimic. After 48 hours luciferase activity was measured. There was no difference between the miR-200a mimic transfected cells compared to the controls. For each replicate 3 wells were transfected per condition and pooled prior to luciferase assay. Experiments were conducted for a total of 2 replicates (n=2). Error bars represent S.T.D.

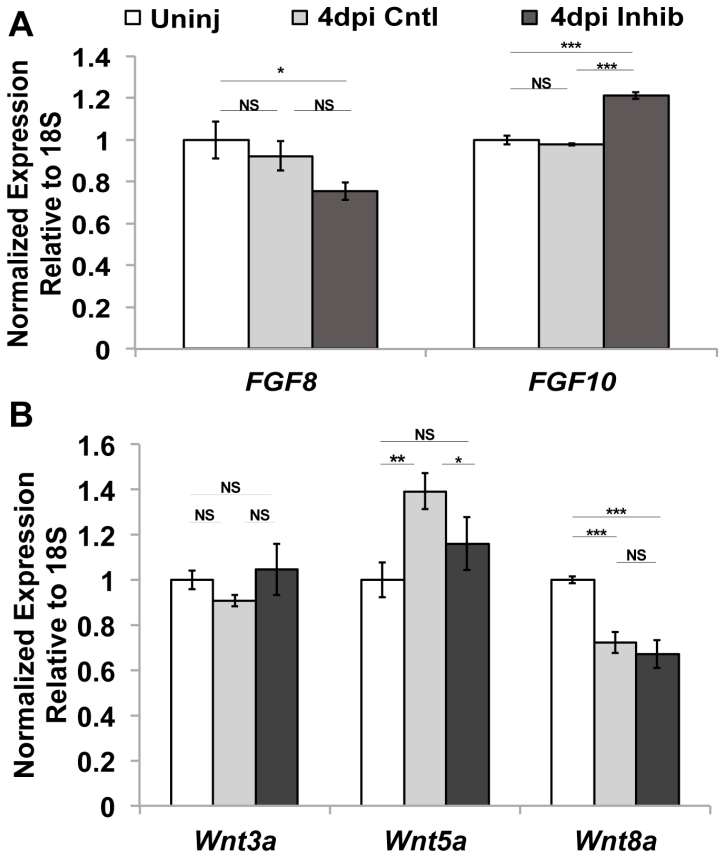

Figure 4-4: miR-200a does not have a robust affect on expression of FGF or Wnt ligands.

(A) qRT-PCR analysis of two FGF ligands associated with Brachyury expression and NMP induction during development reveal only a slight increase in *FGF10* levels (n=3). (B) qRT-PCR analysis of three Wnt ligands previously associated with *Brachyury* expression, NMP induction, and axis elongation during development reveal that miR-200a inhibition does not lead to increased expression at 4 days post injury. RNA from 7-10 spinal cords were pooled per condition per replicate for a total of 4 replicates (n=4). Error bars represent STD. * p≤0.05 ** p≤0.01, ***p≤0.001.

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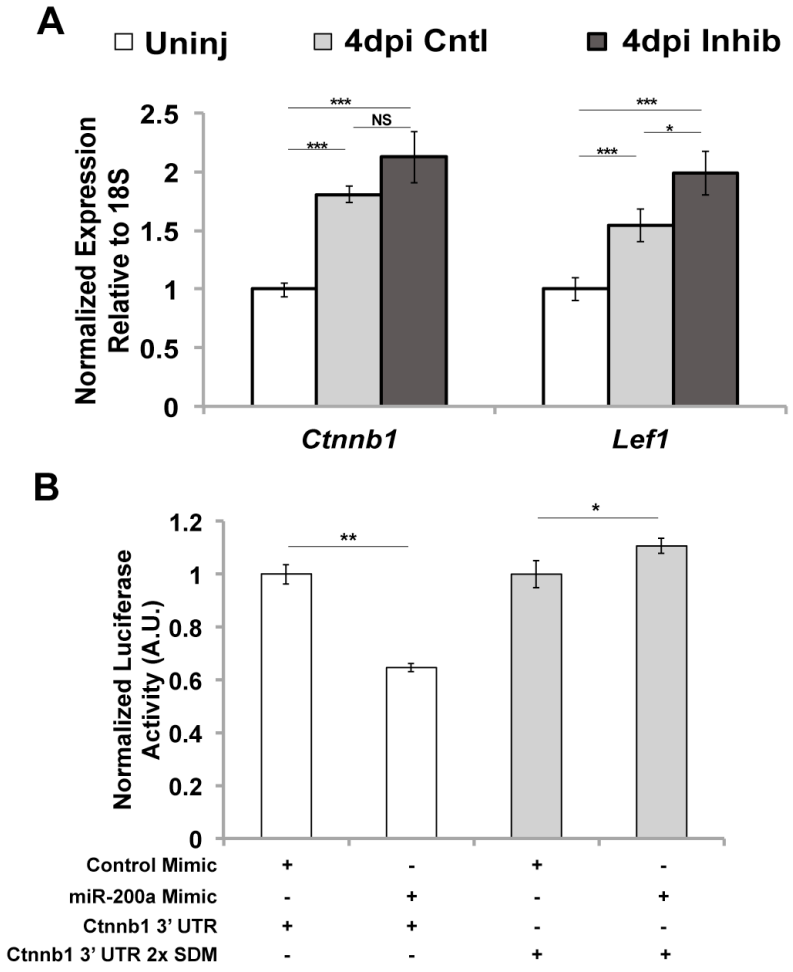

Figure 4-5: miR-200a regulates Wnt signaling activity by directly modulating β-catenin mRNA levels.

(A) qRT-PCR analysis of *Lef1* and *Ctnnb1* (β-catenin) transcript abundance revealed increased levels of expression in control 4 days post injury compared to uninjured spinal cords. *Lef1* expression was further increased at 4 days post injury after miR-200a inhibition compared to control 4 days post injury and control spinal cords. *Ctnnb1* were elevated at 4 days post injury after miR-200a inhibition compared to control 4 days post injury and uninjured spinal cords. (B) The axolotl β-catenin 3' untranslated region was cloned from RNA isolated from embryos at various developmental stages and subcloned into a 3' UTR luciferase reporter. HEK293 cells were transfected with the luciferase reporter and either a control

mimic or miR-200a mimic. After 48 hours luciferase activity was measured. miR-200a mimic transfected cells had significantly less normalized luciferase activity compared to controls. Mutation of both miR-200a seed sequences in the *Ctnnb1* 3' UTR alleviated that repression. For qRT-PCR, RNA from 7-10 spinal cords were pooled per condition per replicate for a total of 3 replicates (n=3). For luciferase experiments, 3 wells were transfected per condition for each replicate and pooled prior to luciferase assay. Experiments were conducted for a total of 3 replicates (n=3). Error bars represent STD. * $p \leq 0.05$ ** $p \leq 0.01$, *** $p \leq 0.001$.

Chapter 5: Discussion of Findings and Project Summary

Dissertation Discussion

Salamanders are unique among tetrapod vertebrates in that they are able to completely regenerate central nervous system tissue (CNS) after injury throughout their lives (Diaz Quiroz and Echeverri, 2013; Tanaka and Ferretti, 2009; Tazaki et al., 2017). Another common tetrapod vertebrate used to study CNS regeneration is frogs. However, frogs are only able to regenerate as tadpoles and lose this ability following metamorphosis (Beattie et al., 1990; Gibbs et al., 2011). Additionally, across regeneration-competent frog species there appears to be differences in the ability of some species of tadpoles to regenerate specific neuroanatomical regions (Clarke et al., 1986a; Forehand and Farel, 1982). Collectively, these reports highlight the axolotl as the premier model to study spinal cord regeneration in tetrapod vertebrates.

Early reports provided extensive characterization of the cellular and tissue level events after spinal cord transection (Butler and Ward, 1965, 1967; Clarke et al., 1988; Piatt, 1955) or tail amputation (Chernoff, 1996; Egar and Singer, 1972b; Nordlander and Singer, 1978; Singer et al., 1979). After spinal cord transection or tail amputation, glial cells respond to injury by increasing proliferation, remodeling the local tissue, driving regenerative outgrowth, and differentiation into new glia and neurons (Butler and Ward, 1965, 1967; Diaz Quiroz et al., 2014; Egar and Singer, 1972b; Fei et al., 2016; Fei et al., 2014; McHedlishvili et al., 2007; Nordlander and Singer, 1978; O'Hara et al., 1992; Piatt, 1955; Sabin et al., 2015; Sehm et al., 2009). Glial cells in the salamander spinal cord are molecularly defined by their expression of the intermediate filament protein glial fibrillary acidic protein (GFAP) and the neural stem cell marker Sox2 (Echeverri and Tanaka, 2002; Fei et al., 2016; Fei et al., 2014; Holder et al., 1990; McHedlishvili et al., 2012; O'Hara et al., 1992; Rodrigo Albors et al., 2015). Morphologically, the glial cell body lines the central canal and projects a process to the pial surface of the spinal cord. Horseradish peroxidase based labeling schemes identified morphological heterogeneity among glial cells

in the axolotl spinal cord (Holder et al., 1990; Zamora, 1978). However, within all morphological subgroups, each cell maintained a contact with both the central canal and pial surface (Holder et al., 1990; Zamora, 1978). Whether differences in morphology relate to functional diversification of subpopulations of glial cells remains unclear. Other regeneration competent organisms (fish and tadpoles) harbor spinal cord glial cells with similar molecular and morphological characteristics (Becker and Becker, 2015).

After spinal cord transection, glial cells seal the injured spinal cord, creating ependymal bulb-like structures on the rostral and caudal stump (Butler and Ward, 1965, 1967; Piatt, 1955; Sabin et al., 2015; Zukor et al., 2011). Over the next days to weeks the glial cells in the two ends of the severed spinal cord migrate towards one another and eventually reconnect, creating a continuous central canal (Butler and Ward, 1965, 1967; Piatt, 1955; Sabin et al., 2015; Zukor et al., 2011). Ultrastructural investigation of the uninjured and regenerated spinal cord showed that regenerating axons are intimately associated with glial cell end feet near the pial surface (Singer et al., 1979; Zamora, 1978). Similar observations have been made in newt following spinal cord transection (Zukor et al., 2011). These observations suggest a pro-regenerative or permissive role for glial cells in guiding axon regeneration. Experimental perturbations of injury-induced glial cell signaling pathways (Wnt, FGF, EGF, microRNAs, Shh) after tail amputation or spinal cord transection lead to a failure of spinal cord outgrowth, spinal cord reconnection, neuronal differentiation, and axon regeneration (Diaz Quiroz et al., 2014; Fei et al., 2016; Fei et al., 2014; Rodrigo Albors et al., 2015; Sabin et al., 2015; Schnapp et al., 2005; Sehm et al., 2009; Zhang et al., 2000). These observations provide further evidence that glial cells play an integral role in promoting functional spinal cord regeneration. However, the early injury signal(s) that activate the pro-regenerative glial cell response after injury were not clear.

Changes in early injury signals, such as reactive oxygen species (ROS), membrane potential, and the cytoskeleton, occur within minutes to hours after

injury (Adams et al., 2007; Beane et al., 2011; Bradke et al., 2012; Levin, 2009a; Love et al., 2013; Niethammer et al., 2009; Rieger and Sagasti, 2011; Tseng et al., 2010; Yoo et al., 2012). Therefore, these signaling pathways are uniquely positioned as early regulators of the pro-regenerative glial cell response to spinal cord injury in axolotl. Changes in membrane potential of cells at the injury site regulate zebrafish fin regeneration and Xenopus tail regeneration (Adams et al., 2007; Monteiro et al., 2014; Tseng et al., 2010). Consistent with these reports, we found that the membrane potential of axolotl glial cells is rapidly and transiently depolarized after spinal cord transection (Sabin et al., 2015). Pharmacologic or genetic perturbation of glial cell depolarization after transection leads to decreased glial cell proliferation and/or migration and blocks axon regeneration through the lesion (Sabin et al., 2015). Transcriptional profiling and biochemical analysis showed that the ERK/c-Fos signaling pathway was downstream of glial cell depolarization. Perturbation of the transient glial cell depolarization blocked c-Fos expression and inhibited ERK activation after injury. Specific inhibition of ERK signaling inhibited glial cell proliferation and blocked axon regeneration, phenocopying what we observed after perturbing glial cell depolarization (Sabin et al., 2015). These results highlight a rapid change in glial cell membrane potential as an early injury signal up-stream of the pro-regenerative response to spinal cord transection in axolotl. Furthermore, it identified the ERK/c-Fos signaling pathway as a critical downstream pathway regulating the glial cell response.

Following these experiments, it was not clear how changes in glial cell membrane potential regulate ERK activation was not clear. Recent reports in axolotl used qRT-PCR and showed that modulation of cellular membrane potential during tail regeneration led to decreased transcript abundance of several kinases, including ERK (Franklin et al., 2017). The authors assume that because there is less ERK mRNA there is less ERK protein, which would result in an overall decrease in ERK signaling. However, when we perturbed glial cell depolarization after SCI there was no change in the overall ERK protein levels

compared to controls, and only the levels of phosphorylated ERK (i.e. active ERK) were affected (Sabin et al., 2015). Therefore, after spinal cord transection, it is likely that the pathway up stream of ERK activation is affected, rather than the levels of ERK. Previous reports have shown an important role for receptor tyrosine kinase signaling in regulating diverse processes in salamander glial cells. Specifically, EGF and FGF signaling are essential for glial cell proliferation and migration in vivo and in vitro (O'Hara and Chernoff, 1994; Zhang et al., 2000; Zhang et al., 2002). Furthermore, the establishment of ex vivo cultures of axolotl and newt glial cells absolutely depend on the addition of FGF and EGF to the culture media (Chernoff et al., 1998; Chernoff et al., 1990; Kirkham et al., 2014; McHedlishvili et al., 2012). A major signaling pathway downstream of receptor tyrosine kinases is the MAP kinase cascade and ERK activation. In the future, it would be interesting to see whether perturbed glial cell depolarization leads to changes in expression of various FGF or EGF ligands and/or their receptors. Alternatively, the protein phosphatases that function to attenuate ERK signaling could be inactivated by prolonged glial cell depolarization. These pathways could provide a mechanistic link between membrane potential and ERK signaling during spinal cord regeneration.

While the exact mechanism by which membrane potential can affect gene expression is still not clear, there is evidence that the electrical state of a cell could affect specific chromatin marks. Specifically, membrane potential regulates the directionality of the sodium/butyrate transporter, leading to accumulation of butyrate within the cell (Miyauchi et al., 2004). Butyrate binds to and inhibits histone deacetylase enzymes, which could have profound affects on global gene transcription (Archer et al., 2005). Additionally, increased cellular membrane potential can lead to activation of voltage gated calcium receptors and of the calcium sensitive kinase, which can lead to changes in gene expression (Sasaki et al., 2000). These observations were made in other systems (i.e. not axolotl), but these lines of questioning could provide interesting future directions to tease

apart the pro-regenerative biochemical signaling pathways regulated by glial cell depolarization after spinal cord transection in axolotl.

While our data clearly shows ERK/c-Fos signaling is necessary for the pro-regenerative glial cell response to spinal cord injury, the identity of the c-Fos binding partner was not clear. Our transcriptional profiling and biochemical analysis revealed that axolotl a majority of glial cells do not express c-Jun, the canonical c-Fos binding partner, but instead express JunB. Biochemical pull-down experiments confirm that axolotl versions of c-Fos and JunB proteins are able to heterodimerize, as has been described in other species (Chinenov and Kerppola, 2001). In mammals, the canonical AP-$1^{cFos/cJun}$ promotes reactive gliosis in damaged astrocytes and formation of the glial scar after injury (Cao et al., 2015; Gadea et al., 2008; Gao et al., 2013). Therefore, we hypothesized that differential composition of AP-1 in axolotl glial cells could promote the pro-regenerative glial cell response to injury.

Reactive gliosis is a complex process involving many different signaling pathways (Rolls et al., 2009; Silver and Miller, 2004; Sofroniew, 2009). However, one unifying aspect of reactive astrocytes after spinal cord injury is the up-regulation of GFAP expression (Yang and Wang, 2015). Therefore, we performed luciferase reporter experiments and used GFAP promoter activity as a proxy for reactive gliosis. Consistent with our hypothesis, AP-$1^{cFos/JunB}$ significantly represses the GFAP promoter compared to controls while AP-$1^{cFos/cJun}$ activates the GFAP promoter, consistent with reports in the mammalian spinal cord injury literature (Gao et al., 2013). Ectopic overexpression of AP-$1^{cFos/cJun}$ in axolotl glial cells, thus mimicking mammalian reactive astrocytes, led to defects in axon regeneration, consistent with an inhibitory role for AP-$1^{cFos/cJun}$ during regeneration. Collectively, these experiments support a role for AP-$1^{cFos/JunB}$ in repressing reactive gliosis and glial scar related genes to promote a pro-regenerative glial cell response.

Data mining gene sets from other regeneration competent organisms suggest that differential expression of FOS and JUN family members could be

important regulators of the pro-regenerative response to spinal cord injury across species. Similar to axolotl, zebrafish up-regulate homologues of *c-Fos* and *JunB* 1 day post spinal cord injury while lamprey up-regulate *c-Fos* and *JunD* by 6 hours (Herrmann et al., 2008; Hui et al., 2010). What should be further investigated is the cell types in which these genes are expressed (i.e. glial cells/astrocytes, oligodendrocytes, neurons) and whether or not, in lamprey, AP-$1^{cFos/JunD}$ functions similarly to AP-$1^{cFos/JunB}$ in axolotl. During oral regeneration in the sea anemone *Nematostella vectensis*, various FOS and JUN homologues are differentially expressed in the early compared to late stages of regeneration (Warner et al., 2018). Perhaps the early expressed FOS and JUN function to promote dedifferentiation or the proliferative and pro-regenerative response of cells at the amputation site while the later expressed FOS and JUN could function to pattern the regenerate and/or regulate tissue remodeling of oral structures. However the precise biological function of these genes during *Nematostella* regeneration remains unknown.

Apart from regeneration of nervous tissue, the differential combination of FOS and JUN proteins regulates regeneration of other tissues as well. Several studies have identified the zebrafish homologue of JunB as an important regulator of fin regeneration (Hasegawa et al., 2017; Ishida et al., 2010). While JunB is specifically up-regulated in blastemal cells, the zebrafish homologue of c-Jun is not expressed. Furthermore, activation of JunB during fin regeneration is dependent on Jun N-terminal Kinase (JNK), and JNK inhibition blocks fin regeneration (Ishida et al., 2010). This is reminiscent of axolotl spinal cord regeneration where JNK inhibition blocks the glial cell response to injury and inhibits axon regeneration (Sabin et al., 2015). The reports on zebrafish fin regeneration did not address whether FOS proteins were similarly involved. However, unlike FOS proteins, JunB is able to homodimerize and could regulate fin regeneration independent of a FOS binding partner (Chinenov and Kerppola, 2001). Interestingly, JunB expression after fin amputation in zebrafish is regulated by immune cell-derived IL-1β (Hasegawa et al., 2017). It is not clear if

this same mechanism regulates JunB expression in axolotl glial cells. Finally, a comparative approach looking at heart regeneration in zebrafish and the regeneration-incompetent Medaka showed that the zebrafish homologues of *c-Fos* and *JunB* are up-regulated at 3 days post amputation in zebrafish. Intriguingly, there is no differential expression of *FOS* or *JUN* family members in Medaka (Lai et al., 2017). In the future, it will be interesting to determine which cells (cardiomyocytes, endothelial cells, epicardial cells etc.) in the zebrafish heart express c-Fos and JunB. Furthermore, would forced, temporally regulated overexpression of c-Fos and JunB in the Medaka heart impart a more regenerative outcome after heart amputation?

While axolotl glial cells up-regulate JunB instead of c-Jun during spinal cord regeneration, the mechanism by which c-Jun expression is repressed in axolotl glial cells was not clear. Over the past 20 years microRNA (miRNA) post-transcriptional regulation of gene expression has proven to be an important regulator of various cellular processes (Bartel, 2009, 2018). Not surprisingly miRNA signaling is a potent regulator of cellular response to injury (reviewed in (Sabin and Echeverri, 2017)). Therefore, we wanted to test the hypothesis that glial cells repress c-Jun expression via a miRNA-mediated process. Subsequent cloning of the axolotl c-Jun 3' UTR identified a miR-200a seed sequence. Through a series of biochemical and molecular approaches, we confirmed that axolotl c-Jun is a direct target of miR-200a, and inhibition of miR-200a 1) leads to expression of c-Jun in $GFAP^{+}$ glial cells, and 2) inhibits spinal cord regeneration similar to $AP\text{-}1^{cFos/cJun}$ overexpression experiments. These experiments are consistent with previous reports that perturbations in the glial cell response to injury lead to gross defects in overall spinal cord regeneration (Diaz Quiroz et al., 2014; Fei et al., 2016; Fei et al., 2014; Rodrigo Albors et al., 2015; Sabin et al., 2015; Schnapp et al., 2005; Sehm et al., 2009).

Unexpectedly, this analysis revealed that a small proportion of glial cells in control spinal cords at 3 days post injury express c-Jun. This is different than our observations at 1 day post injury where only neurons express c-Jun. Therefore,

up-regulation of miR-200a might be necessary to reduce c-Jun levels to allow JunB to outcompete with c-Jun for c-Fos binding. Collectively, this would favor the formation of AP-1$^{cFos/JunB}$ and the pro-regenerative glial cell response to injury. These c-Jun^{+}/GFAP^{+} cells could be newly differentiated neurons that have not degraded the GFAP protein. This would be consistent with our observations at 1 day post injury that c-Jun is only expressed in neurons.

To gain a comprehensive view of changes in gene expression during spinal cord regeneration, we performed RNA sequencing of control and miR-200a inhibitor electroporated spinal cords at 4 days post injury, one day after peak miR-200a expression. Consistent with our hypothesis, a subset of genes that had previously been implicated in reactive gliosis or glial scar formation were differentially expressed at 4 days post injury after miR-200a inhibition. Specifically, the intermediate filament proteins *GFAP* and *Vimentin* were significantly higher after miR-200a inhibition compared to controls. Increases in GFAP and vimentin expression are hallmarks of reactive astrocytes (Rolls et al., 2009; Silver and Miller, 2004; Sofroniew, 2009; Yang and Wang, 2015). In a mouse model of spinal cord injury, double knockout of GFAP and vimentin genes led to decreased reactive astrocytes and increased functional regeneration compared to wild type controls (Menet et al., 2003; Ribotta et al., 2004). Whether or not GFAP and vimentin directly promote reactive gliosis in astrocytes is still not clear. However, the composition of astrocytic intermediate filaments and glial scar ECM components affect tissue stiffness at and around the lesion. Changes in the biomechanical properties of the lesion can affect axon migration and has been proposed as one reason axons do not regenerate through the glial scar (Moeendarbary et al., 2017). This suggests that perturbation of intermediate filament content in reactive astrocytes at the glial scar could affect the stiffness of the glial scar, making it more mechanically favorable to support axon regeneration. Another marker of reactive astrocytes, *LGALS1*, or *Galectin-1*, is significantly up-regulated after miR-200a inhibition. Galectin-1 specifically plays a

role in the up-regulation of GFAP expression and hypertrophy of reactive astrocytes (Sirko et al., 2015).

Apart from markers of reactive astrocytes, molecules involved in glial scar formation were also differentially regulated after miR-200a inhibition. Deposition of chondroitin sulfate proteoglycans (CSPGs) by reactive astrocytes is a major component of the glial scar and a barrier to axon regeneration (Lang et al., 2015; McKeon et al., 1999; Rolls et al., 2009; Silver and Miller, 2004). CSPGs are synthesized by the addition of glycosaminoglycan sugar groups onto a proteoglycan protein core (Kwok et al., 2012). Interestingly, the proteoglycan core protein *PRG2* is significantly up-regulated after miR-200a inhibition. This suggests that, in general, CSPG synthesis could be increased. Consistent with this hypothesis, the chondroitin sulfate proteoglycan *CSPG4*, or *NG2*, a highly repulsive axon migration factor, is only up-regulated after miR-200a inhibition in 4 days post injury spinal cords.

Oligodendrocyte progenitor cells, pericytes, macrophages, microglia, and Schwann cells all express CSPG4 after spinal cord injury. Lineage tracing experiments using a CSPG4 reporter revealed that after spinal cord injury CSPG4 expressing cells can differentiate into astrocytes that undergo reactive gliosis and contribute to the glial scar in mammals (Filous et al., 2014; Hackett et al., 2018; Hesp et al., 2018; Huang et al., 2018). Additionally, after SCI in mice, severed axons associate with CSPG4 in vivo and form synaptic-like structures on CSGP4 expressing cells (Filous et al., 2014). While this close association of dystrophic axons with $CSPG4^{+}$ cells limits axonal retraction after injury, the tight association also prevents regeneration of the trapped axons (Filous et al., 2014).

Paradoxically, our RNA sequencing data revealed that the pro-regenerative proteoglycan decorin (Logan et al., 1999; Minor et al., 2008) is up-regulated and the glial scar associated proteoglycan brevican (Jones et al., 2003; Thon et al., 2000) is down regulated after miR-200a inhibition. Given the severe defects in axon regeneration after miR-200a inhibition, one would predict the opposite expression profile. However, comprehensive biochemical

characterization of CSPGs, their receptors, and downstream signaling pathways is lacking in axolotl. Therefore, these proteins could have a different affect on regenerating axons in axolotl compared to mammals.

Immune modulation is a major component regulating the formation of the glial scar as well as the extent of neuronal death and axonal die off after injury. Interestingly, expression of the innate immune system receptor *Toll-like 2* (*TLR2*) is up-regulated after miR-200a inhibition at 4 days post injury compared to controls. TLR2 has previously been implicated in activation of microglia after stereotactic transection of entorhinal axons (Babcock et al., 2006). TLR2-null animals fail to up-regulate expression of injury-induced cytokines known to promote reactive gliosis in astrocytes after injury (Babcock et al., 2006). This suggests that TLR2 signaling can indirectly promote reactive gliosis via induction of specific injury associated cytokines. However, the precise role of TLR2 or generalized Toll-like receptor signaling after spinal cord injury in mammals remains poorly understood. Other immune cell modulators such as *CCL3L3* and *TNFAIP8*, a secreted cell signaling ligand and TNF receptor adaptor protein, respectively, are also up-regulated at 4 days post injury after miR-200a inhibition. While these factors haven't been specifically studied in the context of spinal cord regeneration, they are involved with immune cell trafficking, phagocytosis, and proliferation (Chihara et al., 2017; Zhang et al., 2018). Dysregulation of these pathways leading to increased immune cell infiltration, clonal expansion, and phagocytosis could lead to increased axonal degeneration and inhibit spinal cord regeneration.

A cohort of genes involved with extracellular matrix (ECM) and ECM remodeling were differentially expressed at 4 days post injury after miR-200a inhibition. A major pro-regenerative feature proposed for axolotl glial cells is their ability to remodel the damaged tissue and secrete growth inductive matrix (Chernoff et al., 2000; Chernoff et al., 2003; Egar and Singer, 1972b; Nordlander and Singer, 1978; O'Hara et al., 1992). The collagen *COL21A1* is up-regulated at 4 days post injury after miR-200a inhibition compared to controls. Additionally,

several genes that code for integrin scaffolding components are up-regulated (*ITGB1BP1, ITGB3BP*) or down regulated (*ITGAD*), suggesting that miR-200a inhibition could alter the formation or signaling of integrin based focal adhesions. Integrins act as biomechanical sensors that bind to matrix components, like collagen, and transduce that information into the cell (Gasparski and Beningo, 2015; Goldmann, 2012). Altered integrin signaling could affect the ability of glial cells to sense the stiffness of the environment around them, which could in turn affect glial cell migration during regeneration. Furthermore, the collagen-remodeling enzyme *LOXL1* is downregulated after miR-200a inhibition. This suggests that not only is there a potential increase in collagen deposition after miR-200a inhibition, but that collagen might not be appropriately remodeled, which is a major contributor to fibrotic scar formation after wound healing (Erickson and Echeverri, 2018; Erickson et al., 2016; Seifert and Maden, 2014; Seifert et al., 2012b).

Apart from collagen remolding, additional matrix remodeling enzymes (*MMP2, ADAM23 and SERPINE1*) are also down regulated after miR-200a inhibition, suggesting that there could be gross defects in matrix turnover. Indeed, MMP2 is one of the major MMPs secreted by axolotl glial cells during matrix remodeling after spinal cord transection (Chernoff et al., 2000). Finally, the pro-migratory substrate, fibronectin, which is specifically up-regulated during spinal cord regeneration (Chernoff, 1996; O'Hara et al., 1992), is significantly down regulated at 4 days post injury after miR-200a inhibition compared to controls. Collectively, these events could lead to a growth inhospitable environment for axon regeneration or glial cell migration, culminating in failed spinal cord regeneration. This could explain why the two ends of the severed spinal cord fail to reconnect after 10 days, or why axons do not fully grow through the lesion but instead sprout randomly after miR-200a inhibition.

Apart from a growth repulsive microenvironment, axon regeneration could fail due to decreased expression of axon growth molecules. The cell adhesion molecule *L1CAM* is down regulated after miR-200a inhibition. L1CAM is

expressed along axons, is involved with axon sprouting and branching (Schafer and Frotscher, 2012), and plays an important role for functional spinal cord regeneration in zebrafish (Becker et al., 1998; Chen et al., 2016). Additionally, the small GTPases *NEF-H* and *NEF-M*, which are involved in growth cone migration, are similarly down regulated. The gene for a kinesin-associated protein *CLSTN1* is also down regulated, suggesting a possible decrease in anterograde vesicle transport (Araki et al., 2007; de Ramon Francas et al., 2017; Konecna et al., 2006; Ponomareva et al., 2014). A decrease in vesicle transport, and presumably fusion to the growth cone membrane, would severely limit the amount of membrane material present to support regenerative axon outgrowth.

Inhibition of miR-200a leads to differential expression of genes involved with reactive gliosis and the glial scar. However, there are additional genes that have been previously reported as necessary for functional spinal cord regeneration that are similarly affected. Collectively, these results provide strong evidence for a pro-regenerative role of miR-200a in regulating the glial cell response to injury. Whether or not all these genes are affected due to perturbed AP-1 composition in axolotl glial cells is not clear. It is known that AP-$1^{cFos/cJun}$ promotes reactive gliosis and glial scar formation in mammals, but how AP-1 signaling relates to the specific subset of genes differentially expressed after miR-200a inhibition is not clear. In the future, it will be important to interrogate the binding patterns of these factors along the genome of axolotl glial cells during regeneration. However, we have not found an antibody that cross-reacts with axolotl c-Fos, c-Jun or JunB that is viable for chromatin immunoprecipitation. Based on genomic sequences, there are binding motifs in the 5' *cis*-regulatory regions of the GFAP, vimentin, CSPG4, and CSPG5 genes but their function has not been experimentally validated. This suggests the possibility that a subset of these genes could be differentially regulated based on the composition of the AP-1 transcriptional complex.

Further analysis of our RNA sequencing dataset revealed that apart from reactive gliosis, the glial scar, and pro-regenerative pathways, many other genes

were differentially expressed in both the uninjured and regenerating spinal cord after miR-200a inhibition. This is consistent with the role miRNAs play in regulating hundreds of transcripts in various cell signaling processes to fine tune cellular responses to injury. Gene ontology (GO) analysis on the subset of genes up-regulated after miR-200a inhibition in uninjured and 4 days post injury spinal cords were enriched for nucleotide metabolism and processing, including mRNAs and ncRNAs, as well as protein localization and translation. This was surprising because, to date, miR-200a has been associated with regulating pathways related to epithelial-to-mesenchymal (EMT) transition, neural progenitor identity, and neurogenesis, but has not been considered a major regulator of macromolecular metabolism (Trumbach and Prakash, 2015; Zaravinos, 2015). However, the process of EMT requires massive rearrangement of protein localization, specifically the remodeling of adherens junctions and the breakdown of epithelial apicobasal polarity, as well as transitioning to a completely different transcriptional state. Indeed, transitioning from a neural progenitor to a post-mitotic neuron also requires massive transcriptional changes. Therefore, it makes sense that miR-200a inhibition could affect expression of genes involved with these processes. However, the precise relationship between miR-200a function, nucleotide metabolic processes, and protein localization is not clear. Whether these genes, or a subset of these genes, are direct miR-200a targets would be an interesting question to test in the future.

Further subdivision of differentially expressed genes identified a core set of genes that were similarly differentially expressed in both uninjured and regenerating spinal cords after miR-200a inhibition. This likely represents a common set of signaling networks regulated by miR-200a in axolotl spinal cord, independent of injury status. Apart from the core set of genes, a subset was only differentially expressed after miR-200a inhibition in either the uninjured or regenerating spinal cord. These sets of genes likely represent the signaling networks regulated by miR-200a in an injury dependent manner. Further analysis of these different gene sets will allow us to decipher the role of miR-200a in

homeostatic compared to regenerating spinal cords and will prove a valuable resource in further defining context specific (uninjured versus injured) aspects of miRNA signaling.

As previously mentioned, the subset of regeneration specific genes was enriched for GO terms involved with retinoic acid (RA) signaling and retinoid metabolism. RA signaling plays a complex role in regulating neural progenitor identity and neuronal differentiation (Duester, 2008; Maden, 2007; Rhinn and Dolle, 2012), and is specifically required during spinal cord development to inhibit FGF signaling and promote spinal cord neurogenesis (Diez del Corral et al., 2003). This same report showed that FGF signaling specifically interferes with patterning of the ventral spinal cord. Our gene expression profiling supports this observation as miR-200a inhibition potentially leads to increased FGF signaling and decreased expression of ventral patterning molecules (discussed in more detail later). Previous studies have established that FGF signaling is necessary for glial cell proliferation and neuronal differentiation during spinal cord regeneration in newts (Zhang et al., 2000; Zhang et al., 2002). Whether perturbation of FGF signaling has an additional effect on patterning the regenerated spinal cord was not determined. In the future, it will be interesting to test if increased FGF signaling directly affects ventral spinal cord patterning during regeneration as it does during development.

Further investigation of the expression dynamics of RA related genes revealed that the RA synthetic enzyme *Aldh1a2* (Molotkov et al., 2005; Sirbu et al., 2005) was down regulated, and the putative RA catabolic enzyme *Cyp1a1* (Chen et al., 2000a; McSorley and Daly, 2000) was up-regulated. These findings suggest that miR-200a inhibition might lead to decreased RA signaling, specifically during spinal cord regeneration. Whether these genes are direct targets of miR-200a or if miR-200a inhibition truly affects RA signaling after spinal cord transection remains to be tested. Transgenic analysis of RA signaling during axolotl development revealed that RA signaling is active during nervous system development and spinal cord regeneration after tail amputation

(Monaghan and Maden, 2012; Nguyen et al., 2017). Importantly, treatment of uninjured axolotls or animals undergoing tail regeneration with exogenous RA led to accumulation of GFP^+ glial cells in the spinal cord (Nguyen et al., 2017). This is consistent with reports that newt glial cells express various RA receptor isoforms and that this pathway is necessary for spinal cord regeneration after tail amputation (Carter et al., 2011; Lepp and Carlone, 2014). Collectively, these reports indicate that salamander glial cells respond to RA signaling, although the identity of *Aldh1a2* and *Cyp1a1* expressing cells remains to be determined.

Exploration of the GO terms enriched in down regulated genes after miR-200a inhibition revealed a role in organismal development, nervous system development and function, as well as cell differentiation. This is more consistent with the published role of miR-200a in regulating neurodevelopment (Trumbach and Prakash, 2015). As mentioned above, RA signaling regulates expression of neural development genes, but whether or not potential changes in RA signaling levels after miR-200a inhibition are responsible for decreased expression of these genes is not clear. Ingenuity Pipeline Analysis revealed that transcription factors, cell signaling molecules, and extracellular matrix remodeling enzymes were significantly down regulated after miR-200a inhibition. Specifically, transcription factors involved with specifying ventral identities in the neural tube (*Olig1/2, Mnx1, Nkx6.1*) and general regulators of neurogenesis (*Neurogenin1, NeuroD4*) were down regulated. Apart from neural progenitor markers and regulators of neurogenesis, there was also a decrease in expression of mature neuronal markers, like neurotransmitter transporters and genes involved with neurotransmission, synaptic function, and neuronal polarization, as well as decreased levels of the neuron specific β-tubulin (*TUBB3*). Taken together, these results suggest that miR-200a inhibition leads to a loss of neural progenitor identity and/or neurogenic function after injury compared to controls.

In the axolotl spinal cord, $GFAP^+$ glial cells co-express Sox2 and function as *bona fide* neural progenitor cells (NPCs) in the homeostatic and regenerating spinal cord (Fei et al., 2016; Fei et al., 2014; Rodrigo Albors et al., 2015).

Interestingly, miR-200a is specifically expressed in these glial cells in the axolotl spinal cord, which function as NPCs. This highlights a potential role for miR-200a in regulating signaling pathways required for the stabilization or maintenance of NPC identity and/or function during spinal cord regeneration. This would be consistent with miR-200a function during mouse midbrain/hindbrain development. Inhibition of miR-200a expression or function during mouse brain development leads to precocious NPC differentiation, decreased number of $Sox2^+$ NPCs, and an overall decrease in the number of neurons (Peng et al., 2012). These observations are consistent with our RNA sequencing data, further supporting our hypothesis that miR-200a inhibition leads to depletion of the NPC pool and decreased neuronal differentiation. We can directly test this hypothesis by assaying changes in the number of $Sox2^+$ glial cells or new neurons after miR-200a inhibition compared to controls.

If $Sox2^+$ glial cells lose the neural progenitor identity following miR-200a inhibition, then perhaps they revert or dedifferentiate to a more developmentally primitive cell type. For a long time, it was thought that committed spinal cord NPCs were solely responsible for the development of the posterior spinal cord. However, within the last 20 years it is clear that a population of transient progenitors, called neuromesodermal progenitors (NMPs), contribute to the developing spinal cord (Garriock et al., 2015; Gouti et al., 2015; Henrique et al., 2015; Kimelman, 2016; Martin, 2016). This population of progenitor cells resides within the caudal lateral epiblast/tail bud of vertebrate embryos (Brown and Storey, 2000; Cambray and Wilson, 2002, 2007; Iimura and Pourquie, 2006; Martin and Kimelman, 2008, 2012; McGrew et al., 2008; Wymeersch et al., 2016), is competent to contribute to paraxial mesoderm or spinal cord (Bonnerot and Nicolas, 1993; Tzouanacou et al., 2009), and co-expresses the transcription factors Brachyury and Sox2 (Martin and Kimelman, 2012; Olivera-Martinez et al., 2012; Tsakiridis et al., 2014; Wymeersch et al., 2016). Genetic analyses using tamoxifen inducible Brachyury-driven Cre have estimated that as many as 65% of posterior, trunk spinal cord NPCs are derived from NMPs in mice

(Chalamalasetty et al., 2014; Garriock et al., 2015; Perantoni et al., 2005). Therefore, many of the Sox2^{+} glial cells in the axolotl spinal cord could presumably be developmentally derived from NMPs. Interestingly, miR-200a inhibition leads to *Brachyury* expression in Sox2^{+} glial cells adjacent to the lesion in 4 days post injury spinal cords, but *Brachyury* is not normally expressed in uninjured or control 4 days post injury spinal cords. This data suggests that miR-200a inhibition leads to dedifferentiation of Sox2^{+} glial cells to *Brachyury*$^{+}$/*Sox2*$^{+}$ NMP-like cells. An important future direction will be to determine if ectopic expression of Brachyury in Sox2^{+} glial cells is sufficient to completely reprogram the NPC potential and stimulate contribution to mesoderm-derived tissues (muscle/cartilage) during spinal cord regeneration. However, until we test this hypothesis, this cell population will be referred to as NMP-like cells.

We showed that *Brachyury* is not a direct miR-200a target in axolotl. Therefore, the precise mechanism by which miR-200a inhibition leads to *Brachyury* expression in glial cells was not clear. Genetic studies in mouse, chick, zebrafish, Xenopus and in vitro systems have identified Wnt, FGF and RA signaling pathways as key regulators of Brachyury expression and NMP differentiation (Henrique et al., 2015; Martin and Kimelman, 2008, 2012; Nowotschin et al., 2012; Olivera-Martinez et al., 2012; Olivera-Martinez and Storey, 2007; Shum et al., 1999; Yamaguchi et al., 1999). Therefore, we sought to determine if miR-200a inhibition led to the up-regulation of various Wnt and FGF ligands. None of the Wnt ligands we assayed were up-regulated after miR-200a inhibition, as would be necessary to induce *Brachyury* expression. However, further investigation revealed that miR-200a inhibition appeared to lead to increased Wnt signaling activity, potentially due to the direct regulation of β-catenin levels. Furthermore, FGF signaling might also increase after miR-200a inhibition, because *FGF10* was significantly up-regulated compared to controls. Taken together, these results suggest that miR-200a does not directly repress *Brachyury* expression in axolotl glial cells. However, miR-200a inhibition potentially creates a permissive signaling environment for the induction of

Brachyury expression and dedifferentiation of glial cells into NMP-like cells. In the future, it will be important to determine if over activation of one or both of these signaling pathways is sufficient to induce *Brachyury* expression in Sox2$^+$ glial cells. To test that possibility we could ectopically overexpress a constitutively active β-catenin and/or FGF10 in axolotl glial cells and assay for induction of *Brachyury* expression.

Dedifferentiation of Sox2$^+$ glial cells to *Brachyury*$^+$/*Sox2*$^+$ NMP-like cells would not be sufficient to explain the decreased expression of neural progenitor and mature neuronal markers after miR-200a inhibition. During development NMPs are fully competent to differentiate into NPCs and contribute to neuronal differentiation (Chalamalasetty et al., 2014; Diez del Corral et al., 2003; Garriock et al., 2015; Gouti et al., 2017; Gouti et al., 2015; Gouti et al., 2014). Therefore, additional factors must be affected that would subvert NMPs away from neural and towards the mesodermal identity. While Wnt and FGF signaling are necessary to induce Brachyury expression, they also function to specify NMPs as mesoderm progenitors and commit NMPs to the paraxial mesoderm fate (Gouti et al., 2017; Gouti et al., 2014; Martin, 2016; Turner et al., 2014). Therefore, increased Wnt and FGF signaling activity caused by miR-200a inhibition could induce both 1) *Brachyury* expression and the dedifferentiation of Sox2$^+$ glial cells into NMP-like cells, and 2) the differentiation of those *Sox2*$^+$/*Brachyury*$^+$ NMP-like cells towards a mesoderm fate (i.e. muscle or cartilage). Furthermore, RA signaling directly opposes Wnt and FGF signaling to induce NMP differentiation into neural progenitors that commit to spinal cord development (Diez del Corral et al., 2003; Olivera-Martinez et al., 2012). However, as discussed above, miR-200a inhibition potentially results in decreased RA signaling compared to controls. Therefore, if miR-200a inhibition leads to high Wnt, high FGF, and low RA signaling activities this would create a permissive environment to induce dedifferentiation of glial cells into *Brachyury*$^+$/*Sox2*$^+$ NMP-like cells and subsequent differentiation into mesoderm derivatives. Commitment of dedifferentiated glial cells towards a mesoderm fate would deplete the Sox2$^+$ glial

cell pool resulting in decreased neurogenesis, consistent with our RNA sequencing data. As mentioned before, whether forced *Brachyury* expression in $Sox2^{+}$ glial cells is sufficient to fully induce an NMP identity and stimulate the contribution of those glial cells to muscle/cartilage during regeneration needs to be addressed.

Project Summary

The pro-regenerative glial cell response to spinal cord injury in axolotls has long been known to be an important factor promoting functional recovery. However, the injury-induced signals that regulate the pro-regenerative response have proved elusive. My thesis research identified a rapid and transient depolarization of the glial cell membrane potential after injury as an important up-stream regulator of the glial cell response to injury. Perturbations of glial cell depolarization inhibited the glial cell response to injury and blocked spinal cord regeneration. Furthermore, molecular and biochemical analysis identified the ERK/c-Fos signaling pathway as a key voltage-sensitive pathway downstream of glial cell depolarization necessary for regeneration.

Investigation into the identity of the c-Fos binding partner showed that JunB, not c-Jun, is expressed in a majority of axolotl glial cells after injury to form the $AP\text{-}1^{cFos/JunB}$ transcriptional complex. This non-canonical AP-1 complex acts as a transcriptional repressor at the GFAP promoter and could differentially regulate expression of other reactive gliosis and glial scar-related genes. Reactive mammalian astrocytes express $AP\text{-}1^{cFos/cJun}$, which functions to promote reactive gliosis and glial scar formation. Consistent with an inhibitory role during spinal cord regeneration, the ectopic overexpression of these factors in axolotl glial cells led to defects in spinal cord regeneration. The expression of c-Jun is repressed in axolotl glial cells due to high levels of miR-200a signaling, and miR-200a inhibition led to severe defects in spinal cord regeneration. Transcriptomic approaches revealed that miR-200a inhibition led to differential expression of

genes related to reactive gliosis, glial scar formation, and a set of genes previously shown to support spinal cord regeneration in fish and frogs. Collectively, these results suggest that miR-200a signaling represses reactive gliosis and glial scar formation in axolotl and promotes expression of pro-regenerative genes.

Our transcriptomic analysis also showed that miR-200a inhibition led to Brachyury expression and decreased expression of markers of neural progenitor and mature neurons. This suggests that miR-200a stabilizes or maintains glial cell neural progenitor identity by suppressing the neuromesodermal progenitor identity. Inhibition of miR-200a leads to high Wnt, high FGF, and low retinoic acid signaling, creating a permissive environment for induction of *Brachyury* expression and dedifferentiation of $Sox2^+$ glial cells into NMP-like cells.

Collectively, these experiments have identified membrane potential as a key injury signal regulating the pro-regenerative glial cell response to injury in axolotl. We went on to show that ERK/c-Fos signaling is one of the signaling pathways downstream of glial cell depolarization and identified miR-200a as a negative regulator of c-Jun expression in axolotl glial cells. These experiments provide a framework for why axolotls regenerate after spinal cord injury while mammals form a glial scar. Finally, we have begun to elucidate a potential role for miR-200a in regulating the neural versus neuromesodermal identity in $Sox2^+$ glial cells during spinal cord regeneration.

Chapter 6: Materials and Methods

Animal Handling and Spinal Cord Injury

All axolotls used in these experiments were obtained from the Genetic Stock Center at the University of Kentucky or bred at the University of Minnesota in accordance with IACUAC protocol No. 1710-35242A. Prior to all in vivo experiments animals (3-5cm) were anesthetized in 0.01% p-amino benzocaine (Sigma). Spinal cord ablations were performed as previously described (Diaz Quiroz et al., 2014; Sabin et al., 2015). Briefly, a 26 gage needle was used to clear away skin and muscle and expose the spinal cord 6-10 muscle bundles caudle to the cloaca. Then, using the needle, a segment of spinal cord 1 muscle bundle thick, approximately 500μm, was removed. Animals were placed in cups and monitored for the duration of the experiment.

Imaging Membrane Potential Dynamics After Injury

The fluorescent voltage indicator dye, $DiBAC_4(3)$ (bis-[1,3-dibutylbarbituric acid]trimethine oxonol)(Invitrogen) was used to determine the change in membrane potential during axolotl spinal cord regeneration. Fluorescent images were taken in uninjured, immediately after injury, 7 hours post injury and 24 hours post injury. DiBAC was injected 10 minutes prior to imaging for each point at a final concentration of 10μM+PBS+1% Fast Green. Fluorescent images were taken using a Leica DMI 6000B Scope and Leica DFC 365 FX camera. The excitation/emission spectra is 495/519 nanometers and subsequent images were exported as TIFF files and were pseudocolored using ImageJ. The average fluorescence intensity of injured spinal cords was measured using ImageJ and normalized to the average fluorescence intensity of uninjured spinal cords.

In vivo modulation of Membrane Potential using Pharmacologic Agents

Ivermectin (Sigma) was pressure injected at a final concentration of 10μM in PBS+1% Fast Green into the spinal cord central canal immediately prior to injury. Control axolotls were injected with PBS+1% Fast Green. After injection

and injury, the spinal cords were allowed to regenerate for 7 days then animals were harvested for immunohistochemistry as described above.
All plasmids drove expression of the transgene with the cytomegalovirus (CMV) promoter. All injections were performed using PV820 Pneumatic PicoPump microinjection setup (Echeverri and Tanaka, 2003b).

In vivo Chloride Measurement

Ivermectin and vehicle treated animals were injured as described above. Uninjured or 1day post injury spinal cord tissue was collected and chloride concentration was determined using the calorimetric Chloride Assay Kit (Abnova) as per the manufacturers instructions. Tissue from 5 animals was pooled and homogenized in autoclaved deionized water representing one biological replicate. Absorbance readings were taken at 610 nanometers with a SpectraMax M2 plate reader (Molecular Devices). Absorbance readings were taken in triplicate and averaged for each treatment and chloride concentration was calculated using a standard curve.

Immunohistochemistry and BrdU

Tissue was harvested and fixed in fresh 4% paraformaldehyde (Sigma) overnight at 4°C. Then tails were washed three times in phosphate buffered saline + 0.1% Tween 20 (PBST). Next the tails were incubated in a 50:50 solution of PBST and 30% sucrose. Finally, tails were transferred to 30% sucrose solution and allowed to equilibrate overnight at 4°C. The next day samples were embedded for longitudinal or cross-sectioning in TissueTek (Sakura) and stored at -20°C.

For immunohistochemistry, tails were sectioned at either 10 or 20 micrometers using a Leica CM1850 cryostat. The following primary antibodies were used for immunofluorescent staining: anti-c-Fos (1:100, Santa Cruz), anti-glial fibrillary acidic protein (1:100 Chemicon), anti-NeuN (1:100 Chemicon), anti-c-Jun (1:100 Cell Signaling), anti-5-bromo-2'-deoxyuridine (BrdU)(1:100 Sigma)

or anti- glycine receptor (1:100 Millipore). All sections were incubated in 70° PBS for 20 minutes and subsequently washed with phosphate buffered saline +0.1% Triton-X (PBSTx) 3 times. To prevent non-specific binding of the antibodies the sections were blocked for 1 hour at room temperature in blocking buffer (PBSTx+2% bovine serum albumin +2% goat serum). Primary antibodies were diluted in blocking buffer and slides were stained overnight at 4°C. The next day, slides were washed four times with phosphate buffered saline plus 0.1% Tween 20 and then incubated with secondary antibody (Invitrogen) diluted in blocking buffer (1:200) for 2 hours at room temp and cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:10,000). After secondary incubation the slides were washed four times with phosphate buffered saline plus 0.1% Tween 20 and mounted in 80% glycerol.

For BrdU samples, animals were injected intraperitoneal with BrdU at a concentration of 2.5µg/µL in PBS+1% Fast Green 24 hours prior to tissue harvest. After harvesting the tissuse it was prepared and described above for IHC with minor adjustments. Prior to addition of the anti-BrdU antibody the slides were treated with 4N hydrochloric acid (HCl) for 10 minutes at room temperature and washed three times in PBST. All samples were imaged using an inverted Leica DMI 6000B fluorescent microscope.

Acid Fuschin Orange G (AFOG) Staining

Tissue samples were collected at various time points throughout regeneration and were fixed in fresh made 4% paraformaldehyde (Sigma) overnight. Samples were embedded in OCT for longitudinal sectioning with a Leica CM1850 cryostat. After sectioning, the tissue samples were post fixed in Bouin's solution (Sigma) overnight and then washed with running distilled water for 30 minutes. After washing, the samples were stained by successive 5 minute incubations in 1% phosphomolybdic acid (Sigma), acid fuchsin orange G solution (0.5% aniline blue (Waldeck-Chroma), 1% orange G (Flucka), 1.5% acid fuchsin (Sigma)) and 0.5% acetic acid. Between incubations in staining solutions the

slides were washed for 5 minutes in distilled water. Upon completion of the staining protocol the samples were dehydrated by successive incubations in 96% ethanol for 2 minutes then 100% ethanol for 2 minutes then xylene for 5 minutes. Finally, the slides were embedded in 80% glycerol and imaged using an Olympus BX40 inverted microscope.

Whole Mount Immunohistochemistry

Tissue were harvested 7 days post injury and fixed in freshly made 4% paraformaldehyde (Sigma) overnight at 4°C. Tails were subsequently washed three times in PBST and treated with 10 µg/mL of Proteinase K (Roche) for 10 minutes. After Proteinase K treatment the tails were washed an additional three times in PBST. To further permeabilize the tissue, the tails were washed with PBS+0.1% Triton X100, three times. To block non-specific binding of the antibodies the tails were blocked for 1 hour at room temp in blocking buffer (PBST+10% goat serum). Then the tails were incubated with mouse anti-β-III tubulin antibody (Sigma) diluted 1:500 in blocking buffer overnight at 4°C. The next day tails were washed four times in PBST before being incubated with goat anti-mouse Alexa Fluor 568 (Invitrogen) secondary antibody for 2 hours at room temperature in blocking buffer. After the secondary incubation the nuclei were stained with DAPI (1:1000) for 10 minutes in PBST. To wash off excess secondary antibody and DAPI the tails were washed four times in PBST. After washing, tails were gradually stepped into methanol by incubating with 25%, 50%, 75% then 100% methanol: PBST solution and then stored at -20°C until imaged. Prior to imaging, the tails were cleared using 1:2 solution of benzyl alcohol and benzyl benzoate (BABB) (Sigma) for 20 minutes and mounted onto a cover slip using 1:2 solution of benzyl alcohol and benzyl benzoate (BABB) as the mounting medium.

Plasmid and microRNA Inhibitor electroporation

Plasmids were diluted in PBS to a final concentration of 0.1-1μg/μL+1% Fast Green and microinjected into the central canal of axolotls and were electroporated (5 square pulses, 50ms, 50V using an ECM830 Electro Square Porator BTM Harvard Apparatus) twice. . All injections were performed using PV820 Pneumatic PicoPump microinjection setup (Echeverri and Tanaka, 2003b).

To induce hyperpolarization we overexpressed the constitutively active potassium channel Kir2.1 harboring Y242F mutation and a green fluorescent protein tag (Hinard et al., 2008). To induce depolarization we overexpressed the GABA-gated cation channel Exp-1 and co-injected a plasmid that contained a green fluorescent protein tagged H2A construct, which allows the electroporated cells to be identified using in vivo fluorescent microscopy (Beg and Jorgensen, 2003; Echeverri and Tanaka, 2003b).

To overexpress AP-$1^{cFos/cJun}$ we subcloned the axolotl full length sequences of c-Fos and c-Jun (described below) into pCMV-GFP overexpression vector (Clontech). The resulting plasmids were diluted 1:1 molar ratio in PBS+1% Fast Green and co-electroporated as described above. Animals were screened for GFP expression and GFP^{+} were subjected to spinal cord injury and harvested 7 days later for whole mount β-III tubulin staining.

To inhibit miR-200a, a chemically synthesized anti-sense inhibitor or scrambled control inhibitor (Dharmacon) was diluted to 20μM in PBS+1%Fast Green and pressure injected into the central canal. Animals were electroporated as described above to facilitate up-take of the inhibitor or control. Animals were electroporated on the day of injury and every other day for the duration of the experiments to ensure efficient microRNA knock down.

Western Blot Analysis

Control or ivermectin injected samples were harvested and placed directly into radioimmunoprecipitation assay buffer (RIPA buffer) containing a

protease/phosphatase inhibitor cocktail (Cell Signaling Technology). The tissue was homogenized using the pestle mortar mixer (Argos Technologies), centrifuged and the supernatant was placed into a new tube. The protein concentration was determined using a micro BCA assay. Samples were separated on a 4-12% Bis-Tris NuPAGE gel (Life Technologies) and transferred onto a nitrocellulose membrane. Membranes were probed with anti-Erk, anti-phospho-Erk, anti-Sox2 (Cell Signaling Technologies), anti-c-Fos (Millipore) or anti-actin (Sigma) antibodies overnight at 4°C. After washing, membranes were incubated with HRP conjugated goat anti-rabbit secondary (Thermo Scientific) and developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

The spinal cords 500μm rostral and 300μm caudal to the lesion from 7-10 control or miR-200a inhibitor electroporated animals were microdissected and pooled for each biological replicate. Total RNA was isolated using Trizol according to the manufacturer. Subsequent cDNA was synthesized from 1ug of DNaseI (NEB) treated RNA using either High Capacity cDNA Reverse Transcription kit (Applied Biosystems) or miScript II RT kit (Qiagen). The qRT-PCR was carried out using LightCycler 480 SYBR Green I Master (Roche). MicroRNA qRT-PCR was carried out with custom designed primers to conserved miRNAs (Qiagen) and custom primers from IDT were used to quantify axolotl mRNAs:

18S_F: CGGCTTAATTTGACTCAACACG
18S_R: TTAGCATGCCAGAGTCTCGTTC
cFos_F: TCCCTCTACACCTCCGAC
cFos_R: AAAGCGTCCGATTCAGGG
cJun_F: CTCTGCCCCAAGAATGTGAC
cJun_R: GAAGTTGCTGAGGTTGGCAT

JunB_F: CTCCTTCCTGCCTGGCTATG

JunB_R: ACTGTCCGAGCCAAAGTAGC

GFAP_F: ACAGAGCCTAAACAGTGATG

GFAP_R: GTCTTTAAGGTTCCGGATGT

Vimentin_F: AACACTCTCCAGTCTTTCAG

Vimentin_R: TCTTCGTCGTGTAGTTTCTT

CSPG4_F: ATTCCATTACCCCACCTAGT

CSPG4_R: AGCTGCCCCTCATTAATATG

CSPG5_F: CATGATGACCGTTTTCTTCG

CSPG5_R: GATGGTGGACAGAGAAAAGT

Col4a1_F: GTGGCTATCTCTCTGGATTG

Col4a1_R: CCATGGCACTCAATAAATGG

Brachyury_F: GAAGTATGTCAACGGGGAAT

Brachyury_R: TTGTTGGTGAGCTTGACTTT

Sox2_F: TTGTGCAAAATGTGTTTCCA

Sox2_R: CATGTTGCTTCGCTTTAGAA

Wnt3a_F: AAGACATGCTGGTGGTCTCA

Wnt3a_R: CCCGTACGCATTCTTGACAG

Wnt5a_F: ACCCTGTTCAAATCCCGGAG

Wnt5a_R: GGTCTTTGCCCCTTCTCCAA

Wnt8a_F: TTGCTGTCAAATCAACCATG

Wnt8a_R: TGCCTATATCCCTGAACTCT

Ctnnb1_F: ACCTTACAGATCAAAGCCAG

Ctnnb1_R: GGACAAGTGTTCCAAGAAGA

Lef1_F: GTCCCACAACTCCTACCACA

Lef1_R: TAGGGGTCGCTGTTCACATT

FGF8_F: TTTGTCCTCTGCATGCAAGC

FGF8_R: GTCTCGGCTCCTTTAATGCG

FGF10_F: AAACTGAAGGAGCGGATGGA

FGF10_R: TCGATCTGCATGGGAAGGAA

microRNA In Situ Hybridization

Samples were fixed in fresh 4% PFA at 4°C overnight. The following day they were washed 3 times for 10 minutes in PBS. Then they were gradually stepped into 100% methanol and stored at -20°C. The samples were gradually stepped back into PBS and processed for cryosectioning, as described above. To remove OCT after sectioning the slides were washed 3 times for 10 minutes in PBST before being incubated with Proteinase K (2ug/uL) for 10 minutes. The slides were rinsed once and then washed for 2 minutes in a glycine solution (2mg/mL). Then slides were rinsed 3 times with PBST and incubated in a 1:1 solution of hybridization buffer:PBST for 10 minutes. Following that the slides were incubated in hybridization buffer plus yeast tRNA extract for 3 hours at room temperature. After the pre-hybridization step miR-200a LNA probe (Exicon) was added to the slides at a final concentration of 40nM. Slides were hybridized over night at 53°C. The following day slides were washed in 5x SSC buffer for 10 minutes then 0.2% SSC buffer for one hour. Both washes were carried out at 60°C. The slides were then stepped back into PBST and washed one final time in PBST for 10'. Then slides were blocked in PBSTw+2% BSA+ 2% sheep serum for 1 hour at room temperature and anti-DIG antibody (Roche) was diluted 1:1000 in blocking buffer for 2 hours. Slides were then rinsed 2 times in PBST and washed 3 times for 10 minutes in PBST before being incubated in fresh AP buffer 3 times for 5 minutes. After the final wash BM Purple was added and slides were checked often for color development. Finally, upon completion of the reaction sections were fixed in 4% PFA for 10 minutes, rinsed 3 times in PBST then embedded with 80% glycerol and imaged using an Olympus BX40 inverted microscope.

JunB Probe Synthesis

Axolotl specific JunB probes were created by PCR amplification with the addition of the T7 and Sp6 promoter into the PCR primers:

T7 JunB ISH For AGAtaatacgactcactatagggAATGTGCCGTGCAGCGGATA
Sp6 JunB ISH Rev AGAtatttaggtgacactatagAAGAGGTAGAGGGAGCCCAGTC

The resulting PCR product was used to synthesize in situ probe by the addition of DIG-labeled UTP (Roche) plus the appropriate RNA Polymerase T7 or Sp6 (NEB). Probes were purified with RNA Clean Up kit (Qiagen) and resuspended in 100uL of hybridization buffer.

Brachyury and Sox2 Probe Synthesis

Approximately 500bp fragments of axolotl Brachyury and Sox2 were PCR amplified using OneStep PCR Kit (Qiagen) from RNA extracted from axolotl embryos at various developmental stages using the following primers:

Brachyury ISH For: CCCCAACGCCATGTACTCTT
Brachyury ISH Rev: GGCCAAGCGATATAGGTGCT

Sox2 ISH For: TGGCAATCAGGAAGAAAGTC
Sox2 ISH Rev: GCAAATGACAGAGCCGAACT

The resulting PCR fragments were separated by gel electrophoresis on a 1% agarose gel and gel purified using the Monarch Gel Purification Kit (New England Biolabs). The subsequent PCR fragments were TA cloned into pGEM-T Easy (Promega) and transformed into DH5α competent *E. coli* and streaked onto X-gal containing plates. Blue/White positive selection was used to pick clones and send recovered plasmids for sequencing. Positive clones were digested with the appropriate enzyme to linearize the plasmid and anti-sense ribonucleoprobe synthesis was carried out using Sp6 or T7 (New England Biolabs)+DIG labeled UTP (Roche). Subsequent probes were cleaned up using the RNA Clean Up kit (Qiagen) and resuspended in hybridization buffer.

In Situ Hybridization

Samples were prepared as described above. After sectioning slides were incubated in 70°C PBS for 15 minutes to remove OCT. Then the slides were incubated in 1:1 PBS:EtOH for 5 minutes before being washed in 100% EtOH for 5 minutes. Then the slides were incubated in Xylene for 30 minutes before being washed twice in 100% EtOH. After washing, the slides were transferred to 100% MeOH for 5 minutes and gradually stepped into PBST. Then slides were incubated in 1:1 PBST:Hyb for 10 minutes and pre-hybridized for 30 minutes. JunB probes were diluted into hybridization buffer and slides were allowed to hybridize overnight at 55°C. The following day the slides were washed 3 times in Wash Buffer (50% Formamide, 5x SSC and 0.1% Tween) for 30 minutes each, once in 1:1 Wash Buffer:PBST for 30 minutes and once in PBST at 55°C. Slides were rinsed in room temperature PBST 3 times for 5 minutes each before blocking buffer was added (2% goat serum, 2% BST in PBSTx) for 1 hour. Anti-DIG F_{AB} (Roche) was diluted 1:1000 in blocking buffer and slides were incubated for at least 1 hour. Slides were washed 3 times for 10 minutes each before addition of fresh AP Buffer for at least 10 minutes. Finally slides were incublated in BM Purple (Roche) until colored reaction was observed. The reaction was stopped by several quick rinses in PBS and were fixed in 4% PFA for 10 minutes. Slides were mounted in 80% glycerol and images were taken with a BX40 Olympus Inverted microscope.

Cloning and Plasmids

The full-length axolotl JunB and axolotl c-Jun 3' UTR were cloned using rapid amplification of cDNA ends (Clontech) as per the manufacturers instructions.

JunB 5' GSP 1: GCTGCACCACTGTCCGAGCCAAAGT
JunB 5' NGSP 1: TGGGTCAGTGAGGTTAAGGGCCAAGC

JunB 3’ GSP 1: CCTCAACCCCACTACTCCACCTCGG
JunB 3’ NGSP 1: GACCAAGAGCGCATTAAGGTGGAGCG

cJun 3’ GSP 1: GAACCGCATCGCCGCCTCCAAGTG
cJun 3’ NGSP 1: GCAGAACTCGGAGCTGGCTTCCACG

The coding sequences of axolotl c-Fos and c-Jun were cloned based on Trinity assembled RNA sequencing contigs from axolotl-omics.org. The coding sequence of axolotl JunB was amplified using primers based on the full-length transcript from our RACE experiments. PCR fragments for c-Fos, c-Jun and JunB were digested with the indicated restriction enzymes and ligated into the pCMV:GFP expression vector (Clontech). Restriction fragments were ligated together using T4 DNA Ligase (NEB) overnight at 4°C and heat shock transformed into DH5α competent E. coli (Promega)

cFos For Axolomics NheI ATTGCTAGCACCATGTTCCAGGGCTTCTCGGG
cFos Rev Axolomics SacII ATCCCGCGGCAGAGCAAGCAAAGTAGGCG

cJun For XhoI ATTCTCGAGACCATGGAGCCTACGTTCTACG
cJun Rev SalI ATTGTCGACACATGAACGTCTGCAGCTGCTG

JunB For NheI ATTGCTAGCACCATGTGCACCAAGATGGACG
JunB Rev SacII ATTCCGCGGAAAGGGCTGCATCTTGGCA

BioID Pull Down Experiments

The axolotl JunB ORF was subcloned into the EcoRV and BamHI sites of BioID Myc tag vector (Addgene #35700).

JunB_BioID_Myc_For ATAGATATCTACCATGTGCACCAAGATGGAG

JunB_BioID_Myc_Rev	AGGGGATCCTCAAAAGGGCTGCATCTTG

293 cells were plated in 24-well plate (1x10^5 cells/well) and transfected with empty BioID+c-Fos, JunB-BioID alone or JunB-BioID+cFos. After 24 hours biotin (50uM) was added to the cell culture media and allowed to incubate for an additional 24 hours. Finally, cells were lysed in boiling RIPA buffer and sonicated to lyse cells and sheer DNA. Then 50uL of pre-washed Dynabeads MyOne Streptavidin T1 (ThermoFischer) was added per lysate and allowed to incubate at 4°C. The following day the beads were washed 4 times in RIPA buffer and bound proteins were released from beads by addition of 25uL of LSB, an excess of biotin and incubated at 95°C for 5 minutes. Subsequent protein fractions were seperated on SDS-PAGE and Western blot was performed as described above.

Cloning of the axolotl GFAP promoter

Genomic DNA was isolated from tails of 2-3cm axolotls using Jetflex Genomic DNA Purification kit (Invitrogen). Following purification of genomic DNA, we used GenomeWalker Universal kit (Clontech) to determine the DNA sequence up stream of the GFAP transcription start site. Two rounds of “walking” were performed using the below primers:

GFAP Promoter GSP1	TTTCCCAACTTCAGCCTCGCAAGACTC
GFAP Promoter NGSP1	ACTTTTGAGGAGGGCCGTTAGAGAAC
GFAP Promoter GSP2	TTCACTGTGGCGTCATGTGGATCGGTAACC
GFAP Promoter NGSP2	TTCCAGCACACTCTGCGTCCCTTTGTTTGC

Distinct PCR bands TA cloned into pGEM-T EZ (Promega) and inserts were sequenced. Based on this analysis we designed primers to amplify ~1.3kb of the axolotl GFAP promoter using Phusion High Fidelity polymerase (NEB):

pAxGFAP For 2 XhoI ATACTCGAGTACCTGGCATTGACATTATCTGGTC
pAxGFAP Rev1 HindIII ATAAAGCTTTTTCAGAGTTTCCCAACTTCAGCCT

The resulting PCR product was ligated as a XhoI and HindIII fragment into pGL3 Enhancer luciferase reporter plasmid (Promega).

Cloning 3' Untranslated Regions for miRNA Luciferase Assays

For 3' UTR luciferase experiments, primers were designed to amplify the c-Jun 3' UTR based off our RACE sequences. Primers were designed to the Brachyury and Ctnnb1 3' UTR based on sequences available at axolotl-omics.org. All the 3' UTRs were amplified with a 5' SpeI and 3' HindIII restriction site.

cJun 3' UTR Luc For ATAACTAGTGGAGGGAAGCGCGG
cJun 3' UTR Luc Rev AGCAAGCTTGTTTGTAGTTTAGTTTGTAATAC

cJun 3' UTR SDM For GATCTGTTTgaattcACCAAGAACTGCAT
cJun 3' UTR SDM Rev GTTCTTGGTgaattcAAACAGATCTCGC

Brachyury 3' UTR For 1 AGCACTAGTATGTGAAATGAGACTTCTAC
Brachyury 3' UTR Rev 1 TGCAAGCTTCTTATTCTTCCCATTTAACTTAAA

Ctnnb1 3' UTR For 1 ATAACTAGTTTGTGTAATTTTTCTTAGCTGTCATAT
Ctnnb1 3' UTR Rev 1 ATCAAGCTTAATTGCTTTATAGTCTCTGCAGAT

Ctnnb1 3' UTR SDM1 For AGTGCCTGATgaattcAACCAAGCTGAG
Ctnnb1 3' UTR SDM1 Rev CTCAGCTTGGTTgaattcATCAGGCACT

Ctnnb1 3' UTR SDM2 For ATTTAATGGTGTAGgaattcAATAGTATAA
Ctnnb1 3' UTR SDM2 Rev TTATACTATTgaattcCTACACCATTAAAT

The PCR fragments and pMiR Report (Life Technologies) were digested with SpeI and HindIII (NEB) and the fragments were ligated over night at 4°C with T4 DNA Ligase (NEB) and heat shock transformed into DH5α competent E. coli (Promega).

3' UTR luciferase experiments

HEK 293 cells were plated in a 96 well plate (Celltreat Scientific Products) at a concentration of 1.0 x 10^5 cells/mL and allowed to adhere over night. The next day cells were co-transfected with 80ng of pMiR Report (Life Technologies) with c-Jun, Brachyury or Ctnnb1 3' UTR, β-Galatosidase internal control and 100nM of miR-200a or control mimic (Qiagen) per well using Lipofectamine 3000 (Invitrogen). After 48 hours luciferase activity was determined using Dual Light Reporter system (Thermo) according to the manufacturers protocol.

GFAP Promoter Luciferase Assays

B35 neuroblastoma cells were co-transfected with pGL3-GFAP promoter plasmid (described above), β-Galatosidase internal control plasmid, and axolotl c-Fos, c-Jun and/or JunB subcloned into pCMV:GFP plasmid (Clontech) or 100ng of control pCMV:GFP with Lipofectamine 3000. After 48 hours luciferase activity was determined using Dual Light Reporter system (Thermo) according to the manufacturers protocol.

RNA Sequencing Sample Preparation

For RNA sequencing animals (3-5cm) were electroporated with miR-200a inhibitor or control inhibitor and a spinal cord trasection was performed, as described above. Animals were electroporated on the day of injury and 2dpi. Spinal cords were microdissected at 4dpi for RNA extraction using TRIzol (Thermo Scientific) according to the maufacturers protocol. Each sample (Uninjured, 4dpi Control and 4dpi Inhibitor) consisted of 6 pooled spinal cords and samples were submitted in biological triplicates. Library preparation and

RNA sequencing was performed by the Genomics Center at the University of Minnesota.

RNA Sequencing Data Analysis

RNA seq data were comprised of 2x 126bp paired-end reads. The RNA-seq reads from all samples were combined and then assembled by using Trinity (Grabherr et al., 2011) (v2.3.2) with default parameteres. We used cd-hit-est(v4.6) (Fu et al., 2012) with parameter “c 1” to removed shorter contigs with 100% identity with aligned longer contigs. These non-redundant contigs were mapped to Ensembl human proteins (v85) by BLASTX (v2.2.18) Contigs were assigned to human protens by taking the best BLASTX hit with E-value $<10^{-5}$.

We used Bowtie (v0.12.1) (Langmead et al., 2009) to map the reads against all non-redundant contigs. Quantification of each contig was performed by RSEM v1.1.6 (Li and Dewey, 2011). RSEM employs an expectation maximization algorithm, so that for reads that match to multiple contigs, RSEM assigns a fraction of each read to each contig based on estimated abundances of contigs based on unique reads (Li and Dewey, 2011).

For each sample, the exprected fragment counts for each contig (as computed by RSEM), were then converted to comparative transcript counts by summing the frament counts of contigs mapped to the same trascritp. Similarly, gene-level counts were obtained by summing the gragment counts of transcripts that were annotated with the same gene symbol. Relative abdunacnes, in terms of transcripts per million (TPM) , for genes were computed by first normalizing each gene’s fragment count by the sum of the “effective lengths” (weighted average of contigs’ lenght based on contigs abundance) of the contigs mapped to that gene and then scaling the resulting values such that they summed to one million over all genes.

To identify differentially expressed genes the nornalied counts from RSEM were imported into DESeq2 (v1.16.1) as integers to determine moderated

sestimates of log2 fold changes and Bejamini-Hochberg adjused p-values between conditions.

Gene Ontology (GO) Analysis

GO terms were determined using GOrilla (Eden et al., 2009). We used 2 unranked list of genes: a background list, all genes that were differentially regulated in our data set, and a target list, the list of genes differentially regulated in a given comparison. Using this approach GOrilla generated a list of enriched GO terms and we selected the top 9-13 terms with the lowest p-value and generated representative bar graphs using Excel.

Pie Chart and Venn Diagram Generation

Pie charts were generated to represent the total number of differentially expressed genes in a given comparison using Excel. Venn Diagrams were generated with Venny (v2.1.0) (Oliveros 2007-2015) and saved as .csv files to be modified in Adobe Illustrator.

Statistical Analyses

All results are presented as mean +/- s.e.m. unless otherwise stated. Analyses were performed using Microsoft Excel or GraphPad Prism. Data set means were compared using ANOVA for three or more tests with a Tukey test (for multiple comparisons) or Dunnett test (to compare to a control mean). When two groups were compared a Students *t*-test was used. Differences between groups was considered significant at three different levels (p-values of *<0.05, **<0.01 and ***<0.001) and are indicated in the figure legends.

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