Characterization of the cellular and molecular mechanisms that drive perineurial glial bridging after spinal motor nerve injury in zebrafish

Kimberly A. Arena

Cold Spring Harbor, New York

B.S., Duke University, Durham, North Carolina, 2016

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Department of Biology

University of Virginia

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ABSTRACT

Spinal motor nerves are essential for proper organismal locomotion and survival. Motor nerves consist of bundles of motor axons ensheathed by layers of connective tissue and supporting glial cells. Remarkably, in most vertebrates these motor nerves are able to naturally regenerate following injury, though full functional recovery in mammals is limited. The same glial cells that support the motor nerve during development are activated to allow for efficient and effective regeneration. However, the cellular and molecular interactions that underlie successful motor nerve regeneration remain poorly understood. Studies have classically focused on the regenerative responses of Schwann cells and macrophages; however, little is known about the injury responses of other cells that make up the motor nerve. Recently, studies from our lab identified that in the early hours following spinal motor nerve injury in zebrafish, perineurial glia form a glial bridge across the injury site and phagocytose axonal debris. Though perineurial glia are essential for successful motor nerve development and regeneration after injury, very little is known about their injury response. In this dissertation, using zebrafish as a model organism, I present the first known signaling pathway to drive perineurial glial bridging after spinal motor nerve injury, explore cellular interactions between perineurial glia and Schwann cells, and establish tools for future studies to continue to elucidate the signals that drive successful regeneration. This work fills gaps in our knowledge about the cellular and molecular interactions that occur between essential glial cells to drive injury responses that are necessary for successful motor nerve regeneration and presents potential targets for future therapeutics.

DEDICATION

Over the years, many people have equated getting a PhD to running a marathon. Having run multiple marathons and completed a PhD in the past six years, I can attest that running a marathon is much easier than getting a PhD. With a marathon, you know what to expect. While there will always be uncontrollable variables, such as race day weather, stomach issues, overuse injuries, even those are to be expected. You know going into that race that there will be aid stations every two miles. You know it will hurt, but you have done the training to get through it. And you know at what point in the race the physical challenge will give way to a mental challenge. I would say that the process of obtaining a PhD is more similar to going on a trail run with my friend Taylor. You go into the run expecting a casual 10 mile run on local trails, then 15 miles later you find yourself chest deep in the Rivanna River in the middle of February wondering how you ever ended up here and why you ever thought this would be fun. While running a marathon might feel more attainable than getting a PhD, the process of getting to that finish line is indeed very similar. When you hit that sign up button you feel a mix of excitement and dread. You have your expectations, but they won't end up being what you thought they were. You know it's going to be hard, but you won't know that pain until you are 20 miles into a long run at the end of a 40-mile week. There are days where it feels easy and you feel as though you are on top of the world and have so much love for what you do, and there are days where putting on your sneakers and getting out the door feels impossible. There are days where you doubt yourself, where you question whether you have any business even being on that start line. Days where you see other people doing more intense training, running faster times, and you convince yourself that you're not good enough. There are times where you

feel exhausted by the work you are putting in but it still doesn't seem like you are progressing. And in the end, many people will see you cross the finish line but few will have witnessed the days it took to get you there. While I have experienced these sentiments both while training for a marathon and training for this degree, the biggest similarity between the two is that you don't get to that finish line on your own. I would never have finished any of my marathons or ultramarathons without every person who I did a training run with, without every person who talked me through injuries and training plans and race day plans, without everyone who listened to me complain about my weekend workout or without everyone who joined me for a blissful long run through the mountains. And getting my PhD was no different. I would not be where I am today without the guidance and support and love that I have felt over the past six years. I am really lucky to have so many wonderful people from all phases of my life on my support crew, and this dissertation is dedicated to every one of them who helped get me to my PhD finish line.

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friends, who have taught me all I need to know and made lab a place I wanted to be even when I didn't want to be there. To Kendra, a wonderful friend and a beautiful scientist whose confidence and passion inspires me on a daily basis. And to Ashtyn, I have been so lucky to have a co-worker who is also one of my best friends, and I truly cannot imagine the past five years without you. From late nights in lab just chatting to art nights, movies, puzzles, and long walks during a pandemic, to book clubs and working through everything life threw at us together I am so grateful that you joined our lab so I could have a best friend to work with every day. To my high school friends, especially Kacey, Liv, Bridget, and Riley. We have been together since our very first science fair in 2001, and from CSH to college and now grad school you all have been with me through it all, from graduations and marriages to babies and PhDs. To the extended CSH group, thank you for always making me feel like I never left despite being basically the only one to leave. To the Five Gals, Simone, Jpop, Jenna, and Jenny, there's no friend group quite like us. Thank you for ten years of laughs, adventures, and endless love, from bombarding group FaceTimes to time capsules and believing in me to finish my PhD when I didn't believe in myself. To Kersky (and Gwen) and Muhammad, for every shenanigan, FaceTime call, and visit over the past six years you both bring such joy into my life and have my back always. To Alessandra, my twin, for every phone call and visit while also being an incredible doctor, you are an inspiration and I love you so much. To my friends here at UVA, Audrey, Phil, Luke, Chris, Taylor, and Jamie. You guys are what made doing this whole thing fun. I could not have done this without every walk to Starbucks, bitch and snitch in the sauna, night of NyQuil and cookie cakes, family dinner, race weekend, beach and camping trip, run club, and everything Audrey bought me that I needed for myself but was too lazy to

get. I love you all and am so lucky to have had such an amazing group of people to procrastinate with over the years. To Edie, for every Friday morning call, every funny text, and for being one of the best things to come out of the pandemic. To Champion Run Club, for every Wednesday night life chat and laugh over miles and beers. To Rocky Top, my home away from home. There are too many people to name but I am so thankful for every person I have climbed with over the years, from the Tuesday night regulars to Brooke's Babes, the establishment of the Fastbois and getting regularly sandbagged on runs with Taylor, to finding my other half, Kim, and every awesome run we have had together, to every trip out to the New, I climb because of you all. You guys are what make Charlottesville feel like home. To Abby, I am so beyond thankful that our dear friend Mark introduced us when he did. Our friendship, and every friendship I have made through our friendship, is the best gift he could have given us. From every weekday Ragged run to date nights at the AAC and life chats 200 ft up a wall in Yosemite, you are such an incredible and inspirational friend on and off the wall, and I truly could not ask for a better person to call my climbing partner and friend, or to start a commune with. To my family, I am proud to come from a large New York Italian (with a little Irish) family, and though I am the first doctor and scientist in the family and no one really knows what I do, I have felt nothing but endless support and excitement about my work and my passions since I was a kid and I am so thankful for all of you. With every phone call and card and care package you have made me feel so loved from afar and always reminded me of how proud you were of me. To the Westfall-Yee family, Audrey, Jack, and Noah, you have all become my family in the past six years and I am so thankful for the guidance and love you have given me. To my parents and AJ, you guys are the backbone of my support. I might be the only scientist

in the family but I am because of you, because of every science book you bought me, of every nature camp you put me in even though I was the only girl, of encouraging me to attend my dream college and working hard so that I could, and supporting me through every uncertainty and doubt that I have encountered as I navigate through my 20s and grad school. I love you all so much and am who I am today and where I am today because of you. To Matt, my best friend, for being by side through all of this even when you couldn't be by my side. For every sacrifice you had to make over the years to support me, and for loving me unconditionally through all my highs and lows. I could not ask for a better life partner and I would not be the doctor I am today without you, even though I beat you to it. And finally, to Jaak Raudsepp, for being the first to recognize that I could be a scientist, for encouraging me to pursue that path, for supporting me years after I graduated high school, and for being a friend beyond my teacher, thank you.

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CHAPTER I

Introduction

The vertebrate nervous system is composed of two main parts: the central nervous system (CNS), which consists of the brain and the spinal cord, and the peripheral nervous system (PNS), which contains all the nerves and glial cells that relay signals to and from the CNS to the rest of the tissues and organs in the body. Together, the nervous system controls all of an organism's movements and sensations. Peripheral nerves consist of axons, supportive glial cells, and connective tissues. Layers of these glia protect the nerve and ensure fast signal transmission (Kristjan R. Jessen & Mirsky, 2005; Kristján R. Jessen, Mirsky, & Lloyd, 2015; Kucenas, 2015). Loss or disruption of glial cells and their associated behaviors results in perturbed nerve development (Binari, Lewis, & Kucenas, 2013; Clark et al., 2014; Fledrich, Kungl, Nave, & Stassart, 2019; Kucenas, Snell, & Appel, 2008; K. R. Monk, Oshima, Jors, Heller, & Talbot, 2011; A. D. Morris, Lewis, & Kucenas, 2017; Reed, Feltri, & Wilson, 2021). Studies of *Drosophila glial cells missing (gcm)* mutants, which do not possess glial cells, demonstrate the essential role of glial cells in guiding developing axons, generating survival signals for neuronal cells, and regulating fasciculation (Garratt, Britsch, & Birchmeier, 2000).

The same glia that are crucial for normal development play essential cellular and molecular roles in modulating regenerative responses after injury in both the CNS and PNS (Cattin & Lloyd, 2016; Clements et al., 2017; Goldshmit et al., 2012; Gonzalez & Allende, 2021; Hsu et al., 2021; G. M. Lewis & Kucenas, 2014; Mokalled et al., 2016; Murphy, Isaacman-Beck, & Granato, 2021). While injury to the CNS results in limited recovery, peripheral

nerves possess the remarkable ability to regenerate and, in some vertebrates, achieve full functional recovery (Gonzalez & Allende, 2021; Huebner & Strittmatter, 2009; Mokalled & Poss, 2018). Coordination of the same glia that are crucial for nerve development and survival are also essential for proper nerve regeneration. In particular, macrophages, Schwann cells, and perineurial glia respond to peripheral nerve injury and aid in regeneration. Though distinct pro-regenerative properties of each cell type are known, the interactions between these cell types as well as the factors driving injury-induced behaviors remain a mystery.

While peripheral nerves possess regenerative capabilities, regaining full function following injury is extremely limited in humans. Currently, less than 10% of peripheral nerve injury patients achieve full functional recovery (Lopes et al., 2022; Witzel, Rohde, & Brushart, 2005; Zochodne, 2012), with patients commonly facing lifelong functional impairment and neuropathic pain (Balakrishnan et al., 2021; Lopes et al., 2022; Menorca, Fussell, & Elfar, 2013). Understanding the cellular interactions between glial cells and the molecular drivers of regeneration is crucial for developing new therapies and elucidating the regeneration process.

In this introduction, I will review what is known about the development and regeneration of spinal motor nerves and the role that glial cells play in these processes. In the following chapters, I will describe novel molecular drivers of perineurial glial bridging in zebrafish and discuss how elucidating the cellular and molecular mechanisms that modulate

perineurial glial behaviors during regeneration is crucial to further understanding successful motor nerve regeneration.

Spinal Motor Nerve Development

Spinal motor nerve and Schwann cell development

Peripheral nerves contain both motor and sensory axons. Though structurally and functionally similar, these distinct axons transmit different information to and from the CNS and undergo different developmental processes. The zebrafish spinal motor nerve root, the subject of my studies, is a region containing only motor fibers, therefore I will focus specifically on motor nerve development. In zebrafish, spinal motor neurons are specified in the ventral ventricular zone, or pMN domain, of the spinal cord by a variety of morphogens and transcription factors, such as *olig2* and *pax6* (Enriquez et al., 2015; Park, Mehta, Richardson, & Appel, 2002). The cell bodies of these neurons remain in the CNS and their axons project ventrally out into the periphery through the motor exit point (MEP) transition zones. The axons then pathfind distally and are guided to their target cells by environmental cues (Masahira et al., 2006). In zebrafish, primary and secondary spinal motor neurons innervate muscle fibers. Primary motor neurons are specified first and establish the first functional circuits, which control survival functions such as swimming. Three primary motor neurons per segment project axons down through the same MEP into the periphery at approximately 16 hours post fertilization (hpf) (Eisen, 1991). Two of these axons, the caudal primary motoneuron (CaP) and rostral primary motoneuron (RoP), will project ventrally and form the caudal and rostral tracts of the nerve, respectively, whereas the third axon, the middle primary motoneuron (MiP), will project dorsally and form the

middle tract. The CaP motor axon is the first of the primary motoneurons to exit the spinal cord, followed shortly after by the RoP and MiP. Secondary motor neurons project axons through the same MEP a few hours later, following similar paths as the primary axons (Eisen, 1991; Myers, Eisen, & Westerfield, 1986). In total, approximately 70 motor axons will exit through each MEP during motor nerve development (Eisen, 1991; Myers et al., 1986). As these axons project into the periphery and begin forming bundles, various glial cells begin their developmental journeys towards these axon bundles to establish protective layers (Figure 1-1).

One of the first glial cells to interact with developing motor axons are Schwann cells. At the completion of neurulation, the neural crest delaminates from the dorsal neural tube and neural crest cells migrate ventrally along the neural tube, splitting off laterally to become melanocytes and endoneurial fibroblasts, or ventrally to become glial cells, such as Schwann cells, or neurons (Kristjan R. Jessen & Mirsky, 2005). The neural crest cells that eventually associate with axons are Schwann cell precursors. Schwann cell precursors are multipotent, migratory, proliferative, and dependent on survival signals from axons. These cells then differentiate into immature Schwann cells, begin to form a basal lamina and invade the axon bundle. As they mature and undergo radial sorting, individual immature Schwann cells will differentiate into either myelinating or non-myelinating Schwann cells. Myelinating Schwann cells associate with single large diameter axons (>1μm) in a 1:1 ratio and begin to myelinate. Non-myelinating Schwann cells will form Remak bundles with groups of smaller diameter axons (D'Rozario, Monk, & Petersen, 2017; Kristjan R. Jessen & Mirsky, 2005; Kristján R. Jessen et al., 2015). As the cells

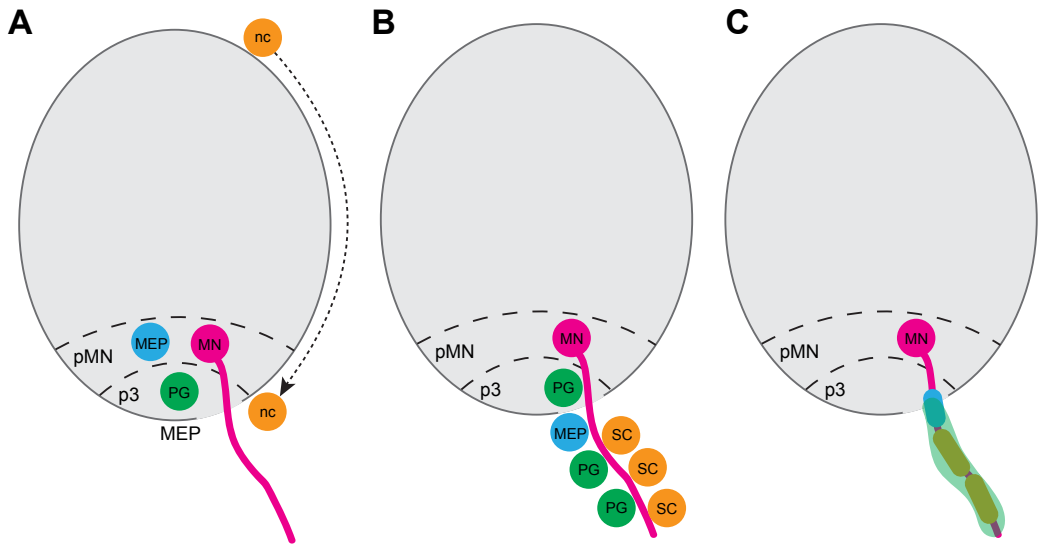


Figure 1-1. Zebrafish spinal motor nerve development.

A-C) Cross section of the spinal cord during development. B) Motor neurons (MN, magenta) and MEP glia (blue) are specified in the pMN domain. Perineurial glia (PG, green) are specified in the p3 domain of the lateral floor plate. As neural crest cells (nc, orange) delaminate ventrally, the motor axons (magenta) exit the spinal cord through the MEP into the periphery. C) PG exit through the MEP and migrate along the motor axons. Neural crest cells associate with the axons and differentiate into Schwann cells (SC, orange). MEP glia traffic cells exiting the spinal cord. D) SC begin forming myelin along axons and PG ensheath axon-SC bundles into fascicles. MEP glia myelinate the dorsal root of the nerve.

differentiate, they begin to rely on autocrine survival as opposed to external cues. The transcription factor Sox10 (SRY(sex determining region Y)-box 10), which is essential for Schwann cell development, is initially expressed in all neural crest cells and remains turned on in Schwann cell precursors (Britsch et al., 2001; Kristjan R. Jessen & Mirsky, 2005). Accordingly, Sox10 null mice and zebrafish *colourless* mutants, which are deficient for *sox10*, do not have Schwann cells along their peripheral nerves (Britsch et al., 2001; Dutton et al., 2001; Kucenas, Takada, et al., 2008).

Later Schwann cell development relies on signaling from motor axons. Neuregulin 1 (Nrg1) is one of the best studied of these signals. Present on peripheral motor axons, the type III isoform is an important signal of Schwann cell survival, growth, proliferation, and migration. Such axonal guidance cues are transmitted to Schwann cells by the receptor tyrosine kinase complex ErbB2/ErbB3, which is expressed by Schwann cells (Garratt, Britsch, et al., 2000). Null mutations of *Nrg1, erbB2,* or *erbB3* in zebrafish and mice cause severe defects in Schwann cell development and myelination (Garratt, Voiculescu, Topilko, Charnay, & Birchmeier, 2000; Lyons et al., 2005). In zebrafish, ErbB2 receptor inhibitors reduced Schwann cell division and perturbed Schwann cell migration (Lyons et al., 2005). ErbB2/ErbB3 works as a heterodimer, with ErbB2 containing the Nrg1 binding domain and ErbB3 possessing intrinsic kinase activity. Mature Schwann cells express ErbB2 and ErbB3, though they become independent of Nrg1 once they have differentiated into a myelinating phenotype (Woodhoo & Sommer, 2008). Though temporally expressed in Schwann cell, expression of *Nrg1* continues into adulthood in both sensory and motor neurons (Velasquez, St John, Nazareth, & Ekberg, 2018). Additionally, Notch signaling is

a positive regulator of Schwann cell precursor maturation (Garratt, Britsch, et al., 2000; Woodhoo & Sommer, 2008). Schwann cell precursors express both Jagged 1 and Notch receptors on their surface while additional Notch ligands are expressed by embryonic axons (Kristján R. Jessen et al., 2015). In mice with inactivation of *Notch1,* Schwann cell generation from precursors is delayed, whereas Schwann cell generation is increased in mice with enhanced Notch signaling (Woodhoo et al., 2009). Thus, Notch likely promotes conversion of Schwann cell precursors into mature Schwann cells. This process may occur indirectly, as Notch increases the levels of ErbB3 receptors in Schwann cell precursors, elevating the effectiveness of Nrg1 signaling (Kristján R. Jessen et al., 2015). Further, G-protein coupled receptor (Gpr126) is located on the surface of Schwann cells and mediates later stages of Schwann cell development, including radial sorting and myelination. In both mouse and zebrafish *Gpr126* mutants, Schwann cells do not undergo terminal differentiation and fail to myelinate axons. However, Schwann cells are still present along the peripheral nerves, indicating that early migration and proliferation are not affected by Gpr126 (Mogha et al., 2013; K. R. Monk et al., 2011; Kelly R. Monk et al., 2009). This data suggests that Gpr126 is essential for later stage maturation of Schwann cells and subsequent myelination of axons (Fernandez, Iyer, & Low, 2017).

The Perineurium

The perineurium is a cellular sheath made up of interdigitated perineurial cells that bundle axons, Schwann cells, and endoneurium into fascicles. Perineurial cells are connected via tight junctions and form concentric layers that function as a component of the blood-nerve-barrier, protecting the nerve from toxins, damage, infection, and ionic changes (Kucenas,

2015; Peltonen, Alanne, & Peltonen, 2013). These perineurial cells characteristically contain pinocytic vesicles and have a double basal lamina, in contrast to fibroblasts which have a single basal lamina (Akert, Sandri, Weibel, Peper, & Moor, 1976; Burkel, 1967; Kucenas, 2015). The transcription factor *NK2 homeobox 2a (nkx2.2a)* is expressed in spinal motor nerve perineurial cells in both mice and zebrafish and is required for perineurial glial specification (Clark et al., 2014; Kucenas, Snell, et al., 2008). Perineurial glia originate in the p3 domain of the spinal cord, also known as the lateral floor plate, and exit the spinal cord at MEP transition zones at approximately 52 hpf in zebrafish and E17.5 in mice (Clark et al., 2014; Kucenas, 2015). *nkx2.2a*$^{+}$ cells migrate peripherally along outgrowing motor axons and are found outside of myelin basic protein (MBP)$^{+}$ Schwann cells. Thus, the motor nerve perineurium is composed of CNS-derived glial cells, perineurial glia, that differentiate into the mature spinal motor nerve perineurium (Kucenas, 2015). These findings were confirmed in mice, indicating conservation of the origin of perineurial cells in mammals (Clark et al., 2014). CNS-derived *nkx2.2a*$^{+}$ perineurial glia are specific to spinal motor nerves and the origin of the sensory nerve perineurium remains unknown. *In vivo,* time-lapse imaging in zebrafish and transgenic imaging in mice shows early migration of perineurial glia from the spinal cord into the periphery during spinal motor nerve development, suggesting that perineurial glia might direct spinal motor nerve development (Clark et al., 2014; Kucenas, 2015). After blocking perineurial glial specification by injecting a morpholino oligonucleotide (MO) to *nkx2.2a* into single-cell embryos, motor axons exit the CNS ectopically and are defasciculated. Motor axon cell bodies were also observed outside the spinal cord, indicating that perineurial glia play an essential role in axon path-finding and transition zone formation. Schwann cells in zebrafish embryos

injected with a *nkx2.2a* MO demonstrate delayed migration along motor nerves and fail to form myelin sheaths, indicating that perineurial glia are important for Schwann cell migration and differentiation during development (Kucenas, 2015).

Very few molecular signals driving perineurial glial development have been identified. *Nkx2.2a* is necessary for perineurial glial specification, as described above. Two other signaling cascades, Notch and Desert Hedgehog (Dhh)-Patched (Ptc), drive perineurial glial development. Notch signaling is essential for early perineurial glial migration from the spinal cord into the periphery (Binari et al., 2013; H. Kim et al., 2008). *Fabp7a,* a brain lipid binding protein essential for differentiation of radial glial progenitor cells and perineurial glia, is expressed in *nkx2.2a*$^{+}$ lateral floor plate cells as well as in perineurial glia that have migrated into the periphery. Further, Notch signaling maintains *fabp7a* expression in perineurial glia during development (H. Kim et al., 2008). Disruption of Notch signaling prior to perineurial glial exit through the MEP disrupts perineurial glial ability to migrate into the periphery. Reciprocally, disruption of Notch signaling after perineurial glial exit leads to defects in perineurial glial differentiation and sheath production (Binari et al., 2013). Therefore, Notch signaling is necessary for perineurial glial migration into the periphery and proper differentiation. Additionally, Dhh-Ptc signaling is important later in perineurial glial development. Dhh is a secreted factor expressed by Schwann cells in both mice and zebrafish (Bitgood & McMahon, 1995; Geuna et al., 2009; Kucenas, Takada, et al., 2008; Parmantier et al., 1999). While *Dhh* is expressed by Schwann cells, its receptor, *Ptc*, is expressed by perineurial glia. *Dhh* mutant mice exhibit normal perineurial glial development and ensheathing of motor axons.

However, older mutant mice exhibit a thin and disorganized perineurium as well as abnormal tight junctions and disturbed blood-nerve-barrier (Parmantier et al., 1999). Interestingly, Gli1, a transcription factor activated downstream of *Dhh,* is necessary for development of the endoneurium but not the perineurium. While *Dhh* mutants disrupt perineurial development, *Gli1* mutants have a normal perineurium. Therefore, perineurial glial development is requires *Dhh* signaling via a canonical, Gli1-independent pathway (Zotter et al., 2022). Together, these studies demonstrate the importance of Notch for proper perineurial glial development and of Dhh-Ptc for perineurium maturation (Binari et al., 2013; Parmantier et al., 1999).

Schwann cell and perineurial glial interactions during development

Perineurial glia and Schwann cells are both essential for spinal motor nerve development (Kristján R. Jessen et al., 2015; Kucenas, Snell, et al., 2008). During development, Notch signaling is required for perineurial glial migration into the periphery, and perturbation to perineurial glial migration by inhibition of Notch signaling adversely affects Schwann cell development and differentiation (Binari et al., 2013). Similarly, perturbing Schwann cell development prevents or delays perineurial glial migration into the periphery. Interestingly, in *erbb3b* mutants, which prevent Schwann cells from associating with axons, perineurial glia exit the spinal cord normally, even in the absence of Schwann cells (A. D. Morris et al., 2017). In the absence of perineurial glia, motor axons exit the spinal cord ectopically and Schwann cells fail to wrap motor nerves (Kucenas, Takada, et al., 2008). Similarly, in *colourless* zebrafish mutants, which lack Schwann cells, perineurial glial migration into the periphery is severely delayed and perineurial glia fail to properly ensheath motor axons

(G. M. Lewis & Kucenas, 2014). In mice lacking Dhh, a Schwann cell-derived morphogen, the perineurium is thin and disorganized and fails to express tight junction proteins, suggesting that Dhh secreted from Schwann cells acts to differentiate the perineurium during development (Parmantier et al., 1999). This is supported by the expression of *ptc,* the *Dhh* receptor, on perineurial glia. Further, perineurial phenotypes in *sox10* mutants were very similar to those in *Dhh* mutants (Kuspert et al., 2012). Because sox10 normally activates Dhh expression in Schwann cells, this data suggests that the similar phenotypes are likely due to lack of *Dhh* expression (Kucenas, 2015; Kuspert et al., 2012). Together, these studies indicate that there are reciprocal interactions between perineurial glia and Schwann cells that are essential for proper spinal motor nerve development.

Organization of the peripheral spinal motor nerve

Peripheral spinal motor nerves are necessary for motor functions and transmit essential signals through action potentials along their axons. Schwann cells, a type of glial cell that can be either myelinating or non-myelinating, protect these axons. Myelin is a lipid rich sheath that insulates the nerve, allowing for fast saltatory conduction of action potentials. Myelinating Schwann cells wrap segments of large diameter axons (>1μm) whereas non-myelinating Schwann cells associate with groups of smaller diameter axons (D'Rozario et al., 2017; Kristjan R. Jessen & Mirsky, 2005; Woodhoo & Sommer, 2008). Non-myelinating Schwann cells create Remak bundles by surrounding the axons and buffering them from each other with their own cytoplasm (Bunge, 2017; Kristján R. Jessen et al., 2015). Smaller diameter axons are not myelinated because they are thought to not require myelin sheaths to increase the speed of axon potential propagation (Kristján R. Jessen et

al., 2015). These axon-Schwann cell complexes are encased by the endoneurium, perineurium, and epineurium (Geuna et al., 2009; Kucenas, 2015; Peltonen et al., 2013; Woodhoo & Sommer, 2008). These three distinct layers all act to protect the nerve and are key components of the blood-nerve barrier and the nerve-tissue barrier. The endoneurium is the innermost layer, encompassing the axon-Schwann cell bundles. It is derived from the neural crest and composed of loose connective tissue embedded with a capillary network . Next, the perineurium, a cellular sheath composed of flat, interdigitated perineurial glia, bundles the endoneurium and axon-Schwann cell complexes into fascicles (Kucenas, Snell, et al., 2008; Peltonen et al., 2013). The number and size of fascicles within a nerve vary considerably from one nerve to another (Stewart, 2003). The perineurium is the main barrier component of the nerve, controlling diffusion between the endoneurium and extrafascicular tissues. This blood-nerve-barrier isolates the endoneurium from circulating blood, thus preventing molecular and ionic molecules from leaking from the blood stream to the peripheral nerve. Perineurial glial protected axon-Schwann cell fascicles are surrounded by the epineurium, the outermost layer of the nerve composed of dense connective tissue and fibroblasts. The epineurium, however, does not act as a barrier. It is composed of collagenous extracellular matrix and contributes to the tensile strength of the nerve (Geuna et al., 2009; Kucenas, Snell, et al., 2008; Peltonen et al., 2013). The final product is an epineurium surrounding nerve fascicles, which contain a perineurium surrounding endoneurial connective tissue, macrophages, blood vessels, fibroblasts, non-myelinating (Remak)-Schwann cells, and axon-myelin Schwann cell bundles (Clark et al., 2014; Kristján R. Jessen et al., 2015; Kucenas, Takada, et al., 2008) (Figure 1-2). The development of spinal motor nerves involves orchestrated interplay of the cell types

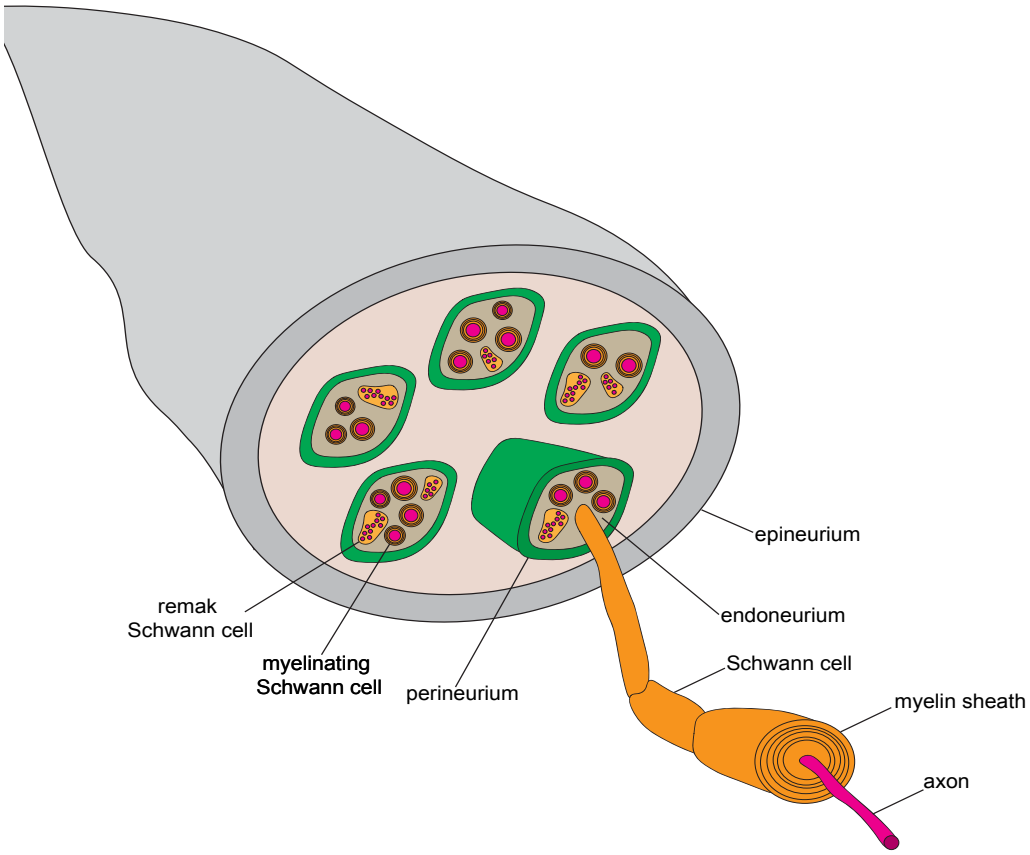


Figure 1-2. Structure of a peripheral spinal motor nerve.

Diagram of a cross section of a peripheral spinal motor nerve illustrating major nerve components. Axons (magenta) are wrapped by myelin sheaths (orange, concentric circles) produced by Schwann cells (orange). These Schwann-cells and myelinated axons, along with unmyelinated axons surrounded by Remak Schwann cells (orange) and the endoneurium (beige), are bundled into fascicles by perineurial glia (green). The outermost layer, the epineurium (gray), surrounds these fascicles.

discussed above. These same cell types that are crucial for spinal motor nerve development are also essential for regeneration of spinal motor nerves after injury.

Peripheral spinal motor nerve injury and regeneration

Injury and motor axon degeneration

Immediately following peripheral spinal motor nerve injury, axons undergo acute axonal degeneration (AAD), where the proximal and distal stumps begin to fragment away from the injury site. Distal stumps then undergo Wallerian degeneration (Waller, 1850), a pattern of anterograde degeneration of both the axon and myelin sheaths resulting in rapid fragmentation of the axon. Axon fragmentation is initiated by an increase in $Ca2^{+}$ influx (Ghosh-Roy, Wu, Goncharov, Jin, & Chisholm, 2010; Wang, Medress, & Barres, 2012) which activates adenylate cyclase to generate cAMP. Increased cAMP levels along with axonal translation of mRNAs initiate the regeneration response mediated by regeneration-associated genes (RAGs) (Chandran et al., 2016; Qiu et al., 2002; Rigoni & Negro, 2020; Twiss, Kalinski, Sachdeva, & Houle, 2016). Such genes are c-Jun (Huebner & Strittmatter, 2009; Raivich et al., 2004) and ATF3, which activate downstream pathways such as JAK-STAT3, PI3K-Akt, Ras-ERK, and Rho-RKO (Chan, Gordon, Zochodne, & Power, 2014; Chandran et al., 2016; Rishal & Fainzilber, 2014). These RAGs are essential for neurite outgrowth and axonal regeneration. While the distal axon stump undergoes Wallerian degeneration, the proximal stump, too, prepares for regeneration. Changes in gene expression in the neuron downregulate constitutively active genes and upregulate RAGs.

New growth cones begin to emerge from the proximal stump axons and regrow along the basal lamina of Schwann cells to re-innervate their targets (Zochodne, 2012). This process of Wallerian degeneration followed by glial support of regeneration is conserved across mammals, *Drosophila*, zebrafish, and other invertebrates (Coleman & Freeman, 2010; G. M. Lewis & Kucenas, 2014; Waller, 1850).

Debris clearance after injury

The final step of Wallerian degeneration is debris clearance, during which phagocytes clear axonal debris and establish a growth permissive environment. Axonal debris is removed predominantly by macrophages, Schwann cells, and perineurial glia (Huebner & Strittmatter, 2009; G. M. Lewis & Kucenas, 2014; Lutz et al., 2017; Nazareth, St John, Murtaza, & Ekberg, 2021; Waller, 1850). In *Drosophila,* glia upregulate expression of engulfment receptors to aid in debris clearance, undergo changes in morphology, and recruit additional cells to the severed axons (Coleman & Freeman, 2010; MacDonald et al., 2006). In zebrafish, macrophages are recruited to the injury site immediately following axonal fragmentation. Macrophages invade the nerve and engulf axonal and myelin debris, effectively removing inhibitory regeneration signals (P. Chen, Piao, & Bonaldo, 2015; K. R. Jessen & Mirsky, 2016). Additionally, macrophages are anti-inflammatory and release regeneration-related factors such as ECM proteins, growth factors, and cytokines, which stimulate regeneration and vascularization and facilitate Schwann cell migration across the wound (Cattin et al., 2015; P. Chen et al., 2015). Delaying nerve fragmentation using Wld^S mutants has no effect on macrophage recruitment, suggesting that invasion of Schwann cells into the injury site and recruitment of macrophages are two distinct mechanisms.

Similarly, macrophage recruitment occurs independently of Schwann cells, though it is thought to be dependent on Schwann cell-derived signals (A. F. Rosenberg, Wolman, Franzini-Armstrong, & Granato, 2012). While macrophages directly infiltrate the injury site, perineurial glia clear axonal debris on both the proximal and distal axonal stumps, with a primary focus on the proximal stump (G. M. Lewis & Kucenas, 2014). Therefore, perineurial glia, macrophages, and Schwann cells spatially coordinate to support rapid debris clearance and establish a growth permissive environment allowing for axonal regeneration.

Perineurial glial bridging

Perineurial cells have long been known as the first cells to enter the injury site following peripheral nerve injury, yet little is known about the cellular or molecular mechanisms that drive their injury response (Schröder, May, & Weis, 1993; Weis, May, & Schröder, 1994). Our lab has demonstrated that following axonal injury in zebrafish, perineurial glia migrate into the injury gap and form a glial bridge that is essential to regeneration. Perineurial glia form this glial bridge early after injury, prior to Schwann cell formation of bands of Bungner and infiltration into the injury site. Perineurial glial bridging is dependent upon the presence of Schwann cells, however it is independent of the presence of macrophages or Wallerian degeneration (G. M. Lewis & Kucenas, 2014). Similarly, studies in mice show that "perineurial fibroblasts" accumulate in the injury site following axotomy (B. Chen, Banton, Singh, Parkinson, & Dun, 2021; Mathon, Malcolm, Harrisingh, Cheng, & Lloyd, 2001; Parrinello et al., 2010; Rigoni & Negro, 2020). Later studies demonstrated that the perineurium includes not fibroblasts, but CNS-derived perineurial glia (Clark et al., 2014;

Kucenas, 2015). Perineurial glial bridging observed in zebrafish is consistent with the immediate response of the "perineurial fibroblasts", indicating that these cells are likely perineurial glia (G. M. Lewis & Kucenas, 2014). Further, perineurial cells may also direct Schwann cell migration across the injury. After nerve injury, EphB2 is activated in Schwann cells and interacts with ephrin-B on "perineurial fibroblasts", allowing for proper migration and accumulation of Schwann cells along the glial bridge. This interaction between Schwann cells and "perineurial fibroblasts" is essential to regeneration (Clements et al., 2017; Parrinello et al., 2010). Therefore, there are clear interactions occurring between perineurial cells and Schwann cells following injury to drive their respective injury responses. However, the mechanisms that drive perineurial bridging, and subsequently the impact that this bridge has on the injury response of Schwann cells, remains poorly understood.

The Schwann cell injury response

After injury, the distal stump of the injured axon degenerates, leaving myelin ovoids behind. As these axons degenerate, Schwann cells convert from myelinating-Schwann cells to a progenitor-like state, producing a distinct repair (Bungner) Schwann cell (Clements et al., 2017; Fernandez et al., 2017; K. R. Jessen & Mirsky, 2016). This transformation switches Schwann function from myelination of axons to phagocytosis of myelin and axonal debris, secretion of neurotrophic factors that promote axonal survival, expression of axonal guidance and adhesive cues for regenerating axons (Clements et al., 2017; K. R. Jessen & Mirsky, 2016), and recruitment of macrophages to clear debris (Cattin et al., 2015; K. R. Jessen & Mirsky, 2016). Following completion of perineurial glial bridging,

Schwann cell processes from both the proximal and distal stumps traverse the injury gap together slightly ahead of the re-growing axons to form Bands of Bungner and guide the regenerating axons across the injury gap (Clements et al., 2017; K. R. Jessen & Mirsky, 2016; G. M. Lewis & Kucenas, 2014; Min, Parkinson, & Dun, 2021; Parrinello et al., 2010). Following regeneration, Schwann cells regain myelinating abilities and begin to re-myelinate the newly established axons (Jeanette et al., 2021; K. R. Jessen & Mirsky, 2016; MacDonald et al., 2006).

The signals driving the Schwann cell injury response and transformation to a repair phenotype are only just beginning to be understood. Schwann cell transdifferentiation is thought to be driven by Ras/Raf/ERK signaling (Harrisingh et al., 2004; Napoli et al., 2012). NRG1/ErbB signaling pathway is also known to regulate myelination during development as well as Schwann cell proliferation, migration, and survival (Min et al., 2021; Nocera & Jacob, 2020). Following injury, Schwann cells express soluble NRG1, and NRG1 and ErbB2/3 receptor levels increase significantly in Schwann cells of the distal nerve stump (Carroll, Miller, Frohnert, Kim, & Corbett, 1997; Nocera & Jacob, 2020). Concurrently, Sox2 regulation of the Slit3/Robo1 pathway drives Schwann cell migration into the nerve bridge and is critical for axon pathfinding following injury (Dun et al., 2019; Nocera & Jacob, 2020). In Schwann cells located distally to the nerve injury, c-Jun is rapidly upregulated facilitates the transformation of myelinating-Schwann cells to Bungner repair Schwann cells (Arthur-Farraj et al., 2017; Kristján R. Jessen et al., 2015). In *c-Jun* knockout mice, Schwann cells in injured distal stumps fail to upregulate key factors driving neural growth and demonstrate delay in myelin breakdown, leading to a failure in

regeneration and functional recovery (Arthur-Farraj et al., 2012). Additionally, without c-Jun, bands of Bungner are structurally disorganized (K. R. Jessen & Mirsky, 2016). c-Jun may be a downstream target of ERK signaling. After nerve injury, Schwann cells have strong activation of the ERK pathway and sustained activation of the ERK pathway in absence of nerve injury drives demyelination and induces an inflammatory response. Further, p38MAPK promotes Schwann cell demyelination and transdifferentiation into repair Schwann cells through downregulation of myelin proteins and upregulation of c-Jun expression. However, it is suggested that MAPK/ERK signaling regulates several processes across multiple pathways during development and following injury, thus the exact role in c-Jun activation remains unknown (Nocera & Jacob, 2020). Gpr126, an essential myelin regulator during development, may also be involved in regulating c-Jun levels after injury and is critical for Schwann cell function during peripheral nerve regeneration. Additionally, intrinsic axonal growth factors encourage regrowth of the proximal stump so the newly regenerating axon makes contact with Schwann cells, which re-differentiate and begin to remyelinate the axon (Fernandez et al., 2017).

In addition to c-Jun, several neurotrophic factors regulate the SC repair program. Neurotrophin mRNA, such as nerve growth factor (NGF), vascular endothelial growth factor (VEGF), and brain derived neurotrophic factor (BDNF), is upregulated by Schwann cells following injury (Pandey & Mudgal, 2021). Low affinity NGF receptor, p75NGFR, increases in both the nerve and Schwann cells following injury, suggesting that these neurotrophic factors secreted by Schwann cells directly interact with axons to promote regrowth. NGF receptors are localized on the surface of Schwann cells in bands of Bungner

and NGF may dissociate from low-affinity Schwann cell receptors to be bound and internalized by high-affinity neuronal receptors following injury. This receptor-mediated intercellular transfer of NGF from Schwann cell receptors to neurons may promote neuronal outgrowth (Frostick, Yin, & Kemp, 1998). The density of NGF receptors diminishes with a time course parallel to axonal regeneration, further implicating NGF and communication between Schwann cells and axons in nerve regrowth (Taniuchi, Clark, & Johnson, 1986). Both c-jun and p75NGFR are also upregulated in human peripheral nerves following injury (Wilcox et al., 2020).

Transforming growth factor-β (TGFβ) is another important regulator of the Schwann cell repair program. Schwann cells both secrete TGFβ-1 following injury and require it for their own regenerative response (Schira et al., 2018). TGF-β and NGF contribute to increased Schwann cell death following injury to neonatal nerves (Kristján R. Jessen et al., 2015). Additionally, interactions between Schwann cells and fibroblasts are crucial for their migration cross the injury site (Clements et al., 2017; Z. Zhang et al., 2016). Fibroblast-derived TGFβ crosstalk with Eph signaling drives collective Schwann cell migration. Loss of TGFβ results in delayed nerve regeneration, while loss of EphB2 impairs Schwann cell migration and exhibits misdirected axonal regrowth (Clements et al., 2017).

Following formation of bands of Bungner and guidance of regenerating axons across the injury gap, Schwann cells re-differentiate into a myelinating phenotype to myelinate the newly regenerated axons (K. R. Jessen & Mirsky, 2016). This transition from repair Schwann cell back into myelinating Schwann cells is regulated by YAP and TAZ signaling

(Jeanette et al., 2021) and maintained by Lrp4-expressing regenerating axons (Gribble, Walker, Saint-Amant, Kuwada, & Granato, 2018). Together, c-Jun, neurotrophic factors, TGFβ, and others signal to Schwann cells to transdifferentiate into Bungner repair cells following axonal injury. Following transdifferentiation, Schwann cells from both proximal and distal ends of the severed stumps clear debris, guide the regenerating axons across the perineurial glial bridge, and remyelinate regenerating axons (Clements et al., 2017; Jeanette et al., 2021; Nocera & Jacob, 2020; Parrinello et al., 2010).

Though many of the signals driving the Schwann cell and perineurial glial injury response remain unknown, it is clear that both cell types play a crucial role in motor axon regeneration. Impairments in either cell type adversely affect the others' ability to respond to injury and leads to defects in axonal regrowth (Clements et al., 2017; G. M. Lewis & Kucenas, 2014; A. D. Morris et al., 2017). Schwann cells are necessary for perineurial glial development and injury response while perineurial glia are necessary for Schwann cell migration, differentiation, and myelination (G. M. Lewis & Kucenas, 2014; A. D. Morris et al., 2017; Reed et al., 2021). Schwann cells and perineurial glia are at the center of cell-cell interactions that are necessary for successful regeneration, yet how these cells are communicating after injury and the signals involved in this process remain a mystery.

Summary of spinal motor nerve injury

In summary, injury to a peripheral spinal motor nerve divides the nerve into a proximal and distal stump. The axonal ends closest to the injury site will quickly undergo AAD, causing the axons to fragment and begin to degenerate away from the injury site. Schwann

cells become activated and begin to transdifferentiate into repair cells that will proliferate and aid in phagocytizing myelin, establishing a growth permissive environment, and guiding axonal regrowth. Distal stumps undergo Wallerian degeneration and the subsequent debris is cleared by Schwann cells, perineurial glia, and macrophages. Perineurial glia begin to extend towards the injury site, eventually establishing a glial bridge across the gap between the two severed stumps. Axon growth cones sprout at the proximal stump and are guided across the injury gap by Schwann cells and perineurial glia. Schwann cells in the distal stump form bands of Bungner, supporting axonal regrowth. Axons re-innervate their targets, Schwann cells re-differentiate and myelinate the new axons, perineurial glia ensheath the axon-Schwann cell bundles, and functional recovery is achieved. Though robust in zebrafish, less than 10% of patients with peripheral nerve damage attain full functional recovery (Gonzalez & Allende, 2021; Zochodne, 2012). Thus, understanding the cellular and molecular mechanisms that underlie effective motor nerve regeneration is critical to developing therapies to enhance regenerative capabilities in humans.

Transforming growth factor-beta (TGFβ) signaling in injury and regeneration

TGFβ signaling plays an important role in regenerative processes (Abarca-Buis, Mandujano-Tinoco, Cabrera-Wrooman, & Krötzsch, 2021; Katsuno & Derynck, 2021). Upon retinal injury in zebrafish, TGFβ is necessary for the expression of essential pro-regenerative transcription factor genes (Conedera et al., 2021; Lenkowski et al., 2013; Sharma et al., 2020). TGFβ-1 also regulates cell proliferation necessary for tissue regeneration in *Xenopus* tails through activation of Smad2/3 (Nakamura et al., 2021).

Similarly, TGFβ signaling promotes cardiac regeneration in zebrafish by enhancing progenitor cell proliferation and regulating epithelial-mesenchymal transition of responsive cells (Bensimon-Brito et al., 2020; Chablais & Jaźwińska, 2012; Peng et al., 2021). TGFβ signaling is also important for regulating inflammation and allowing for regeneration following zebrafish spinal cord injury (Keatinge et al., 2021). Following sciatic nerve injury in mice, TGFβ signaling reprograms wound Schwann cell to invasive mesenchymal-like cells through cross-talk with Ephrin signaling. TGFβ expressed by fibroblasts in the injury site enhances EphB2-mediated cell sorting to promote collective, directional migration of Schwann cells following injury (Clements et al., 2017; Katsuno & Derynck, 2021; Rigoni & Negro, 2020; Wu, Rockel, Lagares, & Kapoor, 2019). Prior studies demonstrate that sox2-mediated Ephrin-B/EphB2 signaling between "perineurial fibroblasts" and Schwann cells following peripheral nerve injury results in cell sorting, followed by directional collective migration of Schwann cells out of nerve stumps to guide re-growing axons across the injury site. Loss of EphB2 impairs Schwann cells migration and results in misdirected axon regrowth (Parrinello et al., 2010). Intriguingly, Schwann cells secrete TGFβ following peripheral nerve injury to enhance debris clearance and suppress fibroblast proliferation, providing a potential source for TGFβ signaling (Schira et al., 2018; Sulaiman & Nguyen, 2016). TGFβ signaling aids in recruitment of macrophages following injury by stimulating the migration of macrophages through RhoA signaling (J. S. Kim et al., 2006). Therefore, TGFβ signaling is crucial for Schwann cell reprogramming, debris clearance, and subsequently proper nerve regeneration.

Connective tissue growth factor (CTGF) is a secreted protein involved in cellular events such as angiogenesis, skeletogenesis, and wound healing (Mokalled et al., 2016; Mukherjee et al., 2021). CTGF is made up of four modules: an amino terminal insulin-like growth factor binding domain (IGFB), a cysteine-rich (CR) domain, a thrombospondin type 1 repeat (TSP1), and a carboxy terminal cysteine knot domain. CTGF directly binds BMP4 and TGFβ-1 through its CR domain. CTGF enhances receptor binding on TGFβ-1 and inhibits BMP4, working in a positive feedback loop to drive TGFβ signaling. At low TGFβ-1 concentrations, CTGF potentiates the phosphorylation of Smad2 induced by TGFβ-1 (Abarca-Buis et al., 2021; Abreu, Ketpura, Reversade, & De Robertis, 2002). CTGF is actively involved in wound healing, and its expression increases following CNS injury in rodents. After injury, CTGF is secreted by M2 macrophages and promotes fibrosis and wound healing (S.-M. Zhang et al., 2021). In zebrafish, the CTGF homolog (CTGFA) is expressed in the floorplate, notochord, and somites (Abreu et al., 2002). Similar to rodent models, CTGF expression is involved in several injury responses in zebrafish. *ctgfa* regulates proliferation and repopulation of damaged tissue in zebrafish cardiomyocytes following injury. Following heart injury, CTGFA is secreted into the extracellular matrix by endocardial cells in the wound site, where it modulates TGFβ/pSmad3 signaling to promote cardiomyocyte proliferation and migration into the injury site. It remains unknown whether this regulation of TGFβ signaling by *ctgfa* is direct or indirect (Mukherjee et al., 2021). Additionally, *ctgfa* expression is both necessary and sufficient for CNS glial bridging following spinal cord injury in zebrafish. This pro-regenerative capacity of CTGFA was mapped to its C-terminal domain (Mokalled et al., 2016). Following sciatic nerve injury, CTGF is secreted by Schwann cells, providing a potential source for CTGF

signaling following spinal motor nerve injury (Schira et al., 2018). Together, *ctgf* is an important regulator of TGFβ signaling and cellular responses following injury. Though the crucial roles of TGFβ-1 and *ctgfa* in regeneration are known, it remains unknown how these signals affect the perineurial glial injury response.

Zebrafish as a model organism for peripheral nerve regeneration

Zebrafish (*Danio rerio)* are a well-established vertebrate model originally used primarily in embryonic studies due to their transparent, ex utero development. Now, zebrafish are one of the most commonly utilized vertebrate model organisms for biomedical research (Grunwald & Eisen, 2002; Meyers, 2018), as they have quick reproduction cycles that produce hundreds of transparent embryos, which are easily manipulated both genetically and pharmacologically and allow for unrivaled *in vivo* imaging (Lin, Chiang, & Tsai, 2016). I used zebrafish in my studies because they have the ability to regenerate injured peripheral spinal motor nerves naturally, making them an optimal nerve injury study model (Gonzalez & Allende, 2021). Further, their transparency and ease in genetic and pharmacological manipulation allows for quick and efficient tagging of specific structures, cell types, and factors, inhibition of signals, performance of injuries, and acquisition of live *in vivo* time lapses of regeneration and the injury response (Gwendolyn M. Lewis & Kucenas, 2013).

In this dissertation, I characterize the cellular and molecular mechanisms that drive perineurial glial bridging after spinal motor nerve injury in zebrafish. Though we know perineurial glial bridging is essential for proper regeneration, little is known about the

signals that drive this bridging (G. M. Lewis & Kucenas, 2014). In Chapter III, I expand upon what is known about perineurial glial bridging by delving into the specifics of bridging dynamics. Additionally, I demonstrate that TGFβ signaling drives perineurial glial bridging initiation using pharmacological perturbation and *in vivo* fluorescent confocal imaging after injury to the spinal motor nerve. Finally, I describe how this TGFβ signaling is in part regulated by a positive feedback loop with *ctgfa* signaling.

In Chapter IV, I investigate the influence Schwann cells have on perineurial glial bridging and begin to develop tools that will facilitate deeper exploration of the role of TGFβ signaling in perineurial glial bridging.

Taken together, my studies presented in this dissertation identify the first known molecular drivers of perineurial glial bridging and offer a novel role for TGFβ signaling in peripheral spinal motor nerve regeneration.

CHAPTER II

Materials and Methods

Fish Husbandry

All animal studies were approved by the University of Virginia Institutional Animal Care and Use Committee. Zebrafish strains used in this study include: AB*, *Tg(nkx2.2a:megfp)*^{vu17} (Kirby et al., 2006; Kucenas, Snell, et al., 2008), *Tg(olig2:dsred2)*^{vu19} (Kucenas, Snell, et al., 2008), *Tg(nkx2.2a(-3.5):nls-egfp)*^{uva1} (Fontenas & Kucenas, 2021), *Tg(nkx2.2a(-3.5):nls-mcherry)*^{uva2} (Zhu et al., 2019), *Tg(sox10(-7.2):megfp)*^{sl3} (Kirby et al., 2006), *Tg(mpeg1:egfp)*^{gl22} (Ellett, Pase, Hayman, Andrianopoulos, & Lieschke, 2011), *Tg(nkx2.2a(-3.5):gal4-VP16;UAS:NTR-mcherry)*^{uva4}, *Tg(ctgfa:egfp)*^{pd96} (Mokalled et al., 2016), *ctgfa*^{bns50} (Mokalled et al., 2016), *Tg(nkx2.2a(-3.5):gal4-VP16;UAS:lifeact-GFP)*^{mu271} (Helker et al., 2013), *Tg(sox10:gal4;UAS:NTR-mcherry)*^{el159} (Das & Crump, 2012), *Tg(12xSBE:egfp)*^{ia16} (Casari et al., 2014), and *Tg(nkx2.2a(-3.5):rtTA;cmlc2:egfp)* (this paper) (Table 2-1). Embryos were produced by pairwise natural matings, raised at 28.5ºC in egg water, staged according to hours or days post fertilization (hpf or dpf, respectively), and screened for correct fluorescence of transgenic lines. Embryos of either sex were used for all experiments (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Embryos used for microscopy or immunohistochemistry were treated at 24 hpf with 0.003% phenylthiourea (PTU) in egg water to reduce development of pigmentation.

Table 2-1: Zebrafish lines used in this study and their genotypes

Full Name	Abbreviation	Reference
AB*	AB*	
Tg(nkx2.2a:megfp)^{vu17}	*nkx2.2a:megfp*	Kirby et al., 2006; Kucenas et al., 2008a
Tg(olig2:dsred2)^{vu19}	*olig2:dsred*	Kucenas et al., 2008a
Tg(nkx2.2a(-3.5):nls-egfp)^{uva1}	*nkx2.2a:nls-egfp*	Fontenas & Kucenas, 2021
Tg(nkx2.2a(-3.5):nls-mcherry)^{uva2}	*nkx2.2a:nls-mch*	Zhu et al., 2019
Tg(sox10(-7.2):megfp)^{sl3}	*sox10:megfp*	Smith et al., 2014
Tg(mpeg1:egfp)^{gl22}	*mpeg1:egfp*	Ellett et al., 2011
Tg(nkx2.2a(-3.5):gal4-VP16;UAS:NTR-mcherry)^{uva4}	*nkx2.2a:gal4;UAS:NTR-mch*	Arena et al., 2022
Tg(nkx2.2a(-3.5):gal4-VP16;UAS:NTR-lifeact-GFP)^{mu271}	*nkx2.2a:gal4;UAS:lifeact*	Helker et al., 2013
Tg(ctgfa:egfp)^{pd96}	*ctgfa:egfp*	Mokalled et al., 2016
ctgfa^{bns50}	*ctgfa*	Mokalled et al., 2016
Tg(sox10:gal4;UAS:NTR-mcherry)^{el159}	*sox10:gal4;UAS:NTR-mch*	Das & Crump, 2012
Tg(12xSBE:egfp)^{ia16}	*12xSBE:egfp*	Casari et al., 2014
Tg(nkx2.2a(-3.5):rtTA;cmlc2:egfp)	*nkx2.2a:rtTA*	This paper

Generation of transgenic lines

All constructs were created using the Tol2kit Gateway-based cloning system (Kwan et al., 2007). Vectors for making the expression constructs were p5E-nkx2.2a(-3.5) (Pauls, Zecchin, Tiso, Bortolussi, & Argenton, 2007), p5E-sox10(-4.9) (Carney et al., 2006), p5E-b-actin2 (Kwan et al., 2007), p5E-dA_nls-mCherry-biTRE (L. J. Campbell, Willoughby, & Jensen, 2012), pME-Gal4-VP16 (Kwan et al., 2007), pL1L2-rtTA (L. J. Campbell et al., 2012), pME-DNtgfbr2b (this paper), p3E-EGFPpA (Kwan et al., 2007), and p3E-polyA (Kwan et al., 2007), which were inserted into either a pDesTol2CG2 (Kwan et al., 2007) or pDesTol2pACryGFP (Berger & Currie, 2013) destination vector through an LR reaction (Kwan et al., 2007). Final constructs were amplified and sequenced using Sanger sequencing to confirm correct insertion. All Sanger sequencing described in this paper was conducted through GENEWIZ (Azenta Life Sciences; https://www.genewiz.com/en). To generate a stable transgenic line, plasmid DNA was microinjected at a concentration of 20 ng/µL in combination with 100 ng/µL *Tol2* transposase mRNA into zebrafish embryos at the one-cell stage. Injected embryos were then screened for founders (Kawakami, 2004).

To generate the *Tg(nkx2.2a:gal4-VP16;UAS:NTR-mcherry)* line, *UAS:NTR-mcherry* plasmid DNA was microinjected at a concentration of 20 ng/µL in combination with 100 ng/µL *Tol2* transposase mRNA into *Tg(nkx2.2a:gal4-VP16;cmlc2:egfp)*^{uva4} embryos at the one-cell stage. Injected embryos were then screened for founders (Kawakami, 2004).

Generation of DNtgfbr2b mutation

To generate the dominant negative mutation, the kinase domain in the TGFβ receptor-II was deleted by introducing an early stop codon, rendering the receptor inactive but retaining its ability to bind TGFβ-1 and form a heterodimer with TGFβ receptor-I. This was achieved through polymerase chain reaction (PCR) using Phusion High-Fidelity DNA polymerase (ThermoFisher Scientific; catalog #F-530S) and *tgfbr2b* cDNA, synthesized using RT-PCR as described below. Though zebrafish have two *tgfbr2* alleles, *tgfbr2a* and *tgfbr2b*, *tgfbr2b* was targeted because *tgfbr2b* has known expression in the nervous system whereas *tgfbr2a* does not (ZFIN). The primers used for the PCR reaction to induce an early stop codon in *tgfbr2b* were: forward: 5'-ATGGAGCGATATCAGCTTTCTG-3' and reverse: 5'-TAAGGGCAGCGGATCCATGTTG-3'. The expected PCR product, further labeled DNtgfbr2b, is 696 bp. The product size was confirmed by running the PCR products on a 1% agarose gel and the sequence was confirmed using the above primers and Sanger sequencing.

To generate the pME:DNtgfbr2b construct, 3' overhangs were added to the DNtgfbr2b PCR product using Amplitaq DNA polymerase with Buffer I (ThermoFisher Scientific; catalog #N8080160) and incubating for 15 minutes at 72ºC. The PCR product was then sub-cloned into an 8/GW/topo vector (Invitrogen; catalog #K250020) to create the pME:DNtgfbr2b vector. The resulting pME:DNtgfbr2b vectors were then transformed into chemically competent *E. Coli* for amplification and isolated with the QIAprep spin miniprep kit (QIAGEN; catalog #27106). The total construct size is ~3,400 bp and was confirmed on a 1% agarose gel. pME:DNtgfbr2b clones with correct construct size were

sequenced using Sanger sequencing with a M13 primer to confirm correct insertion direction.

RT-PCR

mRNA was extracted from 3 dpf AB* zebrafish larvae with the use of the RNeasy mini kit (QIAGEN; catalog #74104). cDNA libraries were synthesized from the extracted mRNA (Peterson & Freeman, 2009) with the use of the High-capacity cDNA Reverse Transcription kit (Applied Biosciences; catalog #4368814). Using the cDNA as a template, PCR was performed using GoTaq green master mix (Promega; catalog #M7122) and the following primers to amplify *tgfbr2b*: forward: 5'-ATGCTGTGCTCTGCTGGTGGAC-3' and reverse: 5'-TAGAAGGCACAGTCGAGG-3'. The product was analyzed on a 1% agarose gel and sequenced using the above primers and Sanger sequencing.

***In vivo* imaging**

At 24 hpf, all embryos used for imaging were treated with 0.003% PTU in egg water to reduce pigment formation. Embryos were screened for correct fluorescence and manually dechorionated. At specified stages, embryos and larvae were anesthetized using 3-aminobenzoic acid esther (Tricaine) and mounted in 0.8% low-gelling point agarose in 4-well glass bottom 35 mm Petri dishes (Fisher, Greiner Bio-One). Following mounting, Petri dishes were filled with egg water containing Tricaine. *In vivo* imaging was conducted on a motorized Zeiss AxioObserver Z1 microscope equipped with Quorum WaveFX-XI (Quorum Technologies) or Andor CSU-W (Andor Oxford Instruments Plc.) spinning disk confocal system. A 40X water objective (NA=1.1) was used to capture all images and time-

lapses. Time-lapses were set to image in 5-minute intervals for 6 to 12 hours, depending on the experiment. For experiments using *ctgfa*^{bns50} larvae, time-lapses were set to image in 2-hour intervals for 10 hours. Z stacks of 1 to 2 μm were collected for each image in a time-lapse. Image processing and limited adjustments were made using MetaMorph software and FIJI (ImageJ).

Nerve Transection

Nerve transections were preformed using a nitrogen-dye (435nm) pumped MicroPoint laser (Andor technology) connected to a spinning disk confocal system (Quorum Technologies) controlled by MetaMorph as previously published (G. M. Lewis & Kucenas, 2014; Gwendolyn M. Lewis & Kucenas, 2013; A. D. Morris et al., 2017; A. F. Rosenberg et al., 2012). Injuries were conducted using either a 40X water (NA=1.1) or 63X water (NA=0.8) objective. Ablation power ranged from 40 to 60 depending on the size of the nerve, the mounting of the larvae, the age of the larvae, and the age of the nitrogen-dye. For all experiments, one to three nerves were injured per larva within hemisegments 4 to 16, creating an approximately 10 μm injury. Nerves with injuries larger than 10 μm or without a full transection were not included in analyses. To transect nerves, an ellipse was virtually drawn around the desired injury site on an image of the nerve in MetaMorph. The laser was pulsed within the designated region of interest (ROI) until the nerve was injured. Injuries were confirmed by presence of axonal debris and lack of return of motor neuron fluorescence in the ROI after 20 seconds. *In vivo* imaging of transected nerves was conducted as described above. In fish that were fixed for antibody staining following nerve transection, the first ten nerves in each fish were injured.

Drug treatments

Drug treatments were performed in 24-well plates with up to 5 larvae per well. Unless otherwise noted, larvae were treated with either drug or control dimethyl sulfoxide (DMSO) (Sigma, catalog #D2438) solutions beginning at 4 dpf and placed in an incubator in dark conditions at 28.5ºC for approximately 24 hours. Fresh drug or DMSO solutions were replaced at 5 dpf and larvae were placed back in the 28.5ºC incubator in dark conditions for an additional 24 hours. At 6 dpf, larvae were treated with fresh drug or DMSO solutions and mounted for imaging. Tricaine was added to drug or DMSO solutions for imaging, and larvae were submerged in these solutions for the entirety of imaging.

TGFβ Inhibition

Larvae were incubated in either 1.5 mL of 10 μM SB431542 (Fisher; catalog #16141), a selective inhibitor of TGFβ receptor-I (A. D. Morris et al., 2017; Sun et al., 2006), in 1% DMSO in PTU egg water or 1.5 mL of 1% DMSO in PTU egg water.

ErbB2/ErbB3 Inhibition

Larvae were incubated in either 1.5 mL of 4 μM of AG1478 (Sigma; catalog #658552), a selective inhibitor of the ErbB2/ErbB3 heterodimer (Lyons et al., 2005), in 1% DMSO in PTU egg water or 1.5 mL of 1% DMSO in PTU egg water.

Ronidazole treatment

Larvae were incubated in either 1.5 mL of 2 mM of ronidazole (Lai et al., 2021) (Sigma; catalog #R7635-5G) in PTU egg water or 1.5 mL of PTU egg water at 28.5ºC in dark conditions for 6 hours prior to imaging and for the duration of imaging.

Doxycycline treatment

Larvae were incubated in either 1.5 mL of 20 μM doxycycline hyclate (L. J. Campbell et al., 2012) (Sigma, catalog #D9891) in 1% DMSO in PTU egg water or 1.5 mL of 1% DMSO in PTU egg water at 28.5ºC in dark conditions for 24 hours prior to imaging and for the duration of imaging.

Whole mount immunohistochemistry

Following injury, 5 to 6 dpf larvae were fixed in AB Fix (4% PFA, 0.1% Triton X-100, 1X PBS) for either 3 hours at 23ºC or overnight at 4ºC. Fixed larvae were then washed for 5 minutes with PBSTx (1% Triton X-100, 1X PBS) followed by a 5-minute wash with DWTx (1% Triton X-100, distilled water), a 5-minute wash with acetone at 23ºC, a 10-minute wash with acetone at -20ºC, and three 5-minute washes with PBSTx. Larvae were pre-blocked in 5% goat serum/PBSTx for a minimum of 1 hour at 23ºC and incubated in primary antibody for 1 hour at 23ºC and overnight at 4ºC. The primary antibodies used in these studies include the following: a rabbit antibody to anti-phospho-Smad3 (1:175, Abcam; catalog #ab52903) (Casari et al., 2014) and a chicken antibody to anti-GFP (1:200, Abcam; catalog #ab13970). Larvae were washed with 1X PBSTx, pre-blocked in 5% goat serum/PBSTx for a minimum of 1 hour at 23ºC and incubated in secondary antibody for 1

hour at 23ºC and overnight at 4ºC. Secondary antibodies used in these studies include the following: goat anti-rabbit IgG (H+L) cross-absorbed secondary antibody, Alex Fluor 647 (ThermoFisher Scientific; catalog #A-21244) and goat anti-chicken IgY (H+L) cross-absorbed secondary antibody, Alexa Fluor 488 (ThermoFisher Scientific; catalog #A-11039). Larvae were washed with 1X PBSTx and stored in 50% glycerol-PBS at 4ºC until imaging. Larvae were mounted on their sides in 0.8% low-gelling point agarose on glass-bottom 35 mm Petri dishes and imaged using the confocal microscope and techniques described above. Image processing and alterations were limited to contrast enhancement and level settings were made using MetaMorph software and FIJI (ImageJ). 3D renderings were produced using Imaris (Oxford Instruments).

In Situ Hybridization

Larvae were fixed in 4% PFA at 4ºC overnight and stored in 100% methanol at -20ºC and processed for *in situ* RNA hybridization as previously described (Hauptmann & Gerster, 2000). Plasmids were linearized with appropriate restriction enzymes and cRNA preparation was carried out using Roche DIG-labeling reagents and RNA polymerases (NEB). For these studies, I used the previously published *krox20* probe (Kelly R. Monk et al., 2009). Images were obtained using a Zeiss AxioObserver inverted microscope equipped with Zen. Image processing and alterations were limited to contrast enhancement and level settings and were conducted in FIJI (ImageJ).

Genotyping

DNA samples were prepared for individual *ctgfa*^{bns50} larvae after imaging by digesting larvae using HotSHOT (hot sodium hydroxide and tris) (Meeker, Hutchinson, Ho, & Trede, 2007) and were amplified using the primers: forward 5'-CATCTCCGTCCCACAGCCA-3' and reverse 5'-ACAGCACCGTCCAGACACG-3' (Mokalled et al., 2016).

Table 2-2: Key Resources

REAGENT or RESOURCE	SOURCE	IDENTIFIER	Additional Information
Antibodies			
Rabbit anti-phospho-Smad3	Abcam	CAT#ab52903	1:175
Chicken anti-GFP	Abcam	CAT#ab13970	1:200
Goat anti-rabbit IgG (H+L) cross-absorbed secondary antibody, Alexa Fluor 647	ThermoFisher Scientific	CAT#A-21244	1:200
Goat anti-chicken IgY (H+L) cross-absorbed secondary antibody, Alexa Fluor 488	ThermoFisher Scientific	CAT#A-11039	1:200
Chemicals, Peptides, and Recombinant Proteins			
Dimethyl sulfoxide (DMSO)	Sigma	CAT#D2438	1%
SB431542	Fisher	CAT#16141	10 µM, 1% DMSO
AG1478	Sigma	CAT#658552	4 µM, 1% DMSO
Ronidazole	Sigma	CAT#R7635-5G	2 mM
Doxycyline hyclate	Sigma	CAT#D9891	20 µM, 1% DMSO
1-Phenyl-2-thiourea (PTU)	Sigma	CAT#P7629	
Oligonucleotides			
5'-ATGGAGCGATATCAGCTTTCTG-3'	This paper	DNtgfbr2b forward primer	

5'-TAAGGGCAGCGGATCCATGTTG-3'	This paper	DNtgfbr2b reverse primer
5'-ATGCTGTGCTCTGCTGGTGGAC-3'	This paper	*tgfbr2b* cDNA forward primer
5'-TAGAAGGCACAGTCGAGG-3'	This paper	*tgfbr2b* cDNA reverse primer
5'-CATCTCCGTCCCACAGCCA-3'	(Mokalled et al., 2016)	*ctgfa*^{bns50} forward primer
5'-ACAGCACCGTCCAGACACG-3'	(Mokalled et al., 2016)	*ctgfa*^{bns50} reverse primer

Recombinant DNA		
p5E-nkx2.2a(-3.5)	(Pauls et al., 2007)	
p5E-sox10(-4.9)	(Carney et al., 2006)	
p5E-b-actin2	(Kwan et al., 2007)	
p5E-dA_nls-mCherry-biTRE	(L. J. Campbell et al., 2012)	
pME-Gal4-VP16	(Kwan et al., 2007)	
pME-DNtgfbr2b	This paper	
pL1L2-rtTA	(L. J. Campbell et al., 2012)	
p3E-EGFPpA	(Kwan et al., 2007)	
p3E-polyA	(Kwan et al., 2007)	
pDesTol2CG2	(Kwan et al., 2007)	
pDesTol2pACryGFP	(Berger & Currie, 2013)	
Software		
MetaMorph	Molecular Devices	http://moleculardevices.com/

FIJI (ImageJ)	NIH	https://imagej.net/
Prism 9.2	GraphPad	http://Graphpad.com
Imaris 9.8.0	Oxford Instruments	https://imaris.oxinst.com/
Illustrator	Adobe	https://adobe.com

Data quantification and statistical analyses

Data Collection

None of the data collected in these studies were blinded, with the exception of the experiments conducted in *ctgfa*^{bns50} larvae. For studies conducted with *ctgfa*^{bns50} larvae, 5 or 6 dpf larvae were injured using the nerve transection assay described above. For live imaging experiments, injured *ctgfa*^{bns50} larvae were time-lapse imaged every 2 hours for 6 hpi. At 6 hpi, the larvae were unmounted and digested using HotSHOT (Meeker et al., 2007) for genotyping. Time-lapse movies were analyzed prior to completion of genotyping. For pSmad3 antibody staining assays, injured *ctgfa*^{bns50} larvae were unmounted and fixed at 3 hpi for antibody staining. Following completion of antibody staining and imaging of injured nerves, *ctgfa*^{bns50} larvae were digested using HotSHOT for genotyping. *ctgfa*$^{-/-}$ larvae are morphologically indistinguishable from *ctgfa*$^{+/-}$ and *ctgfa*$^{+/+}$ larvae and therefore, require genotyping for identification.

Quantification of perineurial glial and Schwann cell bridging

To quantify and compare the efficacy of bridging in either *nkx2.2a:mefp* or *sox10:megpf* larvae treated with either 10 μM SB431542 or 1% DMSO, I calculated the ratio of the size of the bridge across the injury gap compared to the size of the original injury gap. Values greater than 1 indicated that perineurial glia (Figure 3-3G) or Schwann cells (Figure 3-3H) had fully bridged the original injury gap and continued to bridge past the initial distal

stump. Values less than 1 indicated that perineurial glia or Schwann cells had not fully bridged the original injury gap, with negative values indicating the cells had retracted away from the injury site. The size of the initial injury gap was measured using the manual measure tool in FIJI (ImageJ) from a still image from an 8-hour time-lapse movie at 0 HPI. The injury gap bridged was determined by measuring the length of the bridge using the manual measure tool in FIJI (ImageJ) at each time interval in each time-lapse movie. Measurements were taken from still images taken in 30-minute intervals over an 8-hour time-lapse movie. The bridging measurements at each 30-minute interval were then compared to the initial injury gap measurement of the corresponding time-lapse movie (measured bridge size/measured size of initial injury gap). This process was repeated for each time-lapse movie captured for each group. These values were then plotted over time in 30-minute intervals for the duration of the 8-hour time-lapse movie, with each dot representing the ratio of *injury gap bridged:initial injury gap* at a given time-point and with each line connecting the individual time-points from a single time-lapse movie. Each line represents an 8-hour time-lapse movie of a single injury spinal motor nerve in a single 5 or 6 dpf larvae. Lines that end prior to the 8-hour mark indicate time-lapse movies in which the injured larva died or the injured nerve shifted out of the imaging plane before the 8-hour time point.

Quantification of perineurial glial velocity

To quantify and compare the velocity of perineurial glial bridging in larvae treated with either 10 μM SB431542 or 1% DMSO, the position of the edge of either the proximal or distal perineurial glial stump from an individual time-lapse movie were tracked over each

time point using the manual tracking plugin in FIJI (ImageJ). The positioning of the perineurial glial proximal and distal stumps was independently measured in 5-minute intervals for the first 200 minutes post injury (mpi). The manual tracking plugin provides velocity measurements for each time point tracked. Each tracking was repeated three times and an average of the velocities was taken for each time point. This process was repeated for each time-lapse movie captured for each group (SB431542 or DMSO treated larvae in proximal (proximal only), proximal (proximal and distal), and distal (proximal and distal) groups). The average velocity from each time-lapse movie in a group was then averaged at each time point and plotted over time, with each dot representing the average velocity at each time point in 5-minute intervals and the lines connecting each group. Individual graphs were plotted for average velocity measurements over time from the proximal stump in nerves that had proximal only bridging (Figure 3-6C) or both proximal and distal bridging (Figure 3-6D). Average velocity measurements over time from the distal stump in nerves that had both proximal and distal bridging were plotted separately as well (Figure 3-6E). To compare the average velocities between DMSO and SB431542-treated groups over the first 200 mpi, the average velocities for each group at each time point over the 200 mpi time-lapse movie were plotted for both proximal and distal measurements. An unpaired *t*-test was conducted for each plot to determine any significance between the average velocities of DMSO or SB431542-treated groups (Figure 3-6F-H).

Statistical analyses

All graphically presented data represent the mean of the analyzed data. Statistical analyses and graphing were performed using GraphPad Prism software. P-values involving only two

groups were calculated using an unpaired student's *t*-test. Significance levels were determined using a confidence interval of 95%. The data in the plots and in the text are presented as means ± SEM. Other statistical details, such as sample size and p-value, are labeled on the figures or can be found in the figure legends.

CHAPTER III

Transforming growth factor-beta signaling modulates perineurial glial bridging following peripheral spinal motor nerve injury in zebrafish

Abstract

Spinal motor nerves are necessary for organismal locomotion and survival. In zebrafish and most vertebrates, these peripheral nervous system structures are composed of bundles of axons that naturally regenerate following injury. However, the cellular and molecular mechanisms that mediate this process are still only partially understood. Perineurial glia, which form a component of the blood-nerve barrier, are necessary for the earliest regenerative steps by establishing a glial bridge across the injury site as well as phagocytosing debris. Without perineurial glial bridging, regeneration is impaired. In addition to perineurial glia, Schwann cells, the cells that ensheath and myelinate axons within the nerve, are essential for debris clearance and axon guidance. In the absence of Schwann cells, perineurial glia exhibit perturbed bridging, demonstrating that these two cell types communicate during the injury response. While the presence and importance of perineurial glial bridging is known, the molecular mechanisms that underlie this process remain a mystery. Understanding the cellular and molecular interactions that drive perineurial glial bridging is crucial to unlocking the mechanisms underlying successful motor nerve regeneration. Here, using laser axotomy and *in vivo* imaging in zebrafish, I show that transforming growth factor-beta (TGFβ) signaling modulates perineurial glial bridging. Further, I identify *connective tissue growth factor-a (ctgfa)* as a downstream effector of TGFβ signaling that works in a positive feedback loop to drive perineurial glial

bridging. Together, my studies present a the first known signaling pathway involved in the perineurial glial injury response and further characterize the dynamics of the perineurial glial bridge.

Introduction

Motor nerves, the peripheral nerves that control locomotion, require perineurial glia, the protective barrier of the nerve, and Schwann cells, peripheral myelinating cells, for development (Binari et al., 2013; Clark et al., 2014; Kristján R. Jessen et al., 2015; Kucenas, 2015; Kucenas, Takada, et al., 2008). Following axonal projection from the central nervous system (CNS) through motor exit point transition zones into the peripheral nervous system (PNS), individual neural crest-derived Schwann cell precursors hone towards axons in the periphery, associate with them in a 1:1 ratio, and differentiate into myelinating Schwann cells (Jessen and Mirsky, 2005; Jessen et al., 2015; D'Rozario et al., 2017). In zebrafish and mice, the perineurium, made of floor plate-derived perineurial glia, ensheaths these axon-Schwann cell bundles into fascicles, protecting the nerve by establishing a component of the blood-nerve-barrier (Clark et al., 2014; Kucenas, 2015; Peltonen et al., 2013). Perturbation to perineurial glial migration into the periphery adversely affects Schwann cell development and differentiation (Binari et al., 2013). Reciprocally, perturbing Schwann cell development prevents or delays perineurial glial migration into the periphery (A. D. Morris et al., 2017). In the absence of perineurial glia, motor axons exit the spinal cord ectopically and Schwann cells fail to ensheath motor nerves (Kucenas, Takada, et al., 2008). Similarly, in *colourless* zebrafish mutants, which lack Schwann cells due to a mutation in *sox10*, perineurial glial migration into the periphery

is delayed and these cells fail to properly ensheath motor nerves (G. M. Lewis & Kucenas, 2014). Therefore, reciprocal cellular and molecular interactions between Schwann cells and perineurial glia during development are essential for motor nerve development. These same glial cells that are crucial for proper spinal motor nerve development play essential cellular and molecular roles in modulating regenerative responses after nerve injury to drive successful and effective regeneration (Cattin & Lloyd, 2016; Gonzalez & Allende, 2021; Kristjan R. Jessen & Mirsky, 2019; G. M. Lewis & Kucenas, 2014).

Immediately following peripheral motor nerve injury, axons experience acute axonal degeneration, where the proximal and distal stumps of the nerve degenerate away from the injury site. The distal stump then undergoes Wallerian degeneration, a highly conserved pattern of anterograde degeneration of both the axon and associated myelin sheaths, resulting in rapid fragmentation of the axon (Coleman & Freeman, 2010; G. M. Lewis & Kucenas, 2014; Villegas et al., 2012; Waller, 1850). Axonal and myelin debris is then removed by macrophages, Schwann cells, and perineurial glia, establishing a growth permissive environment (Cattin & Lloyd, 2016; Huebner & Strittmatter, 2009; G. M. Lewis & Kucenas, 2014; Waller, 1850).

The perineurium has long been suspected to play an active role in motor nerve regeneration (Behrman & Acland, 1981; Popović, Bresjanac, & Sketelj, 1994; Schröder et al., 1993). Previous studies from our lab discovered that following spinal motor nerve injury in zebrafish, perineurial glia clear debris and form a glial bridge across the injury site that is essential for regeneration, and this process precedes Schwann cell infiltration into the

injury site (G. M. Lewis & Kucenas, 2014). As axons degenerate and perineurial glia begin to bridge, Schwann cells transform into a progenitor-like state, producing a distinct repair (Bungner) Schwann cell. Schwann cell function switches from myelination of axons to phagocytosis of myelin and axonal debris as well as collective migration, forming Bands of Bungner that guide regenerating axons across the injury site. Following regeneration, Schwann cells re-differentiate and begin to myelinate the newly established axons (Fernandez et al., 2017; Kristjan R. Jessen & Mirsky, 2019; Parrinello et al., 2010; Webber & Zochodne, 2010). Together, Schwann cells and perineurial glia provide essential physical structures that are necessary for proper motor nerve regeneration. Though many studies have explored the Schwann cell injury response (Clements et al., 2017; K. R. Jessen & Mirsky, 2016; Allison F. Rosenberg, Isaacman-Beck, Franzini-Armstrong, & Granato, 2014; Schira et al., 2018), the cellular and molecular mechanisms that drive perineurial glial behaviors essential for motor nerve regeneration remain unknown.

Transforming growth factor beta-1 (TGFβ-1) is an important regulator of regenerative processes (Abarca-Buis et al., 2021; Katsuno & Derynck, 2021; Sulaiman & Nguyen, 2016). In particular, Schwann cells both secrete TGFβ-1 following peripheral nerve injury to enhance debris clearance and suppress fibroblast proliferation (Schira et al., 2018; Sulaiman & Nguyen, 2016) as well as require TGFβ signaling to successfully migrate across the injury site (Clements et al., 2017). Therefore, TGFβ signaling is crucial for Schwann cell reprogramming, debris clearance, and subsequent nerve regeneration. Connective tissue growth factor (CTGF) is another secreted protein involved in angiogenesis and wound healing (Mokalled et al., 2016; Mukherjee et al., 2021). CTGF

directly binds TGFβ-1 and enhances receptor binding, working in a positive feedback loop to drive TGFβ signaling. At low TGFβ-1 concentrations, CTGF even potentiates the phosphorylation of Smad2 induced by TGFβ-1 (Abarca-Buis et al., 2021; Abreu et al., 2002). Previous studies demonstrate that CTGF is actively involved in regenerative processes including driving glial bridging after spinal cord injury (Mokalled et al., 2016) and modulating TGFβ/pSmad3 signaling to promote cardiac regeneration (Mukherjee et al., 2021). Additionally, CTGF is secreted by Schwann cells following peripheral nerve injury (Schira et al., 2018). Therefore, CTGF is an important regulator of TGFβ signaling and cellular responses following injury. Though crucial roles of both TGFβ-1 and CTGF in regeneration are known, it remains unknown how these signals might be affecting perineurial glial responses following peripheral motor nerve injury.

While peripheral motor nerves possess regenerative capabilities, regaining full function following injury is extremely limited in humans. Currently, less than 10% of peripheral nerve injury patients achieve full functional recovery (Lopes et al., 2022; Witzel et al., 2005; Zochodne, 2012) with patients commonly facing lifelong functional impairment and neuropathic pain (Balakrishnan et al., 2021; Lopes et al., 2022; Menorca et al., 2013). Understanding the cellular and molecular mechanisms between glial cells and the molecular drivers of their regenerative processes is crucial for developing new targeted therapies to enhance regenerative capacity.

Here, I identify the first signaling pathway known drive perineurial glial bridging, an essential regenerative process, after spinal motor nerve injury. I demonstrate that TGFβ

signaling modulates perineurial glial dynamics after injury by initiating bridging behaviors. We then show that *connective tissue growth factor a* (*ctgfa*), a component of this signaling cascade, is also required for perineurial glial bridging. Together, my studies present TGFβ signaling as a novel driver of perineurial glial bridging, a process that is crucial for successful and functional motor nerve regeneration. Elucidating the cellular and molecular mechanisms that regulate perineurial glial behaviors following injury is crucial to further understanding successful motor nerve regeneration.

Results

Perineurial glia bridge and phagocytose debris after spinal motor nerve injury

In zebrafish, peripheral motor nerve axons originate in the ventral ventricular, or pMN, domain of the spinal cord, migrating through the motor exit point transition zone into the periphery during early development (Myers et al., 1986; Park et al., 2002). Following the growing axons, Schwann cell precursors migrate from the dorsal neural tube into the periphery to differentiate into mature Schwann cells and myelinate axons within nerves (Lyons & Talbot, 2015). Perineurial glia then exit the spinal cord and begin to ensheath the Schwann cell-axon bundles at approximately 52 hpf (Kucenas, Snell, et al., 2008). By 5 days post-fertilization (dpf), zebrafish spinal motor nerves and their associated glia have matured past major developmental processes (Binari et al., 2013; G. M. Lewis & Kucenas, 2014; A. F. Rosenberg et al., 2012). This, along with the highly regenerative capacity of zebrafish (Ghosh & Hui, 2016; Marques, Lupi, & Mercader, 2019; Mokalled & Poss, 2018; Poss, Wilson, & Keating, 2002; Shi, Fang, Li, & Luo, 2015), makes the larval zebrafish an optimal model for studying motor nerve regeneration.

Using *nkx2.2a:megfp* to label perineurial glia and *olig2:dsred* to label motor axons (Kucenas, Snell, et al., 2008; G. M. Lewis & Kucenas, 2014) (Figure 3-1A), I injured peripheral spinal motor nerves in 5 or 6 dpf zebrafish larvae on either the caudal or rostral tract of the nerve using laser axotomy as previously described (Gwendolyn M. Lewis & Kucenas, 2013; A. F. Rosenberg et al., 2012; Allison F. Rosenberg et al., 2014) and time-lapse imaged every five minutes for 8 hours post injury (hpi). Consistent with previous studies (G. M. Lewis & Kucenas, 2014), I observed perineurial glia with phagocytic vesicles primarily proximal to the injury site between 1 and 6 hpi, a behavior that was previously observed via labeling with LysoTracker (Figure 3-1B; *n=11 nerves in 5 larvae*) (G. M. Lewis & Kucenas, 2014). Additionally, I observed perineurial glia form a complete bridge across the injury gap within the first 6 hpi in both rostral and caudal nerve tract injuries (Figure 3-1B; *n=11 nerves in 5 larvae*). Perineurial glial bridging and phagocytosis is crucial for motor nerve regeneration (G. M. Lewis & Kucenas, 2014). However not much is known about the dynamics of these processes nor the identity of the signals that drive these cellular behaviors.

To begin to characterize perineurial glial bridging, I analyzed the time at which perineurial glia initiated and completed bridging. I defined bridging initiation as perineurial glial membranes crossing beyond the proximal or distal injury stump and bridging completion as perineurial glial membranes fully bridging across the original injury gap. For these analyses, I used time-lapse videos of motor nerves injured on either the rostral or caudal tract in 5 or 6 dpf *nkx2.2a:megfp;olig2:dsred* larvae. My results showed that the majority

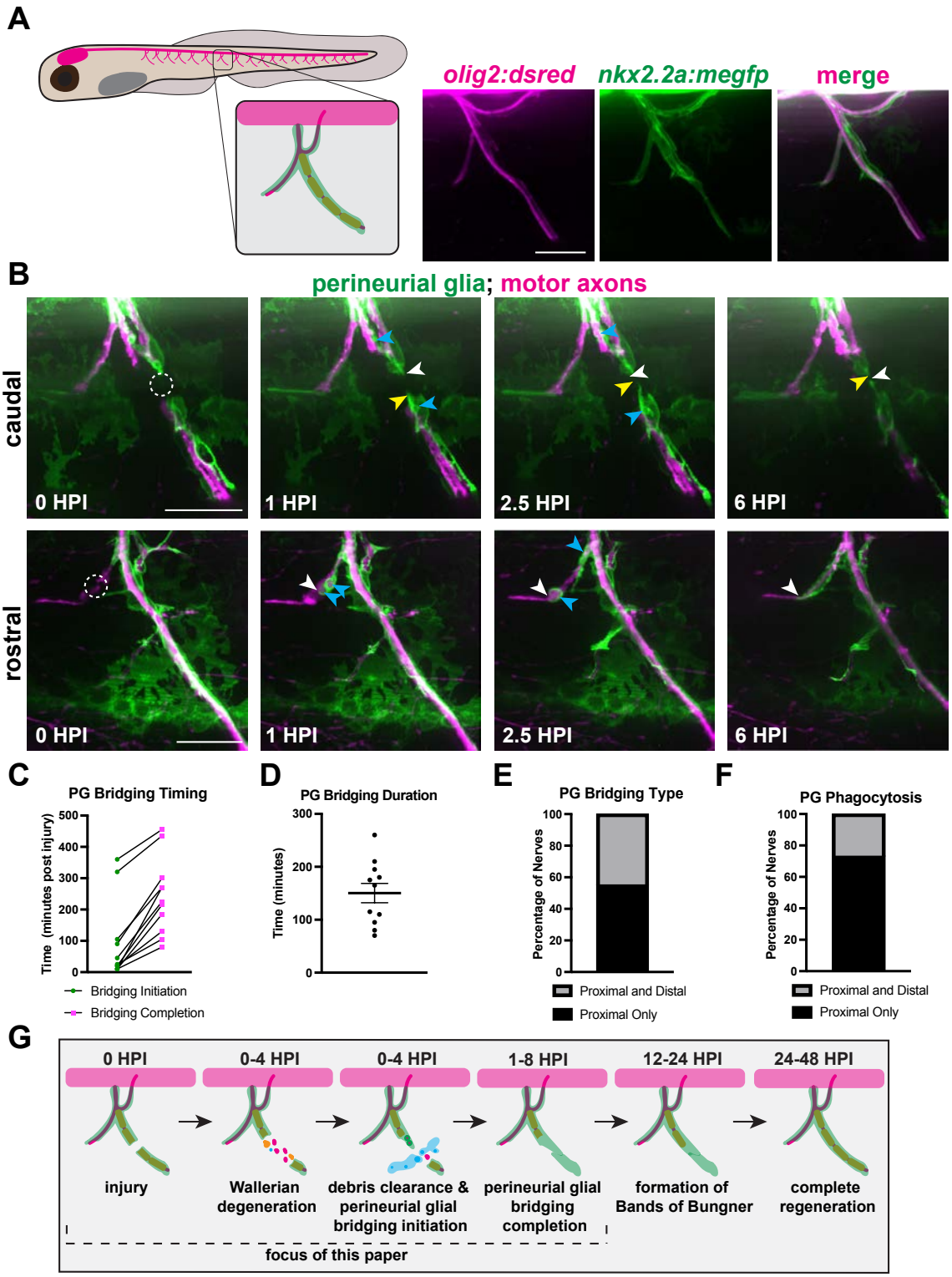

A
olig2:dsred
nkx2.2a:megfp
merge
B
perineurial glia; motor axons
caudal
rostral
0 HPI
1 HPI
2.5 HPI
6 HPI
C
PG Bridging Timing
Time (minutes post injury)
Bridging Initiation
Bridging Completion
D
PG Bridging Duration
Time (minutes)
E
PG Bridging Type
Percentage of Nerves
Proximal and Distal
Proximal Only
F
PG Phagocytosis
Percentage of Nerves
Proximal and Distal
Proximal Only
G
0 HPI
0-4 HPI
0-4 HPI
1-8 HPI
12-24 HPI
24-48 HPI
injury
Wallerian degeneration
debris clearance & perineurial glial bridging initiation
perineurial glial bridging completion
formation of Bands of Bungner
complete regeneration
focus of this paper

Figure 3-1. Perineurial glia form a bridge and phagocytose debris after spinal motor nerve injury.

A) (Left) Diagrammatic representation of a 6 dpf zebrafish larva with a single peripheral spinal motor nerve (inset) displayed. Motor axon (magenta), ensheathed Schwann cells (orange), perineurial glia (green). (Right) Representative images of an *in vivo* 6 dpf spinal motor nerve, where *olig2* labels motor axons (magenta) and *nkx2.2a* labels perineurial glia (green). (B-F) *n=11 nerves in 5 larvae*. B) Representative stills taken from time-lapse movies of perineurial glia (green) and motor axons (magenta) in 5 or 6 dpf larvae injured on either the caudal or rostral side of the spinal motor nerve. Stills are shown in the first 6 hours post injury (hpi). The dashed circle indicates the injury site. Blue arrows denote phagocytic vesicles in perineurial glia. White arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge. C) Quantification of perineurial glial (PG) bridging timing. Timing of bridging initiation (green) and completion (magenta) was recorded in minutes post injury. D) Quantification of perineurial glial (PG) bridging duration in minutes (mean: 150.54±18.25 min). E) Quantification of the type of perineurial glial (PG) bridging observed. Proximal and distal bridging (45% of nerves, gray) or proximal only bridging (55% of nerves, black). F) Quantification of perineurial glial (PG) phagocytosis after injury. Phagocytic vesicles on both proximal and distal perineurial glial stumps (27% of nerves, gray) or only on proximal perineurial glial stumps (73% of nerves, black). G) Representative timeline of the first 48 hours following spinal motor nerve injury in 5 to 6 dpf zebrafish. Motor axon (magenta), Schwann cells (orange), perineurial glia (green), macrophages (blue). Scale bars, 25 μm.

of perineurial glia initiate bridging within the first 2 hpi (*n=9 nerves in 5 larvae*), with all perineurial glia initiating bridging within the first 6 hpi (Figure 3-1C; *n=11 nerves in 5 larvae*). Additionally, the majority of perineurial glia complete bridging by 5 hpi (*n=9 nerves in 5 larvae*), with all bridging complete by 8 hpi (*n=11 nerves in 5 larvae*) and with an average total bridging time of approximately 2.5 hours (Figure 3-1D; mean: 150.54 min). In my studies, I also observed that when perineurial glia bridge, they either bridge from both the proximal and distal sides of the injury, with perineurial glia meeting in the middle (*n=6 nerves in 5 larvae*), or from only the proximal side of the injury, with perineurial glia extending from the proximal stump fully across the injury site (*n=5 nerves in 5 larvae*). Each type of bridging is equally likely to occur, and there is no difference in bridging timing between the two different types (Figure 3-1C, E).

In addition to bridging the injury gap, perineurial glia form phagocytic vesicles to clear axonal debris shortly after injury (G. M. Lewis & Kucenas, 2014). To further characterize perineurial glial phagocytosis, I investigated whether there was a difference in where phagocytic vesicles formed in perineurial glia using the same time-lapse videos of perineurial glial bridging used in the previous analyses (Figure 3-1B-D). Although half of the injured nerves had bridging from perineurial glia on both the proximal and distal side of the injury site, I observed that the majority (73%) of perineurial phagocytic vesicles were formed only on the proximal side of the nerve (Figure 3-1F, *n=8 nerves in 5 larvae*). While some injured nerves did have vesicles on both the proximal and distal side of the injury (*n=3 nerves in 5 larvae*), no injured nerves had phagocytic vesicles in only perineurial glia distal to the injury (Figure 3-1F). These analyses confirm that perineurial

glia play a pivotal role early in the regeneration process by forming a bridge across the injury gap and phagocytosing debris.

Together, my data establishes a timeline during which perineurial glial bridging occurs (Figure 3-1G). During the first 4 hpi, perineurial glia initiate bridging and phagocytose debris, providing by 6 to 8 hpi, a scaffold upon which the injured nerve can regenerate. During this time, macrophages infiltrate the injury site to clear debris and Schwann cells begin to activate their repair program, which occurs during the first 12 hpf (P. Chen et al., 2015; K. R. Jessen & Mirsky, 2016; Kristján R. Jessen et al., 2015; G. M. Lewis & Kucenas, 2014). Between 12 to 24 hpi, following perineurial glial bridge completion, Schwann cells form Bands of Bungner to guide regenerating axons across the injury site to re-innervate target tissues (K. R. Jessen & Mirsky, 2016; Min et al., 2021). Importantly, Schwann cells do not form Bands of Bungner across the injury site until after perineurial glial bridging is complete and full spinal motor nerve regeneration takes between 24 and 48 hpi in zebrafish larvae (G. M. Lewis & Kucenas, 2014; A. F. Rosenberg et al., 2012; Allison F. Rosenberg et al., 2014). For our studies in this manuscript, we focused on the first 8 hpi during which perineurial glia are bridging and phagocytosing debris (Figure 3-1G). While these perineurial glial behaviors are critical for efficient regeneration, not much is known about bridging dynamics or the molecular signals that regulate these events.

Perineurial glia do not proliferate after motor nerve injury

To further elucidate perineurial glial bridging dynamics, I explored the role that the positioning of perineurial glial cells along the nerve plays in bridging. I first examined the

number of *nkx2.2a*$^{+}$ nuclei present on the nerve. Using *nkx2.2a:nls-egfp* or *nkx2.2a:nls-mch* to visualize perineurial glia nuclei (Fontenas & Kucenas, 2021; Kucenas, Snell, et al., 2008; Zhu et al., 2019), I imaged *nkx2.2a*$^{+}$ nuclei at both 5 and 6 dpf. The number of *nkx2.2a*$^{+}$ nuclei as well as the position of the nuclei along the nerve varied per nerve, but there was no significant change in the number of cells between 5 and 6 dpf (Figure 3-2A; *5 dpf: n=10 nerves in 5 larvae, mean: 4.8 nuclei; 6 dpf: n=17 nerves in 5 larvae, mean: 6.1 nuclei*). Next, I asked whether perineurial glia proliferated in order to form the bridge after injury. Using *nkx2.2a:nls-mcherry;olig2:dsred* larvae to label perineurial glial nuclei and motor axons, respectively, I performed laser axotomy and time-lapse imaged for 6 hpi. In these studies, I observed Wallerian degeneration, however *nkx2.2a*$^{+}$ nuclei did not migrate or divide (Figure 3-2B; *n=4 nerves in 3 larvae*). This indicates that when perineurial glia form a bridge, the cell bodies do not migrate and perineurial glia do not proliferate. Instead, perineurial glia extend membrane processes across the injury site.

Finally, I analyzed the role that perineurial glial nuclei position plays in the types of perineurial glial bridging that I observed. I hypothesized that positioning of perineurial glia nuclei relative to the injury site would affect the type of bridging that occurs. Following injury to larvae expressing *nkx2.2a:megfp* and *nkx2.2a:nls-mcherry* to label perineurial glial membranes and nuclei, respectively, I observed two distinct types of bridging on both the rostral and caudal side of the nerve, consistent with my previous imaging (Figure 3-1E). I observed that when an injury is created with no *nkx2.2a*$^{+}$ nuclei distal to the injury

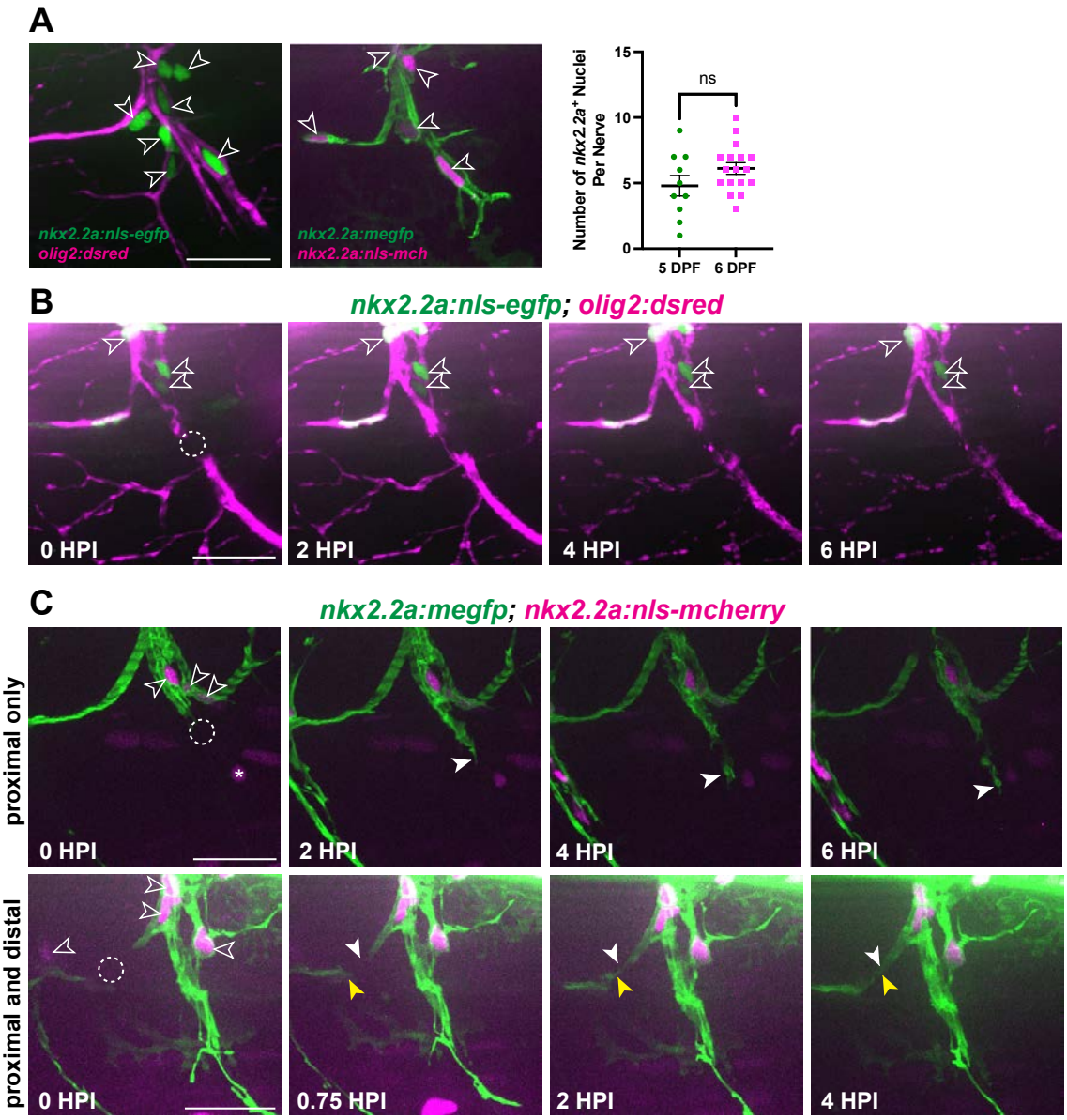

A
nkx2.2a:nls-egfp
olig2:dsred
nkx2.2a:megfp
nkx2.2a:nls-mch
Number of nkx2.2a+ Nuclei Per Nerve
15
10
5
0
ns
5 DPF
6 DPF
B
nkx2.2a:nls-egfp; olig2:dsred
0 HPI
2 HPI
4 HPI
6 HPI
C
nkx2.2a:megfp; nkx2.2a:nls-mcherry
proximal only
0 HPI
2 HPI
4 HPI
6 HPI
proximal and distal
0 HPI
0.75 HPI
2 HPI
4 HPI

Figure 3-2. Perineurial glia do not proliferate following spinal motor nerve injury.

A) (Left) Representative images of perineurial glial nuclei (open arrows) imaged with either motor axons (magenta, left) or perineurial glial membrane (green, right) at 5 dpf. (Right) Quantification of the number of *nkx2.2a*$^{+}$ nuclei on a single spinal motor nerve in 5 (green, *n=10 nerves in 5 larvae,* mean: 4.8±0.79 nuclei) and 6 dpf (magenta, *n=17 nerves in 5 larvae*, mean: 6.1±0.44 nuclei) larvae (p=0.1272). B) Representative images from a time-lapse movie of *nkx2.2a*$^{+}$ nuclei (green) in an injured 6 dpf larva. The dashed circle indicates the injury site (*n=4 nerves in 3 larvae*). C) Representative still images from a time-lapse movie of *nkx2.2a*$^{+}$ nuclei (magenta, open arrowheads) in injured 5 to 6 dpf larvae exhibiting either proximal only bridging (top panels, *n=6 nerves in 4 larvae*) or proximal and distal bridging (bottom panels, *n=4 nerves in 3 larvae*). Solid white arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge (green). Asterisks indicate *nkx2.2a*$^{+}$ cells that are not perineurial glial nuclei. Scale bars, 25 μm.

site, perineurial glia proximal to the injury site extend their membrane fully across the injury gap (Figure 3-2C; *n=6 nerves in 4 larvae*). This likely occurs because when no *nkx2.2a*$^{+}$ nuclei are distal to the injury site, the remaining perineurial glial membrane distal to the injury degenerates away with the nerve. Conversely, perineurial glial membranes both proximal and distal to the injury site actively bridged when *nkx2.2a*$^{+}$ nuclei were present on both sides of the injury site (Figure 3-2C; *n=4 nerves in 3 larvae*). Both types of bridging occurred on a similar time scale, with bridging initiating by 2 hpi and completing by 6 hpi (data not shown). Therefore, the difference in bridging I observed was due to location of the injury site relative to the position of *nkx2.2a*$^{+}$ nuclei. Similar to the *nkx2.2a*$^{+}$ nuclei imaged with *olig2:dsred* (Figure 3-2B), *nkx2.2a*$^{+}$ nuclei did not migrate or divide along nerves that exhibited either type of bridging (Figure 3-2C, *n=10 nerves in 7 larvae*). Overall, these studies demonstrate that perineurial glial bridging behaviors are not due to cell migration or division. Instead, the positioning of *nkx2.2a*$^{+}$ nuclei relative to the injury influences how perineurial glia bridge.

Inhibition of TGFβ signaling perturbs perineurial glial bridging but not phagocytosis

It is known that perineurial glia form a bridge within the first 1 to 4 hpi and complete their membrane extension across the bridge by 6 to 8 hpi, phagocytosing axonal debris as they bridge (Figure 3-1 C,D) (G. M. Lewis & Kucenas, 2014). However, it remains unknown what molecular signals drive this process. Transforming growth factor-beta (TGFβ) signaling is an established regulator of regenerative processes (Keatinge et al., 2021; J. S. Kim et al., 2006; Lenkowski et al., 2013; Nakamura et al., 2021; Sharma et al., 2020; Sulaiman & Nguyen, 2016), specifically during motor nerve regeneration (Clements et al.,

2017; Frostick et al., 1998; Schira et al., 2018). TGFβ-1 is important for the Schwann cell repair program, and Schwann cells both secrete TGFβ-1 following injury (Schira et al., 2018) and require it to successfully migrate across the injury site (Clements et al., 2017). Additionally, TGFβ-1 is secreted by macrophages responding to injury, providing a source of TGFβ-1 for Schwann cells (B. Chen et al., 2021). Finally, TGFβ signaling is also important for perineurial glial development (A. D. Morris et al., 2017). Therefore, because TGFβ signaling is present during motor nerve regeneration and perineurial glia require TGFβ for development, I hypothesized that TGFβ signaling might also drive perineurial glial bridging after injury.

To investigate the role of TGFβ signaling in perineurial glial bridging, I treated 4 dpf *nkx2.2a:megfp;olig2:dsred* larvae with either 10 μM SB431542, a selective inhibitor of the TGFβ-1 receptor, dissolved in 1% DMSO, or 1% DMSO alone for 48 hours (A. D. Morris et al., 2017; Sun et al., 2006) prior to injury. In these studies, larvae were treated from 4 to 6 dpf, a window after which perineurial glia have fully ensheathed the nerve and undergone their major developmental processes (Kucenas, 2015; Kucenas, Snell, et al., 2008; Kucenas, Takada, et al., 2008; G. M. Lewis & Kucenas, 2014). Larvae in both SB4315342 and DMSO-treated groups showed no changes in overall morphology, motor nerve development, or perineurial glial ensheathment of the nerve after treatment (Appendixes-Figure 1; *n=11 nerves in 5 fish, 14 nerves in 6 fish, respectively*) (A. D. Morris et al., 2017; Sun et al., 2006). I then used laser axotomy to injure spinal motor nerves in both SB431542 and DMSO-treated groups. After time-lapse imaging for 8 hpi, I observed that perineurial glia in 1% DMSO-treated control larvae formed phagocytic vesicles and initiated bridging

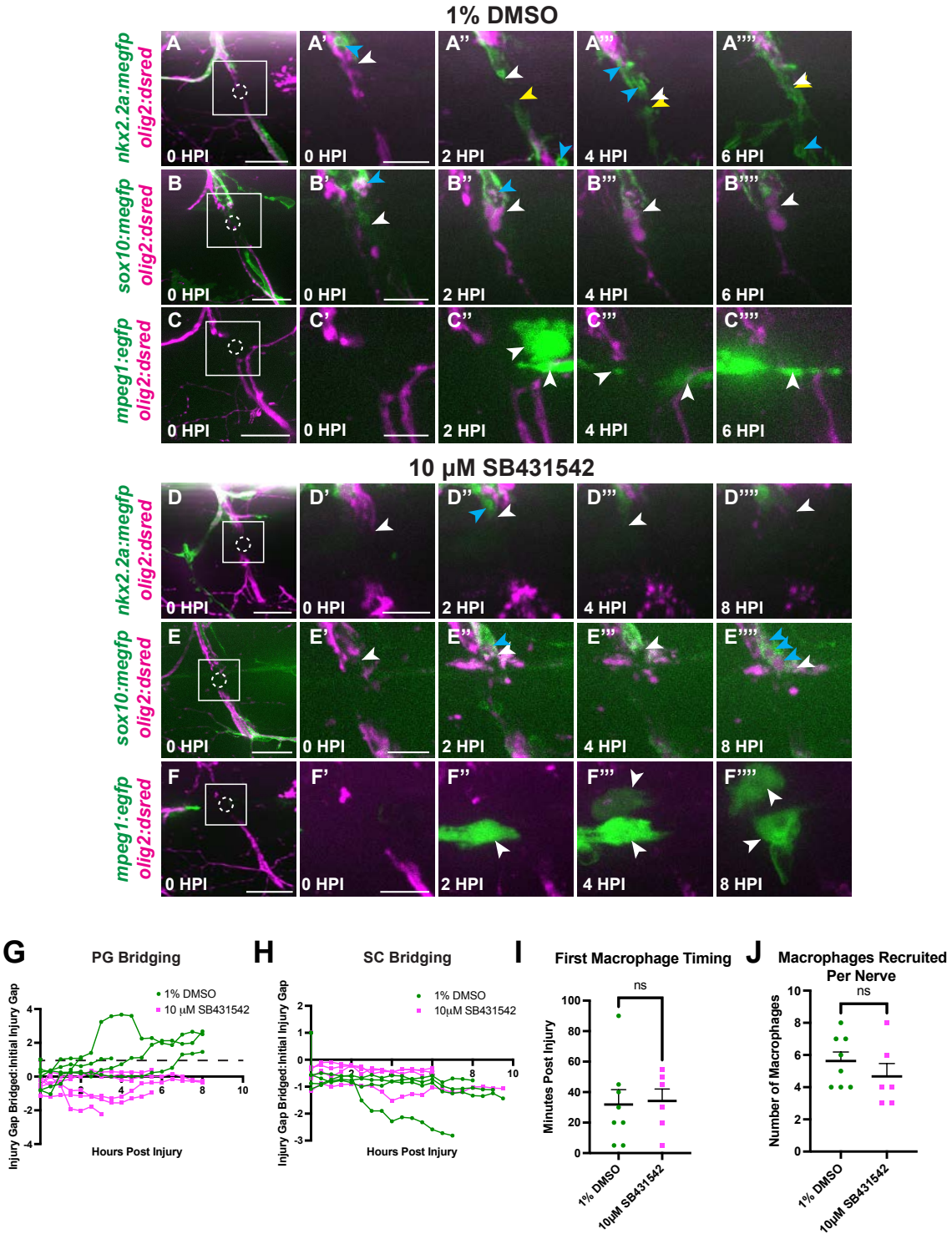

1% DMSO
nkx2.2a:megfp olig2:dsred
sox10:megfp olig2:dsred
mpeg1:egfp olig2:dsred
A
A'
A''
A'''
A''''
B
B'
B''
B'''
B''''
C
C'
C''
C'''
C''''
0 HPI
2 HPI
4 HPI
6 HPI
10 µM SB431542
D
D'
D''
D'''
D''''
E
E'
E''
E'''
E''''
F
F'
F''
F'''
F''''
8 HPI
G
PG Bridging
1% DMSO
10 µM SB431542
Injury Gap Bridged:Initial Injury Gap
Hours Post Injury
H
SC Bridging
1% DMSO
10µM SB431542
Injury Gap Bridged:Initial Injury Gap
Hours Post Injury
I
First Macrophage Timing
ns
Minutes Post Injury
1% DMSO
10µM SB431542
J
Macrophages Recruited Per Nerve
ns
Number of Macrophages
1% DMSO
10µM SB431542

Figure 3-3. Inhibition of TGFβ signaling perturbs perineurial glial bridging.

A-F) Representative stills from time-lapse movies of 5 to 6 dpf larvae with injured spinal motor nerves (magenta) treated with either 1% DMSO (A-C) or 10 μM SB431542 (D-F). The dashed circle indicates the injury site while the solid line box indicates the regions of interest highlighted in adjacent insets. Blue arrows specify phagocytic vesicles. A, D) White arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge (green). B, E) White arrows follow the proximal Schwann cell stump (green). C, F) White arrows show macrophages (green) present in the injury site. G-H) Quantification of either perineurial glial (PG) bridging (G) or Schwann cell (SC) bridging (H) over 8 hpi in both DMSO (green) and SB431542-treated (magenta) groups. Measurements of perineurial glial membrane (G) or Schwann cell (H) extension into the injury site were compared to the size of the initial injury gap, with values over 1 (dotted-line) indicating complete bridging and values less than zero indicating retraction away from the injury site. G) All DMSO-treated larvae (*n=5 nerves in 4 larvae*) demonstrated complete bridging while perineurial glia in all SB432542-treated larvae (*n=6 nerves in 4 larvae*) failed to bridge the injury gap. H) Schwann cells in both DMSO (*n=4 nerves in 3 larvae*) and SB431542-treated larvae (*n=4 nerves in 3 larvae*) did not bridge across the injury site. I) Quantification of the timing of the first macrophage to enter the injury site in both DMSO (green, *n=8 nerves in 5 larvae;* mean: 31.88±0 minutes) and SB431542-treated (magenta, *n=6 nerves in 4 larvae*; mean: 34.175.62±0 minutes) larvae (p=0.3335). J) Quantification of the number of macrophages per nerve recruited to the injury site over 8 hpi in both DMSO (green, *n=8 nerves in 5 larvae*; mean: 5.62±0 macrophages) and

SB431542-treated (magenta, *n=6 nerves in 4 larvae*; mean: 4.67±0 macrophages) larvae (p=0.8655). Scale bar, (A-F) 25 μm; (A'-F'''') magnified insets, 10 μm.

within 2 hpi, with bridging complete by 4 to 6 hpi, consistent with my previous data (Figure 3-3A-A''''; *n=5 nerves in 4 larvae*). In contrast, perineurial glia in larvae treated with 10 μM SB431542 formed phagocytic vesicles but did not initiate bridging or cross over the injury site by 8 hpi (Figure 3-3D-D'''', *n=6 nerves in 4 larvae*). I observed bridging in all DMSO controls (*n=5 nerves in 4 larvae*) but in none of the TGFβ inhibitor-treated larvae (*n=6 nerves in 4 larvae*) (Figure 3-3G). Additionally, in some cases, perineurial glia in SB431542-treated larvae that failed to bridge began to degenerate along with the nerve, resulting in a larger gap between the perineurial glial stump and the original injury site (Figure 3-3G). From these studies, I conclude that inhibition of TGFβ signaling blocks perineurial glial bridging after injury.

To determine if these changes in perineurial glial bridging behaviors were specific to perineurial glia and not due to a change in behavior of other cells involved in the injury response (Figure 3-1G), I repeated these experiments using either *sox10:megfp* to label Schwann cells or *mpeg1:egfp* to label macrophages with *olig2:dsred* to label motor axons. Both groups of transgenic larvae were treated with either 10 μM SB431542 in 1% DMSO or 1% DMSO alone for 48 hours prior to injury and time-lapse imaged for 8 hpi. Previous studies demonstrate that immediately following injury, Schwann cells extend processes towards the injury site, but do not migrate into it within the first 8 hpi (G. M. Lewis & Kucenas, 2014). Consistent with this, I observed no difference in the migration of Schwann cells in *sox10:megfp* larvae treated with DMSO (*n=4 nerves in 3 larvae*) or SB431542 (*n=4 nerves in 3 larvae*) (Figure 3-3B-B'''', E-E'''', H). Similarly, *mpeg1:egfp* larvae treated with DMSO (*n=8 nerves in 5 larvae*) or SB431542 (*n=6 nerves in 4 larvae*) showed

no difference between the number of macrophages recruited to the injury site nor the timing of macrophages entering the injury site (Figure 3-3C-C'''', F-F'''', I, J). These results demonstrate that inhibition of TGFβ signaling disrupts perineurial glial bridging but not phagocytosis, without perturbing the early injury responses of either Schwann cells or macrophages during the first 8 hpi. Therefore, perineurial glial bridging and phagocytosis are two behaviors controlled by separate molecular signals and inhibition of TGFβ signaling perturbs perineurial glial bridging independent of Schwann cell and macrophage behaviors.

To further confirm that the vesicles observed in SB431542-treated perineurial glia were indicative of phagocytosis of axonal debris, I utilized Imaris (Oxford Instruments) to render 3D images of 5 or 6 dpf *nkx2.2a:megfp;olig2:dsred* larvae treated with either 1% DMSO (*n=5 nerves in 4 larvae*) or 10 μM SB431542 in 1% DMSO (*n=6 nerves in 4 larvae*). 3D rendering demonstrated that axonal debris observed in perineurial glial vesicles in my time-lapse imaging in the first 3 hpi were fully engulfed by perineurial glial membranes in both DMSO-treated (Figure 3-4A) and SB431542-treated (Figure 3-4B) larvae. Therefore, although SB431542-treated perineurial glia were unable to form a glial bridge across they injury site, I continued to observe them phagocytosing debris.

TGFβ signaling is present during early phases of perineurial glial bridging

I next wanted to explore the timing during which TGFβ signaling is impacting perineurial glial bridging. Because inhibition of TGFβ signaling prevented perineurial glia from crossing into the injury site, I hypothesized that TGFβ signaling is important for early

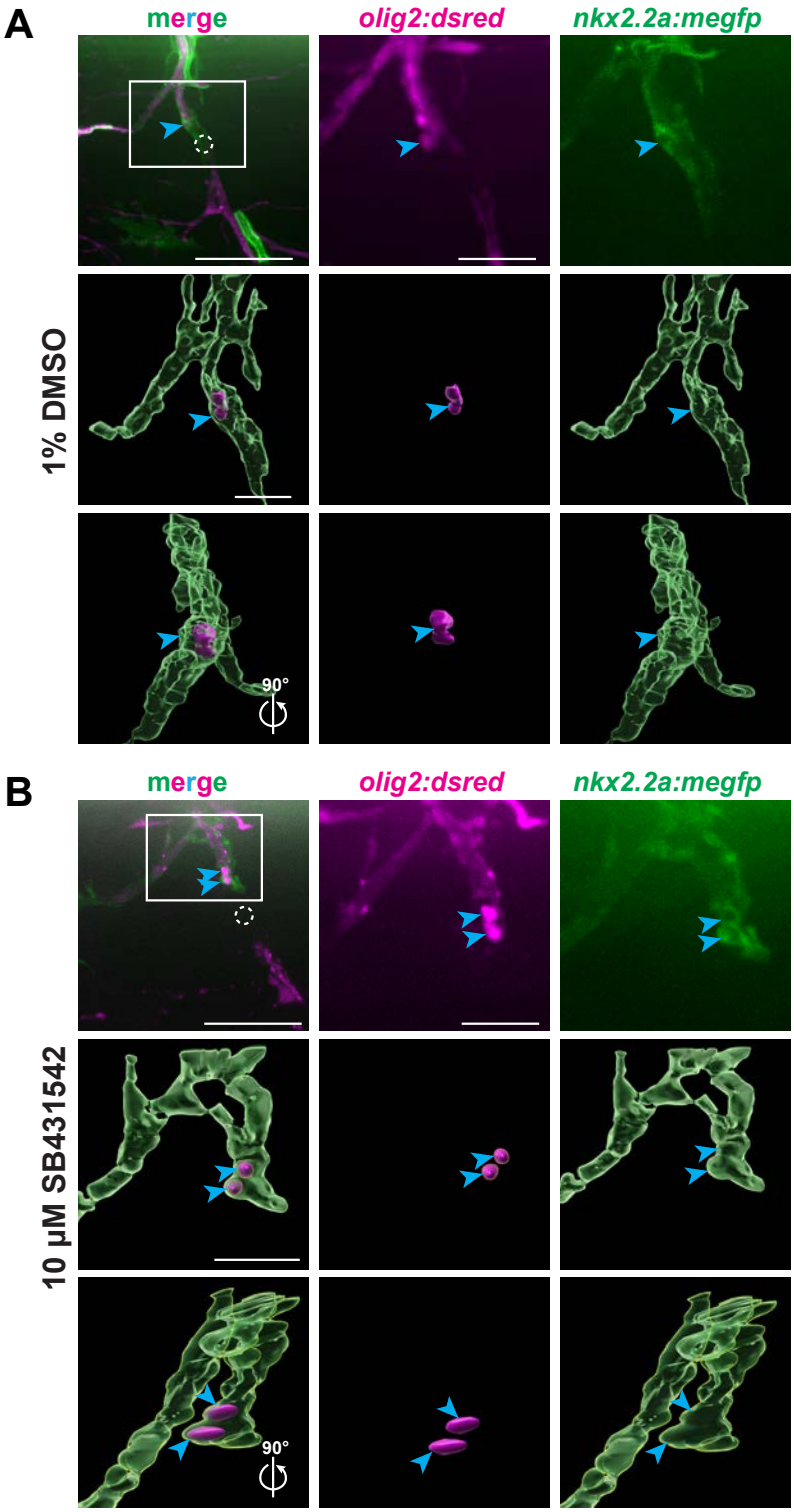

A
merge
olig2:dsred
nkx2.2a:megfp
1% DMSO
90°
B
merge
olig2:dsred
nkx2.2a:megfp
10 µM SB431542
90°

Figure 3-4. Phagocytosis is observed in perineurial glia with inhibition of TGFβ.

A-B) Representative images of 5 dpf *nkx2.2a:megfp;olig2:dsred* injured larvae treated with either 1% DMSO (A) or 10 μM SB431542 (B) at 3 hpi. The dashed circle indicates the injury site. Axonal debris and perineurial glial vesicles are specified by blue arrows. 3D renderings of these representative images are shown in both a 0-degree and 90-degree rotated views (Imaris; *n=5 nerves in 4 larvae, 6 nerves in 4 larvae, respectively*). Scale bars, 25 μm; magnified insets and 3D renderings, 10 μm.

initiation of this behavior. To explore this, I injured 5 or 6 dpf *nkx2.2a:nls-egfp;olig2:dsred* larvae and fixed the larvae at 2, 3, or 6 hpi. For these studies, I used uninjured sibling larvae as a control. I then stained all groups with an antibody specific to phosphorylated Smad3 (pSmad3), an indicator of active TGFβ signaling (Casari et al., 2014; Kitisin et al., 2007; A. D. Morris et al., 2017). In these studies, using Imaris (Oxford Instruments) to render 3D images of *nkx2.2a*$^+$ nuclei and pSmad3 signal, I did not observe any pSmad3-positive *nkx2.2a*$^+$ nuclei along uninjured spinal motor nerves (Figure 3-5A, *n=13 nerves in 9 larvae)*. However, I did see pSmad3 labeling in *nkx2.2a*$^+$ nuclei at 2, 3, and 6 hpi (Figure 3-5B-D; *2 hpi, n=20 nerves in 4 larvae; 3 hpi, n=21 nerves in 6 larvae; 6 hpi, n=17 nerves in 4 larvae*). The percentage of nerves with pSmad3$^+$/*nkx2.2a*$^+$ nuclei peaked at 3 hpi and decreased by 6 hpi (Figure 3-5F). From these data I observed the most pSmad3$^+$ perineurial glial nuclei at 3 hpi, the time at which perineurial glia are actively bridging. Importantly, I did not observe pSmad3 staining in *nkx2.2a*$^+$ nuclei at 3 hpi in larvae treated with 10 μM SB431542 (Figure 3-5E, *n=15 nerves in 5 larvae*). This is consistent with my data demonstrating that perineurial glia do not initiate bridging into the injury gap with inhibition of TGFβ signaling (Figure 3-3D).

In addition to pSmad3 staining in *nkx2.2a*$^+$ nuclei, I also observed pSmad3 staining along the injured nerve in linear structures, but not along uninjured nerves or nerves treated with 10 μM SB431542 (Figure 3-5A-E; *uninjured, n=13 nerves in 9 larvae; 2 hpi n=20 nerves in 4 larvae; 3 hpi, n=21 nerves in 6 larvae; 6 hpi, n=17 nerves in 4 larvae; SB431542 treatment, n=15 nerves in 5 larvae*). The percentage of nerves with pSmad3 staining along the injured nerve increased over time, peaking at 6 hpi, with pSmad3 staining largely

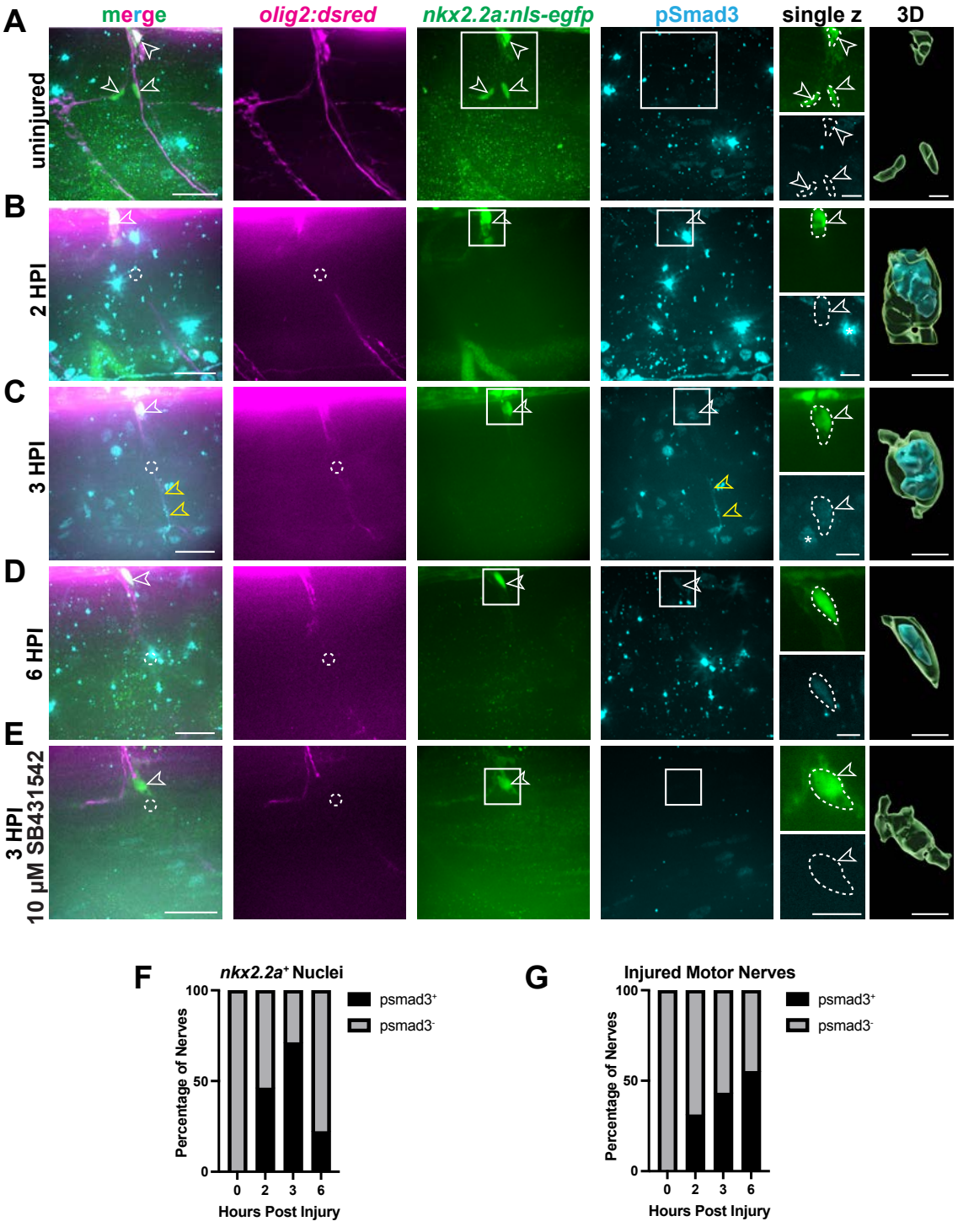

A
merge
olig2:dsred
nkx2.2a:nls-egfp
pSmad3
single z
3D
uninjured
B
2 HPI
C
3 HPI
D
6 HPI
E
3 HPI
10 μM SB431542
F
nkx2.2a+ Nuclei
psmad3+
psmad3-
Percentage of Nerves
100
50
0
0
2
3
6
Hours Post Injury
G
Injured Motor Nerves
psmad3+
psmad3-
Percentage of Nerves
100
50
0
0
2
3
6
Hours Post Injury

Figure 3-5. pSmad3 expression is present in perineurial glia early after injury.

A-E) Representative images of 5 or 6 dpf larvae with motor axons (magenta), *nkx2.2a*$^{+}$ nuclei (green, white open arrows), and anti-pSmad3 (cyan) in uninjured (A), 2 (B), 3 (C), 6 (D) hpi larvae, and larvae treated with SB431542 at 3 hpi (E). Anti-pSmad3 labeling is observed in *nkx2.2a*$^{+}$ nuclei (open white arrows) and along the motor nerve (open yellow arrows) at 2, 3, and 6 hpi. 6 dpf larvae treated with 10 μM SB431542 show loss of anti-pSmad3 staining at 3 hpi (bottom panels). Dashed circles indicate injury sites. Solid-line boxes indicate the area represented in single z plane and 3D rendered images (Imaris). White dotted-lines outline *nkx2.2a*$^{+}$ nuclei and anti-pSmad3 labeling. F, G) Quantification of the percentage of nerves that had anti-pSmad3^{+} labeling (black) co-localized with *nkx2.2a*$^{+}$ nuclei (F) or motor nerves (G). B) 0% of uninjured nerves, 46% of 2 hpi nerves, 71% of 3 hpi nerves, and 22% of 6 hpi nerves had anti-pSmad3^{+} labeling in *nkx2.2a*$^{+}$ nuclei (*n=13 nerves in 9 larvae, 20 nerves in 4 larvae, 21 nerves in 6 larvae, 17 nerves in 4 larvae, respectively*). G) 0% of uninjured nerves, 31% of 2 hpi nerves, 43% of 3 hpi nerves, and 55% of 6 hpi had anti-pSmad3^{+} labeling along the nerve (*n=13 nerves in 9 larvae, 20 nerves in 4 larvae, 21 nerves in 6 larvae, 17 nerves in 4 larvae, respectively*). Scale bars, 25 μm; magnified insets, 10 μm; 3D renderings, 5 μm.

present along both the proximal and distal stump (Figure 3-5G; *2 hpi*, *n=20 nerves in 4 larvae; 3 hpi, n=21 nerves in 6 larvae; 6 hpi, n=17 nerves in 4 larvae*). Because pSmad3 staining along the injured nerve peaks at 6 hpi, when perineurial glia complete bridging, it is possible that this pSmad3 labeling I observed was present in bridged perineurial glial, as the distal axon has mostly degenerated by that time but has not yet begun to regenerate. Therefore, I conclude that TGFβ signaling is important for early initiation of perineurial glial bridging.

Inhibition of TGFβ signaling alters perineurial glial bridging dynamics

Taken together, my data demonstrates that TGFβ signaling is essential for perineurial glial bridging. To further elucidate how TGFβ signaling is affecting perineurial glial bridging, I decided to explore the effect of TGFβ inhibition on bridging dynamics. To do this, I measured the velocity of perineurial glial membrane processes after injury. Velocity was measured by manually tracking perineurial glia on both proximal and distal stumps (Figure 3-6A) over a period of 8 hpi from time-lapse movies taken from injured 6 dpf *nkx2.2a:megfp;olig2:dsred* larvae treated with either 1% DMSO or 10 μM SB431542 in 1% DMSO. My tracking demonstrated that in larvae treated with SB431542, perineurial glia did not bridge the injury gap (Figure 3-6C). In contrast, I observed both perineurial glial stumps meeting in the middle of the injury gap in DMSO-treated larvae (Figure 3-6B). I then plotted average velocity in μm/second against time in minutes post injury for the first 200 minutes post injury, the period during which perineurial glial bridging initiates. Velocities were measured for the proximal stump in nerves that only had proximal bridging, for the proximal stump in nerves that had both proximal and distal bridging, and

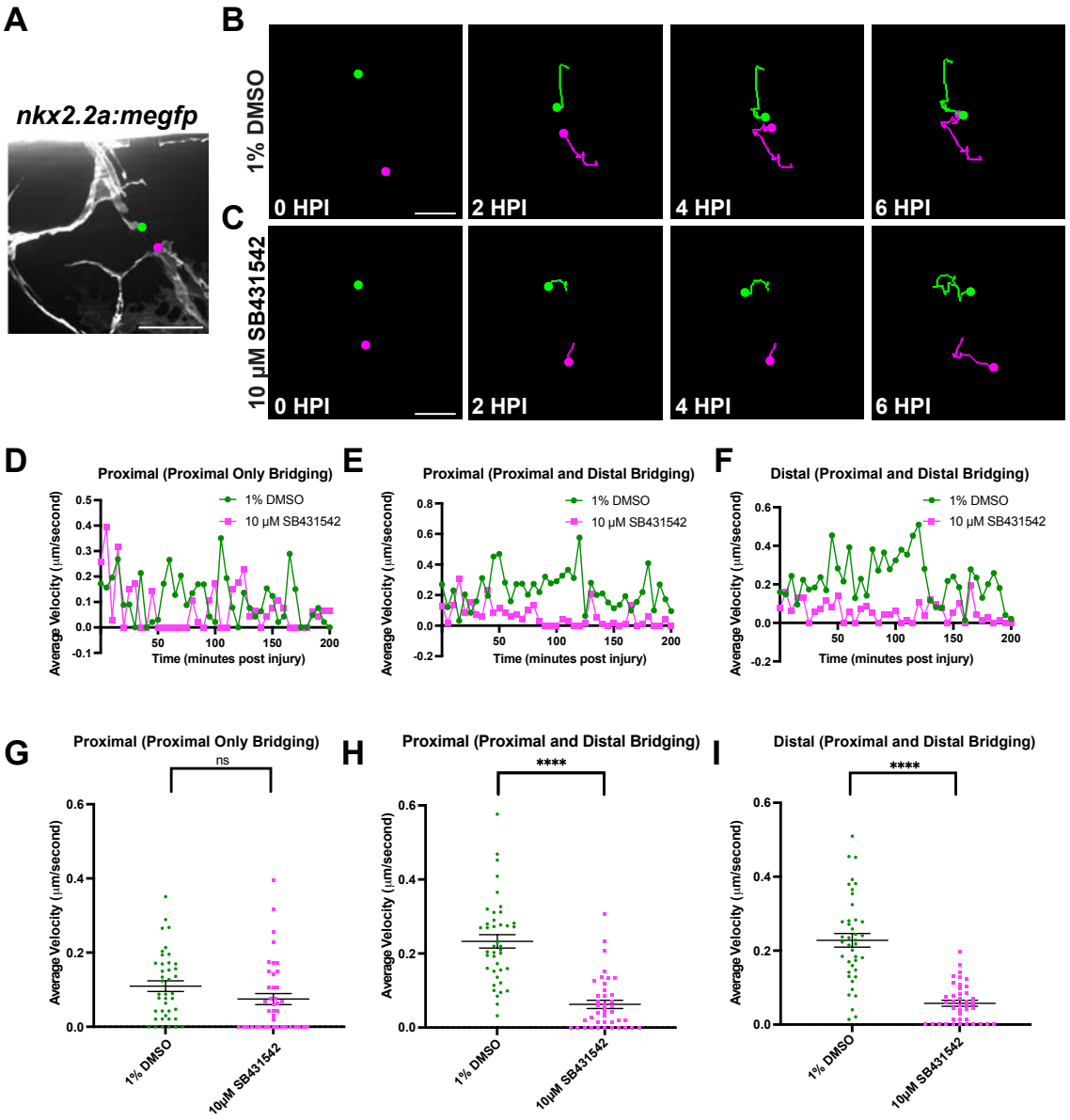

A
nkx2.2a:megfp
B
1% DMSO
0 HPI
2 HPI
4 HPI
6 HPI
C
10 µM SB431542
0 HPI
2 HPI
4 HPI
6 HPI
D
Proximal (Proximal Only Bridging)
1% DMSO
10 µM SB431542
Average Velocity (µm/second)
Time (minutes post injury)
E
Proximal (Proximal and Distal Bridging)
1% DMSO
10 µM SB431542
Average Velocity (µm/second)
Time (minutes post injury)
F
Distal (Proximal and Distal Bridging)
1% DMSO
10 µM SB431542
Average Velocity (µm/second)
Time (minutes post injury)
G
Proximal (Proximal Only Bridging)
ns
Average Velocity (µm/second)
1% DMSO
10µM SB431542
H
Proximal (Proximal and Distal Bridging)
****
Average Velocity (µm/second)
1% DMSO
10µM SB431542
I
Distal (Proximal and Distal Bridging)
****
Average Velocity (µm/second)
1% DMSO
10µM SB431542

Figure 3-6. Inhibition of TGFβ signaling alters perineurial glial bridging dynamics.

A) Representative image of a recently transected spinal motor nerve in a 6 dpf *nkx2.2a:megfp* larva with the proximal (green dot) and distal (magenta dot) stumps labeled. B, C) Representative images from tracking of both the proximal (green dot) and distal (magenta dot) stumps from time-lapse movies of perineurial glia in injured 5 or 6 dpf larvae treated with either 1% DMSO (B) or 10 μM SB431542 (C). D-I) Quantification of the average velocity of perineurial glial membrane proximal or distal stumps in μm/second plotted over time (D-F) or for the first 200 minutes post injury (mpi) (G-I). Velocities were calculated every five minutes using FIJI. Each dot represents the average velocity of all larvae within a group at that specific time point. D, G) There is no significant difference in proximal only perineurial glial bridging velocity between DMSO and SB431542-treated larvae (mean: 0.116±0.02 μm/s, 0.063±0.015 μm/s, respectively; p=0.1078) (*n=3 nerves in 3 larvae, n=3 nerves in 3 larvae, respectively*). E, F, H, I) There is a significant difference in perineurial glial bridging velocity between DMSO and SB432542-treated larvae, with DMSO-treated larvae having a higher average velocity, (*n=4 nerves in 3 larvae, n=3 nerves in 3 larvae, respectively*) in both proximal (mean: 0.139±0.03 μm/s, 0.08±0.02 μm/s, respectively; $p<0.0001$) and proximal and distal (mean: 0.18±0.03 μm/s, 0.06±0.02 μm/s; $p<0.0001$) bridging in larvae that had bridging from both the proximal and distal perineurial glial ends. Scale bars, (A) 25 μm; (B&C) tracking images, 10 μm.

for the distal stump in nerves that had both proximal and distal bridging in both DMSO and SB431542-treated larvae. Intriguingly, there was no difference in bridging velocity between SB431542-treated and DMSO-treated larvae for nerves where only the proximal side bridged (Figure 3-6D, G; *DMSO-treated, n=3 nerves in 3 larvae; SB431542-treated, n=3 nerves in 3 larvae*). However, there was a significantly higher velocity in both proximal bridging velocity and distal bridging velocity in DMSO-treated larvae compared to SB431542-treated larvae for nerves that had both proximal and distal bridging (Figure 3-6E, F, H, I; *DMSO-treated*, *n=4 nerves in 3 larvae, SB431542-treated, n=3 nerves in 3 larvae*). These differences in perineurial glial bridging velocity in DMSO-treated larvae compared to SB431542-treated larvae further demonstrate that TGFβ signaling is essential for initiation of perineurial glial bridging across the injury gap.

connective tissue growth factor-a is expressed in the periphery following injury

Because inhibition of TGFβ signaling perturbs perineurial glial bridging initiation, I next wanted to identify other components of TGFβ signaling that may also be involved in this process. I decided to explore the expression of *connective tissue growth factor a* (*ctgfa*), a gene that is actively involved in wound healing (Mokalled et al., 2016; Mukherjee et al., 2021; S.-M. Zhang et al., 2021). *ctgfa* is expressed downstream of TGFβ signaling and works in a positive feedback loop to enhance receptor binding of TGFβ-1 (Abreu et al., 2002; Mukherjee et al., 2021; Zaykov & Chaqour, 2021; S.-M. Zhang et al., 2021). Following peripheral nerve injury, CTGF is secreted by Schwann cells, which provides a potential source for both TGFβ and *ctgfa* signaling (Schira et al., 2018). Additionally, *ctgfa*

is necessary and sufficient for glial bridging following spinal cord injury in zebrafish (Mokalled et al., 2016).

To determine if *ctgfa* is involved in perineurial glial bridging, I injured 6 dpf *ctgfa:egfp;olig2:dsred* larvae and imaged for 6 hpi to determine if *ctgfa* was expressed following injury. I used laser-induced transection to perform either no injury, injury to the muscle, or spinal motor nerve axotomy (Figure 3-7A-C). In larvae with either no injury or injury to the muscle, I saw no change in *ctgfa* expression over 6 hpi (Figure 3-7A-A’’’’, B-B’’’’; *no injury, n=4 nerves in 4 larvae; muscle injury, n=4 nerves in 4 larvae*). However, in larvae where I induced spinal motor nerve injury, I observed an increase in *ctgfa* expression in the injury site over 6 hpi (Figure 3-7C-C’’’’; *n=11 nerves in 5 larvae*). This data demonstrates that *ctgfa* expression is increased only after spinal motor nerve axotomy. Interestingly, I observed *ctgfa* expression within the injury site during the 2 to 6 hpi window that perineurial glia are bridging. Therefore, I hypothesized that *ctgfa* might drive perineurial glial bridging downstream of TGFβ signaling.

To assess the role of *ctgfa* expression in perineurial glial bridging, I created a *nkx2.2a:gal4;UAS:NTR-mcherry* line to visualize both perineurial glia and *ctgfa* expression after injury. I injured motor nerves in 5 or 6 dpf *nkx2.2a:gal4;UAS:NTR-mcherry;ctgfa:egfp* larvae using laser axotomy and time-lapse imaged for 8 hpi. Although I observed both perineurial glial bridging and an increase in *ctgfa* expression along all injured nerves, I did not observe co-localization of *nkx2.2a* and *ctgfa* expression (Figure 3-7D’-D’’’; *n=6 nerves in 4 larvae)*. Although *ctgfa* expression was not observed in

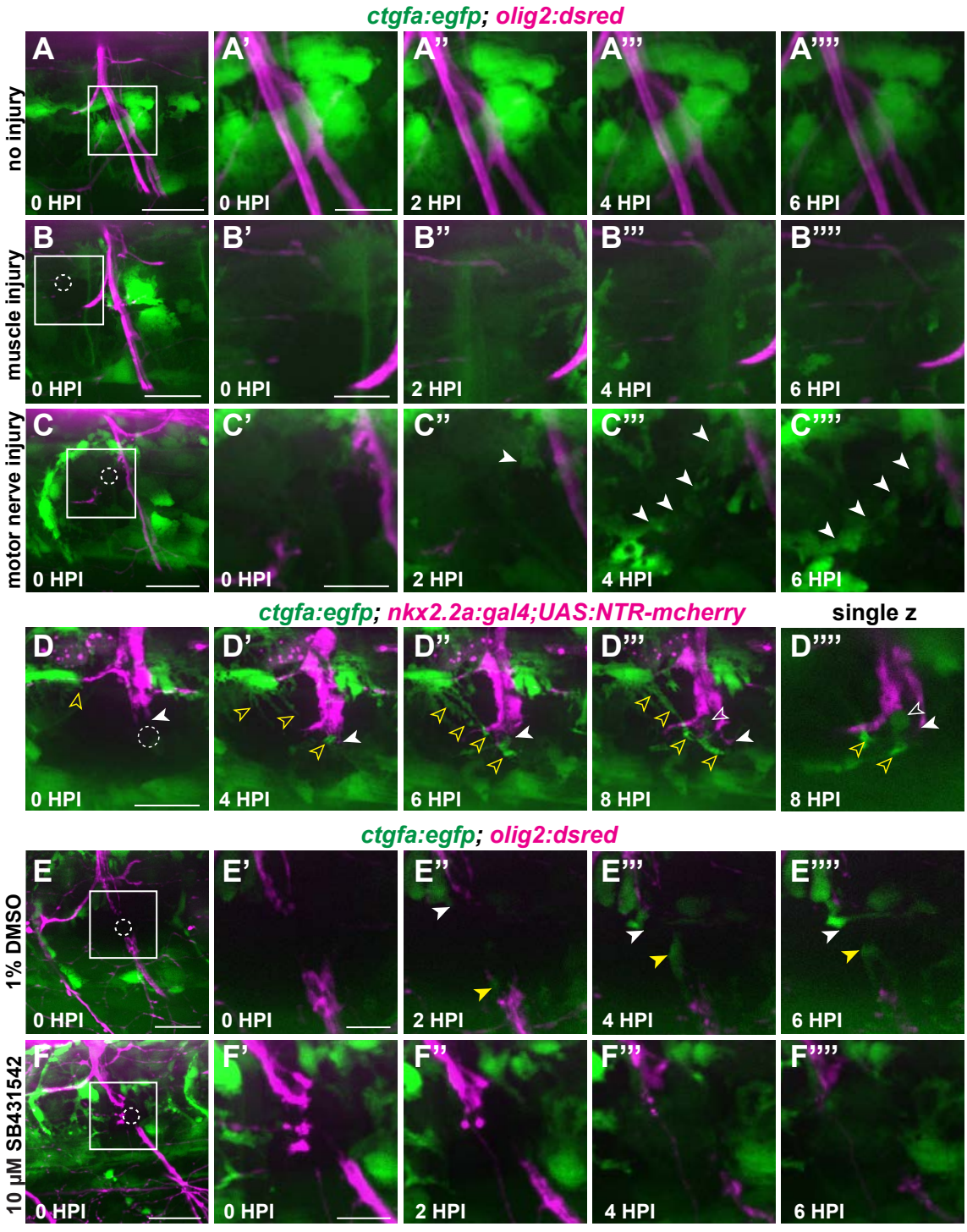

ctgfa:egfp; olig2:dsred
no injury
muscle injury
motor nerve injury
A
A'
A''
A'''
A''''
B
B'
B''
B'''
B''''
C
C'
C''
C'''
C''''
0 HPI
2 HPI
4 HPI
6 HPI
ctgfa:egfp; nkx2.2a:gal4;UAS:NTR-mcherry
single z
D
D'
D''
D'''
D''''
0 HPI
4 HPI
6 HPI
8 HPI
ctgfa:egfp; olig2:dsred
1% DMSO
10 µM SB431542
E
E'
E''
E'''
E''''
F
F'
F''
F'''
F''''

Figure 3-7. *ctgfa* expression increases at the injury site.

A-C) Representative images from stills of time-lapse movies of 5 or 6 dpf *ctgfa:egfp;olig2:dsred* larvae. Larvae were either uninjured (A; *n=4 nerves in 4 larvae*), injured in muscle adjacent to spinal motor nerves (B; *n=4 nerves in 4 larvae*), or injured along the spinal motor nerve (C; *n=11 nerves in 5 larvae*) and time-lapse imaged for 6 hpi. Dashed circles indicate the injury site. White arrows indicate increased *ctgfa* expression. D) Representative images from stills of a time-lapse movie of a 6 dpf *nkx2.2a:gal4;UAS:NTR-mcherry;ctgfa:egfp* larva (*n=6 nerves in 4 larvae*). A single z-plane image demonstrates potential regions of co-localization of perineurial glia (magenta) and *ctgfa* expression (green) at 8 hpi. Solid white arrows follow bridging perineurial glia across the injury site. Open white arrows indicate *ctgfa* expression that might co-localize with perineurial glia. Open yellow arrows specify increased *ctgfa* expression that does not co-localize with perineurial glia. E-F) Representative images from stills of time-lapse movies of 5 or 6 dpf *ctgfa:egfp;olig2:dsred* larvae. Larvae were either treated with 1% DMSO (E; *n=4 nerves in 3 larvae*) or 10 μM SB431542 (F; *n=4 nerves in 3 larvae*). White arrows follow the increase in *ctgfa* expression proximal to the injury site and yellow arrows follow the increase in *ctgfa* distal to the injury site. Scale bars, 25 μm; magnified insets, 10 μm.

perineurial glial cells after injury, it is possible that *ctgfa* drives perineurial glial bridging indirectly through its positive feedback loop with TGFβ signaling.

To determine whether *ctgfa* expression in the injury site was indeed downstream of TGFβ signaling, I treated 4 dpf *ctgfa;egfp;olig2:dsred* larvae with 10 μM SB431542 in 1% DMSO or 1% DMSO alone. Spinal nerves in larvae from both groups were injured and imaged for 6 hpi at 6 dpf. DMSO-treated larvae showed an increase in *ctgfa* expression in the injury site by 2 hpi, with expression in the injury site increasing through 6 hpi (Figure 3-7E-E''''; *n=4 nerves in 3 larvae*). Larvae treated with SB431542 showed no change in expression of *ctgfa* after injury and had no increase in *ctgfa* in the injury site by 6 hpi (Figure 3-7F-F''''; *n=4 nerves in 3 larvae*). Therefore, inhibition of TGFβ signaling negatively regulates *ctgfa* expression in the injury site. Because *ctgfa* expression is changed by TGFβ signaling inhibition but is present in the injury site during the timing of perineurial glial bridging in the presence of TGFβ signaling, I hypothesized that *ctgfa* works within the TGFβ signaling pathway to drive perineurial glial bridging.

Perineurial glial bridging, but not phagocytosis, is perturbed in ctgfa mutant larvae

To determine if *ctgfa* was required for perineurial glial bridging even in the presence of TGFβ signaling, I injured spinal motor axons in *ctgfa*^{bns50} larvae, which have a 7-nt deletion in the third exon of *ctgfa* (Mokalled et al., 2016). *ctgfa*$^{-/-}$ adults are viable and larvae at 6 dpf show typical motor nerve and perineurial glial development (Figure 3-8A). Larvae from an in-cross of *ctgfa*$^{+/-}$ fish expressing both *nkx2.2a:megfp* and *olig2:dsred* were injured at 5 or 6 dpf and time-lapse imaged in 2-hour intervals for 6 hpi. Larvae were then

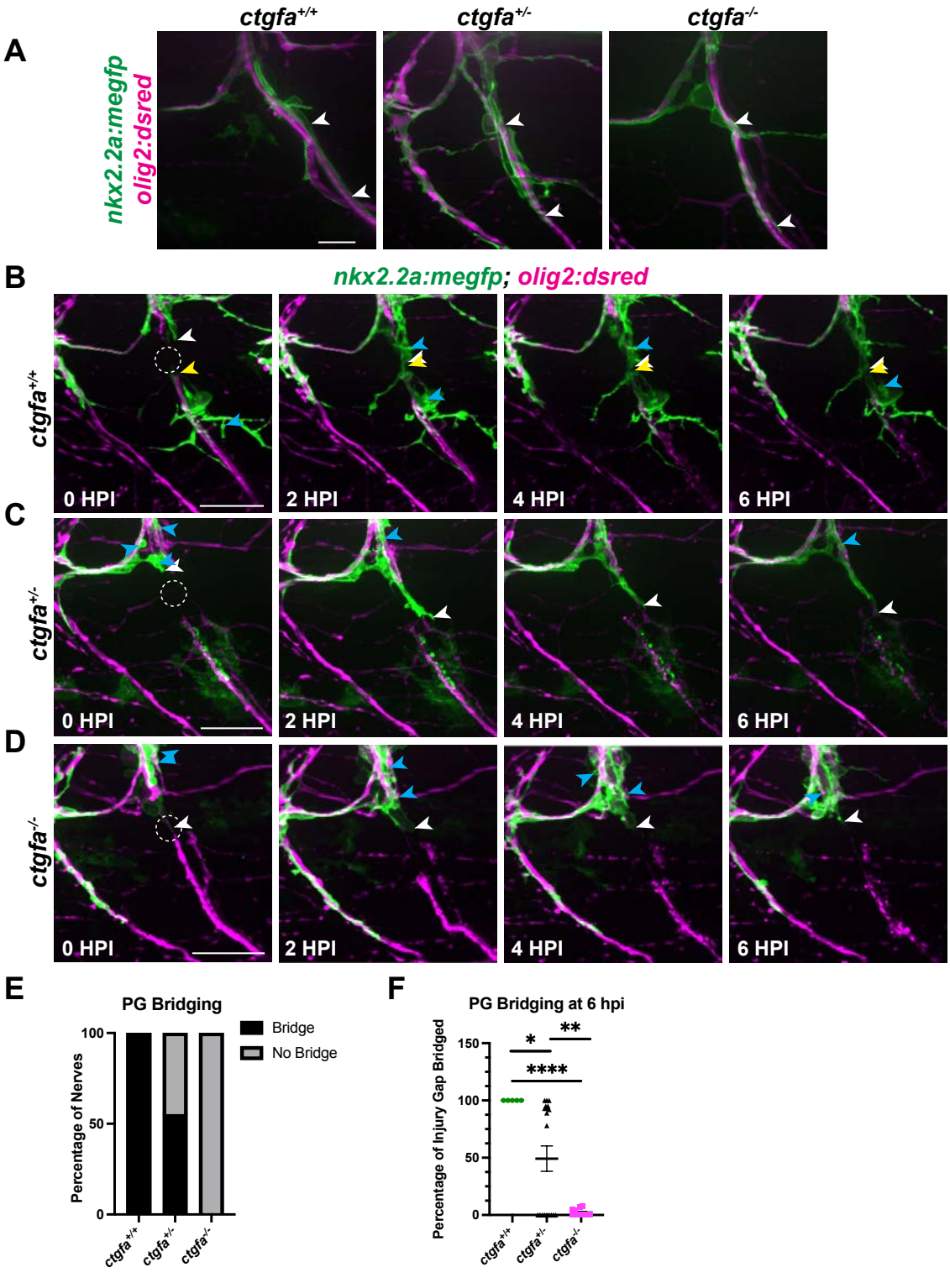

A
ctgfa+/+
ctgfa+/-
ctgfa-/-
nkx2.2a:megfp
olig2:dsred
B
nkx2.2a:megfp; olig2:dsred
ctgfa+/+
0 HPI
2 HPI
4 HPI
6 HPI
C
ctgfa+/-
0 HPI
2 HPI
4 HPI
6 HPI
D
ctgfa-/-
0 HPI
2 HPI
4 HPI
6 HPI
E
PG Bridging
Bridge
No Bridge
Percentage of Nerves
100
50
0
ctgfa+/+
ctgfa+/-
ctgfa-/-
F
PG Bridging at 6 hpi
Percentage of Injury Gap Bridged
150
100
50
0
*
**
****
ctgfa+/+
ctgfa+/-
ctgfa-/-

Figure 3-8. Perineurial glial bridging is perturbed in *ctgfa* mutants.

A) Representative images prior to injury of 5 dpf *ctgfa*$^{+/+}$, *ctgfa*$^{+/-}$, and *ctgfa*$^{-/-}$ *nkx2.2a:megfp;olig2:dsred* larvae. White arrows indicate healthy perineurial glia fully ensheathing motor nerves. B-D) 5 or 6 dpf larvae with *ctgfa*$^{+/+}$ (*n=5 nerves in 4 larvae*), *ctgfa*$^{+/-}$ *(n=19 nerves in 10 larvae*), or *ctgfa*$^{-/-}$ (*n=11 nerves in 6 larvae*) genotypes. B-D) Representative images from time-lapse movies of 5 or 6 dpf *ctgfa*$^{+/+}$ (B), *ctgfa*$^{+/-}$ (C), or *ctgfa*$^{-/-}$ (D) larvae where spinal motor nerves were injured. Dashed circles indicate injury site. White arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge. Blue arrows specify perineurial glial phagocytic vesicles. E) Quantification of percentage of nerves in which perineurial glia bridged (black) or did not bridge (gray) across *ctgfa*$^{+/+}$, *ctgfa*$^{+/-}$, or *ctgfa*$^{-/-}$genotypes. F) Quantification of the percentage of the injury gap bridged by perineurial glia at 6 hpi by *ctgfa*$^{+/+}$, *ctgfa*$^{+/-}$, or *ctgfa*$^{-/-}$ larvae (mean ± SEM: *ctgfa*$^{+/+}$: 100±0; *ctgfa*$^{+/-}$: 49.21±11.06; *ctgfa*$^{-/-}$: 2.18±0.96. p-values: *ctgfa*$^{+/+}$ vs. *ctgfa*$^{+/-}$: p=0.0301; *ctgfa*$^{+/+}$ vs. *ctgfa*$^{-/-}$: p<0.0001; *ctgfa*$^{+/-}$ vs. *ctgfa*$^{-/-}$: p=0.0033). Scale bars, (A) pre-injury images, 10 μm; (B-D) 25 μm.

genotyped immediately following the completion of imaging. In *ctgfa*$^{+/+}$ larvae, I always observed perineurial glial bridging within the first 6 hpi (Figure3-8B, E, F; *n=5 nerves in 4 larvae*). In contrast, perineurial glia fully bridged the injury site in only 55% of nerve axotomies in *ctgfa*$^{+/-}$ larvae (Figure 3-8C, E, F; *n=10 bridging nerves, n=9 non-bridging nerves in 10 larvae)*. Finally, I observed no perineurial glial bridging along injured motor nerves in any *ctgfa*$^{-/-}$ larvae (Figure 3-8D, E, F; *n=11 nerves in 6 larvae)*. This phenotype is consistent with what I observed in SB431542-treated larvae (Figure 3-3D). Interestingly, perineurial glia continue to form phagocytic vesicles in all genotypes (Figure 3-8B-D). Therefore, because loss of *ctgfa* produces a similar phenotype to that of TGFβ inhibition, I conclude that *ctgfa* is required for perineurial glial bridging.

Based on my data, I propose a model in which TGFβ signaling and *ctgfa* work in a positive feedback loop to drive perineurial glial bridging (Figure 3-9). My data shows that perineurial glia independently require TGFβ signaling (Figure 3-3D) and *ctgfa* signaling (Figure 3-9D) for bridging following spinal motor nerve injury. Additionally, inhibition of TGFβ signaling leads to a loss of *ctgfa* expression in the injury site after injury (Figure 3-7F). Therefore, I hypothesize that following spinal motor nerve injury, TGFβ signaling, drives an increase in *ctgfa* expression. This increase in *ctgfa* expression then in turn stimulates binding of TGFβ-1 to its receptors to drive the TGFβ signaling necessary for perineurial glial bridging. It is possible that the stimulation of TGFβ signaling by *ctgfa* is necessary to initiate perineurial glial bridging, and that basal availability of TGFβ signaling is insufficient to do so. Therefore, loss of TGFβ signaling would cause a loss of both perineurial glial bridging (Figure 3-3D) and *ctgfa* expression (Figure 3-8D) after injury, as

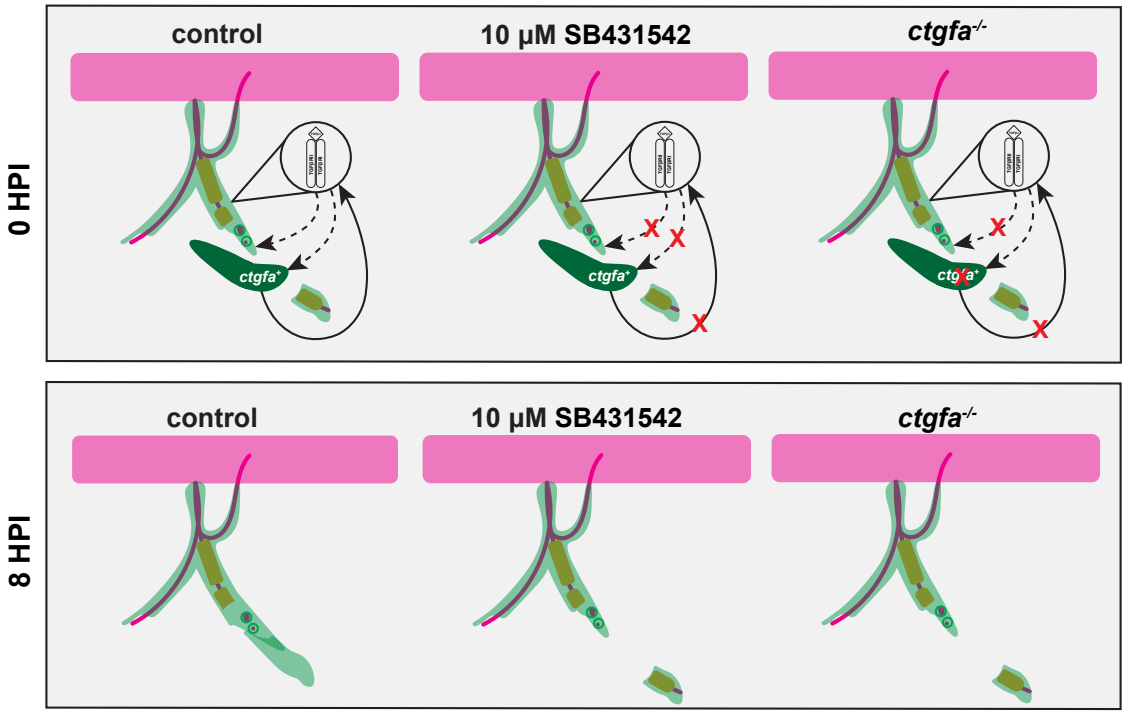


Figure 3-9. Perineurial glial bridging is modulated by a positive feedback loop between TGFβ signaling and *ctgfa*.

Summary diagram of our proposed signaling model that drives perineurial glial bridging after injury in control, 10 µM SB431542, and *ctgfa*$^{-/-}$ conditions at 0 (top panels) and 8 hpi (bottom panels). Motor axons (magenta), Schwann cells (orange), perineurial glia (light green), *ctgfa* expressing cells (dark green). The magnified region designates perineurial glial-specific TGFβ signaling. Dotted-lines indicate TGFβ signaling whereas solid lines indicate *ctgfa* signaling. Red "X"s demonstrate pathways that are inhibited or turned off in either 10 µM SB431542 treatment or *ctgfa*$^{-/-}$ larvae.

observed with my SB431542-treated larvae (Figure 3-9). Subsequently, loss of *ctgfa* expression would cause a loss of perineurial glial bridging (Figure 3-8D) due to insufficient availability of TGFβ signaling, as observed in my imaging with injured *ctgfa*$^{-/-}$ larvae (Figure 3-9). Therefore, I hypothesize that TGFβ signaling drives perineurial glial bridging after spinal motor nerve injury through a positive feedback loop with *ctgfa*.

Discussion

Understanding the cellular and molecular drivers of peripheral motor nerve regeneration is crucial to gaining insight into effective and functional recovery after injury. Most research focuses on the roles that Schwann cells and macrophages play in regeneration (B. Chen et al., 2021; P. Chen et al., 2015; Clements et al., 2017; Kristjan R. Jessen & Mirsky, 2019; A. F. Rosenberg et al., 2012; Schira et al., 2018). However, perineurial glia are also essential for efficient nerve regeneration (G. M. Lewis & Kucenas, 2014). Here, I show that TGFβ signaling drives perineurial glial bridging. I also introduce a factor downstream of TGFβ signaling, *ctgfa*, which is necessary for perineurial bridging. These data unlock novel insights into what drives perineurial glial bridging, a process that is essential for successful nerve regeneration.

TGFβ signaling drives perineurial glial bridging after spinal motor nerve injury

Perineurial glia are one of the first cell types to respond to spinal motor nerve injury in zebrafish and initiate the regeneration process by phagocytosing debris and bridging across the injury site within the first 8 hpi (G. M. Lewis & Kucenas, 2014). My data demonstrates that the location of perineurial glial nuclei relative to the injury site influences whether

perineurial glia bridge from both the proximal and distal side of the injury or just from the proximal side. While the location of perineurial glial nuclei affects the type of bridging that occurs, it is also likely that location of perineurial glial nuclei could affect signaling that occurs during bridging. Following the inhibition of TGFβ signaling, perineurial glial proximal stump velocity is decreased relative to DMSO-treated larvae along nerves that had proximal and distal bridging. However, proximal stump velocity is not decreased relative to DMSO-treated controls along nerves that had proximal only bridging. This data raises the possibility that TGFβ signaling might be acting in a paracrine manner in perineurial glia, and that the lack of distal perineurial glia in proximal only bridging nerves might result in a smaller source of TGFβ signaling in DMSO-treated control larvae, causing their initial velocity to be more similar to that of those with TGFβ inhibition. Therefore, the availability of TGFβ signaling depending on the position of perineurial glial nuclei relative to the injury site might influence the ability of perineurial glia to initiate bridging.

Alternatively, it is possible that TGFβ signaling indirectly drives perineurial glial bridging. Previous studies demonstrate that perineurial glial fail to bridge but continue to phagocytose debris in the absence of Schwann cells (G. M. Lewis & Kucenas, 2014). TGFβ-1 is secreted from Schwann cells after injury and is necessary for their transdifferentiation from myelinating to repair Schwann cells (Clements et al., 2017; Schira et al., 2018). Perineurial glial bridge before Schwann cells form bands of Bungner and physically enter the injury site (G. M. Lewis & Kucenas, 2014). My studies demonstrate that when Schwann cells are present during development but eliminated immediately prior to injury, perineurial glia are unable to bridge. Therefore, it is likely that perineurial glia

are bridging during the time in which Schwann cells secrete TGFβ-1. For this reason, Schwann cells are another possible source of TGFβ signaling. Future studies further investigating the source of TGFβ-1 and whether this signaling is directly or indirectly affecting perineurial glial bridging will shed light on this possibility.

Finally, my studies demonstrate that pSmad3 is present in *nkx2.2a*$^{+}$ nuclei early during perineurial glial bridging behaviors, peaking at about 3 hpi, suggesting that TGFβ signaling in perineurial glia is important for the initiation of bridging. Although it is known that TGFβ signaling is crucial for the initiation of bridging, it remains unknown if acute or sustained inhibition of this cascade is what causes this phenotype. Further understanding the critical period of TGFβ signaling required for perineurial glial bridging could provide insight into what cells might be a direct source of this molecule and reveal potential therapeutic targets for enhancing peripheral nerve regeneration.

Perineurial glial bridging and phagocytosis are controlled by separate molecular mediators

In addition to bridging, perineurial glia phagocytose debris after injury. Consistent with previous work from our lab, I observed that perineurial glial phagocytosis is more prominent on the proximal stump than on the distal stump (G. M. Lewis & Kucenas, 2014). In the absence of Schwann cells, perineurial glia fail to bridge, however they continue to phagocytose debris (G. M. Lewis & Kucenas, 2014). My data demonstrates that while perineurial glia fail to bridge in the absence of TGFβ signaling or depletion of *ctgfa* expression, they continue to phagocytose debris, supporting the hypothesis that perineurial

glial bridging and phagocytosis are controlled by distinct molecular drivers. Further, perineurial glia do not appear to phagocytose Schwann cell debris, suggesting that perineurial glial phagocytosis is specific to axonal debris and supporting previous work demonstrating that perineurial glia, Schwann cells, and macrophages spatially coordinate to clear debris after injury. Future studies aiming to identify potential signaling pathways that control perineurial glial phagocytosis would add to our understanding of the full molecular repertoire that drives the perineurial glial injury response. Such studies could also elucidate the role that perineurial glial phagocytosis plays in debris clearance and whether their phagocytosis is required for perineurial glial bridging as well as full regeneration. Because perineurial glia phagocytose axonal debris primarily on the proximal stump of the axon, it is possible that without perineurial glial phagocytosis clearing debris to create a growth permissive environment, perineurial glia would not be able to establish a bridge.

connective tissue growth factor-a is necessary for perineurial glial bridging

ctgfa is a well-known driver of regenerative responses after injury (Mokalled et al., 2016; Mukherjee et al., 2021). Additionally, *ctgfa* works in a positive feedback loop with TGFβ signaling to increase the production of TGFβ-1 (Abreu et al., 2002). My results support the presence of this feedback loop, as inhibition of TGFβ signaling resulted in a loss of *ctgfa* expression after injury. Similarly, absence of *ctgfa* signaling in *ctgfa* mutants phenocopied the loss of TGFβ signaling, with both resulting in the loss of perineurial glial bridging but the retention of perineurial glial phagocytosis. This suggests that loss of *ctgfa* expression

might be affecting TGFβ signaling levels, therefore indirectly affecting perineurial glial bridging through the downregulation of TGFβ signaling.

In my imaging with *ctgfa:egfp* after injury, I observed expression of *ctgfa* increase in the injury site during the first 6 hpi, the period during which perineurial glia are forming their bridge. While CTGF is secreted by Schwann cells after injury (Schira et al., 2018), I see an increase in *ctgfa* expression in the injury site in the first 6 hpi, before Schwann cells migrate into the injury site and form Bands of Bungner. Therefore, it is unlikely that the cells we observe expressing *ctgfa* during this time are Schwann cells. Bulk RNA-sequencing data from our lab shows that *nkx2.2a*$^{+}$ cells express *ctgfa* during development (unpublished), therefore it is possible that perineurial glia express *ctgfa* during regeneration. Although I do not observe co-localization of *ctgfa* expression with perineurial glia during the first 8 hpi, it is still possible that perineurial glia are expressing *ctgfa* at a low level that is difficult to observe with the tools currently available. Additionally, I observed cells that express *ctgfa* actively respond to motor nerve injury but are not physically found along the nerve. Therefore, additional cells are expressing *ctgfa* as a response to injury, and these cells could be indirectly driving perineurial glial bridging through *ctgfa* expression and enhancement of TGFβ signaling. Schwann cells secrete TGFβ after injury (Schira et al., 2018; Sulaiman & Nguyen, 2016), however it is likely that TGFβ signaling derived from Schwann cells alone is insufficient to drive perineurial glial bridging. It is possible that enhancement of this basal TGFβ signaling by *ctgfa* expression is sufficient to drive bridging. This is supported by my data, where eliminating TGFβ signaling or *ctgfa* expression independently produced similar phenotypes, with perineurial

glia unable to form a bridge but continuing to phagocytose debris. Future studies investigating which cells are expressing *ctgfa* after injury and the role that these cells play in perineurial glial bridging and motor nerve regeneration will be key to closing this loop. These cells could be epineurium or endoneurium cells, of which little is known about their injury responses and of which we currently lack specific markers. Further, perineurial glia act as a component of the blood-nerve barrier in both mice and zebrafish (Clark et al., 2014; Kucenas, Takada, et al., 2008) and *ctgfa* is important for angiogenesis during wound healing (Abreu et al., 2002; Schira et al., 2018). Therefore, *ctgfa* could be driving perineurial glial bridging through stimulation of TGFβ signaling while concurrently aiding in reinnervation of vasculature following injury, with endothelial cells being the *ctgfa*$^{+}$ cells we observe. Future studies investigating the relationship between blood vessel bridging and perineurial glial bridging are important to determine the role that perineurial glia play in reinnervation following injury.

Together, my work establishes TGFβ signaling as an essential driver of perineurial glial bridging after peripheral motor nerve injury in zebrafish. I found that TGFβ signaling is important for early initiation of perineurial glial bridging. Further, I identify *ctgfa* as a downstream effector of TGFβ signaling that is necessary for perineurial glial bridging. Although both TGFβ and *ctgfa* signaling are known to be involved in peripheral nerve regeneration and wound healing (Clements et al., 2017; Mokalled et al., 2016; Mukherjee et al., 2021; Schira et al., 2018; Sulaiman & Nguyen, 2016), this work highlights a novel and crucial role for both of these signals in the perineurial glial injury response. Collectively, my studies increase our understanding of how perineurial glial respond to

injury and provide insight into potential cellular and molecular targets for therapeutics to enhance and promote regeneration.

CHAPTER IV

Elucidation of cellular interactions driving perineurial glial bridging

Abstract

Schwann cells and perineurial glia are crucial for spinal motor nerve development as well as effective and efficient spinal motor nerve regeneration after injury. Though both cell types are known to be essential for regeneration and their individual repair programs have been investigated, little is known about the cellular and molecular interactions between these two cell types following injury. Though previous studies from our lab demonstrated that complete absence of Schwann cells perturbs perineurial glial bridging, these studies lacked tools to investigate these questions without perturbing perineurial glial development. In this chapter, I used pharmacological perturbation of the Schwann cell injury response as well as cell-specific and drug-inducible death of *sox10* cells using genetic ablation to determine that Schwann cells are indeed necessary for perineurial glial bridging without perturbing perineurial glial development.

Though Schwann cells are necessary for perineurial bridging, the mechanism through which Schwann cells communicate to perineurial glia following injury remains unknown. My studies presented in Chapter III indicate that TGFβ signaling drives perineurial glial bridging through a positive feedback loop with *ctgfa*. However, it remains unclear whether this TGFβ signaling acts directly or indirectly on perineurial glial to drive bridging. Schwann cells are known to secrete TGFβ-1 following motor nerve injury and absence of Schwann cells perturbs perineurial glial bridging. Therefore, Schwann cells are a possible

source of TGFβ signaling for perineurial glial bridging. To determine whether TGFβ signaling is directly driving perineurial glial or bridging or if TGFβ signaling is indirectly affecting bridging through interactions with Schwann cells, I developed a cell-specific and drug-inducible dominant negative *tgfbr2b* mutation. This tool will allow future studies to investigate the molecular interactions between Schwann cells and perineurial glia following injury and elucidate whether TGFβ signaling is directly or indirectly driving perineurial glial bridging.

Introduction

Schwann cells and perineurial glia are essential for proper motor nerve development (Clark et al., 2014; Kristjan R. Jessen & Mirsky, 2005; Kristján R. Jessen et al., 2015; Kucenas, Snell, et al., 2008; Kucenas, Takada, et al., 2008; A. D. Morris et al., 2017). Reciprocal interactions between Schwann cells and perineurial glia drive these developmental processes. In both mice and zebrafish, inhibition of perineurial glial migration during development adversely affects Schwann cell differentiation and ability to myelinate motor nerves (Binari et al., 2013; Kucenas, Takada, et al., 2008). Conversely, absence of Schwann cells during development causes perineurial glia to sparsely ensheath motor nerves (G. M. Lewis & Kucenas, 2014; A. D. Morris et al., 2017). Therefore, both perineurial glia and Schwann cells require proper development of the other cell type in order for their own development to occur. Similarly, these cells types are essential for successful motor nerve regeneration after injury. Though interactions between these cell types have not been extensively investigated following regeneration, studies suggest that absence of Schwann cells negatively affects the ability of perineurial glia to form a glial bridge after injury (G.

M. Lewis & Kucenas, 2014). However, these studies were conducted using *colourless* mutants, which are deficient for *sox10* and completely lack Schwann cells during development (Dutton et al., 2001). Perineurial glia in these mutants were sparse and did not fully ensheath nerves, again demonstrating that perineurial glia require Schwann cells for proper development (G. M. Lewis & Kucenas, 2014). However, lack of perineurial glial bridging following injury could be due to developmental defects rather than a direct effect on their regenerative program. Therefore, more precise strategies are necessary to determine if perineurial glia require Schwann cells for bridging following injury.

In the previous chapter, my studies identified TGFβ signaling as a driver of perineurial glial bridging after spinal motor nerve injury. I demonstrate that in the absence of TGFβ signaling perineurial glia are unable to bridge (Figure 3-3), however, whether TGFβ signaling is directly or indirectly driving perineurial glial bridging remains unknown. While it is possible that TGFβ signaling acts in an autocrine manner, being both secreted and received by perineurial glia to drive bridging, it is also likely that perineurial glia are receiving TGFβ-1 from an extrinsic source. It is known that TGFβ-1 is secreted from Schwann cells following injury (Schira et al., 2018; Sulaiman & Nguyen, 2016) and Schwann cells require TGFβ signaling for the early stages of their injury response (Clements et al., 2017), therefore Schwann cells are a possible candidate source of TGFβ signaling for perineurial glial bridging. Alternatively, it is possible that TGFβ signaling is indirectly driving perineurial glial bridging by regulating the cellular behaviors of another cell type, such as Schwann cells, whose regenerative response is necessary for perineurial glial bridging to occur. Inhibiting TGFβ signaling cell-specifically in either Schwann cells

or perineurial glia would elucidate whether TGFβ signaling is directly driving perineurial glial bridging or indirectly affecting bridging through interactions with Schwann cells. However, there are currently no tools that would allow for cell-specific inhibition of TGFβ signaling without perturbing perineurial glial development.

Here, using pharmacological perturbation to inhibit the Schwann cell injury response prior to spinal motor nerve injury without disrupting perineurial glial development, I demonstrate that perineurial glia require Schwann cells to undergo their injury response to bridge. Further, I utilize a *sox10* driven nitroreductase line to show that perineurial glia do not bridge following the death of Schwann cells immediately prior to spinal motor nerve injury, nor do they phagocytose Schwann cell debris. These studies support the hypothesis that perineurial glia require Schwann cells for bridging following injury. However, it still remains unclear whether perineurial glial interactions with Schwann cells after injury are tied to TGFβ signaling. To elucidate whether TGFβ signaling acts directly or indirectly to drive perineurial glial bridging, I created a cell-specific and drug-inducible dominant negative mutant of the TGFβ receptor II to allow for inhibition of TGFβ signaling specifically in either perineurial glia or Schwann cells prior to injury, but after major developmental processes have occurred. I have validated that the dominant negative TGFβRII mutation effectively inhibits TGFβ signaling and preliminary data using this line suggests that TGFβ signaling might be directly driving perineurial glial bridging. Together, my findings indicate that Schwann cells are essential for perineurial glial bridging and I provide a crucial tool for future studies to elucidate the mechanism through which TGFβ signaling drives perineurial glial bridging.

Results

Inhibition of erbB2/erbB3 perturbs perineurial glial bridging

Previous studies from our lab show that in the absence of Schwann cells, perineurial glia fail to bridge (G. Lewis, 2015; G. M. Lewis & Kucenas, 2014). However, these studies were conducted in *colourless* mutants, which are deficient for *sox10* and entirely lack nerve-associated Schwann cells (Dutton et al., 2001). In these mutants, perineurial glia were present but were sparse and did not form continuous sheaths (G. M. Lewis & Kucenas, 2014; A. D. Morris et al., 2017). Therefore, although perineurial glia did not bridge following injury in *colourless* mutants, it is possible that this resulted from perturbed development of perineurial glia due to lack of nerve-associated Schwann cells.

Studies in *erbb3b* mutants show that perineurial glial proliferation and migration into the periphery is normal in the absence of Schwann cells. However, the presence of Schwann cells is necessary for perineurial glial differentiation (A. D. Morris et al., 2017). These findings reinforce that further studies are necessary to truly determine if perineurial glia require the presence of Schwann cells for bridging. To further investigate the relationship between perineurial glia and Schwann cells following injury without perturbing perineurial glial development, I treated 4 dpf *nkx2.2a:megfp;olig2:dsred* larvae for 48 hours with either 4 μM AG1478, a selective inhibitor of the ErbB2/ErbB3 heterodimer as well as the ErbB1 receptor (EGFR) (Lyons et al., 2005), in 1% DMSO in PTU egg water or with 1% DMSO alone in PTU egg water. Inhibition of ErbB2/ErbB3 perturbs Schwann cell migration, proliferation, and terminal differentiation in zebrafish (Lyons et al., 2005).

Larvae were treated with AG1478 or DMSO at 4 dpf, after major developmental processes for both Schwann cells and perineurial glia have occurred (Kristján R. Jessen et al., 2015; Kucenas, 2015). At 6 dpf, after 48 hours of treatment, larvae in both groups had normal ensheathment of nerves by perineurial glia (data not shown). Larvae from both DMSO and AG1478-treated groups were injured using laser axotomy and imaged every five minutes for 6 hpi. Larvae in DMSO-treated groups had complete bridging by 6 hpi and phagocytosis was observed in perineurial glia (Figure 4-1A, *n=4 nerves*). However, perineurial glia in AG1478-treated larvae did not bridge by 6 hpi but did phagocytose debris (Figure 4-1A; *n=3 nerves*). These results support the previous data from our lab suggesting that perineurial glia require Schwann cells for proper bridging (G. M. Lewis & Kucenas, 2014). Though Schwann cell development and ability to migrate and proliferate is perturbed with treatment of AG1478 (Lyons et al., 2005), Schwann cells remain physically present on the nerve in my studies. Schwann cells have begun to associate with axons and myelinate at this time, as I treated my larvae with AG1478 at 4 dpf to avoid perturbing perineurial glial development (Kristján R. Jessen et al., 2015; Lyons et al., 2005). Despite the presence of Schwann cells on the nerve, larvae treated with AG1478 were unable to form a perineurial glial bridge after injury. Therefore, it is likely that signaling coming from Schwann cells as they undergo their own regenerative behaviors is driving perineurial glial bridging rather than physical presence of Schwann cells.

Absence of Schwann cells immediately prior to injury perturbs perineurial glial bridging

Though treatment with AG1478 inhibits Schwann cell migration and proliferation immediately prior to and during the injury response, allowing for normal perineurial glial

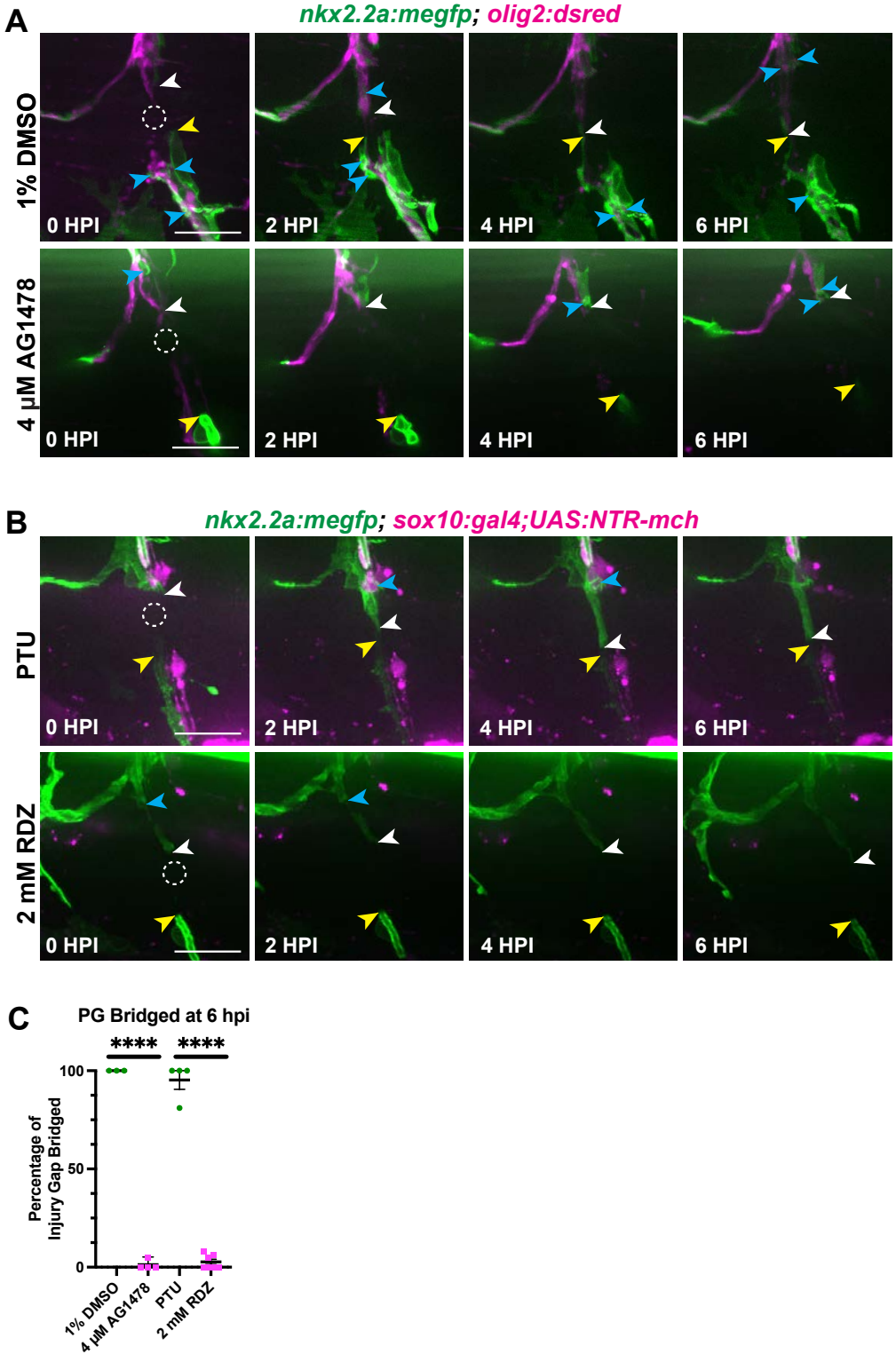

A
nkx2.2a:megfp; olig2:dsred
1% DMSO
4 µM AG1478
0 HPI
2 HPI
4 HPI
6 HPI
B
nkx2.2a:megfp; sox10:gal4;UAS:NTR-mch
PTU
2 mM RDZ
C
PG Bridged at 6 hpi
****
****
Percentage of Injury Gap Bridged
100
50
0
1% DMSO
4 µM AG1478
PTU
2 mM RDZ

Figure 4-1. Perineurial glia require Schwann cells for their bridging injury response.

Representative images from stills of time-lapse movies of injured 5 or 6 dpf *nkx2.2a:megfp;olig2:dsred* (A) or *nkx2.2a:megfp;sox10:gal4;UAS:NTR-mch* (B) larvae. Dashed circles indicate injury sites. White arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge (green). Blue arrows indicate phagocytic vesicles. A) Larvae were either treated with 1% DMSO (top panels; *n=4 nerves in 3 larvae*) or 4 μM AG1478 (bottom panels; *n=3 nerves in 3 larvae*). B) Larvae were treated with either PTU water (top panels; *n=4 nerves in 3 larvae*) or 2 mM ronidazole (RDZ) (bottom panels; *n=7 nerves in 5 larvae*). C) Quantification of the percentage of the injury gap bridged by perineurial glia (PG) at 6 hpi in larvae treated with either 1% DMSO (green; mean: 100±0), 4 μM AG1478 (magenta; mean: 1.25±1.25) ($p<0.0001$), PTU water (green; mean: 95.25±1.32) or 2 mM RDZ (magenta; mean: 2.71±1.32) ($p<0.0001$).

development but preventing perineurial glial bridging, it is possible that the perturbation in perineurial glial bridging observed in AG1478-treated larvae is due to inhibition of ErbB2/ErbB3. Though ErbB2/ErbB3 is not known to drive perineurial glial behaviors after major developmental processes have completed, because the drug treatment is global, it is difficult to discern whether this inhibition would have a direct effect on perineurial glial bridging. To determine that it is specifically Schwann cells that are required for perineurial glial bridging, I injured and imaged 5 or 6 dpf larvae expressing *nkx2.2a:megfp* and *sox10:gal4;UAS:NTR-mcherry* for 6 hpi. Larvae were either kept in PTU egg water or treated with 2 mM ronidazole (RDZ) in PTU egg water to induce the expression of nitroreductase and subsequent cell-specific death in *sox10*$^{+}$ cells (Lai et al., 2021). Larvae were treated with RDZ for only 6 hours prior to imaging to ensure that perineurial glial development occurred and Schwann cells were only eliminated immediately prior to injury. RDZ-treated larvae demonstrated Schwann cell death at the time of imaging, however perineurial glial ensheathment of spinal motor nerves appeared normal and healthy (data not shown). Following spinal motor nerve injury, larvae in only PTU egg water displayed perineurial glial bridging and phagocytosis by 6 hpi (Figure 4-1B; *n=4 nerves*). However, perineurial glia in larvae treated with RDZ did not bridge by 6 hpi (Figure 4-1B; *n=7 nerves*). Therefore, in the complete absence of Schwann cells, perineurial glia are unable to bridge, indicating that perineurial glia require signals from Schwann cells for proper bridging. Intriguingly, I observed that perineurial glia continue to phagocytose debris in RDZ-treated larvae, reinforcing the hypothesis that perineurial glial bridging and phagocytosis are distinct events controlled by different molecular cues (Figure 3-4, 4-1). However, perineurial glia do not phagocytose Schwann cell debris in RDZ-treated larvae.

This indicates that perineurial glial phagocytosis is specific to axonal debris and that the signals that drive phagocytosis are not derived from Schwann cells. This observation is consistent with previous data suggesting that Schwann cells, macrophages, and perineurial glia spatially coordinate their debris clearance following injury (G. M. Lewis & Kucenas, 2014).

Together, these studies further support the hypothesis that perineurial glia require Schwann cells for bridging. My studies in particular suggest that there is chemical communication occurring between Schwann cells and perineurial glia following injury that drives perineurial glial bridging. Schwann cells are known to both secrete TGFβ-1 (Schira et al., 2018) and require TGFβ signaling (Clements et al., 2017) for their own injury response. This data, combined with my studies presented in the previous chapter indicating that TGFβ signaling drives perineurial glial bridging, suggest Schwann cells as a likely source of TGFβ-1 for perineurial glial bridging. However, more tools are required to fully elucidate whether perineurial glial bridging is being directly or indirectly affected by TGFβ signaling and where the source of TGFβ signaling is originating from.

Generation of a cell-specific and drug-inducible dominant negative TGFβRII

In order to investigate whether perineurial glial bridging is being directly or indirectly affected by TGFβ signaling, I created a cell-specific and drug-inducible dominant negative mutation of TGFβRII. The construct containing the dominant negative mutation was made to be cell-specific by utilizing a 3.5 kilobase (kb) *nkx2.2a* promoter (Zhu, 2019) to drive the dominant negative mutation, therefore perturbing TGFβ signaling only in perineurial

glial cells. Previous studies from our lab showed that TGFβ signaling is necessary for perineurial glial development (A. D. Morris et al., 2017). To address this issue and prevent disruption of developmental processes, I utilized the TetOn system to create my dominant negative construct, making expression of the mutation inducible by the addition of doxycycline hyclate (doxy) (L. J. Campbell et al., 2012). By treating larvae expressing the TetOn dominant negative construct with doxy at 5 dpf, after all major perineurial glial developmental processes have occurred (Kucenas, 2015), for 24 hours prior to injury, I could ensure that perineurial glial development would not be perturbed.

To create the dominant negative mutation, I induced an early stop codon in the *tgfbr2b* locus, thus deleting the active kinase domain of the receptor and establishing *DNtgfbr2b*. Elimination of the kinase domain allows for the TGFβRII receptor to continue to form a heterodimer with TGFβRI and bind TGFβ-1, but prevents phosphorylation of pSmad3 and subsequent downstream signaling from occurring (Kitisin et al., 2007; Tanaka, Mori, Mafune, Ohno, & Sugimachi, 2000) (Figure 4-2A). Using the TetOn system to allow this dominant negative mutation to be cell-specific and drug-inducible (L. J. Campbell et al., 2012), I generated a *nkx2.2a:rtTA* construct which would cell-specifically drive the dominant negative mutation in *nkx2.2a*$^{+}$ cells. Further, I inserted the dominant negative mutation into a *biTRE:nls-mch* vector using Tol2kit (Kwan et al., 2007), allowing for bi-directional expression of both my dominant negative mutation and *nls-mcherry*, providing an easy method to screen and confirm that the mutation has been induced after the addition of doxy. Therefore, upon the introduction of doxy, *nkx2.2a:rtTA* binds to *biTRE:nls-mch:DNtgfbr2b* and induces co-expression of *DNtgfbr2b* and *nls-mcherry* in *nkx2.2a*$^{+}$ cells

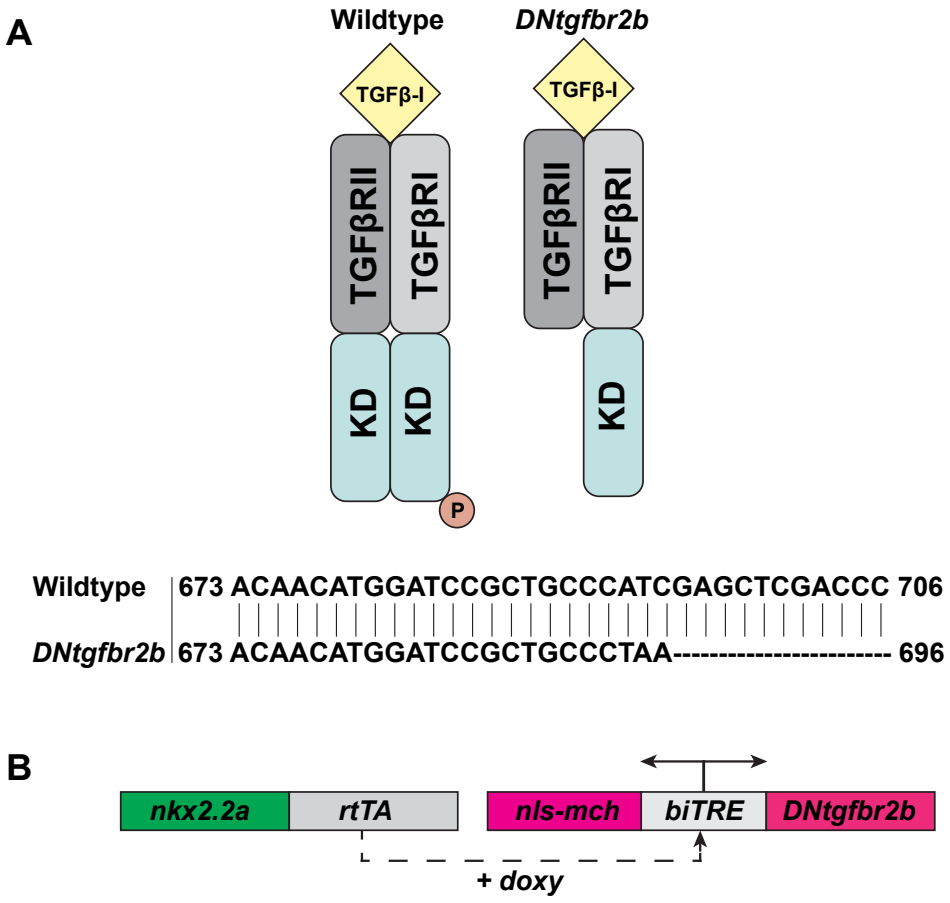


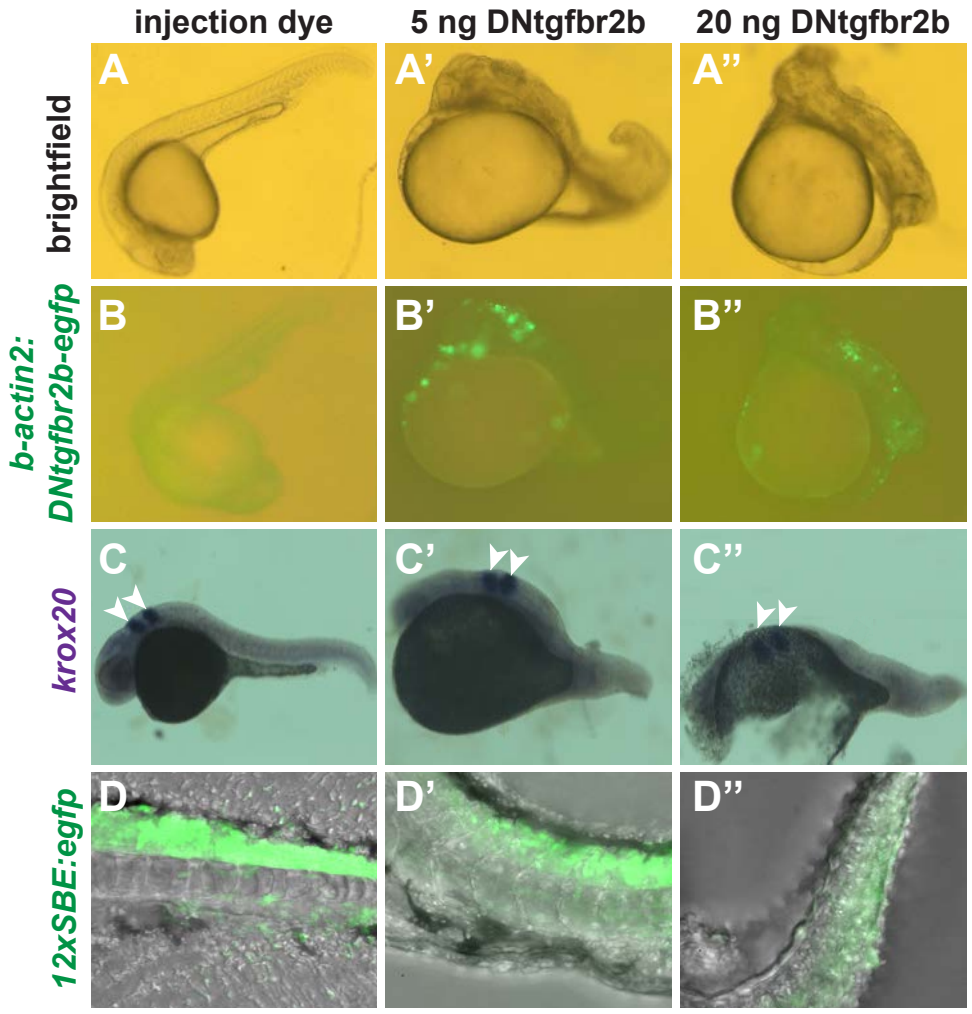
Figure 4-2. Generation of a dominant negative *tgfbr2b*.

A) Diagrammatic representation of the deletion of the kinase domain (KD) in TGFβRII by introduction of an early stop codon to produce *DNtgfbr2b*. Deletion of the kinase domain of TGFβRII allows TGFβ-1 to bind to the TGFβRII/ TGFβRI heterodimer, but prevents TGFβRII from phosphorylating TGFβRI and activating downstream signaling. B) Diagrammatic representation of the cell-specific and doxycycline-inducible *nkx2.2a:rtTA; biTRE:nls-mch:DNtgfbr2b* construct created using the TetOn system.

(Figure 4-2B), allowing for temporally controlled elimination of TGFβ signaling specifically in perineurial glia. Application of this tool will allow future studies to investigate whether TGFβ signaling directly or indirectly affects perineurial glial bridging.

Validation of DNtgfbr2b

To validate that the *DNtgfbr2b* mutation that I created does indeed eliminate TGFβ signaling, I used Tol2Kit to create a construct driving the DNtgfbr2b with a *b-actin2* promoter and an p3E-egfp tag, *b-actin2:DNtgfbr2b-egfp* (Kwan et al., 2007). Embryos expressing this transgene would lack TGFβ signaling globally. I injected this construct into AB* embryos at the one-cell stage, screened larvae for GFP expression, and imaged GFP^+ larvae at 24 hpf. Control larvae were injected with injection dye alone. Though the embryos would have mosaic expression of the *b-actin2:DNtgfbr2b-egfp* construct, it should be sufficient to perturb development, as early inhibition of TGFβ signaling at various concentrations causes developmental deformities, such as loss of mesodermal tissues, the spinal cord, midbrain, and hindbrain, as early as 24 hpf in zebrafish (Sun et al., 2006). Accordingly, larvae injected with either 5 ng or 20 ng of *b-actin2:DNtgfbr2b-egfp* that had GFP^+ cells showed abnormal development consistent with that observed with drug inhibition TGFβ signaling at 24 hpf, with the 20 ng group having a higher percentage of affected larvae (Figure 4-3A'-A", B'-B", E; *n=63, 122 larvae, respectively*) (Sun et al., 2006). Conversely, larvae injected with only injection dye showed no GFP^+ cells and normal development at 24 hpf (Figure 4-3A, B, E; *n=113 larvae*). To validate that the DNtgfbr2b was indeed inhibiting TGFβ signaling, I conducted *in situ* hybridization for *krox20* in 24 hpf *b-actin2:DNtgfbr2b-egfp* or injection dye-injected larvae. In healthy


injection dye
5 ng DNtgfbr2b
20 ng DNtgfbr2b
brightfield
b-actin2:
DNtgfbr2b-egfp
krox20
12xSBE:egfp
A
A'
A''
B
B'
B''
C
C'
C''
D
D'
D''

E

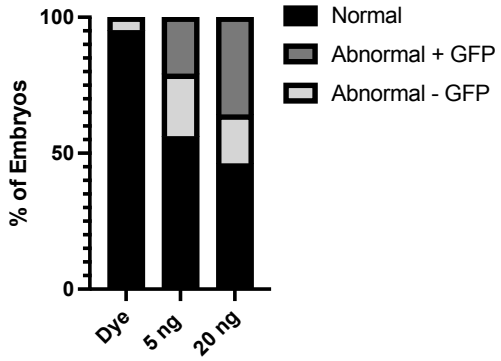

100
50
0
% of Embryos
Dye
5 ng
20 ng
Normal
Abnormal + GFP
Abnormal - GFP

Figure 4-3. Validation of a dominant negative *tgfbr2b*.

Representative still images of 24 hpf larvae injected with either injection dye, 5 ng of *b-actin2:DNtgfrb2b-egfp*, or 20 ng of *b-actin2:DNtgfbr2b-egfp.* A-A") Brightfield imaging of *b-actin2:DNtgfbr2b-egfp* larvae (*n=133, 63, 122 larvae, respectively*). B-B") GFP imaging of *b-actin2:DNtgfbr2b-egfp* larvae (*n=133, 63, 122 larvae, respectively*). C-C') *krox20 in situ* hybridization. White arrows point to *krox20* staining in the 3rd and 5th rhombomeres of the hindbrain (*n=5, 7, 7, larvae, respectively*). D-D") Merge of brightfield and GFP imaging in *12xSBE:egfp* larvae, which fluoresce green in the presence of Smad3 expression (*n=11, 5, 6, larvae, respectively*). E) Quantification of the percentage of 24 hpf injected embryos from A-A") and B-B") that had normal morphology (black), abnormal morphology and GFP expression (dark gray), or abnormal morphology and no GFP expression (light gray). Larvae injected with only injection dye had a majority with normal morphology (95%, *n=113 larvae*). Larvae injected with 20 ng of *b-actin2:DNtgfbr2b-egfp* had more affected larvae (36% abnormal and GFP^{+}, 46% normal; *n=122* larvae) than those injected with only 5 ng (23% abnormal and GFP^{+}, 56% normal; *n=63 larvae*).

larvae, *krox20* expression is observed in the 3rd and 5th rhombomeres of the hindbrain. However, with inhibition of TGFβ signaling, *krox20* expression is either present more posteriorly than in unperturbed larvae or completely absent depending on the concentration of TGFβ inhibition, demonstrating abnormal development of the hindbrain (Sun et al., 2006). In larvae injected with injection dye alone, *in situ* hybridization of *krox20* showed expected hindbrain development, with *krox20* expression in the 3rd and 5th rhombomeres as well as overall normal morphology for 24 hpf larvae (Figure 4-3C; *n=5 larvae*). However, in *krox20 in situ* hybridizations in larvae injected with either 5 ng or 20 ng of *b-actin2:DNtgfbr2b-egfp*, larvae showed *krox20* labeling more posteriorly than in uninjected larvae, indicating abnormal hindbrain development consistent with inhibition of TGFβ signaling (Figure 4-3C', C"; *n=7, 7 larvae, respectively*). *krox20* expression is still present because the injected larvae have mosaic expression of *b-actin:DNtgfbr2b-egfp*, and the *krox20* posterior shift phenotype is consistent with that of inhibition of TGFβ signaling at a lower drug concentration (Sun et al., 2006). Further, these larvae showed developmental defects of mesodermal tissues and the spinal cord. Finally, I injected either injection dye alone or *b-actin2:DNtgfbr2b* without a p3E-egfp tag into *12xSBE:egfp* larvae, which contain Smad3 responsive elements that express GFP in the presence of TGFβ signaling, at the one-cell stage and imaged at 24 hpf (Casari et al., 2014). In larvae injected only with injection dye, there is strong GFP expression in the tail of the larvae, indicative of active Smad3 signaling (Figure 4-3D; *n=11 larvae*) (Casari et al., 2014). However, in larvae injected with either 5 ng or 20 ng of *b-actin2:DNtgfbr2b*, larvae showed decreased levels of GFP expression in the tail as well as morphological abnormalities consistent with TGFβ signaling inhibition (Figure 4-3D', D"; *=5, 6 larvae, respectively*). Because of the

mosaicism associated with injecting *b-actin2:DNtgfbr2b,* there remained some GFP expression in the tail. However less GFP expression was observed in the 20 ng injected group compared to the 5 ng group, indicating that higher expression of *b-actin2:DNtgfbr2b* results in greater inhibition of TGFβ signaling (Figure 4-3D', D"). Taken together, I concluded that the DNtgfbr2b mutation that I created does indeed inhibit TGFβ signaling when expressed. This mutation, when expressed using the TetOn system, provides us with a tool to be able to explore the role of TGFβ signaling in perineurial glial bridging in a cell-specific and drug-inducible manner.

Mosaic expression of DNtgfbr2b may perturb perineurial glial bridging

To determine if inhibition of TGFβ signaling using my *DNtgfbr2b* mutation directly affects perineurial glial bridging, I created *nkx2.2a:rtTA;cmlc2:egfp*, *sox10:rtTA;cmlc2:gfp*, and *biTRE:nls-mch:DNtgfbr2b;cry:gfp* constructs using the Tol2kit and TetOn tool kit (L. J. Campbell et al., 2012; Kwan et al., 2007). I injected these constructs independently into AB* embryos at the one-cell stage and screened for reporter expression. Larvae that contained the reporter specific to the injected construct (*cmlc2:egfp* expression for *nkx2.2a:rtTA* or *sox10:rtTA* injected larvae and *cry:gfp* expression for *biTRE:nls-mch:DNtgfbr2b* injected larvae) were sterilized using ovadine and put into the system to screen for potential founders. DNA from embryos from each injected construct group was extracted using HotSHOT (Meeker et al., 2007) and was sequenced using Sanger sequencing to confirm that the correct constructs had inserted into the larvae genome. Founders were identified for the *nkx2.2a:rtTA* line, however no founders were identified for the *sox10:rtTA* or *biTRE:nls-mch:DNtgfbr2b* lines. *nkx2.2a:rtTA* founders were

identified by presence of the *cmlc2:egfp* reporter in larvae when outcrossed to AB*. To determine that the *nkx2.2a:rtTA* line correctly functioned, *nkx2.2a:rtTA* founders were crossed to *nkx2.2a:megfp* fish and injected with 20 ng of *biTRE:nls-mch:DNtgfbr2b;cry:gfp* at the one-cell stage. Injected larvae were screened at 48 hpf for presence of both the *cmlc2:egfp* and *cry:gfp* reporter. Larvae containing both reporters were treated at 2 or 3 dpf with either 20 μM doxy and 1% DMSO in PTU egg water or 1% DMSO in PTU egg water for 24 hours prior to imaging. At 3 or 4 dpf, larvae were imaged. DMSO-treated larvae showed no nuclear *mcherry* expression in *nkx2.2a*$^{+}$ cells, indicating no activation of the *biTRE:nls-mch:DNtgfbr2b* (Figure 4-4A; *n=5 larvae*). Conversely, larvae treated with doxy displayed mosaic nuclear *mcherry* expression specifically in *nkx2.2a*$^{+}$ cells, indicating that these constructs were correctly activated by addition of doxy (Figure 4-4A; *n=5 larvae*).

To preliminarily investigate whether TGFβ signaling directly affects perineurial glial bridging, I injected *biTRE:nls-mch:DNtgfbr2b;cry:gfp* into *nkx2.2a:rtTA;cmlc2:egfp* F1*;nkx2.2a:megfp* embryos at the one-cell stage. Larvae were screened at 48 hpf for both *cmlc2:egfp* and *cry:gfp* reporters. At 4 dpf, larvae were treated with either 20 μM doxy and 1% DMSO in PTU egg water or 1% DMSO in PTU egg water for 24 hours prior to imaging. Doxy-treated larvae with *nls-mcherry* expression in *nkx2.2a*$^{+}$ cells were injured and time-lapse imaged for 6 hpi. In contrast to DMSO-treated larvae, in which perineurial glia bridged by 6 hpi (Figure 4-4B; *n=3 nerves*), doxy-treated larvae did not bridge by 6 hpi (Figure 4-4B; *n=3 nerves*). This data suggests that TGFβ signaling directly drives perineurial glial bridging. However, because *biTRE:nls-mch:DNtgfbr2b* was injected into

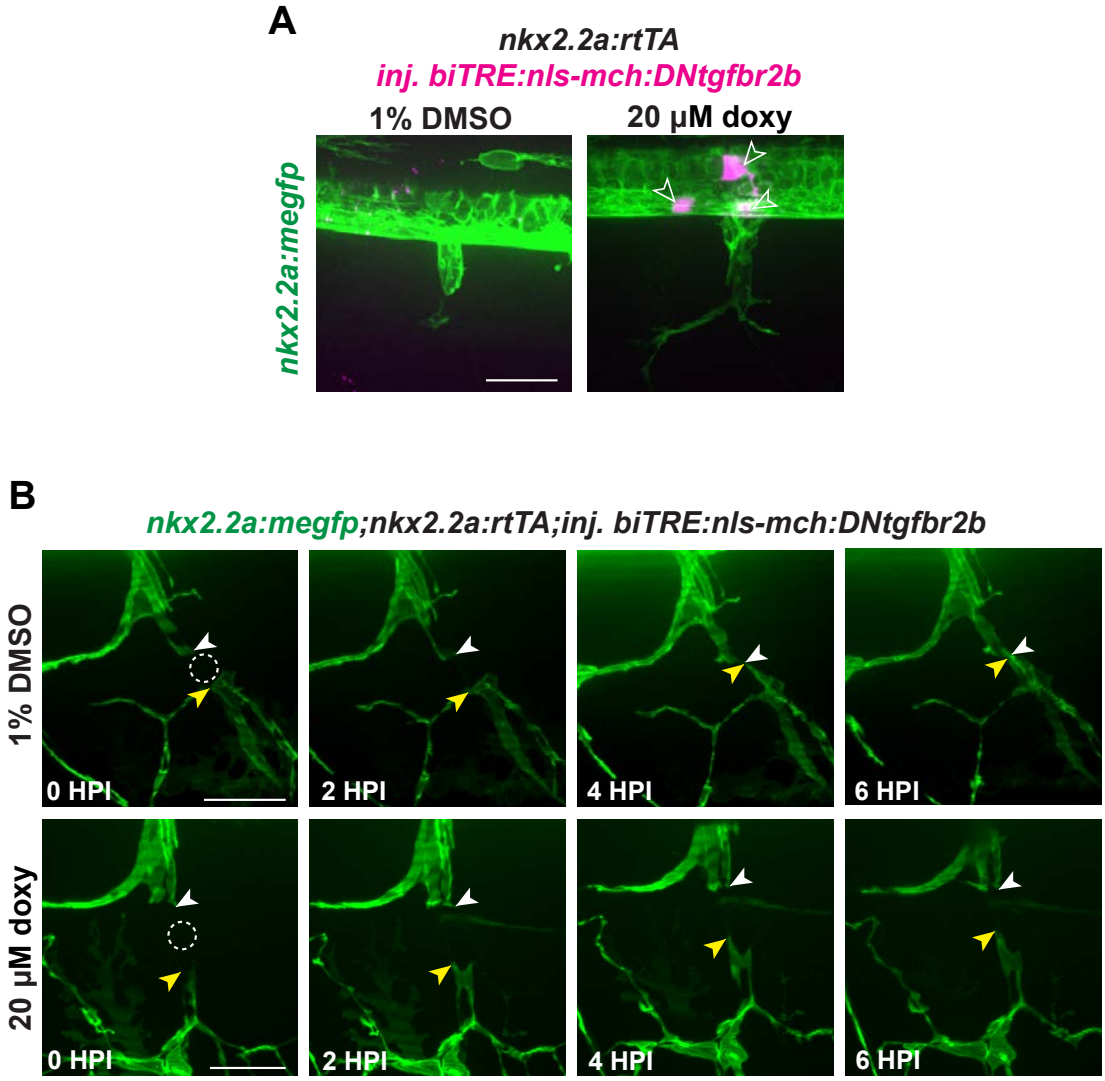

A
nkx2.2a:rtTA
inj. biTRE:nls-mch:DNtgfbr2b
1% DMSO
20 µM doxy
nkx2.2a:megfp
B
nkx2.2a:megfp;nkx2.2a:rtTA;inj. biTRE:nls-mch:DNtgfbr2b
1% DMSO
20 µM doxy
0 HPI
2 HPI
4 HPI
6 HPI
0 HPI
2 HPI
4 HPI
6 HPI

Figure 4-4. Mosaic expression of *DNtgfrb2b* may perturb perineurial glial bridging.

A) Representative still images of 3 dpf *nkx2.2a:megfp;nkx2.2a:rtTA* larvae injected with *biTRE:nls-mch:DNtgfbr2b* and treated with either 1% DMSO or 20 μM of doxycycline (doxy). Open arrows indicate *nkx2.2a*$^{+}$ nuclei expressing *nls-mcherry*. *nls-mcherry* expression was only observed in *nkx2.2a*$^{+}$ nuclei in doxy treated larvae (*n=5 larvae*). B) Representative still images from a time-lapse movie of injured 6 dpf *nkx2.2a:megfp;nkx2.2a:rtTA* larvae injected with *biTRE:nls-mch:DNtgfbr2b* and treated with either 1% DMSO or 20 μM of doxy over 6 hpi. Dashed circles indicate injury sites. White arrows follow the proximal end and yellow arrows follow the distal end of the perineurial glial bridge. Scale bars, 25 μm.

nkx2.2a:rtTA F1 embryos, expression of *DNtgfbr2b* remains mosaic and was not found in all *nkx2.2a*$^{+}$ cells on a particular nerve. Finding a founder for *biTRE:nls-mch:DNtgfbr2b* and generation of a stable line would allow for more accurate studies of how TGFβ signaling drives perineurial glial bridging.

Discussion

Reciprocal interactions between Schwann cells and perineurial glia are necessary for proper spinal motor nerve development (Binari et al., 2013; Kucenas, Takada, et al., 2008; A. D. Morris et al., 2017). Immediately following injury, perineurial glia phagocytose debris and form a bridge across the injury site while Schwann cells begin to transform into a repair phenotype, phagocytosing debris and guiding regenerating axons across the perineurial glial bridge later in the regeneration process (Kristjan R. Jessen & Mirsky, 2019; G. M. Lewis & Kucenas, 2014). While these two cell types are essential for spinal motor nerve development and regeneration, little is known about the cellular and molecular interactions between the two cell types during regeneration. In this chapter, my studies reinforce previous findings from our lab suggesting that perineurial glia require Schwann cells in order to bridge following injury (G. M. Lewis & Kucenas, 2014) by utilizing pharmacological perturbation and nitroreductase to disrupt the Schwann cell injury response or eliminate Schwann cells, respectively, without perturbing perineurial glial development. However, it remains unclear whether signaling from Schwann cells drives perineurial glial bridging. My findings presented in Chapter III determined that TGFβ signaling drives perineurial glial bridging, and previous data suggests Schwann cells as a likely source of TGFβ signaling (Clements et al., 2017; Schira et al., 2018; Sulaiman &

Nguyen, 2016). To elucidate how TGFβ signaling modulates perineurial glial bridging, I created and validated a cell-specific and drug-inducible dominant negative *tgfbr2b* mutation. Future studies utilizing this mutation could determine whether TGFβ signaling directly drives perineurial glial bridging or whether it is extrinsically modulating bridging through interactions with other cell types, such as Schwann cells, following injury. Such insights would illuminate how this signaling pathway regulates crucial injury responses and how interactions between key cell types allow for successful motor nerve regeneration.

Perineurial glia require Schwann cells to form a bridge after spinal motor nerve injury

Previous studies demonstrate that perineurial glia require Schwann cells in order to bridge, however these studies were limited as the *colourless* mutants utilized to eliminate Schwann cells also perturbed perineurial glial development (Dutton et al., 2001; G. M. Lewis & Kucenas, 2014). I utilized a specific inhibitor of ErbB2/ErbB3 to prevent Schwann cell proliferation and migration from 4 to 6 dpf, after major perineurial glial developmental processes have occurred (Kucenas, 2015; Lyons et al., 2005). Following spinal motor nerve injury, perineurial glia treated with the inhibitor do not bridge. This suggests that perineurial glia do indeed require Schwann cells for their injury response. It is unclear whether it is the physical presence of Schwann cells or molecular interactions between perineurial glia and Schwann cells that are required for proper perineurial glial bridging. However, Schwann cells treated with the ErbB2/ErbB3 inhibitor are still present on the nerve and perineurial glia are unable to bridge, suggesting that it is the perturbation to the Schwann cell injury response, and subsequent changes in signaling, that prevents perineurial glia from bridging. Future studies using the ErbB2/ErbB3 inhibitor in a *sox10*

transgenic line could confirm that the Schwann cell injury response is disrupted. During the time that perineurial glia are bridging, Schwann cells are not migrating or actively proliferating, therefore it is likely that inhibition of ErbB2/ErbB3 is perturbing the Schwann cell injury response in a way that disrupts molecular communication between Schwann cells and perineurial glia. This disruption could be a direct effect, where ErbB2/ErbB3 activates downstream signals in Schwann cells that are secreted to perineurial glia to drive bridging. Alternatively, ErbB2/ErbB3 signaling could drive cellular behaviors in Schwann cells that lead to the release of molecular signals necessary for perineurial glial bridging.

There still remains the possibility that inhibition of ErbB2/ErbB3 is directly affecting perineurial glial bridging, though no previous studies suggest that this would be the case. In fact, in *erbb3b* mutants, perineurial glial differentiation during early development is disrupted, however these perineurial morphological defects do not persist into adulthood (G. Lewis, 2015), indicating that if ErbB2/ErbB3 signaling does affect perineurial glial development, it is during a very specific early window. Additionally, bulk RNAseq data from our lab shows little expression of ErbB2/ErbB3 in *nkx2.2a*$^{+}$ cells at 72 hpf (unpublished), after major developmental processes have completed (Kucenas, 2015). Therefore, it is unlikely that inhibition of ErbB2/ErbB3 is directly preventing perineurial glia from forming a bridge after injury. Further, complete elimination of Schwann cells with nitroreductase demonstrated that perineurial glia do not bridge in the absence of Schwann cells. This suggests that it is molecular interactions between perineurial glia and Schwann cells that drive perineurial glial bridging. Because Schwann cell and perineurial

glial interactions are reciprocal during development, it is likely that reciprocal interactions are required for regeneration as well. Future studies utilizing *nkx2.2a:gal4;UAS:NTR-mch* line with a *sox10* transgenic line could determine whether Schwann cells require perineurial glial bridging for their injury response. By utilizing this line and imaging later after injury, additional studies could determine whether Schwann cells are able to form bands of Bungner in the absence of perineurial glia and subsequent bridging. Further understanding the cellular and molecular interactions between perineurial glia and Schwann cells following injury could provide insights into the signaling pathways that drive successful regeneration as well as provide targets for therapeutic approaches to injury recovery.

Using a dominant negative tgfbr2b to elucidate how TGFβ signaling modulates perineurial glial bridging

My studies presented in Chapter III indicated that TGFβ signaling drives perineurial glial bridging after spinal motor nerve injury. However, it remains unclear where the source of TGFβ signaling is coming from and whether TGFβ signaling acts directly or indirectly on perineurial glia to drive bridging. One possibility is that TGFβ signaling acts directly on perineurial glia to drive bridging. This could occur in two manners; one in which TGFβ signaling acts in an autocrine manner, with TGFβ-1 both being produced and received by perineurial glia, or where TGFβ-1 is received directly by perineurial glia but from an extrinsic source, such as neighboring Schwann cells, macrophages, or degenerating axons. A third possibility is that TGFβ signaling drives perineurial glial bridging indirectly, with TGFβ-1 acting directly on another cell type, such as Schwann cells, to initiate a cellular

behavior or molecular cascade that then modulates perineurial glial bridging. My findings analyzing perineurial glial velocity after injury in different types of bridging both with and without inhibition of TGFβ signaling suggest that TGFβ signaling might be working in an autocrine manner following injury, as perineurial glia with nuclei both proximal and distal to the injury site had a larger difference in velocity in both control and TGFβ-inhibited groups, whereas perineurial glia with nuclei only proximal to the injury site did not have a difference in velocity. Though they were still able to bridge, this suggests that perhaps their TGFβ-1 availability was lower because of less nuclei present, therefore slowing down their initial bridging velocity (Figure 3-6). However, my studies presented in this chapter demonstrate that perineurial glia require Schwann cells to bridge, suggesting that Schwann cells might be a source of signaling for perineurial glial bridging. Schwann cells are known to secrete both TGFβ-1 and CTGF following injury (Schira et al., 2018; Sulaiman & Nguyen, 2016), so it is possible that Schwann cells are directly providing a source of TGFβ-1 for perineurial glia. Alternatively, Schwann cells might secrete the CTGF necessary to drive the positive feedback loop with TGFβ signaling to produce the concentration of TGFβ-1 required for perineurial glial bridging, as perineurial glia might use TGFβ-1 in an autocrine manner but are unable to basally produce enough to allow for bridging. Therefore, more specific studies are necessary to elucidate how TGFβ signaling modulates perineurial glial bridging. To address this issue, I created a cell-specific and doxycycline-inducible dominant negative *tgfbr2b* (*DNtgfrb2b*) using the TetOn system (L. J. Campbell et al., 2012) to allow for targeted elimination of TGFβ signaling without disrupting perineurial glial or Schwann cell development. I established a stable line for *nkx2.2a:rtTA*, allowing for perineurial glial-specific expression of *DNtgfbr2b* when paired with the

biTRE:nls-mch:DNtgfbr2b construct. Preliminary mosaic studies where *biTRE:nls-mch:DNtgfbr2b* was injected into stable *nkx2.2a:rtTA* larvae suggested that TGFβ signaling is directly affecting perineurial glial bridging. However, generation of a stable *biTRE:nls-mch:DNtgfbr2b* line will allow for more accurate studies to be conducted. Additionally, I created a *sox10:rtTA* construct so that TGFβ signaling may be specifically eliminated in Schwann cells prior to injury in combination with *biTRE:nls-mch:DNtgfbr2b.* Generation of a stable *sox10:rtTA* line will allow for future studies to determine if elimination of TGFβ signaling in Schwann cells negatively affects perineurial glial bridging. If perineurial glial bridging were perturbed, it would suggest that the TGFβ signaling is indirectly affecting perineurial glial bridging through the Schwann cell injury response. Therefore, the generation of a cell-specific and drug-inducible dominant negative *tgfbr2b* will allow for future studies to determine how TGFβ signaling modulates perineurial glial bridging. While this powerful tool will elucidate how TGFβ signaling is being received to drive bridging, additional tools will need to be established to derive the source of TGFβ signaling.

Together, my studies further implicate that Schwann cells and perineurial glia interact following spinal motor nerve injury to drive regeneration. However, it remains unknown whether the interactions between Schwann cells and perineurial glia are reciprocal and how TGFβ signaling is modulating these processes. Additionally, there is no set timeline for when these cellular and molecular interactions are necessary. By continuing to investigate the cellular behaviors of these two cell types following injury utilizing the tools I established in this chapter, we can begin to establish a timeline of cellular and molecular

interactions between perineurial glia and Schwann cells that will allow us to better understand the interplay of signals that modulate spinal motor nerve regeneration.

CHAPTER V

Discussion and Future Directions

Summary

In this dissertation, I have identified the first known molecular pathways to drive perineurial glial bridging after spinal motor nerve injury in zebrafish and created tools with which future studies can continue to study the cellular and molecular mechanisms that drive perineurial glial bridging.

In Chapter III, I characterized the dynamics of perineurial glial bridging, establishing timing of different phases of bridging and showing that the positioning of perineurial glial nuclei relative to the injury site is essential for determining what type of bridging will occur. Understanding the dynamics of perineurial glial bridging will allow for better interpretation of phenotypes in conditions where perineurial glial bridging is perturbed as well as provide insight into how signals are driving this process. Further, I identified TGFβ signaling as a novel driver of perineurial glial bridging, but not phagocytosis, confirming previous data that bridging and phagocytosis are controlled by separate molecular signals. I demonstrated that inhibition of TGFβ signaling perturbs perineurial glial bridging dynamics, but not the injury responses of Schwann cells or macrophages, therefore this signaling is specific to the perineurial glial injury response. Using an antibody specific to pSmad3, I determined that TGFβ signaling is present in perineurial glial nuclei early after injury, peaking at around 3 hpi. This indicates that TGFβ signaling is essential for early initiation of the perineurial glial bridge into the injury site. Finally, I identified *connective*

tissue growth factor-a as a factor downstream of TGFβ signaling that is necessary for perineurial glial bridging. *ctgfa* is expressed in the injury site following spinal motor nerve injury within the first 6 hpi, during which perineurial glia are bridging. Interestingly, inhibition of TGFβ signaling eliminates *ctgfa* in the injury site, suggesting that *ctgfa* is downstream of TGFβ signaling. In the absence of *ctgfa* signaling, perineurial glia no longer bridge but continue to phagocytose debris. This phenotype is similar to that when TGFβ signaling is inhibited, suggesting that *ctgfa* works in a positive feedback loop to drive perineurial glial bridging in the early hours after spinal motor nerve injury. Together, my findings identify novel signaling pathways driving perineurial glial bridging, filling a gap in our knowledge about how these molecular signals modulate cellular behaviors that are essential for effective spinal motor nerve regeneration after injury and providing future targets for therapeutic approaches.

In Chapter IV, I investigated the cellular interactions between perineurial glia and Schwann cells after injury. Using pharmacological perturbation and cell-specific and drug-inducible expression of nitroreductase to inhibit the Schwann cell injury response and eliminate the presence of Schwann cells, respectively, without perturbing development of either cell type. Under these conditions, I demonstrate that perineurial glia require Schwann cells in order to properly bridge, confirming previous studies. Similarly, perineurial glia fail to bridge with inhibition of the Schwann cell injury response while Schwann cells are present on the nerve, suggesting that it is molecular communication between these two cell types that is necessary to drive the perineurial glial injury response. In order to more precisely determine how molecular signals are driving perineurial glial bridging, I created and

validated a drug-inducible dominant negative *tgfbr2b* mutation which can be driven specifically in either Schwann cells or perineurial glia. This tool will allow for future studies to elucidate whether TGFβ signaling is directly or indirectly driving perineurial glial bridging and will provide additional insight into how perineurial glia and Schwann cells are molecularly communicating to drive efficient and effective regeneration.

Molecular regulators of perineurial glial bridging after spinal motor nerve injury

My studies build upon previous work from our lab to establish three main stages of perineurial glial bridging: initial attraction to the injury site, bridging initiation, and complete bridging (G. Lewis, 2015), with initial attraction occurring immediately, bridging initiation occurring within the first 6 hpi, and complete bridging occurring from 3 to 8 hpi. Evidence suggests that each of these phases of bridging are controlled by separate molecular regulators. When TGFβ signaling is inhibited, perineurial glia are unable to initiate or complete bridging, however they maintain attraction to the injury site, extending processes towards the gap but never crossing over the axonal stump. This phenotype is similarly observed in injured *ctgfa* mutant larvae as well as in injured larvae treated with an ErbB2/ErbB3 inhibitor or lacking Schwann cells. This suggests that initial attraction of perineurial glial processes to the injury site are not derived from interactions with Schwann cells. Rather, these signals could be autocrine, as part of the perineurial glial intrinsic repair program, or received from the degenerating axons themselves. Perineurial glia do not require Wallerian degeneration in order to successfully bridge (G. M. Lewis & Kucenas, 2014), therefore if the signal is coming from axons it could be from injured axons that have not yet undergone rapid degeneration. Perineurial glia extend these initial processes

immediately after injury, before macrophages have entered the injury gap. Therefore, it is unlikely that these signals are macrophages derived. Further, perineurial glia do not bridge when adjacent muscles are injured, only when motor nerve is injured. Therefore, signals driving their initial injury response are likely nerve-derived.

Bridging initiation across the injury site is perturbed when TGFβ signaling is inhibited and in *ctgfa* mutants. pSmad3 presence in *nkx2.2a*$^{+}$ nuclei peaks at 3 hpi and *ctgfa* expression in the injury site peaks at this time. These findings suggest that bridging initiation is regulated by a positive feedback loop between TGFβ signaling and *ctgfa* expression. However, the source of these signals remains unknown. It is likely that these signals are derived from Schwann cells, as Schwann cells are known to secrete both TGFβ-1 and CTGF following motor nerve injury (Schira et al., 2018; Sulaiman & Nguyen, 2016). In *erbb3* mutants, perineurial processes had initial attraction to the injury site but do not bridge on nerves where the injured axons are not fully ensheathed by perineurial glia, as perineurial glia in *erbb3* mutants are sparse. However, perineurial glia on nerves that were fully ensheathed are able to form successful bridges across the injury gap. In *erbb3* mutants, oligodendrocyte precursor cells (OPCs) exit the spinal cord ectopically and myelinate the axon. Therefore, it is possible that similar to how ectopic OPCs rescue Schwann cell myelination of the peripheral motor nerve in their absence, OPCs are rescuing the signaling normally derived from Schwann cells to drive the perineurial glial injury response (G. Lewis, 2015; A. D. Morris et al., 2017). In my studies pharmacologically inhibiting ErbB2/ErbB3 only immediately prior to injury, without perturbing developmental processes, I do not observe perineurial glial bridging. Because

Schwann cells are able to develop properly and are present on the nerve in these assays, ectopic OPCs are not present and cannot rescue bridging. In studies conducted with *colourless* mutants, Schwann cells are absent and perineurial glia are unable to bridge. However, these mutants do not have ectopic OPCs (Dutton et al., 2001; G. M. Lewis & Kucenas, 2014). Therefore, Schwann cells are a likely source of signaling for perineurial glial bridging. My studies entirely eliminating Schwann cells after development but prior to injury resulted in perineurial glia being unable to bridge. Because perineurial glia are unable to bridge in both cases where Schwann cells were present but their function is perturbed and where Schwann cells were entirely eliminated, I hypothesize that Schwann cells are chemically secreting TGFβ-1 directly to perineurial glia to drive bridging. Bridging without presence of Schwann cells or proper Schwann cell function is only observed in *erbb3* mutants in the presence of ectopic OPCs, suggesting these cells are indeed supplementing the signaling lost from Schwann cells. Future studies utilizing the drug-includible dominant negative *tgfbr2b* mutation that I created driven by either *sox10* or *nkx2.2a* could elucidate whether Schwann cells are indirectly driving perineurial glial bridging through TGFβ signaling or if TGFβ signaling is acting directly upon perineurial glia to drive bridging.

Finally, after perineurial glia have initiated bridging across the injury site, perineurial complete bridging by 8 hpi, either fully extending across the injury gap from the proximal end or with the proximal and distal end of the perineurial glial bridge meeting in the middle (G. M. Lewis & Kucenas, 2014). It is likely that once perineurial glia initiate bridging, perineurial-perineurial interactions allow for bridging to be completed. Perineurial glia are

able to bridge both when *nkx2.2a*$^{+}$ nuclei are present distal to the injury site and when they are absent, suggesting that perineurial glia do not need signals distal to the injury gap in order to fully bridge. In cases where perineurial glia bridge from both the proximal and distal side of the injury gap, the two ends move into the injury site with similar velocities and meet roughly in the middle. Upon contact, they maintain adhesion of membrane processes and do not break apart. Together, these observations support the idea that autocrine signaling drives perineurial cells to continue bridging once they have received signals to initiate their bridging response.

Future studies could aim to identify a critical window of signaling for perineurial glial bridging. Though I have identified three main phases of perineurial glial bridging, initial attraction, bridging initiation, and bridging completion, it remains unknown what different signals regulate these individual phases and how these phases can be manipulated while maintaining proper regeneration. For instance, while we know that initiation of perineurial glial bridging is necessary for complete bridging to occur, it remains unclear if inhibition of signals after initiation occurs would affect complete bridging, or if perineurial glia only need extrinsic signals for initiation of bridging and activation of an intrinsic program to drive bridging competition in an autocrine manner. Further, could bridging be halted once it has begun? Or again, once bridging is initiated has it been set on a path to bridging completion that cannot be affected by extrinsic factors? If so, what intrinsic signals are activated to drive complete bridging? And if not, what extrinsic cellular or molecular cues are necessary to ensure that successful bridging occurs even after initiation has begun? Future investigation into what signals drive each of these phases and elucidation of the

timing when these signals are necessary to regulate perineurial glial bridging will establish a precise timeline of cellular and molecular interactions that will allow us to better understand successful regeneration.

Similarly, while my findings have identified TGFβ signaling as essential for initiation of perineurial glial bridging, it remains unclear if there is a critical time where perineurial glia need to receive TGFβ signaling in order for this bridging to occur. Inhibition of TGFβ signaling immediately after initiation occurs could determine whether perineurial glia only require this signal to begin their bridging process and activate intrinsic pathways to complete bridging, or if TGFβ signaling is necessary for the entirety of the bridging process. It would be interesting to investigate if re-introduction of TGFβ signaling after inhibition of the initiation of perineurial glial bridging would drive bridging behavior later in the injury process. If so, studies could investigate if there is an essential window of time during which perineurial glia must bridge in order for proper regeneration to occur, or if bridging could be delayed and still have successful regeneration occur. Such studies could be conducted globally using SB431542 to inhibit TGFβ signaling at specific stages post injury or cell-specifically in either Schwann cells or perineurial glia using my *sox10* or *nkx2.2a* driven *DNtgfbr2b* mutation, respectively, and activating it with doxy at various stages of bridging. Identifying the critical period during which perineurial glia require TGFβ signaling to bridge will give additional insight into how these signals are driving these regenerative processes and well as suggest potential targets for future therapies.

It is likely that perineurial glial bridging is driven by more than just TGFβ signaling. Often in regeneration, developmental programs are reactivated to drive responding cells to a progenitor-like state, increasing cell mobility and proliferation (K. R. Jessen & Mirsky, 2016; Rigoni & Negro, 2020). TGFβ signaling is known to be essential for perineurial glial development (A. D. Morris et al., 2017) and my findings identify TGFβ signaling as a novel driver of perineurial glial bridging after injury. Therefore, it is possible that other signals important for perineurial glial development are driving their regenerative response. My findings indicate that *ctgfa* works in a positive feedback loop to drive perineurial glial bridging, and that *ctgfa* is expressed in the lateral floor plate, where perineurial glia also originate, during early development. While perineurial glial behaviors during development are similar to those during regeneration, with perineurial glia extending membrane processes, some behaviors are unique to their injury response. Unlike development, my studies have shown that perineurial glia do not proliferate after injury nor do the cells migrate down the axon. Rather, they extend existing membrane processes into the injury gap to bridge. Therefore, developmental cues might be activating slightly different pathways during regeneration than they do during development. For instance, perineurial glia bridge before Schwann cells enter the injury gap (G. M. Lewis & Kucenas, 2014), whereas Schwann cells associate with axons before perineurial glia exit the spinal cord and ensheath motor nerves during development. Further, Notch signaling is required for perineurial glial maturation and differentiation (Binari et al., 2013). However, Notch signaling was not observed in perineurial glia following injury (G. Lewis, 2015). Therefore, additional studies are necessary to elucidate which signals are driving

perineurial glial bridging and how differences in cellular interactions between development and regeneration might be influencing how these signals direct their regenerative programs.

My studies demonstrate that perineurial phagocytosis of debris is controlled independently of TGFβ signaling and *ctgfa* expression, suggesting that additional signaling pathways are regulating perineurial glial regenerative responses. While signals that are present during development potentially drive perineurial glial bridging, allowing them to extend processes across the injury gap similarly to how they ensheath Schwann cell-axon bundles during development, perineurial glia are not known to phagocytose debris during development. Therefore, it is likely not signals recapitulated from developmental processes that drive perineurial glial phagocytosis. Future studies could seek to identify which signals drive perineurial glial phagocytosis and determine how critical perineurial glial phagocytosis is to successful regeneration. Single-cell RNA-sequencing of *nkx2.2a*$^{+}$ cells following injury could indicate potential engulfment factors expressed by perineurial glia. Studies in *Drosophila* show that *draper* and *ced-6* interact to drive glial engulfment of degenerating axons during development (MacDonald et al., 2006). Later studies identified a signaling cascade in which activation of Src42A drives Draper-I and a downstream kinase, Shark, which is required for glial migration to wound sites. This Src42A-Draper-Shark mediated signaling axis is homologous to the SFK-ITAM-Syk signaling pathway present in vertebrate immune responses (J. S. Campbell et al., 2021; Evans, Rodrigues, Armitage, & Wood, 2015). Further, following axonal injury in *Drosophila*, TRAF4 works downstream of Draper to activate JNK signaling in glia to drive engulfment of debris (Lu et al., 2017). *gulp1* is a gene analogous to *ced6* that is found in zebrafish and could be a potential

candidate driver of perineurial glial phagocytosis. It also remains unknown whether perineurial glia phagocytosis is driven intrinsically through autocrine signaling or if the signaling is derived from nearby cells. Previous studies have shown that perineurial glia coordinate with macrophages to phagocytose axonal debris primarily on the proximal stump, with macrophages infiltrating the injury gap (G. M. Lewis & Kucenas, 2014). Perineurial glia and Schwann cells both phagocytose debris on the distal stump, however my studies suggest that perineurial glia do not phagocytose Schwann cell debris. Therefore, degenerating axons might be sending signals directly to perineurial glia to initiate phagocytic behaviors.

Cellular interactions following spinal motor nerve injury

The cellular interactions between various cell types immediately following injury are essential for regulating regenerative programs and driving effective motor nerve regeneration (K. R. Jessen & Mirsky, 2016; G. M. Lewis & Kucenas, 2014; Mahar & Cavalli, 2018; Webber & Zochodne, 2010). Though the regenerative responses of individual cell types are well studied, we do not know much about how these cells are interacting with each other to drive axon regeneration. As we learn more about each individual cell type, and identify additional cell types and sub-types, understanding how these cells are communicating physically and molecularly is crucial to understanding the mechanisms that underlie successful motor nerve regeneration.

As I have presented in previous chapters, it is known that perineurial glia require Schwann cells in order to successfully bridge across the injury gap after injury to spinal motor nerves.

Further, Schwann cells and perineurial glia undergo reciprocal communication to drive each other's development (Binari et al., 2013; A. D. Morris et al., 2017). Therefore, it is likely that perineurial glia are communicating with Schwann cells to drive their injury response. Perineurial glia bridge the injury gap before Schwann cells form bands of Bungner and guide regenerating axons across the injury gap (G. M. Lewis & Kucenas, 2014). Therefore, it is possible that perineurial glia communicate with Schwann cells to tell them that a glial bridge has been formed and that they can begin to guide regenerating axons across the bridge. This communication may occur after the bridge has fully formed, or may occur at each phase of perineurial glial bridging, from bridging initiation through completion. Studies in mice have shown that following sciatic nerve injury, nerve fibroblasts derived from the perineurium induce Schwann cells to sort into discrete bands via Ephrin/EphB2 signaling (Parrinello et al., 2010), suggesting that perineurial glia likely do drive Schwann cell injury behaviors. Later studies found that fibroblasts express TGFβ-1 and crosstalk with Ephrin to drive collective Schwann cell migration across the injury gap (Clements et al., 2017). Therefore, if TGFβ signaling works in an autocrine manner to drive perineurial glial bridging, perineurial glial-derived TGFβ-1 might be necessary for Schwann cells to migrate across the injury gap. Future studies could investigate when the presence of perineurial glia and the perineurial glial bridge are necessary for Schwann cells to undergo their injury response. To determine if the presence of perineurial glia is necessary for the Schwann cell development, one could use the *nkx2.2a:gal4:UAS:NTR-mcherry* line that I created to cell-specifically eliminate perineurial glia immediately prior to injury without perturbing development. Live time-lapse imaging later than my studies, closer to 12 hpi when Schwann cells are forming bands of Bungner, could then determine

if Schwann cells are able to complete their injury response without the presence of perineurial glia. It might be the successful formation of the perineurial glial bridge, and the signaling cascades that accompany bridging, rather than the presence of perineurial cells themselves, that is necessary for Schwann cells to properly transdifferentiate and form bands of Bungner. By driving my *DNtgfbr2b* mutation in perineurial glia using the *nkx2.2a* promoter and treating with doxy immediately prior to injury to perturb perineurial glial bridging but not development, one could time-lapse image Schwann cells after 12 hpi and determine whether the inhibition of perineurial glial bridging disrupts formation of bands of Bungner and subsequent regeneration. These studies would further would shed light on how Schwann cells and perineurial glia are interacting after injury to drive each other's responses and could help determine how the signaling I characterized in previous chapters might be modulating these processes.

Macrophages play an important role in debris clearance following motor nerve injury (P. Chen et al., 2015; Villegas et al., 2012). Arriving to the injury site within the first hpi, macrophages spatially coordinate with perineurial glia to ensure inhibitory signals are removed from the axons stumps and the injury gap to allow for proper regeneration to occur (G. M. Lewis & Kucenas, 2014). However, it remains unknown how perineurial glia and macrophages communicate to ensure that debris is cleared efficiently and effectively after injury. Macrophage recruitment to the injury site occurs independently of Schwann cells (A. F. Rosenberg et al., 2012), therefore it is possible that signals from perineurial glia are coordinating with macrophages. Inhibition of TGFβ signaling impairs perineurial glial bridging, but does not affect macrophage recruitment to the injury site or ability to clear

debris. However, my studies demonstrate that perineurial glia still phagocytose debris when TGFβ signaling is inhibited, suggesting perineurial glial bridging and phagocytosis are molecularly distinct events. Further, macrophages are recruited to the injury site in the first hpi, before perineurial glia begin to bridge. As such, I would hypothesize that macrophages do not require perineurial glial bridging to successfully clear debris, but signals driving perineurial glial debris clearance could also be contributing to the macrophage injury response. Such communication might signal to macrophage to specifically clear debris in the injury gap as opposed to entering the proximal and distal axonal stumps. Future studies could utilize the *nkx2.2a:gal4;UAS:NTR-mcherry* line that I created to specifically eliminate perineurial glia prior to injury and time-lapse image macrophages to determine if the presence of perineurial glia is required for macrophages to be clear debris from the injury site and maintain their preferential debris clearance in the injury gap as opposed to the axonal stumps. Reciprocally, macrophages might be a source of signaling driving perineurial glial phagocytosis and communicating with perineurial glia to spatially clear debris only on the axonal stumps. Clearance of debris, and subsequent removal of inhibitory signals from the injury gap, by macrophages might be necessary for perineurial glia to form a bridge. Future studies could determine if the elimination of macrophages prior to injury would prevent perineurial glia from bridging. It would be interesting to see if perineurial glia are able to phagocytose debris in the absence of macrophages and if they would expand their boundaries of debris clearance into the injury gap to compensate for the lack of clearance by macrophages. It remains unknown what signals drive perineurial glial phagocytosis, and elucidating the interactions between perineurial glia and macrophages could provide potential signaling candidates as well as

explore the significance of perineurial glial phagocytosis for successful motor nerve regeneration.

While perineurial glia, Schwann cells, and macrophages have been characterized as playing major roles in successful motor nerve regeneration, other understudied and newly emerging cell types also likely contribute to the regenerative process. Motor exit point (MEP) glia are a new type of glial cell recently characterized by our lab. MEP glia are a centrally-derived glial cells that myelinate motor nerves both in the CNS and in the PNS and regulate trafficking of cells into the periphery. Recently, it was determined that MEP glia also express *nkx2.2a*, though they are present closer to the motor root than perineurial glial cells (Fontenas & Kucenas, 2018, 2021). However, some *nkx2.2a*$^{+}$ cells observed on the dorsal end of the motor nerve might be MEP glial cells. MEP glia also express *sox10* and *foxd3*, which perineurial glia do not. Therefore, identification of a marker more specific to perineurial glia will help to determine which of these cell types is observed on the motor nerve in dorsal regions close to the motor root. Currently, it remains completely unknown whether MEP glia respond to injury. Future studies investigating the role that MEP glia play in motor nerve injury could identify a new cell type involved in the injury response and potentially a new source of signals that could drive perineurial glial bridging.

In Chapter III, I identified *connective tissue growth factor a (ctgfa)* as necessary for perineurial glial bridging. *ctgfa* expression increases in the injury site only following motor nerve injury within the first 6 hpi. However, it does not appear that the *ctgfa* expression observed in the injury site is expressed by perineurial glia. Though Schwann cells secrete

CTGF after injury (Schira et al., 2018), the observed *ctgfa*$^{+}$ cells do not appear to be Schwann cells, as Schwann cells do not enter the injury site during the first 6 hpi. It is possible that this *ctgfa* expression is derived from the epineurium or endoneurium. Little is known about the injury responses of either of these nerve components, but as they are essential to nerve development and structure and are fully reformed after regeneration is complete, it is likely that they contribute to the injury response. Recent single-cell RNAseq datasets have identified genes specific to either the endoneurium and epineurium (B. Chen et al., 2021). Creating markers for the epineurium and endoneurium and live-imaging after injury could elucidate the roles these cells play in regenerative responses and determine if they could the *ctgfa*$^{+}$ cells observed in my imaging. In addition to increased *ctgfa* expression in the injury site, I observed cells that expressed *ctgfa* after motor nerve injury but were not present on the nerve, rather they seemed to be extending from the vasculature towards the nerve. *ctgfa* is known to drive angiogenesis during wound healing, so it is possible that these cells are endothelial (Abreu et al., 2002; Schira et al., 2018). Though is it known that regenerated motor nerves are revascularized after injury, the role that endothelial cells play in regeneration is not well characterized. Still, endothelial cells have been observed in the nerve bridge after injury (Cattin et al., 2015; Clements et al., 2017). Further, perineurial glia act as a component of the blood-nerve-barrier, therefore it is possible that *ctgfa* is simultaneously stimulating TGFβ signaling to drive perineurial glial bridging while also aiding in reinnervation of the nerve as it begins to regenerate. Identification of what cells are expressing *ctgfa* both in the injury site and outside the nerve during this time could specify another cell type that is important for motor nerve

regeneration and elucidate how *ctgfa* expression works with TGFβ signaling to drive perineurial glial bridging.

Fibroblast or glial cell?

Our lab has characterized that perineurial glia bridge after spinal motor nerve injury, phagocytose axonal debris, and are essential for proper regeneration (G. M. Lewis & Kucenas, 2014). Though perineurial glia are present in mammals, express *nkx2.2*, and undergo very similar developmental processes as in zebrafish (Clark et al., 2014), perineurial glial bridging after sciatic nerve injury has yet to be studied in mammalian models. However, studies in rodent models suggest that perineurial glial bridging might be occurring in mammals as well. Ephrin-B ligands on perineurium-derived fibroblasts direct Schwann cell sorting and directional migration by activating EphB2 receptors on Schwann cells, leading to relocalization of N-cadherin to cell-cell contacts (Clements et al., 2017; Parrinello et al., 2010). Further, these fibroblasts express high levels of TGFβ and are abundant in the nerve bridge after injury (Clements et al., 2017). Therefore, it is highly possible that these perineurium-derived fibroblasts are indeed perineurial glia. In a rabbit model of tibial nerve injury, perineurial cells respond to injury by detaching from one another and assuming a fibroblast morphology to surround small fascicles of regenerating axons (Petrova & Kolos, 2022). Previous studies have found that it is hard to distinguish between Schwann cells, endoneurial fibroblasts, and perineurial cells after injury because of the dramatic morphological and molecular changes that these cells undergo for their regenerative programs (J. H. Morris, Hudson, & Weddell, 1972). Future studies identifying the molecular changes that occur in these cells after injury could provide cell-specific

markers for repair phenotypes, allowing for more detailed studies of how these cells are interacting to drive regeneration.

Though recent studies have explored the transcriptomes of various cell types following peripheral motor nerve injury in rodents (B. Chen et al., 2021; Clements et al., 2017; Endo et al., 2022; Ydens et al., 2020), the perineurium is often stripped for these studies. Because very few specific markers exit for perineurial glia, some perineurial glia might be included in other cell type bundles in these studies. Therefore, their signaling might be misattributed to other cell types. Currently, *nkx2.2a* is the only marker specific to perineurial glia. However, this marker is also expressed by MEP glia and OPCs (Fontenas & Kucenas, 2021). Single-cell RNA-sequencing of perineurial glia prior to injury could lead to identification of clusters separate from OPCs, MEP glia, and other cell types that express *nkx2.2a,* and the establishment of more specific markers for perineurial glia. This would allow for separation of perineurial glial expression profiles from those of other cell types. It is possible as well that there are different sub-types of perineurial glia, as only a subset of the perineurium expresses *nkx2.2* in mice (Clark et al., 2014). Identification of such subtypes and specific markers for each would allow for future studies to elucidate how these different subclasses of perineurial cells drive motor nerve development and regeneration. This could also offer insight into how perineurial glia are related to perineurial fibroblasts observed in rodent studies (Parrinello et al., 2010).

Cell-specific transcriptome studies after injury might identify additional signaling pathways involved in the perineurial glial injury response, such as what might be driving

phagocytosis, and could help to elucidate what cells perineurial glia are communicating with following injury. Though our studies show that perineurial glia form a bridge across the injury gap after injury and that this bridge is formed through extension of the perineurial glial membrane rather than cell migration or proliferation, it remains unknown what cellular and molecular changes perineurial glia undergo to drive this injury response. RNA-sequencing analysis of perineurial glial cells after peripheral motor nerve injury could give crucial insights into what these changes might be, and whether perineurial glia, similarly to Schwann cells, undergo a form of transdifferentiation to establish a molecularly distinct repair phenotype. It is possible that perineurial fibroblasts are indeed perineurial glia being misattributed as fibroblasts due to a change in morphology following injury and lack of a specific marker during cluster separation.

Identification of the perineurial glial transcriptome after injury could distinguish whether these cells a form of activated perineurial glia or if they are indeed distinct fibroblasts with similar functions in rodents that perineurial glia have in zebrafish. Though peripheral motor nerves are regenerative in humans and mammals, the ability for these nerves to fully regenerate and gain full functional recovery is not as effective as that of zebrafish. Perhaps there was an evolutionary shift from glia to fibroblasts that sacrificed regenerative ability for increases in structural and immune complexity (Kristjan R. Jessen & Mirsky, 2019; Mokalled & Poss, 2018; Petrova & Kolos, 2022; Zochodne, 2012). Continued studies comparing the similarities and differences between the regenerative processes of perineurial glia in zebrafish and perineurial fibroblasts in rodents could unlock potential

for enhancement of regenerative programs in less regenerative organisms and elucidate evolutionary shifts towards higher organismal complexity and lower regenerative capacity.

Implications for peripheral nerve injury therapeutics

Injury to peripheral motor nerves can lead to motor deficits and neuropathic pain. While motor nerves are naturally regenerative, the ability of these nerves to achieve full functional recovery in humans is extremely limited, and patients are often left with lifelong disability (Balakrishnan et al., 2021; Hussain et al., 2020; Zochodne, 2012). Therapies to improve peripheral nerve regeneration have varied from surgical approaches and nerve grafts to nerve conduits, fibrin glue, cell-based therapy, electrical nerve stimulation, and medications (Hussain et al., 2020). Nerve conduits are utilized as a bridge between the proximal and distal stumps of the injured nerve, serving as a scaffold for regenerating axons. These conduits can be seeded with neurotrophic factors that enhance regenerative capacity, establishing an ideal microenvironment for axonal regrowth. Transplantation of Schwann cells into nerve conduits has been shown to enhance axonal regeneration. Specifically, transplantation of Schwann cells that have already transdifferentiated into a repair phenotype allow for higher axonal regeneration than nerve conduits seeded with myelinating Schwann cells (Balakrishnan et al., 2021; Hussain et al., 2020). Therefore, understanding the signals that drive these cells to adapt to a repair phenotype is essential for enhancing therapeutic strategies. My studies identified TGFβ signaling as a driver of perineurial glial bridging. Because TGFβ signaling is also necessary for directive Schwann cell migration across the injury gap (Clements et al., 2017), it is possible that adding

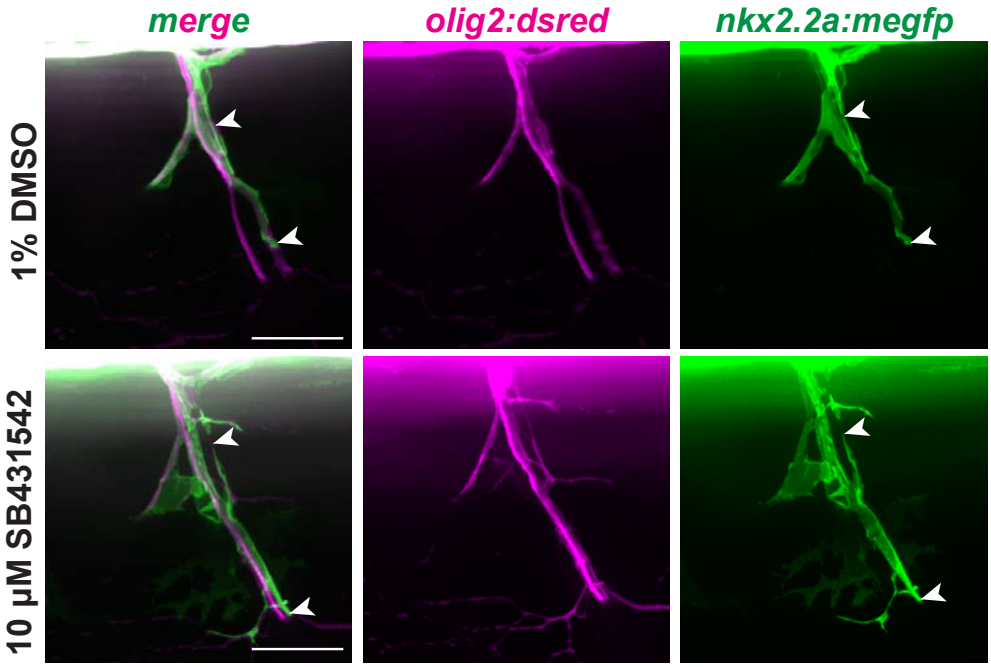
perineurial glial cells and TGFβ-1 to nerve conduits seeded with Schwann cells could enhance the ability of injury motor axons to regenerate.

Together, my studies presented in this dissertation characterizing the cellular and molecular interactions that drive perineurial glial bridging fills a gap in our knowledge about how peripheral motor nerves are able to successfully regenerate and could provide future targets for therapeutic strategies to enhance regenerative capacity of motor nerves after injury.

Appendixes

Perineurial glial morphology is not perturbed by treatment with SB431542

In Chapter III, I utilized TGFβRI inhibitor SB431542 to perturb TGFβ signaling immediately prior to injury. To ensure that treatment with SB431542 is not toxic to perineurial glia, I imaged 5 or 6 dpf *nkx2.2a:megfp;olig2:dsred* larvae treated for 48 hours with either 1% DMSO or 10 μM SB431542 in 1% DMSO. Perineurial glia in both groups appeared healthy and fully ensheathed motor nerves after treatment, therefore SB431542 is not toxic to perineurial glia (Appendixes-Figure 1, *n=11 nerves in 5 fish, 14 nerves in 6 fish, respectively*).

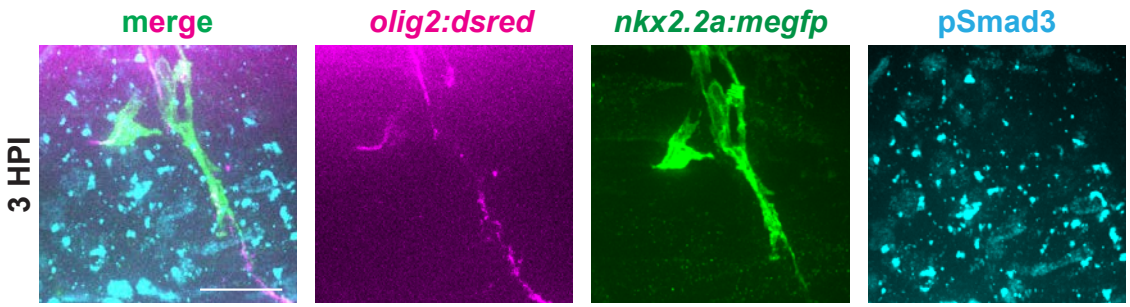


Appendixes Figure 1: Perineurial glial morphology is not perturbed by treatment with SB431542.

Representative images of 5 or 6 dpf *nkx2.2a:megfp;olig2:dsred* larvae treated with either 1% DMSO (top panels) or 10 µm SB431542 (bottom panels) for 48 hours (*n=11 nerves in 5 fish, 14 nerves in 6 fish, respectively)*. Perineurial glia (green) fully ensheath motor nerves (magenta) and have normal morphology in both groups. Scale bars, 25 µm.

Perineurial glia bridge when fixed at 3 hpi

In Chapter III, I utilized antibody staining with anti-pSmad3 to demonstrate that TGFβ signaling is present during early initiation of perineurial glial bridging (Figure 3-6). To confirm that perineurial glia are indeed bridging by 3 hours post injury (hpi), the middle time at which embryos were fixed post injury, I injured 5 or 6 dpf *nkx2.2a:megfp*;*olig2:dsred* larvae and fixed at about 3 hpi. Fixed larvae were stained with anti-pSmad3 and anti-GFP and imaged. Results showed that by 3 hpi, perineurial glia had indeed begun to, if not fully completed, bridging the injury gap (Appendixes-Figure 2, *n=10 nerves*). Further studies were not conducted using the *nkx2.2a:megfp* line, but instead using the *nkx2.2a:nls-egfp* line, to better determine whether pSmad3 was expressed specifically in perineurial glial cells.



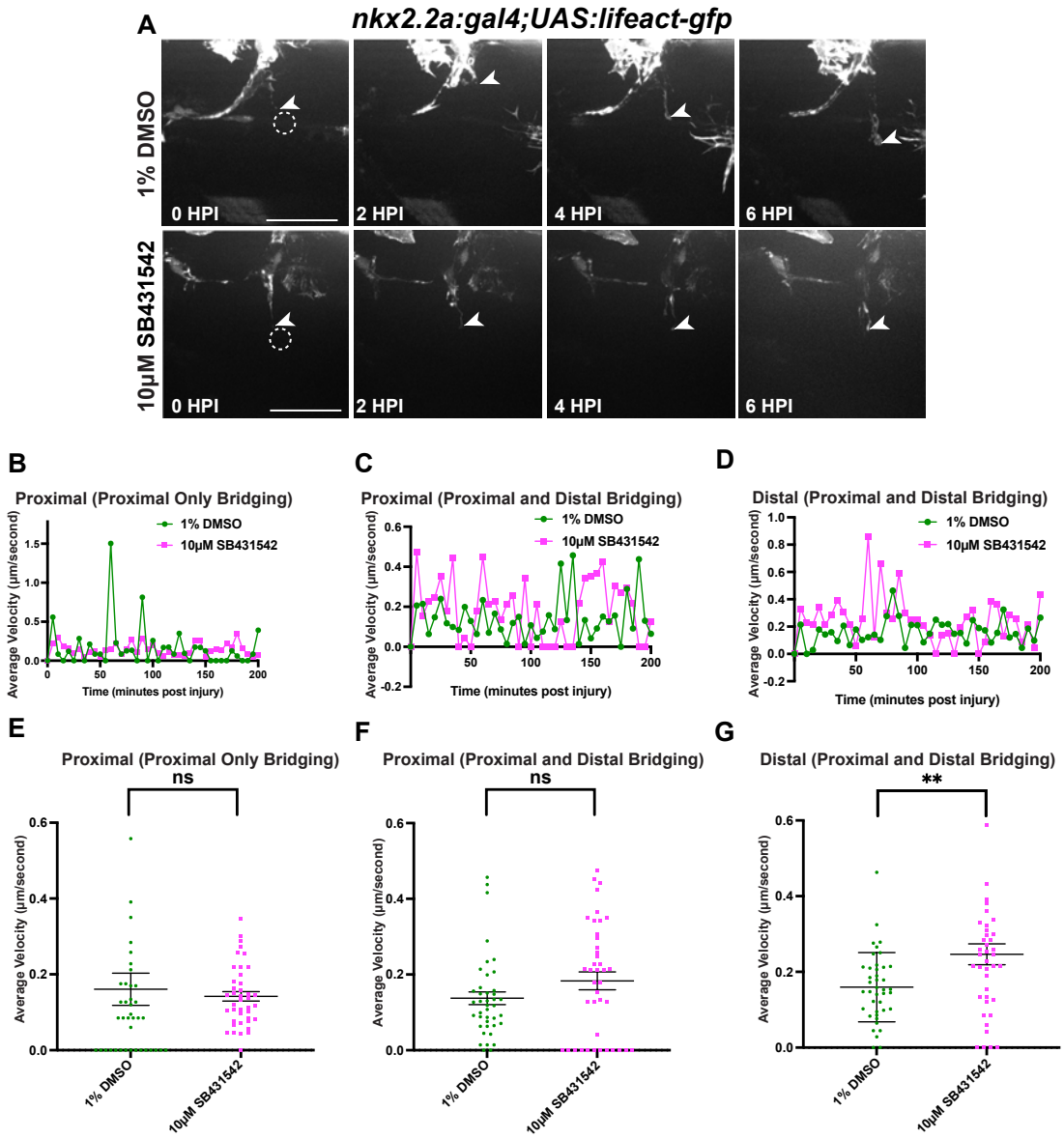
Appendixes-Figure 2. Perineurial glia bridge when fixed at 3 hpi.

Representative images of injured 5 or 6 dpf larvae fixed at 3 hpi with motor axons (magenta), perineurial glia (green), and anti-pSmad3 (cyan). Axonal degeneration is observed (magenta) and perineurial glia form a bridge across the injury site (green) (*n=10 nerves in 5 larvae*). Dashed circles indicate injury sites. Scale bars, 25 μm.

Perineurial glial actin dynamics are perturbed in the absence of TGFβ signaling

In Chapter III, I demonstrated that inhibition of TGFβ signaling alters perineurial glial bridging dynamics, specifically by decreasing the velocity of perineurial glia during the first 200 minutes post injury (mpi) when bridging is initiated (Figure 3-6). To build upon these findings, I decided to explore whether inhibition of TGFβ signaling would affect perineurial glial actin dynamics after injury. To do so, I utilized a *nkx2.2a:gal4;UAS:lifeact-gfp* line to visualize perineurial glial actin after injury. As in Chapter III, velocity was measured by manually tracking perineurial glia on both proximal and distal stumps (Appendixes-Figure 2A) over a period of 6 hpi from time-lapse movies taken from injured 5 or 6 dpf *nkx2.2a:gal4;UAS:lifeact-gfp* larvae treated with either 1% DMSO or 10 μM SB431542 in 1% DMSO. Consistent with previous imaging, perineurial glia did not bridge in larvae treated with SB431542 (Appendixes-Figure 2A). In contrast, I observed both perineurial glial stumps meeting in the middle of the injury gap in DMSO-treated larvae (Appendixes-Figure 3A). The average velocity was plotted in μm/second against time in mpi for the first 200 mpi, the period during which perineurial glial bridging initiates. Velocities were measured for the proximal stump in nerves that only had proximal bridging, for the proximal stump in nerves that had both proximal and distal bridging, and for the distal stump in nerves that had both proximal and distal bridging in both DMSO and SB431542-treated larvae. Intriguingly, there was no difference in bridging velocity between SB431542-treated and DMSO-treated larvae for nerves where only the proximal side bridged (Appendixes-Figure 3B, E; *n=1 nerve, n=4 nerves, respectively*). Intriguingly, there was a significantly higher velocity in distal bridging velocity in SB431542-treated larvae compared to DMSO-treated larvae, but not in proximal bridging velocity, for nerves

that had both proximal and distal bridging (Appendixes-Figure 3C, D, F, G; *n=3 nerves, n=1 nerve, respectively*). This differs slightly from the results observed in the perineurial glial bridging velocity studies described in Chapter III (Figure 3-6) and could indicate that changes in actin dynamics are negatively affecting perineurial glial bridging. However, a higher *n* in both DMSO and SB431542-treated groups is required to form an accurate conclusion to this data.


A
nkx2.2a:gal4;UAS:lifeact-gfp
1% DMSO
10μM SB431542
0 HPI
2 HPI
4 HPI
6 HPI
B
Proximal (Proximal Only Bridging)
C
Proximal (Proximal and Distal Bridging)
D
Distal (Proximal and Distal Bridging)
1% DMSO
10μM SB431542
Average Velocity (μm/second)
Time (minutes post injury)
E
Proximal (Proximal Only Bridging)
F
Proximal (Proximal and Distal Bridging)
G
Distal (Proximal and Distal Bridging)
ns
ns
**
Average Velocity (μm/second)
1% DMSO
10μM SB431542

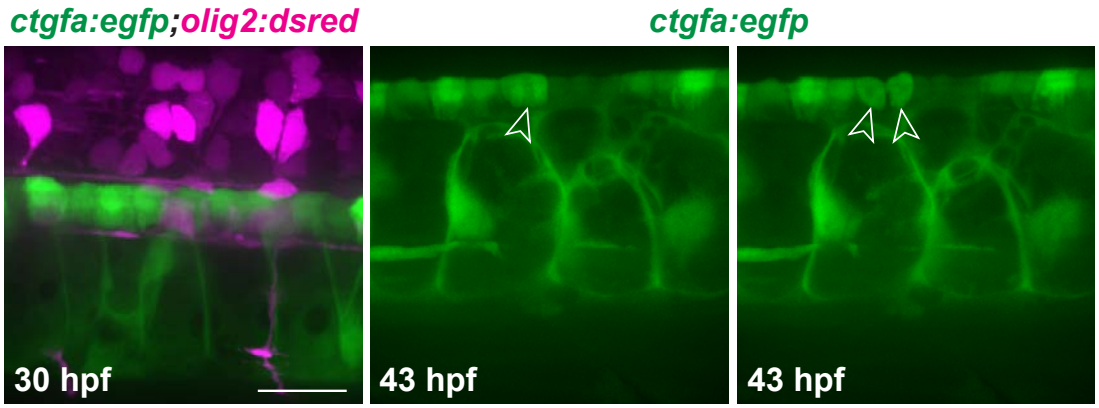
Appendixes-Figure 3. Perineurial glial actin dynamics are perturbed in the absence of TGFβ signaling.

A) Representative images from time-lapse movies of perineurial glia in injured 5 or 6 dpf larvae treated with either 1% DMSO (top panels) or 10 μM SB431542 (bottom panels) for 6 hpi. White arrows follow the proximal end of the perineurial glial bridge. Dashed circles indicate injury sites. B-G) Quantification of the average velocity of perineurial glial membrane proximal or distal stumps in μm/second plotted over time (B-D) or for the first 200 minutes post injury (mpi) (E-G). Velocities were calculated every five minutes using FIJI. B, C, E, F) There is no significant difference in proximal perineurial glial bridging velocity between DMSO and SB431542-treated larvae in both proximal only (*n=4 nerves in 2 larvae, mean: 0.16±0.04 μm/s, 1 nerve in 1 larva, mean: 0.12±0.02 μm/s, respectively*) (p=0.6556) and proximal and distal bridging larvae (p=.1392) (*n=1 nerve in 1 larvae, mean: 0.11±0.02 μm/s, 3 nerves in 2 larvae, mean: 0.18±0.02 μm/s, respectively*). D, G) There is a significant difference in distal perineurial glial bridging velocity between DMSO and SB432542-treated larvae (*n=1 nerve in 1 larva, mean: 0.14±0.03 μm/s, 3 nerves in 2 larvae, mean: 0.25±0.02 μm/s respectively*) (p=0.0065), with SB431542-treated larvae having a slightly higher average velocity. Scale bars, 25 μm.

ctgfa is expressed in the lateral floor plate during perineurial glial development

To explore the role *ctgfa* expression might play in perineurial glial bridging, I investigated whether *ctgfa* is expressed in perineurial glial cells during development, as many signals important for development prove to be equally important for regenerative programs (Mokalled & Poss, 2018). To determine if *ctgfa* is expressed in perineurial glial cells during development, I imaged *ctgfa:egfp;olig2:dsred* larvae from 24-48 hpf, prior to when perineurial glia exit the spinal cord and enter the periphery (Kucenas, 2015; Kucenas, Takada, et al., 2008). I observed *ctgfa* expression in the lateral floor plate from 24-48 hpf, consistent with *nkx2.2a* expression during this time (Appendixes-Figure 4) (Kucenas, Snell, et al., 2008; Kucenas, Takada, et al., 2008). At around 43 hpf, *ctgfa*$^{+}$ cells in the lateral floorplate divide (Appendixes-Figure 4, *n=5 larvae*). This cell division is consistent with that observed by *nkx2.2a*$^{+}$ cells at this time (Zhu, 2019). Further, bulk RNAseq data from our lab indicates that *ctgfa* is highly expressed by *nkx2.2a*$^{+}$ cells at 72 hpf (unpublished). Therefore, I conclude that *ctgfa* is expressed by *nkx2.2a*$^{+}$ cells early in development, suggesting that *ctgfa* might be important for perineurial glial development. Future studies imaging *ctgfa* expression during different developmental time points could elucidate the timing at which *ctgfa* expression is important for perineurial glial development. Imaging *ctgfa* expression with the *nkx2.2a:nls-mcherry* line during these developmental time points would confirm that *ctgfa* is truly expressed in *nkx2.2a*$^{+}$ cells. My studies described in Chapter III utilizing *ctgfa*^{bns50} mutants showed 6 dpf larvae with normal perineurial glial ensheathment of motor nerves (Figure 3-8A). Therefore, though *ctgfa* might be expressed in *nkx2.2a*$^{+}$ cells early in development, it is not essential for proper perineurial glial development. However, TGFβ signaling is important for

perineurial glial development (A. D. Morris et al., 2017), and my studies described in Chapter III indicate that TGFβ signaling drives perineurial glial bridging through a positive feedback loop with *ctgfa*. Therefore, it is possible that *ctgfa* expression perpetuates, but is not necessary for, the TGFβ signaling that is crucial for perineurial glial development. Future studies elucidating the role that *ctgfa* expression plays in TGFβ signaling during perineurial glial development would provide insight into how this signaling pathway regulates perineurial glial behaviors both in development and regeneration.



Appendixes-Figure 4. *ctgfa* is expressed in the lateral floor plate during perineurial glial development.

Representative still images from a 24-hour time-lapse movie of 30 hpf *ctgfa:egfp;olig2:dsred* larvae. Open white arrows indicate actively dividing *ctgfa*$^{+}$ cells (green) in the lateral floor plate. Scale bars, 25 μm.

Abbreviations

Abbreviations are listed in alphabetical order. See table 2-1 for abbreviations of transgenes.

AAD	acute axonal degeneration
AKT	protein kinase B
ATF3	activating transcription factor-3
BDNF	brain derived neurotrophic factor
biTRE	bi-directional tetracycline response element
BMP4	bone morphogenic protein 4
bp	base pair
cAMP	cyclic adenosine monophosphate
CaP	caudal primary motoneuron
cDNA	complementary DNA
ced6	*cell death protein 6*
cRNA	complementary RNA
cmlc2	*myosin light chain 2*
CNS	central nervous system
CTGF	connective tissue growth factor
ctgfa	*connective tissue growth factor a*
CR	cysteine rich domain
cry	*crystallin*
dhh	*desert hedgehog*
DMSO	dimethyl sulfoxide
DN	dominant negative

doxy	doxycycline hyclate
dpf	days post fertilization
ECM	extracellular matrix
egfp	enhanced green fluorescence protein
Eph	ephrin
EphB2	ephrin type-B receptor 2
ErbB2	erythroblastic oncogene B-2
ErbB3	erythroblastic oncogene B-3
ERK	extracellular signal-regulated kinase
fabp7a	*fatty acid binding protein 7*
foxd3	*forkhead box d3*
gcm	*glial cells missing* mutant
gpr126	*g-protein coupled receptor 126*
gulp1	*gulp PTB domain containing engulfment adaptor 1*
hpf	hours post fertilization
hpi	hours post injury
IGFB	insulin-like growth factor biding domain
ITAM	immunoreceptor tyrosine based-activation motif
JAK	janus kinase
JNK	c-Jun N-terminal kinase
krox20	early growth response protein 2
Lrp4	low-density lipoprotein receptor-related protein 4
mbp	*myelin basic protein*

megfp	membrane enhanced green fluorescent protein
MEP	motor exit point
MiP	middle primary motoneuron
MO	morpholino oligonucleotide
mpeg1	*macrophage expressed 1*
mpi	minutes post injury
mRNA	messenger RNA
nkx2.2a	*NK2 homeobox 2a*
NGF	nerve growth factor
nls	nuclear
nrg1	*neuregulin 1*
NTR	nitroreductase
olig2	*oligodendrocyte transcription factor 2*
OPC	oligodendrocyte progenitor cell
p38MAPK	p38 mitogen-activated protein kinase
p75NGFR	p75 nerve growth factor receptor
pax6	*paired box 6*
PCR	polymerase chain reaction
PFA	paraformaldehyde
PI3K	phosphoinositide 3-kinase
PNS	peripheral nervous system
pSmad3	phospho-Smad3
ptc	*patched*

PTU	phenylthiourea
RAGs	regeneration associated genes
Ras	rat sarcoma virus
RDZ	ronidazole
RFP	red fluorescent protein
Rho	ras homologous protein
Robo1	roundabout homolog 1
ROI	region of interest
RoP	rostral primary motoneurono
RT-PCR	reverse transcription polymerase chain reaction
rtTA	reverse tetracycline-controlled transactivator
Slit3	slit homolog 1
Smad3	smad family member 3
sox2	*SRY-box transcription factor 2*
sox10	*SRY-box transcription factor 10*
Src42A	src oncogene at 42a
STAT3	signal transducer and activator of transcription proteins 3
Syk	spleen associated tyrosine kinase
TAZ	WW domain-containing transcription regulator protein
TGFβ	transforming growth factor beta
TGFβRI	transforming growth factor beta receptor I
TGFβRII	transforming growth factor beta receptor II
TRAF4	TNF receptor associated factor 4

TSP1	thrombospondin type 1 repeat
VEGF	vascular endothelial growth factor
YAP	yes-associated protein

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