THE ROLE OF PERIPHERAL NERVES AND MECHANICAL LOADING DURING MOUSE DIGIT TIP REGENERATION

A Dissertation

by

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ABSTRACT

In the past two decades, several groups have shown that the mammalian digit tip regeneration is peripheral nerve-dependent. However, there is a serious gap in this body of literature as no one has characterized the neuroanatomy of the digit. Addressing this important gap in knowledge, my dissertation used several approaches to directly test the hypothesis that peripheral nerves contribute to digit regeneration. Specifically, my specific aims were to 1) characterize the neuroanatomy of the unamputated and regenerated mouse digit, 2) directly test the dependence of peripheral nerves on digit tip regeneration, and 3) to determine whether digit tip regeneration is dependent upon mechanical load.

In Chapter 2, I show that the neuroanatomy of the unamputated mouse digit is organized in a region-specific manner which are distinct in axon and Schwann cell composition. Curiously, I also demonstrate that after digit tip regeneration, nerve and Schwann cell regeneration is impaired. These results suggest that the mouse digit tip regeneration may not be as nerve-dependent as was previously proposed.

In Chapter 3, I re-explore the paradigm that mammalian digit tip regeneration is peripheral nerve-dependent. In reviewing the literature that demonstrates peripheral nerve-dependency, it is apparent that all of the earlier work had utilized sciatic denervation to study regeneration in a nerve-free environment. While this is a robust procedure for removing nerves down stream of the injury, it is also a well-established model of decreasing mechanical loading of the denervated limb. I hypothesized that decreased mechanical loading following sciatic denervation could be confounding the previous work that demonstrates digit tip regeneration is peripheral nerve-dependent.

To test the role of mechanical loading during regeneration without damaging peripheral nerves, a model known as hindlimb unloading was utilized. These studies revealed that when mice were hindlimb unloaded, they were unable to mount a regeneration response after injury. I next set out to test the role of peripheral nerves during digit regeneration without decreasing mechanical loading. To do this, I developed a novel surgical procedure in which denervation was performed at the level of the digit. Digit denervation maintains the animals ability to ambulate, thus minimizing any deficits in mechanical loading. Startlingly, the studies demonstrate that after digit denervation surgery, amputated digits are able to mount a robust regeneration response.

Taken together, these results strongly indicate that mouse digit tip regeneration is a peripheral nerve-independent phenomena, and that previous reports indicating that digit tip regeneration is peripheral nerve-dependent are confounded by the fact that sciatic denervation decreases mechanical loading.

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In 2004, Kanye West released his debut album *The College Dropout*. A lot of people would argue that this is his best work, and with songs like *All Falls Down*, *Jesus Walks*, and *Through the Wire*, who am I to argue? At the end of the album, after all of the hits, on a song called *Last Call*, he does something amazing that has since be copied by the likes of J. Cole and Logic. In the last 8:45 minutes of the song, he takes the time to be humble, and thank everyone who helped him get to where he was.

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So Ken, thank you being my advisor and my friend. I think we have made some meaningful discoveries in the past few years—some pretty important ones that aren't even discussed here. Who knows what the future has in store for me, but I just want to say that you have prepared me for it.

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In Chapter 2, all hypotheses, study designs, and animal protocols were developed jointly by C.P. Dolan and K. Muneoka. Hindlimb unloading studies were performed by C.P. Dolan, and with significant help from R. Bokhari. Immunohistochemical experiments were performed by C.P Dolan and were supported by M. Yan, L.A. Dawson, and F. Imholt. Micro-computed tomography experiments were performed by C.P Dolan, and supported by K. Zimmel, T.J. Yang, and O. Queresi. Mallory's trichrome staining was performed by C.P. Dolan and J. Gregory. Preliminary experiments were performed by E. Leininger. All statistical analyses were performed by C.P. Dolan.

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1. INTRODUCTION*

1.1 Summary

Regeneration Biology is the study of organisms with endogenous regenerative abilities, whereas Regenerative Medicine focuses on engineering solutions for human injuries that do not regenerate (Stocum, 2012). While the two fields are fundamentally different in their approach, there is an obvious interface involving mammalian regeneration models. The fingertip is the only part of the human limb that is regeneration-competent and the regenerating mouse digit tip has emerged as a model to study a clinically relevant regenerative response (Simkin et al., 2015a). In this dissertation I discuss how studies of digit tip regeneration have identified critical components of the regenerative response, and how an understanding of endogenous regeneration can lead to expanding the regenerative capabilities of non-regenerative amputation wounds. Such studies demonstrate that regeneration-incompetent wounds can respond to treatment with individual morphogenetic agents by initiating a multi-tissue response that culminates in structural regeneration. In addition, the healing process of non-regenerative wounds are found to cycle through non-responsive, responsive and non-responsive phases, a phenomena that we call the Regeneration Window. We also find the responsiveness of mature healed amputation wounds can be re-activated by re-injury, thus non-regenerated wounds retain a potential for regeneration. We propose that regeneration-incompetent injuries possess dormant regenerative potential that can be activated by targeted treatment with specific morphogenetic agents. We believe that future Regenerative Medicine-based-therapies should be designed to promote, not replace, regenerative responses.

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1.2 Introduction

In thinking about regeneration, it is important to delineate Regeneration Biology from Regenerative Medicine. Regeneration Biology focuses on endogenous regenerative abilities which includes homeostatic turnover of tissues (Physiological Regeneration) as well as the response to injury regardless of whether repair is partial or complete (Reparative Regeneration) (Stocum, 2012). In reparative regeneration, the capacity to regenerate (Regenerative Ability) is an endogenous tissue or organ specific characteristic and a mechanistic understanding of the regeneration processes is proposed to impact non-regenerative responses (Stocum, 2012). This proposal is justified because regenerative ability is an ancient characteristic that is robust in primitive organisms but limited among animals that have evolved more recently, such as mammals (Sanchez Alvarado, 2000). This general phylogenetic relationship indicates that the inability to regenerate (Regenerative Failure) evolved from a regenerative pre-condition; regenerative failure results from a disruption of an endogenous regeneration process. Thus, cells involved in an injury response are inhibited from following a regeneration path, and since it is well established that the regeneration process involves a complex series of steps (Tanaka, 2016), much like embryonic development, a detailed understanding of the regeneration process can lead to the identification of strategies to stimulate regeneration at sites where regenerative failure is the norm. Recent studies have now demonstrated that regeneration can be stimulated in rodent models (Dawson et al., 2016; Ide, 2012; Johnston et al., 2016; Lee et al., 2013; Masaki and Ide, 2007; Mu et al., 2013; Takeo et al., 2013; Yu et al., 2010; Yu et al., 2014). One general conclusion is that regenerative failure is linked to the microenvironment of the healing wound and not to an inability of cells to respond in a pro-regenerative manner. Thus, cells involved in a non-regenerative injury evince a Regenerative Potential that underlies dormant pro-regenerative

pathways not activated during an injury response. These findings identify an interface in which regeneration biology can play a key role in the field of Regenerative Medicine.

Regenerative Medicine is a relatively new field dedicated to engineering solutions for Regenerative Failure in humans (Stocum, 2012). Broadly speaking, the field encompasses all parts of the human body but utilizes two specific strategies: manipulation of stem cells (e.g. MSCs, iPSCs, etc.) and the engineering of extracellular templates (e.g. synthetic scaffolds, decellularized organs, etc.). These approaches have had an enormous impact on translational medicine, although not all positive. For example, clinical use of engineered trachea have been far from successful (Cyranoski, 2015), and while stem cell based therapies are currently in clinical trials for various diseases (e.g. spinal cord injury, myocardial infarction, age-related macular degeneration (Trounson and DeWitt, 2016), it is acknowledged that scientific evidence supporting the efficacy of these therapies is lacking (Daley, 2017; Marks et al., 2017). On the other hand, regenerative scaffolds have demonstrated clinical success. For instance, peripheral nerve allografts (Axogen, Avance ® Nerve Graft), and natural (Integra, NeuraGen), and synthetic (Polyganics, Neurolac) polymer-based devices have been shown to promote peripheral nerve regeneration across injury gaps ranging from 20mm-3cm (Bertleff et al., 2005; Karabekmez et al., 2009; Lohmeyer et al., 2009; Tian et al., 2015). Moreover, there are a spectrum of regenerative scaffolds used clinically for aiding skin regeneration after mechanical trauma, severe burns, and aging, with current therapies in wide use (Zhong et al., 2010). The field of Regenerative Medicine is beginning to recognize that endogenous regenerative ability plays an important role in the therapeutic outcome (Caplan, 2017), although an understanding of regenerative ability as well as regenerative potential is severely lacking.

The regeneration of the fingertip represents a clear example of human regenerative ability, and this contrasts the regenerative failure of amputations proximal to the fingertip. Fingertip regeneration was first reported in a case involving amputation of an infected adult fingertip treated with repeated dressing changes that resulted in a regenerative outcome documented by X-ray imaging over the next 3 months (McKim, 1932). Fingertip regeneration following conservative treatment of amputations in children has also been documented in the clinical literature (Douglas, 1972; Illingworth, 1974). While the question of whether to treat fingertip amputations that present with exposed bone in a conservative manner remains controversial (Bickel and Dosanjh, 2008), there is clear evidence from regenerative models that closure of the amputation wound with mature skin is inhibitory for a regeneration response (Mescher, 1976). Thus, in some ways, current clinical practices are contraindicated for a successful regenerative response.

The clinical literature demonstrates that the human fingertip has regenerative ability, however it does not bring us closer to understanding the processes underlying this phenomenon. The importance of a mechanistic understanding of regeneration allows for targeted intervention to improve the response (e.g. a reduction in patient variability), and to begin to explore ways to stimulate regeneration where it does not normally occur. The mouse digit responds to amputation in a manner that parallels humans (Borgens, 1982) and has become a premiere testing ground for studying regenerative ability as well as regenerative potential (Said et al., 2004; Singer et al., 1987; Zhao and Neufeld, 1995). The introduction to my dissertation will discuss what is currently known about the regenerative ability of the digit tip, and how we can exploit the regenerative potential of non-regenerative amputations of the digit and limb.

1.3 Part I: Endogenous Regeneration of the Mouse Digit Tip

The distal tip of the terminal phalanx (P3), the last bone in the mouse digit, regenerates after amputation (Figure 1.1). Distal amputation removes 15-20% of the P3 bone, but does not

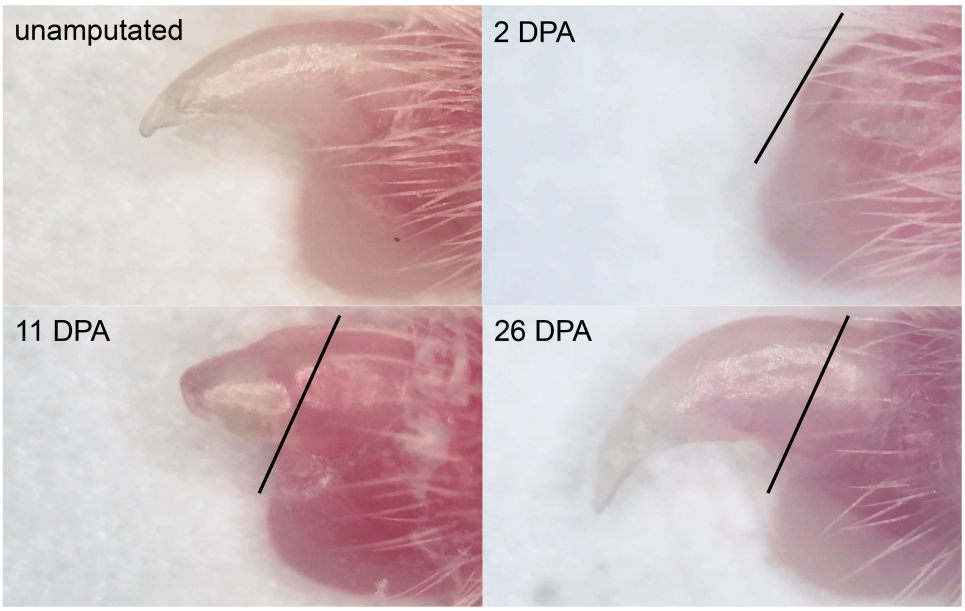

Figure 1.1: Mouse Digit Tip Regeneration. Black line indicates the amputation plane. DPA = days post amputation

damage the bone marrow, fat pad, or proximal nail matrix. Digit tip regeneration is a complex process both resembling and differing from digit development, and occurs through sequential phases that include inflammation, histolysis, epidermal closure, blastema formation, and differentiation to restore amputated structures (Fernando et al., 2011; Simkin et al., 2015a). What distinguishes digit tip regeneration from other tissue-specific regenerative responses, such as fracture repair and skeletal muscle injury, is that it is mediated by the formation of a blastema. The blastema, best characterized in salamander limb regeneration (Tanaka, 2016), is a transient structure comprised of proliferative undifferentiated cells that undergo pattern formation, morphogenesis, and differentiation to regenerate structures lost by amputation (Han et al., 2005; Muneoka et al., 2008). The mouse digit tip blastema is remarkably similar to the blastema

formed in response to axolotl limb and zebrafish fin amputation (Gemberling et al., 2013; McCusker et al., 2015). Blastema-mediated regeneration is defined as an epimorphic response, and is considered a rare event in mammals including, in addition to digit tip regeneration, the regeneration of ear hole punch wounds in rabbits and some rodents, and the annual regeneration of antlers in deer (Kierdorf et al., 2007). In order to ensure a simple understanding of the complex process of digit tip regeneration, I will describe in more detail, each of the specific stages (Figure 1.2).

Inflammation: In response to digit tip amputation, a scab forms over injured tissues. At 3 days post amputation (DPA), F4/80+ macrophages and Ly6B.2+ neutrophils infiltrate the tissues adjacent to the amputation injury (Simkin et al., 2017b). Macrophage and neutrophil levels peak at 7DPA, but are found in spatially distinct regions of the digit tip. Macrophages accumulate along the endosteum of the P3 bone and proximal dermis associated with the nail matrix while neutrophils localize to the P3 bone marrow and connective tissue surrounding the P3 bone (Simkin et al., 2017b). When the blastema forms at 10DPA, neutrophils but not macrophages, are found throughout the blastema (Simkin et al., 2017b). From 14-21DPA, macrophage and neutrophils return to pre-amputation levels.

Macrophages can be both permissive and inhibitory to regeneration. For instance, mice deficient in macrophages are capable of scar free wound healing suggesting that macrophages inhibit the regenerative response (Martin et al., 2003). However, macrophage depletion inhibits regeneration of the digit tip (Simkin et al., 2017b) as it does for the regeneration of zebrafish tail fins, axolotl limbs, and ear holes of the spiny mouse (Godwin et al., 2013; Petrie et al., 2014; Simkin et al., 2017a). During digit tip regeneration, macrophage depletion modifies early events in the regeneration process, including an inhibition of histolysis and wound closure, and this

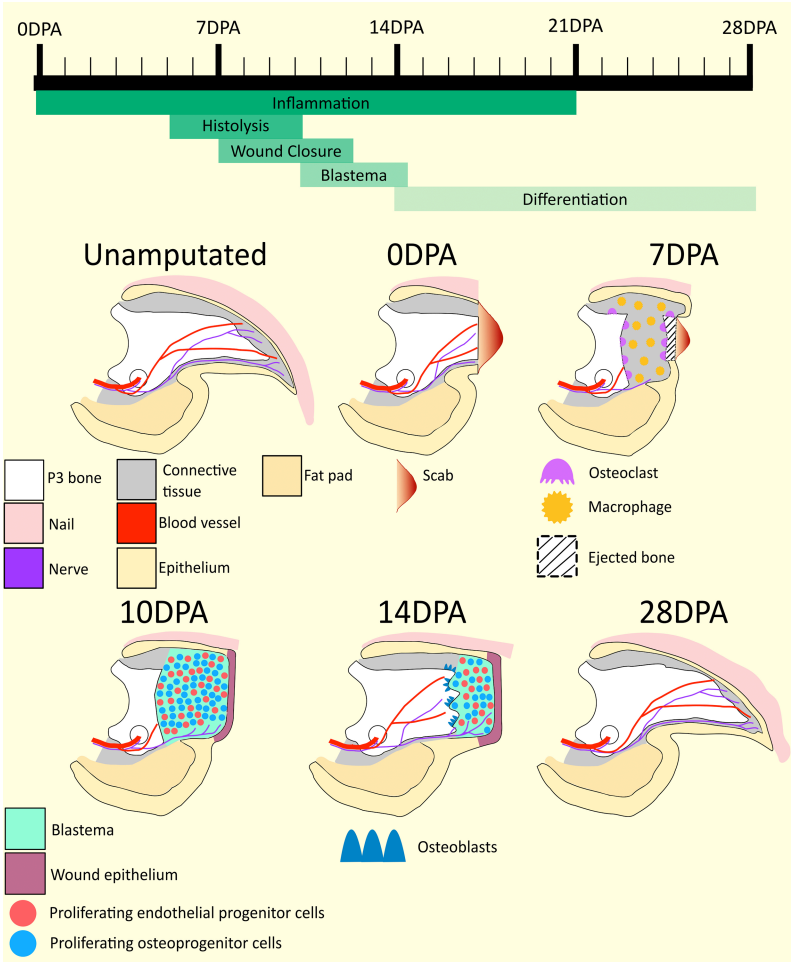

Figure 1.2: Diagram of Digit Tip Regeneration Unamputated: The mouse terminal phalanx (P3), is a triangular shaped cortical bone; wide at its base where it articulates with the second phalanx (P2; not pictured), gradually narrowing until it terminates as a pointed tip. Vasculature and nerves enter the P3 bone marrow via foramen referred to as os holes (shown as circles) located on either side of the ventral base of P3. The nail organ surrounds the entire digit except for the ventral surface where the ventral epidermis is an extension of the digital fat pad. Nerves and blood vessels are localized throughout the connective tissue located between the P3 bone and surrounding epidermis. 0DPA: A scab forms in response to distal P3 amputation. Distal amputation removes 15-20% of the P3 bone volume, but does not damage the bone marrow, fat pad, or proximal nail matrix (not shown). 7DPA: Macrophages and other cells of the innate immune response (not shown) are scattered throughout the connective tissue and P3 bone marrow. Concurrently, large, multinucleated osteoclasts degrade the periosteal and endosteal surfaces of the P3 bone. Osteoclast activity erodes the P3 bone into two segments, thus exposing the P3 bone marrow. The remaining proximal bone stump will be reincorporated into the regenerated digit tip. 10DPA: Epidermal migration proximal to the eroded bone functions to close the wound and eject the eroded bone. Wound closure is associated with subjacent blastema formation and the culmination of histolysis. The blastema is avascular, but is innervated. 14DPA: Intramembranous bone redifferentiation and associated revascularization occurs proximal to distal within the wound environment. As new tissues are regenerated proximally, the distal blastema shrinks in size. 28DPA: Digit regeneration is complete by 28 DPA, resulting in woven bone cosmetically larger than the unamputated digit. The regenerated digit tip is innervated, vascularized, and restores pre-amputation length. Distal is to the right. Reprinted with permission from "Digit tip regeneration: merging regeneration biology with regenerative medicine" by Dolan CP†, Dawson LA†, Muneoka K, 2018. *Stem cells translational medicine* 7, 262-270, Copyright 2018 by John Wiley and Sons. † denotes co-first authorship.

results in a failure of the blastema to form (Simkin et al., 2017b). The result indicates that macrophage recruitment during early wound healing stages is required to set the stage for blastema formation and the regeneration of the digit tip.

Histolysis: Histolysis is the second stage of digit tip regeneration and overlaps with the inflammatory stage (Figure 1.2). Histolysis is the enzymatic degradation of extracellular matrix resulting in the loss of organized tissues (Stocum, 2002). While the histolytic response occurs in all tissues of the amputation wound, the best characterized response is the degradative response of bone because it can be monitored in vivo by micro-computed tomography (μCT) imaging. At 5DPA, large, multinucleated osteoclasts can be seen scattered across the P3 bone and it is thought that these osteoclasts arise from the fusion of monocytes recruited during inflammation. Osteoclast number peaks at 7DPA, but are rapidly depleted by 10DPA. During this 5 day period, osteoclasts lining the endosteum and periosteum degrade through the P3 bone creating a secondary amputation and effectively splitting it into proximal and distal halves. The distal P3 bone is not re-incorporated into the regenerate. Rather, the dorsal and ventral epidermis migrate to close the wound proximal to the distal P3 stump bone, effectively ejecting it along with the wound scab from the digit (Fernando et al., 2011). The role that osteoclast-driven bone degradation plays in regeneration is significant: initially 20% of the P3 bone volume is amputated; however, the histolytic response results in the additional degradation of 2.5 times more stump bone (Fernando et al., 2011). It should be noted that 70% amputation of the digit identifies a non-regenerative proximal amputation (Chamberlain et al., 2017; Neufeld and Zhao, 1993), suggesting that the histolytic phase is acting in a pro-regenerative manner.

Why histolysis occurs remains in question, but there is precedence for it in other organisms such as the axolotl and the mammalian peripheral nerve (Conforti et al., 2014; Maden,

2008). There are a few things that we do know which helps to unravel this mystery. First, specifically inhibiting osteoclasts impairs the regeneration response, although it is not inhibited (Simkin et al., 2017b). Second, osteoclasts and histolysis are regulated in part by dynamically changing oxygen tensions and the blastema itself is known to be transiently avascular and hypoxic (Sammarco et al., 2014; Yu et al., 2014). Hyperbaric oxygen (HBO) treatment to decrease hypoxia during the regeneration response extends the period of osteoclast activity and increases the amount of P3 bone degraded (Sammarco et al., 2015; Sammarco et al., 2014). Conversely, facilitating wound closure with a Dermabond wound dressing enhances hypoxia, reduces osteoclast numbers and decreases bone degradation (Simkin et al., 2015b). Third, blastema size positively correlates with the amount of histolysis; increased histolysis generates larger blastemas while decreasing histolysis creates smaller blastemas (Simkin et al., 2015b). These data support the hypothesis that tissue histolysis releases cells and factors embedded in mature tissues, contributing to a pro-regenerative wound environment (Dawson et al., 2016).

Wound Closure: Rapid wound re-epithelization is a characteristic common to many regeneration models (Maden, 2008), however in the digit tip response, wound closure correlates with the end of histolysis and is significantly delayed (Fernando et al., 2011; Simkin et al., 2015b) (Figure 1.2). After amputation the injured epidermis does not migrate across the exposed P3 bone, but instead the epidermis retracts and establishes connections with the periosteum of the P3 bone stump, effectively sealing the amputation wound during the inflammation and histolytic phases (Simkin et al., 2015b) . After bone degradation, epidermal migration through the region of degraded bone completes wound closure and forms a wound epithelium (WE) that caps the regenerating stump (Fernando et al., 2011). The WE is a transient structure that is required for blastema formation and acts as a signaling center for mesenchymal blastema cells

(Takeo et al., 2013). Inhibiting WE formation or replacing it with mature skin inhibits regeneration in amphibians as well as mammals, including humans (Illingworth, 1974; Simkin et al., 2017b; Thornton, 1957). In digit tip regeneration the WE is a source for stromal cell-derived factor 1 (SDF-1), a known chemoattractant for blastema cells (Lee et al., 2013), and the WE is required for blastema formation (Simkin et al., 2017b). Additional studies are needed before we can fully appreciate the role that the WE plays in regulating the regeneration response, and given current clinical practices that preserve the WE as much as possible for the treatment of amputation wounds, it is an obvious and clinically-relevant area of research to pursue.

Blastema: The completion of wound closure appears to function as a transitional switch that signals the end of the histolytic phase and the initiation of the blastema phase (Figure 1.2). The blastema is a transient aggregation of undifferentiated cells that forms between the proximal P3 bone stump and the distal WE. The mouse digit blastema is characterized as avascular, hypoxic, and highly proliferative (Fernando et al., 2011; Sammarco et al., 2014; Yu et al., 2014). The cells of the blastema are heterogeneous and derived from multiple tissue sources, including the epidermis, bone, vasculature and loose connective tissues (Fernando et al., 2011; Lehoczky et al., 2011; Rinkevich et al., 2011). To date, studies show that blastema cells are lineage restricted (Lehoczky et al., 2011; Rinkevich et al., 2011), i.e. their fate during regeneration does not deviate from their tissue of origin, however cell types known to be multipotent in other injury repair models (Pang et al., 2016) have yet to be studied. As mentioned above, the recruitment of blastema cells is mediated through the SDF-1 signaling pathway, and many blastema cells express CXCR4 and CXCR7, known receptors for SDF-1 (Lee et al., 2013). As cells migrate to form the blastema, they organize themselves by producing an extracellular matrix rich in

Collagen III (Col3) and, as newly regenerated structures differentiate, the blastema ECM is degraded and replaced (Marrero et al., 2017).

A hallmark characteristic of the blastema is its immense proliferative ability. Several signaling pathways have been shown to be critical to maintaining proliferation. Bone morphogenetic proteins (BMPs) have long been identified as potent proliferative molecules and a requirement for digit tip regeneration (Han et al., 2003; Yu et al., 2010). This is best demonstrated in loss-of-function studies in which amputated digits treated with noggin, a BMP-inhibitor, do not regenerate (Yu et al., 2010). Paracrine signaling from surrounding tissues also influences blastema proliferation. Wnt signaling in the proximal nail bed enhances blastema proliferation by regulating FGF secretion from digital nerves (Takeo et al., 2013). Evidence for mitogenic paracrine signaling from nerves is supported by denervation experiments that also reduce blastema proliferation. Recently, it has been suggested that mitogenic neurotrophic factors are not secreted from the axons, but by Schwann cells (Johnston et al., 2016). Blastema signaling networks are highly regulated and exogenous growth factors that increase proliferation do not always enhance regeneration. For instance, the blastema is avascular and correlates with down-regulation of pro-angiogenic (*Vegfa)* and up-regulation of anti-angiogenic (*Pedf*) activity. Inducing precocious angiogenesis with exogenous VEGFA (or BMP9 which induces *Vegfa* expression) inhibits digit tip regeneration but can be rescued if digits are subsequently treated with PEDF (Yu et al., 2014).

Differentiation: The differentiation phase of regeneration is highly coordinated and progresses in a temporal sequence that initiates proximally and progresses to the distal tip (Figure 1.2). In this way the differentiation of newly regenerated bone is graded, first building on the stump bone and progressively adding new bone as the digit tip elongates. The differentiation

of regenerated bone from the blastema is rapid and new bone forms by direct ossification that involves the deposition of osteoid in a pericellular manner to form woven bone (Simkin et al., 2015a). In development, the digit tip is initially formed by endochondral ossification and its maturation involves an extended period of postnatal elongation involving a proximal growth plate and distal appositional ossification to form cortical bone (Han et al., 2008). Thus, the regeneration response does not involve a slow and deliberate reiteration of digit formation, but instead adapted an alternative osteogenic mechanism to effect a similar outcome over a shorter timeframe. While regenerated woven bone is highly porous and considered weaker than the original cortical bone, the regenerated P3 element is 50% larger than the original digit tip while maintaining a constant length (Fernando et al., 2011). We suspect that this overshoot in regenerated bone volume evolved as a way to replace bone function with an inherently weaker structure. With time, regenerated bone increases in density but maintains a histologically distinct microarchitecture (Fernando et al., 2011).

Expanding Regenerative Potential Based on our Current Understanding of Regenerative Ability: So what lessons can we learn from the digit tip's innate Regenerative Ability (Figure 1.1)? First, studying digit tip regeneration has identified cells, factors, and signaling pathways that are necessary for regeneration. All of these represent potential targets to remedy a failed regenerative response, and their deficiency in a non-regenerative wound environment would prompt the development of a pro-regeneration strategy. Second, regeneration involves the recruitment and proliferation of stem/progenitor cells derived from local tissues that maintain spatial information required for an appropriate regeneration response (Lehoczky et al., 2011; Rinkevich et al., 2011). Stem-cell based therapies which have pro-regenerative effects without the direct integration of stem cells into injured tissue are likely functioning by activating the

regenerative potential of local cell sources (Caplan, 2017) in a manner that parallels endogenous regenerative ability. Third, regeneration is a dynamic process involving stepwise phases of overlapping and interdependent events, thus it is clear that manipulating one stage will likely affect later stages (Figure 1.2). Therefore, in designing therapies, it is critical to consider how early treatments will alter later regeneration stages. Finally, the scaffold produced by regenerating cells is transiently modified during blastema formation, then remodeled to reestablish the original scaffold (Marrero et al., 2017). Such dynamic processing of the regenerative scaffold is entirely inconsistent with strategies for engineered scaffold designs that are based on the architecture of the target structure. In the next section, I discuss digit and limb injuries that do not regenerate, and I will identify some strategies for exploring their regenerative potential based on an understanding of regenerative ability.

1.4 Part II: Wound Repair and Induced Digit Regeneration

Induced Regeneration: A conceptually similar but significantly more challenging approach is to focus on a proximal amputation injury that is non-regenerative at all stages of development. It is generally recognized that developing or immature tissues display an enhanced level of regenerative ability when compared to adults (Stocum, 2012). In humans, full thickness skin wounds undergo a defective healing response resulting in scar formation, whereas fetal skin wounds do not form scars but regenerate perfect replacement skin (Ferguson and O'Kane, 2004). Modulation of the transforming growth factor (TGF-β) repertoire in adults to mimic that of fetal skin wounds results in scar free skin regeneration and thus indicates latent regenerative potential of adult skin that can be extrinsically activated (Ferguson and O'Kane, 2004). The embryonic chick limb is another example of regenerative potential; amputated early stage chick limbs fail to

regenerate, yet targeted application of Fibroblast Growth Factors stimulates reprogramming of local mesodermal cells and subsequent induced limb regeneration (Kostakopoulou et al., 1997; Kostakopoulou et al., 1996; Taylor et al., 1994). These examples identify developing or immature tissues as possessing dormant regenerative potential that can be used to characterize defects in the injury response, and to identify agents that can stimulate regeneration.

Amputation of the non-regenerative neonatal mouse digit/limb has been used to demonstrate and characterize BMP induced regeneration (Dawson et al., 2016; Ide, 2012; Masaki and Ide, 2007; Yu et al., 2010; Yu et al., 2012). In amputated neonatal digits, BMP2-targeted treatment stimulates skeletal elongation by inducing the formation of a distal endochondral ossification center (EOC) at the amputation wound that functions as a morphogenetic center to organize the regeneration of new bone onto the stump (Yu et al., 2012). Targeted BMP treatment involves loading BMP onto a vehicle that is engrafted into the amputation wound after wound closure. This allows for transient release of BMP over a 2-3 day period, and during that time the EOC is established. The actual regeneration of new bone is mediated by the EOC and is independent of additional BMP treatment. An important aspect of these studies is that the skeletal structure induced to regenerate is dictated by the amputation level and not by the BMP treatment itself, thus BMP is functioning as a morphogenetic agent to establish the EOC and not as a morphogen to dictate the structure that regenerates (Dawson et al., 2017; Yu et al., 2010; Yu et al., 2012). These findings suggest the cells at differing amputation levels are regeneration responsive and retain a positional memory that can be triggered to determine the appropriate structure of the regenerate (Yu et al., 2012). This conclusion is supported by a number of different studies showing BMP-induced regeneration is specific to distinct amputation levels in mice (Dawson et al., 2016; Ide, 2012; Masaki and Ide,

2007; Yu et al., 2010; Yu et al., 2012). Notably, in vitro studies using fibroblasts isolated from P3 (regeneration-competent) or P2 (regeneration-incompetent) digit regions suggest that these cells retain positional characteristics, but that positional memory alone does not limit or induce a regeneration response (Wu et al., 2013).

Regenerative Failure and Induced Regeneration in Adults: Amputation of the mouse digit at the level of the P2 bone has emerged as a standardized system to investigate regenerative failure (Figure 1.3) (Agrawal et al., 2010; Agrawal et al., 2011a; Agrawal et al., 2011b; Dawson et al., 2016; Miura et al., 2015; Mu et al., 2013; Neufeld, 1985, 1989; Schotte, 1959; Schotte and Smith, 1961; Yu et al., 2012). P2 amputation removes all distal elements and traverses multiple tissue

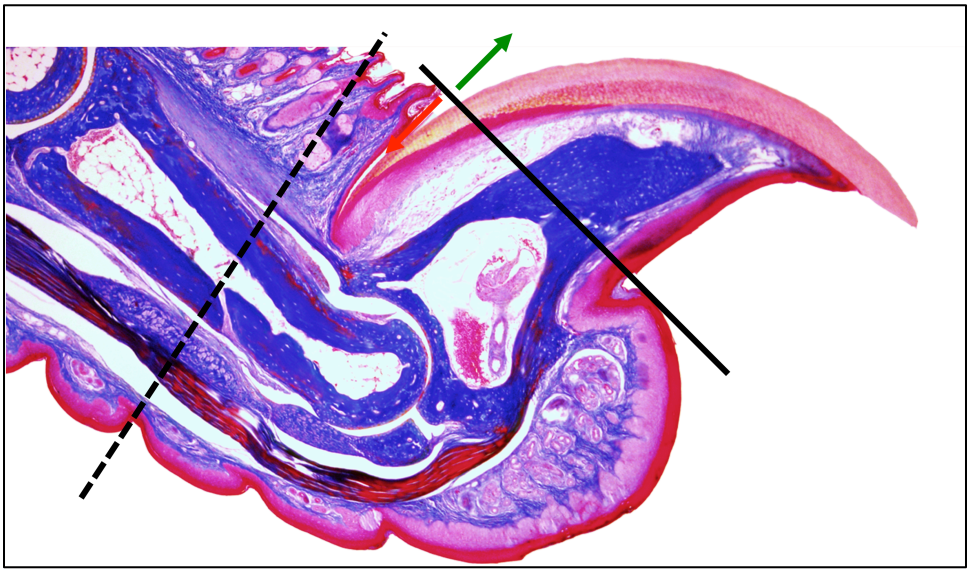

Figure 1.3: Different Amputations Levels on the Mouse Digit. Distal digit tip amputation: Amputations at, or distal to, the solid black line will regenerate (green arrow). Amputations proximal to the solid black line do not regenerate (red arrow). The dashed black line indicates the standard amputation plane to study P2 regenerative failure or induced-regeneration.

types, including the P2 diaphysis, bone marrow, dorsal elastic claw ligament, digital flexor tendon, and skin (Wong et al., 2006), and ultimately results in skeletal truncation covered by a fibrotic scar (Dawson et al., 2016). While the outcome of amputation is skeletal truncation, the

injury response is quite dynamic and initiates a skeletal repair response analogous to the proximal bone segment of long bone fracture repair (Dawson et al., 2016). The well-characterized cellular events of fracture repair are delineated into several stages, including inflammation, cartilaginous callus formation, boney callus formation, and eventual remodeling of the boney callus into a structure that resembles the pre-injury bone (Colnot, 2009; Einhorn, 2005; Gerstenfeld et al., 2003; Schindeler et al., 2008; Shapiro, 2008). Likewise, P2 amputation initiates an inflammatory response that is followed by proliferation of local periosteal cells that differentiate into chondrocytes that form a cartilaginous callus external to the bone surface, i.e. the peripheral callus. Vascular invasion and osteoblast recruitment reorganize the cartilaginous peripheral callus into a woven boney callus, and this is followed by remodeling of the boney callus into a lamellar structure that largely resembles the original bone stump (Dawson et al., 2016). After remodeling, the truncated stump bone appears identical to the amputated P2 element, so there is the impression that the injury response is largely inert. However, the response is dynamic and consistent with the conclusion that the injury response involves an attempt at regeneration that ultimately fails (Dawson et al., 2016). Because this injury response is dynamic it establishes a model to test strategies for enhancing a regenerative response.

The transient formation of the peripheral cartilaginous callus by periosteal cells following amputation identifies a potential cell target for regenerative intervention. Periosteal cells are known to be critical for skeletal regeneration in fracture healing (Colnot, 2009; Tsuji et al., 2006), and are responsive to BMP2 (Minear et al., 2010; Wang et al., 2011; Yu et al., 2010). Targeted BMP2 treatment during the periosteal response was found to induce the formation of a distal cartilaginous callus that paralleled EOC formation in neonatal models (Dawson et al., 2017; Yu et al., 2012). The distal callus is comprised of proliferating chondrocytes undergoing

endochondral ossification and functions as a template for new bone formation, resulting in restoration of amputated skeletal length (Figure 1.4) (Dawson et al., 2017). These studies provide

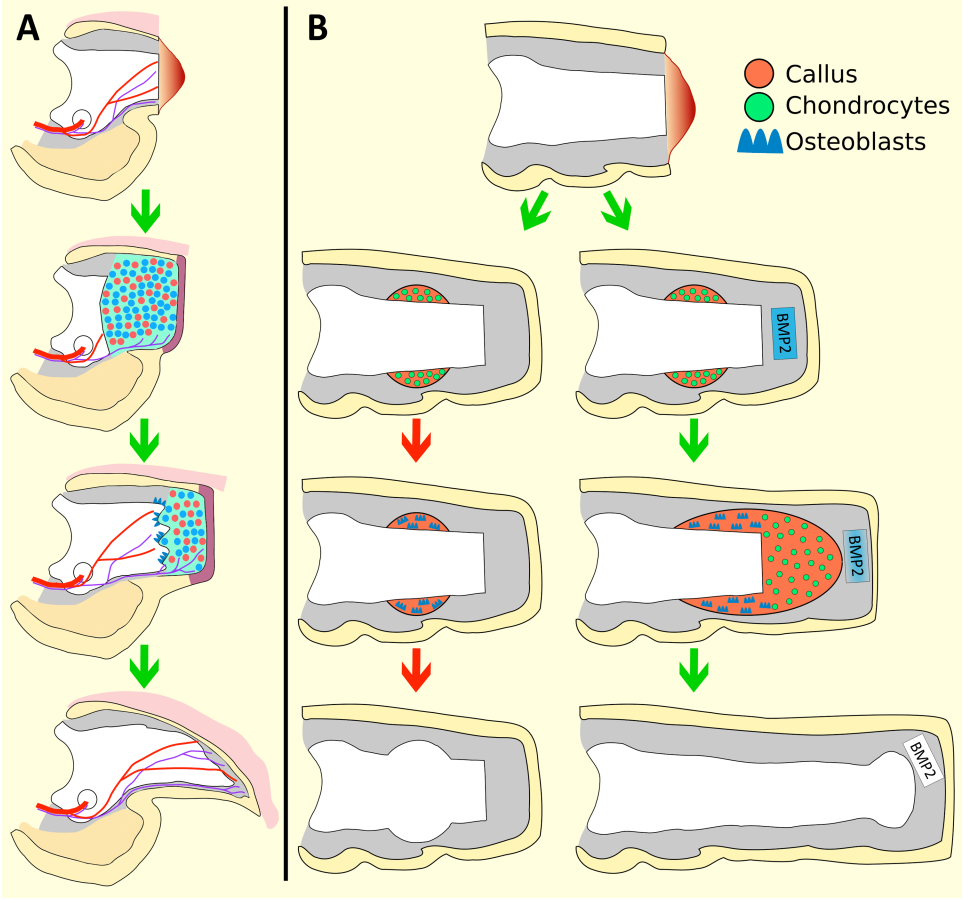

Figure 1.4: Diagram of Induced P2 Regeneration. (A) The regeneration response following amputation of P3 is used as a regeneration competent model to identify factors required for blastema formation and a regeneration response. Refer to Figure 1.1 for full description. A number of factors, including BMP2, have been shown to be essential for the P3 regeneration response. (B) Amputation of P2 induces a dynamic wound repair response characterized by periosteal chondrogenic callus formation, followed by callus conversion to woven bone, and ultimately truncation of the bone at the original amputation plane. The wound healing response is likened to an attempt at regeneration that ultimately fails, indicated by the red arrows. BMP2 treatment to target the periosteal chondrogenic callus induces the formation of a distal chondrogenic callus that functions as a template for subsequent bone regeneration to restore the amputated skeletal length. The change in color gradient of the BMP2-delivery-vehicle reflects the exhaustion of BMP2. Distal is to the right. Reprinted with permission from "Digit tip regeneration: merging regeneration biology with regenerative medicine" by Dolan CP[†], Dawson LA[†], Muneoka K, 2018. *Stem cells translational medicine* 7, 262-270, Copyright 2018 by John Wiley and Sons. † denotes co-first authorship.

a proof of concept that targeted intervention of an injury response that fails to regenerate can be effectively induced to undergo a significant regenerative response. These findings also begin to

characterize the regenerative potential of non-regenerative amputation injuries and show that latent regenerative ability can be activated by a single factor that is administered in a spatiotemporally targeted manner. Therefore, it is reasonable to conclude that the root cause of regenerative failure following amputation injury is not a lack of regeneration-responsive cells, but rather a toxic wound environment that precludes a regeneration response.

The Regeneration Window: In neonatal mice, BMP2 treatment over a 2-3 day period is sufficient to initiate a significant regenerative response. The use of a vehicle that effects transient BMP2 release has allowed an investigation into the temporal responsiveness of cells at the amputation wound. Such studies have identified a peak in responsiveness that correlates with the timing of wound closure and identified reduced responses of treatments at earlier and later time points (Dawson et al., 2017). This illustrates a dynamic of the wound environment with respect to regenerative potential, and identifies a temporally specific Regeneration Window within which BMP2 is effective at activating a regeneration response. As the P2 amputation wound matures it becomes refractory to BMP2, however we show that simple re-injury of the healed P2 stump re-initiates the injury response and recreates a wound environment that cycles through a BMP2 responsive window, complete with distal callus formation and subsequent skeletal regeneration (Figure 1.5A) (Dawson et al., 2017). In other words, mature non-regenerative amputation wounds respond to injury in a manner similar to the initial amputation, thus the process of non-regenerative wound healing does not alter the wound's regenerative potential. We note the clinical significance of this finding with regard to limb amputations; since BMP2 stimulates segment-specific bone regeneration of adult limb (Figure 1.5B) (Yu et al., 2012), we predict that previously healed amputation wounds in humans can be induced to regenerate.

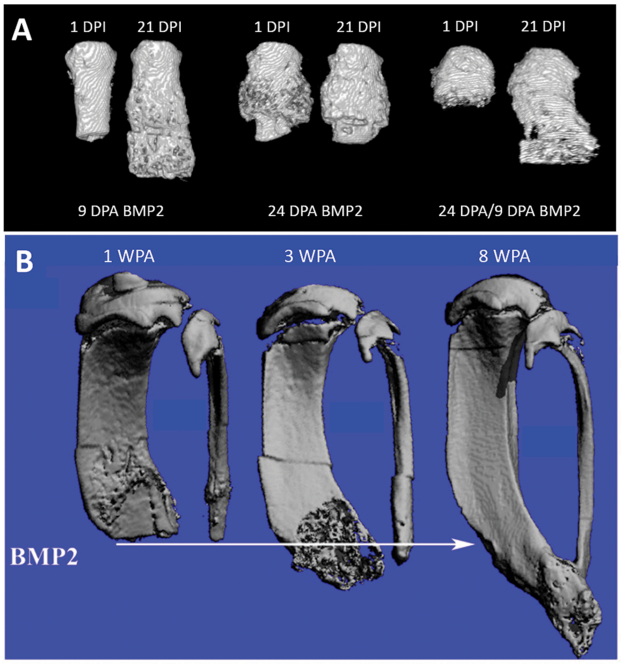

Figure 1.5: BMP2-induced Regeneration (A) Micro-computed tomography 3D renderings of BMP2-treated adult mouse P2 digits. BMP2 treatment during cartilaginous peripheral callus formation, at 9 DPA, induces robust skeletal regeneration, evident by 21 days post implantation (DPI). BMP2-treatment after the cartilaginous peripheral callus conversion to boney tissue at 24 DPA does not induce skeletal regeneration. Re-injury of previously healed 24 DPA P2 digits stimulates a regeneration permissive environment in which BMP2 functions to induce regeneration. (B) Sequential MicroCT 3D renderings of the BMP2-induced hind limb regeneration response in adult mice. Amputation plane shown as an arrow. Hindlimbs were treated with BMP2 at 2 weeks post amputation (WPA). BMP2-induced regeneration is evident by 3 WPA, shown as the formation of woven bone distal to the amputation plane. By 8 WPA, distal skeletal fusion is shown, indicating the regeneration response is associated with the reestablishment of skeletal patterning. (A-B) Distal is to the bottom. Reprinted with permission from "Digit tip regeneration: merging regeneration biology with regenerative medicine" by Dolan CP[†], Dawson LA[†], Muneoka K, 2018. *Stem cells translational medicine* 7, 262-270, Copyright 2018 by John Wiley and Sons. † denotes co-first authorship.

In its simplest form, and at a cellular level, the concept of a Regeneration Window can be viewed in the context of the availability of cells expressing appropriate receptors that can be activated by targeted treatment with a regeneration inducing factor. Indeed, targeted genetic or pharmacological knockdown of individual signaling pathways to inhibit the endogenous regeneration response supports this simplistic model (Han et al., 2003; Takeo et al., 2013; Yu et al., 2010). However, an alternative way to think about the regeneration window is that multiple cell types that function in a coordinated manner are required to effect a regeneration response. For example, BMP2 induced P2 regeneration in neonates involves the action of BMP2 as a

mitogen for cells that become proliferating chondrocytes and establish the EOC (Yu et al., 2012). At the same time, BMP2 also induces expression of *Sdf1a* by cells of the wound epidermis and endothelial cells of the wound mesenchyme, and SDF1 functions to recruit CXCR4 expressing cells for the regeneration response (Lee et al., 2013). Thus, at a minimum, BMP2 is activating 3 different cell types to induce skeletal regeneration, and it is likely that all three are needed to induce the response. It is therefore important to recognize that the Regeneration Window identifies a multi-tissue response that is coordinated by the morphogenetic action of the inducing agent, in this case BMP2. It is anticipated that other key morphogenetic agents will be identified that induce regeneration of other complex tissues that lack regenerative ability but possess regenerative potential.

1.5 Conclusion

Endogenous epimorphic regenerative responses in mammals, such as the mouse digit tip and human fingertip, provide models in which all requirements for a successful regeneration response are intrinsically met, e.g. angiogenesis, neurogenesis, inflammation, trophic factors, and regeneration-competent cells. As we come to understand the regeneration response, it follows that the conceptual application of these requirements to regeneration-incompetent injuries can effectively guide the design of therapeutic strategies for human regeneration. The example of the regeneration-incompetent middle phalanx (P2) is a case in point. Investigating this amputation injury identified a responsive population of cells and targeted application of BMP2 successfully induced regeneration of the skeletal element. These studies lead to three important conclusions that can be generalized to other injury models. First, regeneration-competent cells are present at traumatic injury wound sites, but they undergo dynamic changes and are only responsive during

a restricted period of the healing process, the Regeneration Window. Second, since BMP2 can stimulate appropriately patterned regeneration responses from different amputation levels, it is acting as a morphogenetic agent to elicit patterned responses and not as a morphogen that instructs patterning. Third, regenerative failure is caused by a toxic wound environment that minimally lacks the signaling profile of a morphogenetic agent necessary to coordinate a multi-tissue regenerative response. As we tease apart mammalian regeneration it is becoming apparent that partial regenerative responses can be stimulated from regeneration-incompetent injuries, and continued studies are expected to enhance the diversity of responses. To date, the stimulation of partial regenerative responses has not involved the formation of a blastema, thus a long term goal will be to solve the puzzle of how to build a blastema, a structure that coordinates pattern formation, morphogenesis, and differentiation of a complete regenerative response.

Several laboratories have demonstrated that peripheral nerves are also required for blastema formation during mouse digit tip regeneration (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). Thus, in considering how to induce blastema formation after non-regenerative injuries, peripheral nerves stand to be a reasonable candidate for intense investigation. However, before we can develop strategies that make peripheral nerves stimulate blastema formation at non-regenerative injuries, there must be considerable effort invested into understanding how peripheral nerves induce blastema formation during endogenous digit tip regeneration.

In this Dissertation, I will investigate the role of peripheral nerves during regeneration, with the overarching hypothesis that peripheral nerves are required endogenous mouse digit tip regeneration. In chapter 2, I will investigate the neuroanatomy of the unamputated and regenerated digit tip. In chapter 3, I will determine if mechanical load is required for

regeneration, as well as investigating if digit tip regeneration occurs in the absence of peripheral nerves.

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2. AXONAL REGROWTH IS IMPAIRED DURING DIGIT TIP REGENERATION*

2.1 Introduction

In amphibians, peripheral nerves are highly regenerative and required for the urodele limb regeneration response (Kumar and Brockes, 2012; Stocum, 2011; Todd, 1823). Peripheral nerves are thought to stimulate regeneration via the release of neurotrophic factors including transferrin (Kiffmeyer et al., 1991), FGFs (Satoh et al., 2011), anterior gradient protein (Kumar et al., 2007), neuregulin-1 (Farkas et al., 2016), and BMPs (Makanae et al., 2014). Implanting certain neurotrophic molecules such as neuregulin-1 (Farkas et al., 2016), combinations of FGFs and BMPs (Makanae et al., 2014), or dorsal root ganglia can rescue limb regeneration after denervation (Goldhamer et al., 1992; Kamrin and Singer, 1959; Tomlinson and Tassava, 1987). Additional evidence for a peripheral nerve requirement for limb regeneration come from gain-of-function experiments in which an accessory limb can be formed from a simple skin wound by rerouting nerves to the injury and adding a contralateral piece of dermis (Endo et al., 2004; Makanae and Satoh, 2012; Satoh et al., 2015).

Mice and humans are capable of regenerating the distal tip of their toes and fingers, respectively (Dolan et al., 2018; Douglas, 1972; Illingworth, 1974). In mice, inflammatory cells and osteoclasts are recruited to the injury after amputation and act to remove pathogens and to degrade dead bone (Simkin et al., 2015; Simkin et al., 2017). When the amputation wound heals, a blastema forms between the proximal bone stump and distal epidermis. The blastema is a transient, highly proliferative, heterogeneous, and lineage-restricted collection of

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stem/progenitors cells that organizes the regeneration response (Han et al., 2003; Lehoczky et al., 2011; Muneoka et al., 2008; Rinkevich et al., 2011). Epimorphic regeneration, defined by the formation of a blastema, distinguishes complex multi-tissue regeneration from tissue-specific regeneration responses (e.g. fracture repair, liver regeneration, etc.). By four weeks after injury, amputated structures—including bone, nail, epidermis, and vasculature—have completely regenerated. However, regeneration is restricted to the distal digit tip, and amputation at more proximal levels results in bone truncation, fibrotic healing and scar formation (Dawson et al., 2016; Yu et al., 2010). Similar to the salamander limb, mouse digit tip regeneration is a peripheral nerve-dependent event (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013), however unlike amphibians, transected mammalian peripheral nerves exhibit regenerative deficits (Jonsson et al., 2013). Nevertheless, transection of the sciatic nerve before digit tip amputation inhibits digit tip regeneration (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). Schwann cells have also been implicated in digit tip regeneration, and supplementing a denervated digit with PDGF-AA or Oncostatin-M can rescue the denervated regenerative response (Johnston et al., 2016).

The mammalian peripheral nervous system is a complex organ, comprised of numerous subtypes of nerves and Schwann cells that each have a specific function. Presently, it is unknown what types of nerves and Schwann cells innervate the digit tip, and to what extent these cells regenerate in association with the regenerative response. Given the necessity of peripheral nerves for mammalian regeneration, it is critical to address this gap in knowledge. Here, we characterize the neuroanatomy of the unamputated digit tip and show that there is a significant reduction of both nerves and Schwann cells in the regenerated digit tip. These findings suggest that the

neurotrophic requirement for regeneration is fundamentally different in mammals versus amphibians.

2.2 Specific Aims

Specific Aim 1: *Characterize the neuroanatomy of the unampuated digit.* I hypothesize that the unamputated digit will have a robust, patterned, and organized neuroanatomy.

Specific Aim 1.1: *Describe the organization of axons in the unamputated digit.* I hypothesize that axons will be present throughout the unamputated digit and will innervate the bone marrow vasculature and epidermal structures.

Specific Aim 1.2: *Determine if sensory and autonomic of axons are found in the unamputated digit.* I hypothesize that sensory and autonomic axons will be found in the unamputated digit.

Specific Aim 1.3: *Describe the organization of myelinating and non-myelinating Schwann cells in the unamputated digit.* I hypothesize unamputated digit axons will be ensheathed by myelinating and non-myelinating Schwann cells.

Specific Aim 2: *Determine if the neuroanatomy of the regenerated digit differs from the neuroanatomy of the unamputated digit*. I hypothesize that the neuroanatomy of the regenerated digit will be quantitatively and qualitatively similar to the neuroanatomy of the unamputated digit.

Specific Aim 2.1: *Determine if axons regenerate after digit tip amputation.* I hypothesize that axons will regenerate after digit tip amputation, and that extent of innervation in the regenerated digit will be quantitatively similar to that of the unamputated digit.

Specific Aim 2.2: *Determine if different sensory and autonomic axons regenerate after digit tip amputation.* I hypothesize that sensory and autonomic axons will regenerate after digit tip amputation, and the frequency of each subtype will be similar to what is observed in the unamputated digit.

Specific Aim 2.3: *Determine if myelinating and non-myelinating Schwann cells regenerate after digit tip amputation*. I hypothesize that myelinating and non-myelinating Schwann cells will regenerate after digit tip amputation.

2.3 Materials and Methods

Animals and Amputations: Adult 8-week-old, female CD-1 mice were purchased from Texas Institute for Genomic Medicine. Digit tip amputations of the 2nd and 4th hindlimb digits were performed as described previously (Simkin et al., 2013). In some mice regenerated digits were re-amputated. All animal use and techniques were in compliance with the standard operating procedures of Texas A&M University's Institutional Animal Care and Use Committee.

Digit Processing and Histological Staining: Digits were collected from mice and fixed in buffered zinc formalin (Anatech Ltd) for 24 hours at room temperature. Digits were decalcified using Decalcifier I (Surgipath), a 10% formic acid solution, for 24 hours. Decalcified digits were embedded in paraffin, serially sectioned (4 - 5 μm) and mounted onto microscope slides. For histological staining, slides were incubated at 60ºC (45 minutes), 37ºC (2 hours), deparaffinized to water and stained with Mallory trichrome. Slides were mounted using Permount Mounting Medium (Thermo Fisher Scientific). All slides were imaged using either 1) Olympus BX60 microscope with an Olympus DP72 camera, utilizing the DP2-BSW software, or 2) the Olympus

VS120 microscope with a Pike F-505C camera (Allied Vision) utilizing the VS-ASW FL2.8 software.

Immunohistochemistry: Antigen retrieval was performed using heat retrieval performed in citrate buffer solution (Dako). Slides were blocked using Protein Block (Dako) for 1 hour at room temperature. Incubation with primary antibody was performed overnight at 4ºC; followed by a wash in tris buffered saline with Tween R20 solution (Sigma-Aldrich Co.) and incubated in secondary antibody for 1 hour at room temperature. Slides were then incubated in a phosphate buffered saline (Sigma-Aldrich) and DAPI (Invitrogen) solution, dried, and mounted with Prolong Gold (Invitrogen). Samples were imaged using the either 1) Olympus BX61 microscope with a Hamamatsu ORCA-ER camera via the Slidebook software (Intelligent Imaging Innovations Inc.) or 2) Olympus VS120 microscope with a Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera via VS-ASW FL2.8 software (Olympus). Primary antibodies used were chicken anti-glial fibrillary acidic protein (1:500; Abcam; Ab4674), chicken anti-neurofilament H (1:1000; Millipore; Ab5539), guinea pig anti-substance P (1:250; Abcam; Ab10353), rabbit anti-tyrosine hydroxylase (1:500; Abcam; Ab112), rabbit anti-beta III tubulin (1:500; Abcam; Ab18207), and rabbit anti-protein zero (1:1000; Millipore; Abn363). Secondary antibodies used were goat anti-rabbit, chicken, or guinea pig Alexa Fluor-488, 568 or 647 (1:500; Invitrogen).

Quantification of β3T, P0, and GFAP: Quantitation of immunostained sections was carried out for β3T in the bone marrow and distal tip, and for P0 and GFAP in the connective tissue. 4-8 samples were analyzed and 3 images per sample were averaged for each region of interest. The region of interest was identified (μm^2) and images were acquired at 200X magnification. The immunofluorescent signal identified the area of antibody staining (μm^2) and this was normalized to the region of interest. For statistical analysis, either an unpaired t-test or a

one-way ANOVA with a post-hoc Tukey's multiple comparisons test was performed using GraphPad Prism 7 software (GraphPad Software).

Micro-computed tomography (μCT), repeat amputations, and quantitation of P3 bone volume and length: μCT scanning was performed using a were performed using a vivaCT 40 (SCANCO Medical) as previously described (Fernando et al., 2011). For the first amputation, unamputated (0DPA), amputated (1DPA), and regenerated (28DPA) P3 digits were scanned. Immediately after scanning regenerated digits, mice were anesthetized as described above, and the distal tip of their digits were re-amputated. The following day, amputated digits (1DPA(2)) were scanned, allowed to regenerated for four weeks, and then were scanned to assess regeneration (28DPA(2)). Using GraphPad Prism 7 software, a repeated-measures one-way ANOVA with a post-hoc Tukey's multiple comparisons test was used to assess statistical significance.

2.4 Results

Neuroanatomy of the Unamputated Digit Tip: The terminal phalanx (P3) of the 2nd and 4th hindlimb digits are each innervated by two nerves (digital nerves) that extend the length of the digit and are positioned ventrolaterally to the three phalangeal elements (P1, P2, P3; Figure 2.1A). Axons are identified based on immunostaining for the pan-neuronal markers beta-III-tubulin (β3T) and neurofilament-H (NFH) and these markers are expressed in digital nerve axons (Figure 2.1B; Figure 2.2A-D). While all digit samples analyzed co-express both neuronal markers, β3T expression is more prominent than NFH expression. At the level of the os holes, two prominent skeletal foramens that connect the P3 bone marrow cavity with the surrounding connective tissue, each digital nerve bifurcates to establish two distinct branches: one that

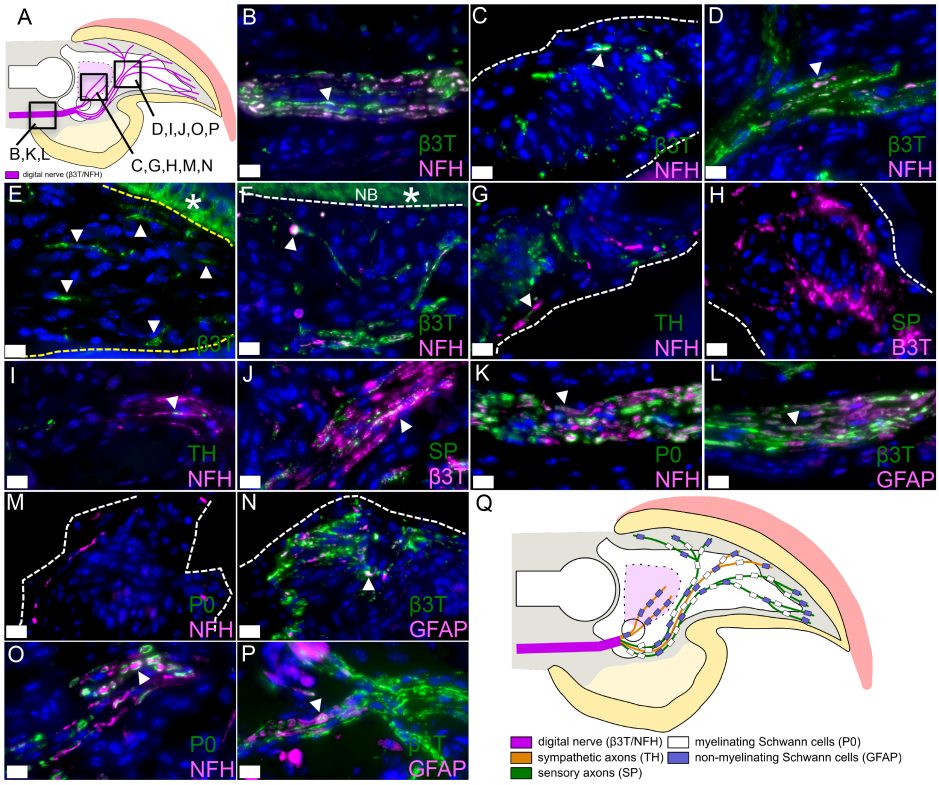

Figure 2.1: Neuroanatomy of the Unamputated Digit Tip (A) Diagram of the digital nerves in the terminal phalanx (P3). The black boxes indicate where images were taken within the P3 region with letters corresponding to the other images in the figure. (B-F) β3T and NFH immunopositive axons (arrowheads) in the proximal digital nerves (B), BM branch (C), CT branch (D), the distal digit tip (E), and subjacent to the nailbed (F). Arrowheads indicate axons; * indicate β3T-immunopositive keratinocytes in the nail bed. (G) Expression of TH and NFH (arrowhead) indicating that the BM branches are composed of sympathetic axons. (H) SP immunopositive axons are absent from the BM branches. (I) Expression of TH and NFH (arrowhead) indicating that the CT branches are composed of sympathetic axons. (J) Expression of SP and β3T (arrowhead) indicating that the CT branches are composed of sensory axons. (K) P0 myelinating-Schwann cells associate with NFH axons (arrowhead) in the digital nerves. (L) GFAP non-myelinating-Schwann cells associate with β3T axons (arrowhead) in the digital nerves. (M) P0 myelinating-Schwann cells are not associated with BM branch axons. (N) GFAP non-myelinating-Schwann cells associate with BM branch axons (arrowhead). (O) P0 myelinating-Schwann cells associate with CT branch axons (arrowhead). (P) GFAP non-myelinating-Schwann cells associate with CT branch axons (arrowhead). (Q) Diagram of the complete neuroanatomy of the terminal phalanx. β3T: beta-3-tubulin; NFH: neurofilament-H; TH: tyrosine hydroxylase; SP: substance P; P0: myelin protein zero; GFAP: glial fibrillary acidic protein; BM: bone marrow; CT: connective tissue; NB: nailbed. Scale bar = 10μm.White dashed lines: delineates BM vasculature; Yellow dashed lines: delineates epidermis. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

innervates the bone marrow (BM branch) (Figure 2.1C; Figure 2.2E-G) via the os hole, and another that remains peripheral to the P3 element and innervates the surrounding connective tissue (CT branch) (Figure 2.1D; Figure 2.2F,G). The BM branches terminate onto vasculature within the P3 marrow compartment (Figure 2.1C; Figure 2.2F,G). The CT branches extend distal

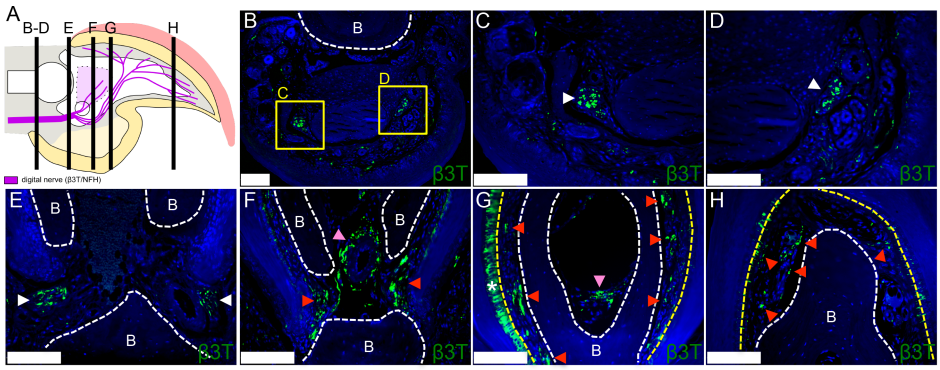

Figure 2.2: Transverse Sections of the Unamputated Digit Tip (A) Diagram of the digital nerves in the unampuated terminal phalanx (P3). The black lines indicate where images were taken within the P3 region with letters corresponding to the other images in the figure. (B-H) Immunohistochemistry for β3T-immunopositive axons (green) in the unamputated digit. (B) β3T-immunopositive axons in the two digital nerves (yellow boxes) that innervate P3. (C,D) Higher magnification images of digital nerves in (B). (E) β3T-immunopositive axons in the two digital nerves (arrowheads) approaching the P3 bone prior to bifurcation. (F,G) β3T-immunopositive axons after digital nerve bifurcation into the bone marrow (BM) branches (pink arrowhead) and the connective tissue (CT) branches (red arrowheads). (H) β3T-immunopositive axons of the CT branches in the distal digit tip (red arrowheads). β3T: beta-3-tubulin. Scale bar = 100µm.White dashed lines: delineates P3 bone; Yellow dashed lines: delineates epidermis;*: β3T-immunopositive signaling in the epidermis that is non-axonal. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

and dorsal through tissues surrounding the P3 bone and extend axons that project to epidermal structures (Figure 2.1E; Figure 2.2H) including the nailbed (Figure 2.1F).

The peripheral nervous system (PNS) is heterogeneous in composition, consisting of a number of axonal subtypes including sympathetic, sensory, and motor neurons. The axons of sympathetic neurons can be identified based on expression of tyrosine hydroxylase (TH) (Lazaroff et al., 1995), sensory neurons based on expression of substance P (SP) (Harrison and Geppetti, 2001), and motor neurons based on expression of choline acetyl-transferase (ChAT) (Misgeld et al., 2002). Coupling one of the pan-neuronal antibodies with subtype specific axonal immunostaining we are able to identify the composition of the BM and CT branches of the digital nerves. Since the digit does not contain any muscle tissue, it is not surprising that $ChAT^{+}$ axons are not observed in the digit tip (data not shown). BM branch axons are immunopositive for TH (100%; n=7/7) and the minority of digits are immunopositive for SP (14%; n=1/7)

indicating that they are sympathetic and not sensory (Figure 2.1G,H). Conversely, CT branch axons are immunopositive for both TH (86%; n=6/7) and SP (100%; n=7/7) indicating that the CT branch contains both sympathetic and sensory axons (Figure 2.1J). CT branch axons that are SP immunopositive terminate at epidermal structures whereas TH immunopositive axons appear to terminate on vasculature.

Schwann cells play a critical role in PNS function, and recently have been shown to be required for digit tip regeneration (Johnston et al., 2016). Within the PNS there are two types of terminally-differentiated Schwann cells: 1) Myelinating-Schwann cells ensheathe a single axon, are critical for proper conduction of nerve impulses and can be identified based on expression of myelin protein zero (P0), a glycoprotein expressed in the myelin sheath (Salzer, 2015); 2) Non-myelinating-Schwann cells ensheathe multiple axons, are commonly found at axon termini and can be identified based on expression of glial fibrillary acidic protein (GFAP), an intermediate filament protein (Jessen et al., 1990). Coupling one of the pan-neuronal antibodies with Schwann cell-specific immunostaining we characterized the BM and CT branches of the digital nerves. The digital nerves proximal to the digital nerve bifurcation are immunopositive for both P0 and GFAP, indicating that this region is associated with both myelinating and non-myelinating Schwann cells (Figure 2.1K,L). BM branch axons are associated with cells immunonegative for P0, but immunopositive for GFAP, indicating that they are only associated with non-myelinating-Schwann cells (Figure 2.1M,N). CT branch axons are immunopositive for both P0 and GFAP indicating that, like the proximal digital nerve, they are associated with myelinating and non-myelinating Schwann cells (Figure 2.1O,P).

In summary (Figure 2.1Q), the digit tip is innervated by two ventrolateral digital nerves that each bifurcate at the base of the P3 bone. The BM branches enter the P3 marrow through the

os holes and consist of sympathetic axons associated with non-myelinating Schwann cells that terminate on marrow vasculature. The CT branches consist of sensory axons that terminate on the epidermal structures and sympathetic axons that terminate on vasculature, and that associate with myelinating and non-myelinating Schwann cells. These data identify clear differences in axon and Schwann cell composition that correlate with physiological functions expected in different regions of the digit tip.

Neuroanatomy of the Regenerated Digit Tip: Amputation of the digit tip removes approximately 25% of the P3 bone length as well as the surrounding connective tissues, skin, nail, vasculature, and axons of the CT branches, but leaves the marrow region intact (Figure 2.3A). During the regenerative response however, the stump bone surrounding the marrow is degraded and the blastema forms from an intermingling of marrow and connective tissue cells (Dawson et al., 2018). Thus, the proximal region of the blastema is contiguous peripherally with the stump connective tissue, and centrally with the distal bone marrow. Twenty-eight days post-amputation (DPA) the bone marrow is restored as is the P3 bone length (Figure 2.3B) (Dawson et al., 2018; Fernando et al., 2011). While the amputated P3 bone is cortical, the regenerate consists of woven bone that contains numerous vascularized pockets that are contiguous with the marrow region and the surrounding connective tissue (Figure 2.3B). To assess how the neuroanatomy of the digit tip had changed during regeneration, we analyzed 28 DPA regenerates and immunostained for axon and Schwann cell markers (Figure 2.3; Figure 2.4).

The BM branch of the digital nerve innervates the regenerated P3 marrow and axons are immunopositive for β3T. Quantitative analysis comparing β3T expression of representative sections of the marrow region between regenerates and unamputated control digits show that the average β3T expression in the regenerates (0.013 ± 0.0005; n=8) was similar to controls (0.011 ±

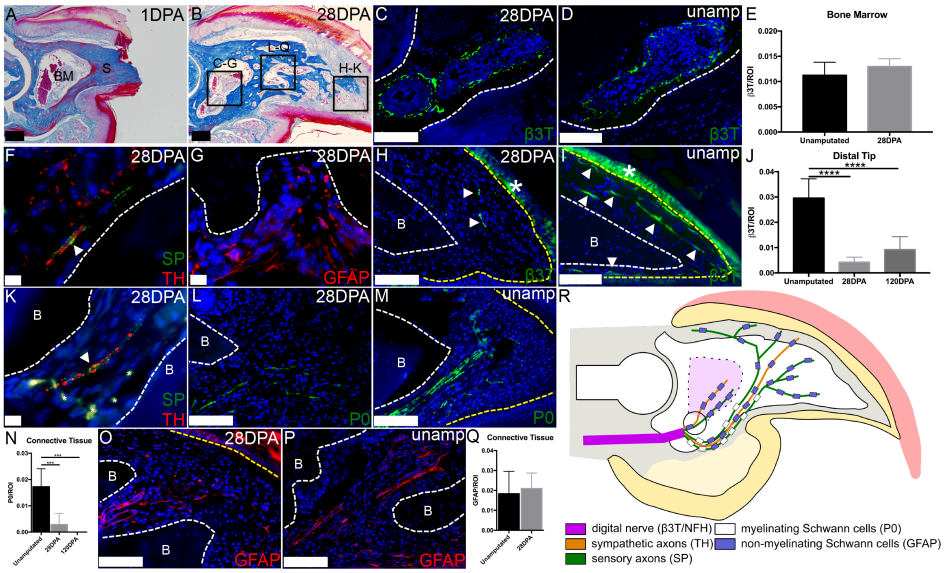

Figure 2.3: Neuroanatomy of the Regenerated Digit Tip (A,B) Mallory's Trichrome staining of the amputated digit at 1 DPA (A) and regenerated digit at 28 DPA (B). (C,D) β3T immunopositive axons in the BM of the regenerated (C) and unamputated digit (D). (E) Quantification of β3T immunopositive axons in the BM (mean ± SD; n=8; P=0.1239). (F) SP (arrowheads) and TH immunopositive axons in the BM of the regenerated digit. (G) GFAP immunopositive non-myelinating-Schwann cells in the BM of the regenerated digit. (H,I) β3T immunopositive axons in the distal digit tip of the regenerated (H) and unamputated digit (I). Arrowheads indicate axons; * indicate β3T-immunopositive keratinocytes in the nail bed. (J) Quantification of β3T immunopositive axons in the unamputated and regenerated distal digit tip at 28 and 120 DPA (mean ± SD; n=5-8; **** = P<0.0001). (K) SP and TH immunopositive axons in the distal tip of the regenerated digit (white asterisks: autoflourescence from red blood cells). (L,M) P0 immunopositive myelinating-Schwann cells in CT of the regenerated (L) and unamputated (M) digit tip. (N) Quantification of P0 immunopositive myelinating-Schwann cells in the CT of the unampuated and regenerated digit tip at 28 and 120 DPA (mean ± SD; n=4-7; *** = P<0.001) (O,P) GFAP immunopositive non-myelinating-Schwann cells in the CT of the regenerated (O) and unamputated (P) digit tip. (Q) Quantification of GFAP immunopositive non-myelinating-Schwann cells in the CT of the unamputated and regenerated digit (mean ± SD; n=6-7; P=0.63). (R) Diagram of the neuroanatomy of the regenerated terminal phalanx. β3T: beta-3-tubulin; NFH: neurofilament-H; TH: tyrosine hydroxylase; SP: substance P; P0: myelin protein zero; GFAP: glial fibrillary acidic protein; S: stump bone; BM: bone marrow; CT: connective tissue; B: bone; unamp: unamputated digit; DPA: days post amputation A,B: Scale bar = 200µm. C,D,H,I,L,M,O,P: Scale bar = 100µm. F,G,K: Scale bar = 10µm. C,D,F,G: White dashed lines: delineates BM vasculature; H,I,L,M,O,P: White dashed lines: delineates P3 bone; H,I: Yellow dashed lines: delineates epidermis. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

0.001; n=8; P=0.1239) (Figure 2.3C-E). Similar to the unamputated digit, axons of the BM branch are immunopositive for TH (100%; n=12/12) (Figure 2.3F). However, in 50% of regenerated digits (n=6/12) SP immunopositive sensory axons are also observed in the bone marrow compartment (Figure 2.3F). Schwann cells in the BM are immunopositive for GFAP

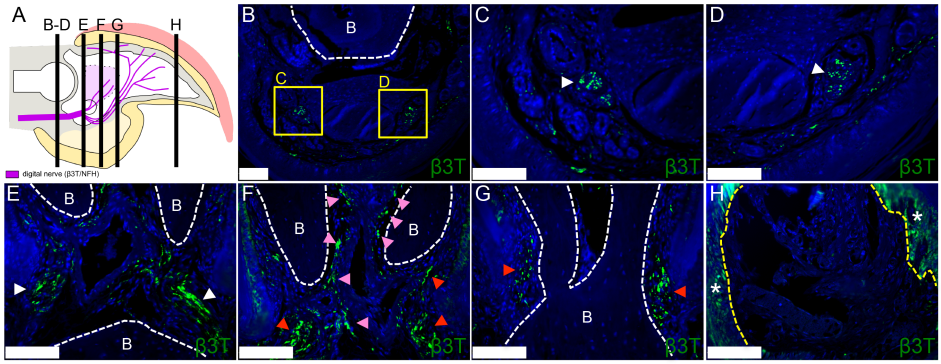

Figure 2.4: Transverse Sections of the Regenerated Digit Tip (A) Diagram of the digital nerves in the regenerated terminal phalanx (P3). The black lines indicate where images were taken within the P3 region with letters corresponding to the other images in the figure. (B-H) Immunohistochemistry for β3T-immunopositive axons (green) in the regenerated digit tip 28 days post amputation (DPA). (B) β3T-immunopositive axons in the two digital nerves (yellow boxes) that innervate the regenerated P3. (C,D) Higher magnification images of digital nerves in (B). (E) β3T-immunopositive axons in the two digital nerves (arrowheads) approaching the P3 bone prior to bifurcation. (F,G) β3T-immunopositive axons after digital nerve bifurcation into the bone marrow (BM) branches (pink arrowheads) and the connective tissue (CT) branches (red arrowheads). (H) β3T-immunopositive axons of the CT branches are absent from the regenerated distal digit tip. Asterisks indicate β3T-immunopositive signaling in the epidermis that is non-axonal. β3T: beta-3-tubulin. Scale bar = 100μm.White dashed lines: delineates P3 bone; Yellow dashed lines: delineates epidermis. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

(Figure 2.3G) and immunonegative for P0 (data not shown), indicating similarity to the unamputated controls.

Digit tip amputation transects CT branch axons and removes the distal tissue that these axons innervate. The regenerated digit tip contains axons that track to the CT branch of the digital nerve however, they do not appear as abundant when compared to control unamputated digits, particularly when considering innervation of the epidermis (Figure 2.3H,I). Quantitative analysis of axons within the connective tissue of the digit tip indicates that the distal tip of the regenerate contains significantly fewer axons when compared to unamputated control digit tips (Figure 2.3J). The average β3T expression in control digits was 0.03 ± 0.003 (n=6) whereas the average β3T expression in regenerated digits was 0.004 ± 0.0007 (n=8; $P<0.0001$) indicating a 7.5-fold decline in the ability to restore digit innervation (Figure 2.3J). To determine if axon regrowth is delayed in digit tip regeneration, we analyzed digits at 120 DPA and found that the

average β3T expression in regenerated digits was 0.009 ± 0.002 (n=5; P<0.0001) indicating a 3.3-fold decline (Figure 2.3J). These data demonstrate that axon regeneration associated with digit tip regeneration is impaired and not simply delayed. SP immunopositive sensory axons are observed in all of the regenerated digits (100%; n=12/12) whereas TH immunopositive sympathetic axons are only observed in 42% of regenerated digits (n=5/12) (Figure 2.3K). Associated with the decrease in regenerated axons the average level of P0 expression in regenerated digits (0.003 ± 0.002; n=7) was significantly lower than unamputated control digits (0.017 ± 0.003; n=4; P<0.001) at 28 DPA (Figure 2.3L-N). This 5.6-fold decline in myelinated Schwann cells correlates with the 7.5-fold decline in regenerated axons and suggest that the reduced number of regenerated sensory axons are appropriately myelinated. Moreover, P0-immunopositive myelinating Schwann cells in the regenerated digit at 120 DPA have not regenerated back to unamputated levels ($4.46e^{-5} \pm 1.43e^{-5}$; n=5; P<0.0001) (Figure 2.3N). Conversely, the average level of GFAP expression in regenerated digits (0.021 ± 0.003; n=7) was not significantly different when compared to unamputated control digits (0.018 ±0.005; n=6; P=0.63), indicating that the non-myelinating Schwann cell population is restored during regeneration (Figure 2.3O-Q).

In summary (Figure 2.3R), after regeneration BM branch axons remain quantitatively similar to unamputated control digits, although we do observe an increase in sensory axons within the bone marrow compartment. CT branch axons display an impaired regeneration response and there is a significant decrease in myelinating Schwann cells whereas the non-myelinating-Schwann cells regenerate to levels similar to unamputated control digits.

Neuroanatomy of the Blastema: The blastema is a transient structure that forms between the wound epidermis and bone stump and distinguishes digit tip regeneration from tissue-specific

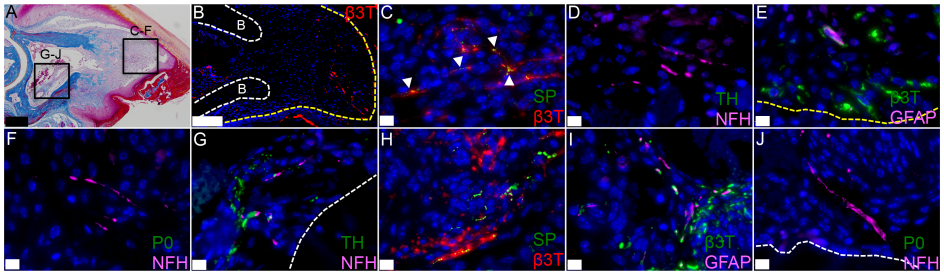

Figure 2.5: Neuroanatomy of the Blastema (A) Mallory's Trichrome staining of the blastema at 10 DPA. (B-J) Immunohistochemical staining of the blastema at 10 DPA. (B) β3T immunopositive axons surround, but are not found in the center of the blastema. (C) Expression of SP (arrowheads) and β3T indicate sensory axons in the distal blastema. (D) TH immunopositive sympathetic axons are not found in the distal digit tip. (E) GFAP immunopositive non-myelinating-Schwann cells in the distal blastema associated with some but not all β3T immunopositive axons. (F) P0 immunopositive myelinating-Schwann cells are not found in the distal blastema. (G,H) TH (G) and SP (H) immunopositive axons are present in the BM at 10 DPA. (I,J) GFAP immunopositive non-myelinating-Schwann cells (I), but not P0 immunopositive myelinating-Schwann cells (J), are found associated with axons in the BM at 10 DPA. β3T: beta-3-tubulin; NFH: neurofilament-H; TH: tyrosine hydroxylase; SP: substance P; P0: myelin protein zero; GFAP: glial fibrillary acidic protein; BM: bone marrow; CT: connective tissue; B: bone; unamp: unamputated digit; DPA: days post amputation. C-J: Scale bars = 100μm. C-J: Scale bars = 10μm. B: White dashed line: delineates terminal phalanx bone; Yellow dashed lines: delineates epidermis. G,J: White dashed lines: delineates BM vasculature. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

regenerative responses in mammals (Figure 2.5A) (Seifert and Muneoka, 2018). The blastema is avascular, hypoxic, derived from multiple tissue sources, and is highly proliferative (Dolan et al., 2018). The central region of the blastema is immunonegative for β3T (Figure 2.5B) indicating that this blastema region is devoid of axons. Rather, β3T immunopositive axons are restricted to peripheral and distal blastema regions indicating that regenerating axons envelop the central core of proliferating pre-osteogenic blastema cells (Figure 2.5B) (Dawson et al., 2018). These regenerating axons are immunopositive for SP and immunonegative for TH (Figure 2.5C,D) indicating that the regenerating axons at this stage are sensory. These sensory axons are associated with GFAP but not P0 expressing cells (Figure 2.5E,F) indicating that only non-myelinating-Schwann cells are supporting the regenerative response. We note that axons at the distal end of the blastema associated with the wound epidermis are not always associated with Schwann cells (Figure 2.5E). TH and SP immunopositive axons are found in the bone marrow

(Figure 2.5G,H) indicating that both sympathetic and sensory axons are present in the bone marrow during the blastema stage of regeneration. Axons in the bone marrow associate with non-myelinating Schwann cells but not myelinating Schwann cells, similar to the unamputated and regenerated digits. (Figure 2.5I,J).

Re-amputation does not inhibit digit tip regeneration: Since mouse digit tip regeneration has been shown to be peripheral nerve-dependent (Takeo et al., 2010; Rinkevich et al., 2013; Johnston et al., 2016) but the regeneration of digit nerves is impaired, we carried out a quantitative micro-computed tomography (μCT) analysis of regeneration following re-amputation of regenerated digits (Simkin et al., 2015) to determine if a second regenerative response was impaired. Digit tips were amputated and allowed to regenerate for four weeks at which time the regenerated digits were re-amputated and analyzed after four additional weeks. For quantifying P3 bone regeneration, unamputated, amputated, and regenerated digits were scanned using in vivo μCT which allows for monitoring the regeneration responses of the same digit following repeated amputation. Representative μCT redendered-3D images clearly show that regeneration is not inhibited following re-amputation (Figure 2.6A). After the first amputation, digits regenerate significantly more bone volume (BV) compared to the unamputated digit ($P<0.0001$; $n=39$) consistent with a BV overshoot previously reported (Figure 2.6B) (Dawson et al., 2018; Fernando et al., 2011). After the second amputation, we observe a similar trend, with a significant increase in BV compared the first regenerate ($P<0.0001$; $n=39$) and producing a P3 bone volume that is approximately 50% larger than the initial unamputated digit tip (Figure 2.6B). These results clearly show that impaired innervation does not restrict the regenerative capabilities of the digit tip.

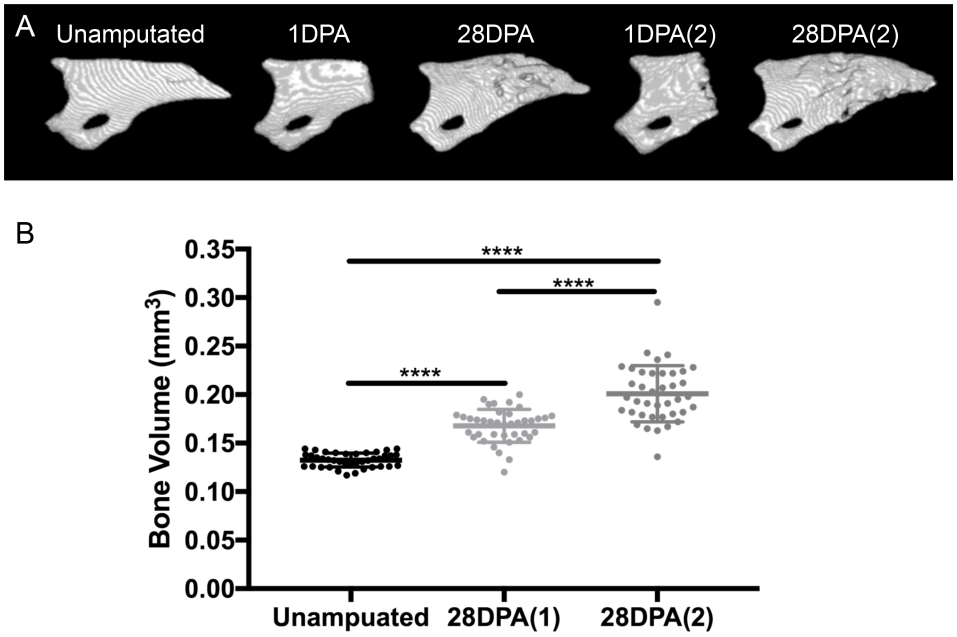

Figure 2.6: Re-amputation does not Inhibit Digit Tip Regeneration (A) Representative μCT redendered-3D images of the unampuated, amputated (1DPA), regenerated (28DPA), re-amputated (1DPA(1)), and 2nd regeneration ((28DPA(2)) P3 digit bone. (B) Quantitative measurements of P3 digit bone volume. **** = P<0.0001. DPA = Days post amputation. Reprinted with permission from "Axonal regrowth is impaired during digit tip regeneration in mice" by Dolan CP, Yan M, Zimmel K, Yang TJ, Leininger E, Dawson LA, Muneoka K, 2019. *Developmental Biology* 445(2), 237-244, Copyright 2018 by Elsevier.

2.5 Discussion

The neuroanatomy of the mouse digit tip is largely predicted from innervation studies of the bone marrow and skin in other parts of the body. The digit tip is innervated by two ventrolateral digital nerves that upon reaching the terminal phalanx bifurcate into two distinct branches: the BM and CT branch. The BM branches are composed of sympathetic axons associated with non-myelinating-Schwann cells which is consistent with published bone marrow studies characterizing innervation of the marrow compartment (Bjurholm et al., 1988). The CT branches are composed of sensory and sympathetic axons that associate with myelinating-Schwann cells and non-myelinating-Schwann cells. These data parallel well-established reports that sensory nerves of the dermis are responsible for receiving and transmitting afferent signals while sympathetic nerves regulate vascular tone primarily by inducing vasoconstriction of small

resistance arteries (Bruno et al., 2012; Zochodne, 2008). The importance of a detailed assessment of the digit tip axons and Schwann cells rests in the fact that this region of the mammalian limb displays endogenous regenerative properties and that the regenerative response is dependent on both nerves and Schwann cells (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013).

One striking result from the results reported in this chapter is that successful digit tip regeneration is associated with a reduction of connective tissue axons when compared to unamputated controls. While this finding is consistent with poor regenerative capabilities of mammalian PNS axons following axotomy (Stankovic et al., 1996), the results are not consistent with studies demonstrating that nerves are required for digit tip regeneration (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). Moreover, a robust regenerative response was observed in digits with impaired innervation. The neurotrophic effect on epimorphic regeneration has been best studied in salamander limb regeneration where there is robust regeneration of limb axons that produce neurotrophic factors essential for the regeneration response (Pirotte et al., 2016; Satoh et al., 2015). Whether there is a similar neurotrophic effect in mammalian regeneration is important with respect to strategies for enhancing regenerative capabilities, particularly in humans. A number of studies show that regeneration of the denervated digit tip is not completely inhibited, but is associated with a delayed healing response, a reduced level of blastema cell proliferation and delayed/reduced bone regrowth (Mohammad and Neufeld, 2000; Rinkevich et al., 2014; Takeo et al., 2013). The central region of the digit blastema is osteogenic and highly proliferative (Dawson et al., 2018), and as shown here it is also devoid of axons making it unlikely that proliferation of pre-osteoblasts within the blastema is a nerve-dependent event. This conclusion is consistent with reports that bone repair is nerve-independent (Miura et al., 2015).

Mammalian regeneration studies utilize transection of the sciatic nerve at the level of the thigh to denervate the digit tip, but damaging the sciatic nerve also paralyzes the hindlimb making it non-functional. This procedure can have multiple off-target effects beyond digit denervation; indeed, sciatic nerve transection is commonly used to study the effect of activity and mechanical load on limb muscle and skeletal tissues (Gross et al., 2010). Thus, it is possible that the nerve influence on mouse digit tip regeneration is indirect and not necessarily related to the absence of regenerating axons during the response. Overall, it is difficult to find clear parallels between the nerve-dependent responses of salamander limb regeneration and mouse digit regeneration, and the available evidence supports the conclusion that there are fundamental differences in the neurotrophic influence on these two epimorphic regeneration models (Mohammad and Neufeld, 2000).

Schwann cells are known to be present during the digit tip regenerative response where they produce PDGF and Oncostatin-M that are both mitogenic for blastema cells (Johnston et al., 2016). In response to PNS axon injury, Schwann cells are known to support and guide the regrowth of severed axons by the modification the wound environment and the production of specific trophic factors (Boyd and Gordon, 2003; Chen et al., 2007; Scheib and Hoke, 2013; Wood and Mackinnon, 2015). Thus, it is conceivable that the digit tip regenerative response has evolved mechanisms that utilize trophic activities produced by these cells. Nevertheless, there is an overall decline in Schwann cells associated with the regeneration response and only non-myelinating Schwann cells are present during the blastema stage. After peripheral nerve injury, myelinating Schwann cells are known to down-regulate genes associated with myelination (e.g. P0, MBP, MAG, etc.) and up-regulate genes associated with immature and/or non-myelinating Schwann cells (e.g. GFAP, L1, NCAM, etc.) (Chen et al., 2007; Jessen and Mirsky, 2008), thus

the absence of myelinating Schwann cells can be attributed to this transformation (Gomez-Sanchez et al., 2017). The restoration of myelinating Schwann cells in the regenerate to numbers that correspond to the reduction in regenerated CT branch axons suggest that myelinating Schwann cells are re-differentiating in accord with axon maturation. The restoration of non-myelinating Schwann cells to pre-amputation numbers despite a significant decline in regenerated axons is more difficult to explain, and suggests that this population of cells is not responding to the regenerated neural environment, but perhaps to the regenerated vascular environment.

The digit blastema is devoid of vasculature and is hypoxic (Fernando et al., 2011; Sammarco et al., 2014). Maintaining the blastema in a hypoxic state plays a critical role in coordinating the regenerative response (Sammarco et al., 2015; Simkin et al., 2015). I show here that the avascular region of the blastema is also devoid of axons and Schwann cells, suggesting that the regenerative responses of these tissues are somehow linked. During digit tip regeneration, blastema cells express *Pedf*, a potent anti-angiogenic factor that inhibits expression of *Vegf*, which creates a pro-regenerative wound environment by inhibiting early revascularization (Yu et al., 2014). Angiogenesis during the healing of full thickness skin wounds is also modulated by the balance between VEGF and PEDF, and in this model excessive angiogenesis is linked to an anti-regenerative wound environment that ultimately leads to the deposition of scar tissue (DiPietro, 2016; Wietecha et al., 2015). During vertebrate development formation of the vascular system precedes and guides subsequent neural innervation (Bates et al., 2003; Bates et al., 2002). Studies of de novo regeneration of peripheral nerve axons following sciatic nerve transection have found that Schwann cells serve as axonal guides but their

migration is directed by blood vessels, and that this multicellular response is initiated by macrophages responding to hypoxia (Cattin et al., 2015).

The digit regenerative response is dependent on macrophages which play a crucial role at multiple phases of the regenerative process (Simkin et al., 2017). It is intriguing that the regenerating mouse digit blastema has all of the components identified for a peripheral nerve-specific tissue regenerative response, but in the context of a blastema that coordinates the regeneration of multiple tissue types, including bone, epidermis and connective tissue. Together the evidence suggest that hypoxia and macrophages play a regulatory role in coordinating digit vasculature as well as epidermal and osteogenic regeneration (Simkin et al., 2015; Simkin et al., 2017), while Schwann cells and regenerating axons utilize regenerating vascular routes to re-innervate the regenerating digit tip.

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3. MOUSE DIGIT TIP REGENERATION IS PERIPHERAL NERVE-INDEPENDENT

3.1 Introduction

Annually, 185,000 people undergo amputation of an upper or lower limb extremity (Owings and Kozak, 1998). The primary approaches in regenerative medicine include the use of native or bioengineered scaffolds, stem cell therapy, or a combination of these two approaches to repair or replace the lost appendage (Stocum, 2012). An alternative and long-range approach is to better understand how to target and enhance endogenous repair capabilities to induce significant regenerative responses. Mice, like humans, are capable of regenerating the distal tips of their digits after amputation (Figure 1.1; Figure 1.2) (reviewed in Dolan et al., 2018; Douglas, 1972; Illingworth, 1974). However, amputation of the digit at more proximal levels results in fibrotic healing and scar formation (Dawson et al., 2016; Yu et al., 2010; Yu et al., 2012) (Figure 1.3; Figure 1.4).

In some regeneration competent non-mammalian animals, peripheral nerves promote regeneration by secreting neurotrophic factors, and removing nerves prior to injury inhibits regeneration (Farkas et al., 2016; Pirotte et al., 2016; Satoh et al., 2015; Stocum, 2011; Todd, 1823). In mice, denervation prior to digit tip amputation inhibits regeneration suggesting that mammals may also require peripheral nerves for regeneration (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). However, as we have shown in chapter 2 (Dolan et al., 2019), peripheral nerve regeneration is impaired during mouse digit tip regeneration, and that even with impaired innervation the digit remains capable of regenerating if it is re-amputated (Dolan et al., 2019). Previous studies demonstrating that digit tip regeneration is peripheral nerve-dependent have been performed using the surgical procedure known as sciatic denervation (or sciatic

neurectomy. (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). While sciatic denervation is a robust surgical procedure for studying regeneration in the absence of peripheral innervation, it is also an established model for decreasing mechanical loading of the denervated limb (Gross et al., 2010).

Mechanical loading is the term used to describe the forces applied to the body, and is critical for the homeostasis of organs—most notably bones and muscles (Gross et al., 2010). Mechanical loading is essential for skeletal development, and in the absence of skeletal loading, limbs develop with only 30-50% of normal bone mass (Robling and Turner, 2009). Conversely, increasing mechanical loading through exercise increases bone mass and strength (Robling and Turner, 2009). Importantly, experimentally decreasing mechanical loading has been shown to have both positive and negative affects on appendage regeneration (Grinfeld et al., 1996; Mitashov et al., 1996; Recidoro et al., 2014). Determining if regenerative failure after sciatic denervation (sciatic neurectomy) is a result of decreased peripheral nerve signaling or decreased mechanical loading, or both, is imperative for understanding the underlying mechanisms driving mammalian regeneration.

In this chapter, I begin by investigating the role of mechanical loading during mouse digit tip regeneration. Hindlimb unloading (HU), is a well-established model in which the hindlimbs of mice are suspended such that they are no longer weight bearing (Ferreira et al., 2011; Globus and Morey-Holton, 2016; Morey-Holton and Globus, 2002). I find that hindlimb unloading mice after amputation inhibits digit tip regeneration by preventing wound closure, blastema formation, new bone formation, and by attenuating histolysis. Moreover, the early stages of digit tip regeneration are more sensitive to mechanical perturbation and require mechanical loading whereas later regenerative processes can occur in the absence of significant

mechanical loading. Further, the data in this chapter convincingly demonstrate that denervating digits prior to amputation at the digit-level, and thereby preserving the animals ability to ambulate and exert normal mechanical forces during regeneration, does not significantly inhibit digit tip regeneration. Taken together, these results provide compelling evidence to demonstrate that mouse digit tip regeneration is peripheral-nerve independent.

3.2 Specific Aims

Specific Aim 1: *Determine if mechanical loading is a critical factor influencing digit tip regeneration*. I hypothesize that mechanical loading is an essential factor influencing digit tip regeneration, and that decreasing mechanical unloading will inhibit digit tip regeneration.

Specific Aim 1.1: *Evaluate the impact that decreased mechanical loading will have on regenerated bone volume and bone length, and bone degradation.* I hypothesize that decreased mechanical loading will inhibit bone volume and bone length regeneration, but will have no effect on bone degradation.

Specific Aim 1.2: *Determine if decreased mechanical loading will impair amputation wound healing.* I hypothesize that decreased mechanical loading will prevent the amputation wound from healing.

Specific Aim 2: *Determine if digit tip regeneration is impaired after digit denervation.* I hypothesize that digit denervation will not impair digit tip regeneration.

Specific Aim 2.1: *Determine if digit denervation impairs bone volume and length regeneration.* I hypothesize that digit denervation will not impair bone volume and length regeneration.

Specific Aim 2.2: *Determine if digit denervation impairs blastema cell-proliferation.* I hypothesize that digit denervation will not impair blastema cell-proliferation.

Specific Aim 2.3: *Determine if digit denervation impairs amputation wound healing.* I hypothesize that digit denervation will not impair amputation wound healing

3.3 Materials and Methods

Animals and digit tip amputations: Adult 8-week-old, female CD-1 mice were purchased from Texas Institute for Genomic Medicine (College Station, TX) and used for all denervation experiments. Adult, 8-week-old, female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) for all hindlimb unloading experiments. Digit tip amputations of the 2nd and 4th hindlimb digits were performed as described previously (Figure 1.2) (Simkin et al., 2013). All animal use and techniques were in compliance with the standard operating procedures of Texas A&M University's Institutional Animal Care and Use Committee.

Digit denervation surgery: Mice were anesthetized with isoflurane and hindlimb paws were sterilized using betadine. Denervations were performed by creating an incision with micro-scissors on the ventral surface of the digit, which stretched from the proximal head of the first phalange to the fat pad. Digit nerves were transected at the distal fat pad, pulled proximally with forceps, and then a second transection was made creating a gap the size of the original incision. Finally, the open wound was closed using Dermabond (Ethicon). The 2nd and 4th left and right hindlimb digits were used as experimental and sham operations, respectively. Five days after digit denervation or sham operation digits tips were amputated.

Tail ring implantation surgery, continuous hindlimb unloading, and staged hindlimb unloading: Tail ring implantation and hindlimb unloading are described in detail by (Ferreira et

al., 2011). Briefly, mice were anesthetized with isofluorane and their tails were cleaned with betadine. A 25-gauge needle was pushed through the intervertebral space between the 5-6 tail vertebra and then removed and a sterile 2-0 surgical steel suture (Ethicon) was pushed through the tail and shaped into a ring. After 5 days of recovery, mice had the distal tips of their digits amputated and after awaking were placed into a hindlimb unloading specific cage for approximately 4 weeks (continuous hindlimb unloading) (Ferreira et al., 2011). For stage specific hindlimb unloading experiments, tail rings were implanted as described above. For one group of mice, after digit amputation, they were placed into hindlimb unloading for 12 days, and then removed for the remainder of the experiment. For the second group of mice, they were allowed to ambulate normally for 12 days following digit tip amputation, and were then placed into hindlimb unloading for the rest of the experiment.

Micro-computed tomography (μCT), image processing, and quantification of bone volume, bone length, and rate of new bone formation: Terminal phalanx (P3) bone volume and length were measured using a vivaCT 40 (SCANCO Medical) as previously described (Dawson et al., 2018). μCT files were saved as a DICOM image stack, and subsequently uploaded to ImageJ where we used the 3D viewer plugin to create 3D renderings of bones. Bone volume was measured using the volume fraction tool in the BoneJ plugin in ImageJ. Bone length was measured using the point tool also in ImageJ. Bone volume and length are normalized to each individuals digit's volume or length at 1 day post amputation to account for variation in amputation. Depending on the experiment, a paired t-test, an unpaired t-test, or a one-way ANOVA with a post-hoc Tukey's multiple comparisons test using GraphPad Prism 7 (GraphPad Software). To determine and compare that rate of new bone volume and bone length regeneration, we performed a linear regression also using GraphPad Prism 7.

Digit processing, Mallory's trichrome staining, and wound closure: Digits were collected from mice and fixed in buffered zinc formalin (Anatech Ltd) for 24 hours at room temperature. Digits were decalcified using Decalcifier I (Surgipath), a 10% formic acid solution, for 24 hours. Decalcified digits were processed through a graded ethanol series, xylenes, and immersed in paraffin wax. Digits embedded in paraffin wax were serial sectioned at 4 - 5 μm thickness. Before to histological staining, slides were incubated at 60C for 45 minutes, followed by incubation at 37C for 2 hours, with subsequent deparaffinization with xylenes, a graded ethanol series, and eventual submersion in water. Mallory trichrome staining was performed to illustrate general histology. Slides were mounted using Permount Mounting Medium (Thermo Fisher Scientific). All slides were imaged using either 1) Olympus BX60 microscope with an Olympus DP72 camera, utilizing the DP2-BSW software, or 2) the Olympus VS120 microscope with a Pike F-505C camera (Allied Vision) utilizing the VS-ASW FL2.8 software. Digits were collected at specified time points for histological analysis to assess for wound closure. Wound closure was assed using Mallory's Trichrome staining. An amputation wound was considered closed if the entire epidermis covered the amputation injury in all sections on the slide

Immunohistochemistry and EdU injections: Antigen retrieval was performed using heat retrieval performed in 1Å~ citrate buffer solution (Dako), except for Runx2 where antigen retrieval was performed using EDTA buffer (pH 8.0; Thermo Fisher Scientific). Slides were blocked using Protein Block (Dako) for 1 hour at room temperature. Incubation with primary antibody/antibodies was performed overnight at 4C; they were then washed in tris buffered saline with Tween R20 solution (Sigma-Aldrich Co.) and incubated in secondary antibody/antibodies for 1 hour at room temperature. Slides were then incubated in a phosphate buffered saline (Sigma-Aldrich) and DAPI (Invitrogen) solution, dried, and mounted with

Prolong Gold (Invitrogen). Samples were imaged using the either 1) Olympus BX61 microscope with a Hamamatsu ORCA-ER camera via the Slidebook software (Intelligent Imaging Innovations Inc.) or 2) Olympus VS120 microscope with a Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera via VS-ASW FL2.8 software (Olympus). EdU (5-ethynyl-2'-deoxyuridine) was injected intraperitoneally into mice 3 hours prior to specimen collection at a dose of 10μL/kg. EdU detection is based on a copper (I) catalyzed reaction between an azide and an alkyne, and was performed after washing of the secondary antibody immunohistochemistry but before DAPI was applied. Primary antibodies used were, rabbit anti-beta III tubulin (1-500; Abcam; Ab18207), rabbit anti-runx2 (1:250; Sigma Aldridge Co.; HPA022040), rat anti-CXCR4 antibody (1:500; R&D Systems; MAB21651); rabbit anti-Cathepsin K (1:100; Abcam; Ab19027); rabbit anti-S100-beta antibody (1:500; Abcam; Ab52642). Secondary antibodies used were goat anti-rabbit, chicken, or rat Alexa Fluor-488 or 568 (1:500; Invitrogen).

Quantification of β3T, S100β, EdU, and Cathepsin K in sham and denervated digits:

Quantification of β3T in the P3 bone marrow was described previously (Dolan et al., 2019). To quantify S100β-Schwann cells in the connective tissue at 21 days post denervation (DPDN) and 42 days post amputation (DPA), EdU-positive cells in the blastema at 10 DPA, and cathepsin-K-osteoclasts on the P3 bone at 6 and 12 DPA, digits were imaged at 200X magnification using a Olympus BX61 microscope with a Hamamatsu ORCA-ER camera. Using the Slidebook software a region of interest (ROI; μm^2) was manually determined, and then the area (μm^2) of S100β or EdU within the ROI were automatically determined. Cathepsin K-immunopositive signaling (μm^2) was manually determined, and only multi-nucleated osteoclasts attached to the P3 bone were included for analysis. Three sections per slide were analyzed, then

S100β/EdU/Cathepsin K was normalized to the ROI, averaged, and combined with other digits for statistical analysis which consisted of an unpaired t-test using GraphPad Prism 7 software.

Quantification of cathepsin K for hindlimb unloading experiments: To quantify cathepsin K-immunopositive osteoclasts, fluorescent images were taken using an Olympus VS120 microscope with a Hamamatsu ORCA-Flash4.0 V2 Digital CMOS camera with VS-ASW FL2.8 software. The VS-ASW FL2.8 software was used to manually outline regions of interest (ROI) in the regenerated digit and to automatically determine the total area of the ROI (μm^2) and cathepsin K-immunopositive signal (μm^2). Three sections per slide were analyzed, normalized to the ROI, averaged, and combined with other digits for statistical analysis which consisted of an ordinary one-way ANOVA with a post-hoc Tukey's multiple comparisons test using GraphPad Prism 7 software. Only multi-nucleated osteoclasts attached to bone were included for quantification.

3.4 Results

Hindlimb unloading inhibits digit tip regeneration: Sciatic denervation is a robust model for depleting peripheral nerves downstream of where the transection is made, but also causes paralysis of the limb (Gross et al., 2010). Limb paralysis, such as after spinal cord injury, causes significant atrophy of bone and muscle (Giangregorio and McCartney, 2006). Thus, it remains in question if regenerative failure of the mouse digit following sciatic denervation is stems from absence of peripheral innervation, or decreased mechanical loading of the paralyzed limb. To begin to untangle the relationship between mechanical loading and denervation, we utilized Hindlimb Unloading (HU), a well-established model in which the hindlimbs of mice are suspended such that they are no longer weight bearing (Ferreira et al., 2011; Globus and Morey-

Figure 3.1: Hindlimb Unloading. Diagram illustrating a mouse in hindlimb unloading. In this procedure, a tail ring is inserted between tail vertebra. Next, the mouse is suspended such that the animal's rear limbs are no longer touching the ground. This image is reprinted with permission from, and kindly provided by, Jennifer Kosniewski.

Holton, 2016; Morey-Holton and Globus, 2002) (Figure 3.1). Hindlimb digit tips were amputated and their regenerative response was analyzed after continuous hindlimb unloading for approximately 4 weeks with micro-computed tomography (μCT) to quantitate changes in bone volume (BV) and bone length (BL). Amputated and hindlimb unloaded digits (Amp + HU) were compared to control unamputated HU digits (HU) and to amputated digits not subjected to HU (Amp).

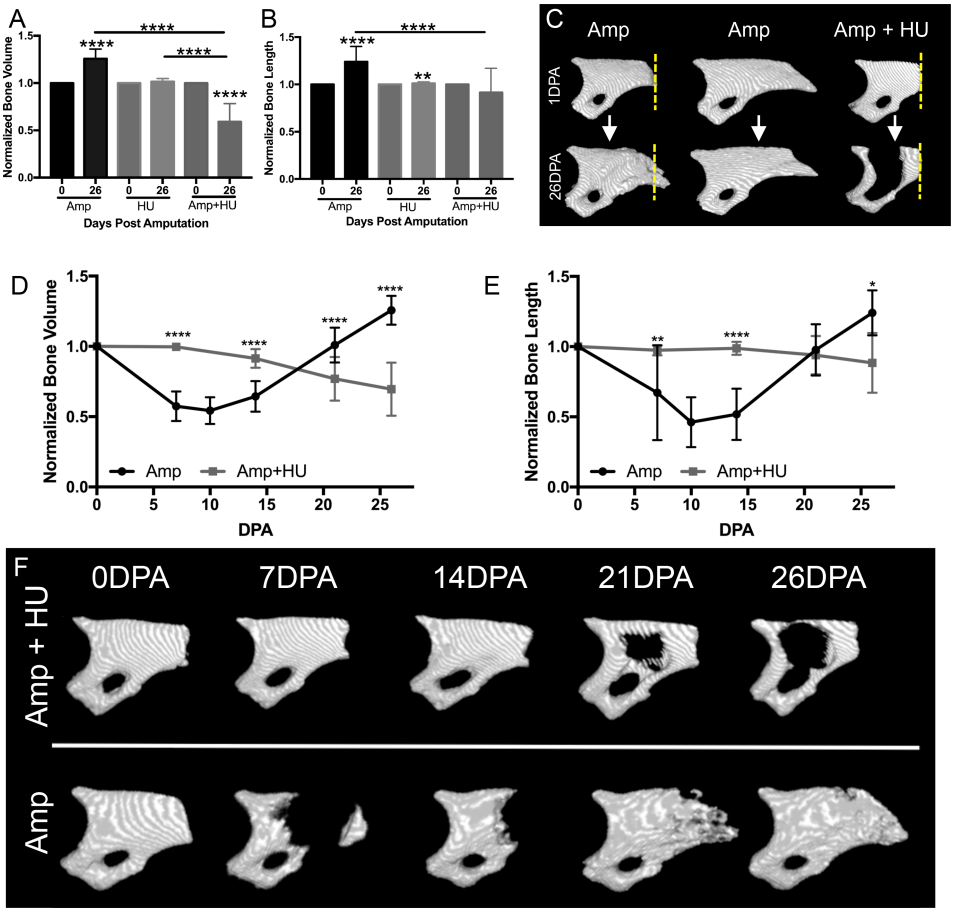

Figure 3.2: Hindlimb Unloading Inhibits Digit Tip Regeneration (A,B) Quantification of bone volume (A) and length (B) measurements normalized to 0 DPA in Amp, HU, and Amp + HU digits at 26 DPA (n=20 digits/group). (C) µCT renderings of Amp, HU, and Amp+HU digits at 0 and 26 DPA. Yellow dashed lines indicate the amputation plane. (D,E) Quantification of bone volume (D) and length (E) over 26 days normalized to 0DPA in Amp and Amp + HU digits. (F) Sequential µCT renderings of Amp and Amp+HU digits over 26 days. Amp = amputation control; HU = hindlimb unloading control; Amp+HU = amputation + hindlimb unloading; µCT = micro-computed tomography; DPA = day post amputation; *=P<0.05; **=P<0.01; ****=P<0.0001.

The regeneration response of control Amp digits display an increase in BV and BL that parallels the regeneration response characterized in previous studies (Figure 3.2A,B) (Dawson et al., 2018; Fernando et al., 2011). BV measurements of control HU digits before and after HU treatment indicates that the P3 bone does not display BV changes and increases in BL when mechanically unloaded (Figure 3.2A,B). At 26 DPA, Amp + HU digits had significantly less BV relative to 0 DPA indicating a degradation response of the P3 bone stump (Figure 3.2A,C). BL measurements were highly variable but on average HU treatment did not influence BL (Figure

3.2B,C). Amp+HU digits also had significantly less BV compared to Amp and HU control digits, as well as being significantly shorter compared to Amp controls (Figure 3.2A-C). These data indicate that HU treatment inhibits digit tip regeneration, and suggests that the bone degradation phase occurred normally, but the transition to new bone formation never occurred (Figure 3.2A-C). To test this, the study was repeated with μCT measurements performed at 0, 7, 14, 21, and 26 DPA. Remarkably, the degradation of Amp + HU digits is much slower than Amp control digits, reaching the minimum recorded BV and BL at 26 DPA (Figure 3.2D-F). Further, there is no evidence of decreasing BV or BL until 14 DPA and 21 DPA, respectively (Figure 3.2D-F), suggesting that HU significantly attenuates the process of bone histolysis (Figure 1.2). In summary, these data show that HU inhibits the regeneration response by slowing the rate of bone degradation and inhibiting new bone formation.

HU prevents wound closure, causes excessive bone degradation, and disrupts the bone marrow organization: Histological studies of control Amp digits at 26 DPA identified evidence of newly formed woven bone formation distal to the amputation plane (Figure 3.3A) that parallels previous descriptions of the regeneration response (Dawson et al., 2018; Fernando et al., 2011). Similar histological studies of control HU digits at 26 DPA validated the μCT BV and BL measurements and provided no evidence of either bone degradation or new bone formation (Figure 3.3B). In contrast, histological studies of Amp + HU treated amputations revealed numerous differences in tissue architecture when compared to control digits (Figure 3.3C). First, epidermal wound closure was incomplete in 95% of the digits analyzed (n=19/20), and in some cases the dorsal/lateral epidermis retracted as far back as the nail matrix (Figure 3.2C,D). Second, the P3 bone marrow of Amp + HU digits is highly cellular and lacks the vascular

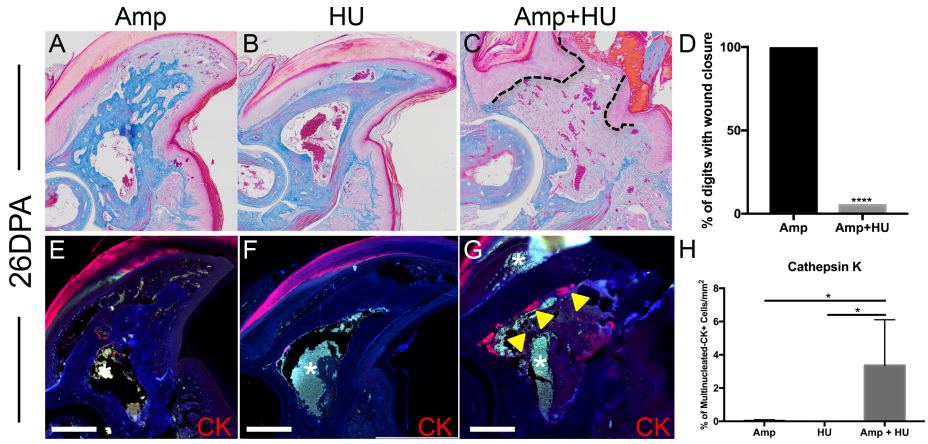

Figure 3.3: Hindlimb Unloading Inhibits Wound Closure (A-C) Mallory's trichrome staining of Amp (A), HU (B), and Amp + HU (C; black dashed lines indicates an open wound) digits at 26DPA. (D) Percentage of Amp and Amp + HU digits with wound closure at 26 DPA (n=20 digits/group). (E-G) Immunohistochemistry for CK-immunopositive multinucleated-osteoclasts at 26DPA in Amp (E), HU (F), and Amp + HU digits (G; yellow arrowheads indicate osteoclasts). (N) Quantification of CK-immunopositive multinucleated-osteoclasts on the surface of the bone at 26 DPA (n=4-5 digits/group). Distal is to the right and dorsal is to the top. (E-G): Blue = DAPI. Amp = amputation control; HU = hindlimb unloading control; Amp + HU = amputation + hindlimb unloading; DPA = day post amputation; * = autoflourescence from red blood cells; CK = cathepsin K. *=P<0.05; ****=P<0.0001; Scale bars = 100μm.

organization seen in control digits (Figure 3.3A-C). Third, the degradation of stump bone is remarkable in that proximal bone is eroded and in some cases the proximal bone plate is degraded to the P2/P3 joint (Figure 3.3C). Bone degradation is primarily endosteal, rather than periosteal, and is associated with Cathepsin K (CK)-immunopositive multinucleated-osteoclasts (Figure 3.3G,H) covering the P3 bone. Importantly, HU treatment does not appear to damage peripheral nerves in the digit tip as β3T-immunopositive axons are present throughout control HU and Amp + HU digits (Figure 3.3A,B). Overall, these results are consistent with the conclusion that HU treatment inhibits multiple regenerative processes without damaging peripheral nerves, and suggests that mechanical loading is required for digit tip regeneration.

Mechanical loading is important for organizing the phases of digit regeneration:

Hindlimb unloading inhibits multiple stages (e.g. histolysis, wound closure, blastema formation, etc.) of digit regeneration (Figure 1.2). However, digit regeneration proceeds through a sequence

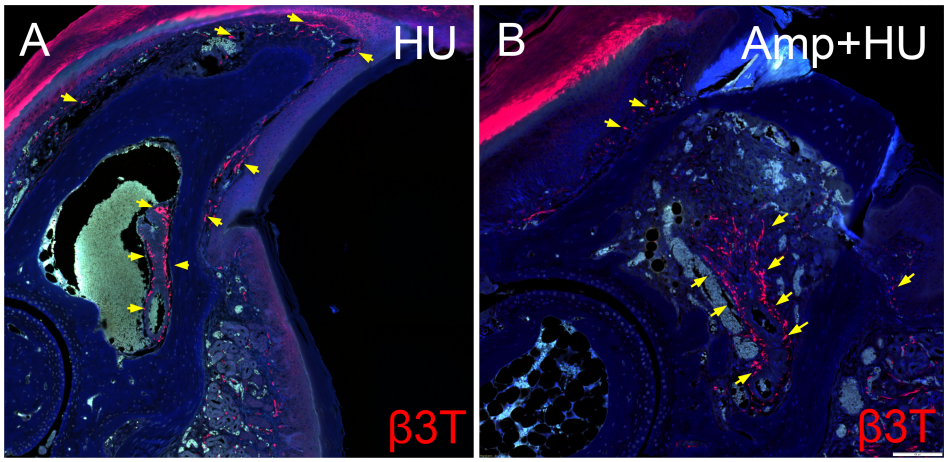

Figure 3.4: Hindlimb Unloading does not Damage Peripheral Nerves (A,B) β3T-immunopositive axons in HU (A) and Amp + HU (B) digits at 26 DPA (yellow arrows indicate β3T-immunopositive axons). β3T = beta-III-tubulin; HU = hindlimb unloading control; Amp + HU = amputation + hindlimb unloading; DPA = day post amputation.

of events, and manipulation of one step can affect later regenerative events (Dolan et al., 2018). Thus, it is unclear if hindlimb unloading inhibits early regenerative events that indirectly impair subsequent regenerative processes, or if hindlimb unloading directly inhibits multiple aspects of regeneration. To determine which stages of regeneration are dependent on mechanical loading, either 1) mice were hindlimb unloaded before (0-12 DPA) wound closure, and then removed from HU (12-40 DPA) or 2) allowed to ambulate until wound closure occurred (0-12 DPA), and then hindlimb unloaded them (12-40 DPA). Similar to previous experiments, mice that were hindlimb unloaded from 0-12 DPA exhibit a stunted histolytic response compared to Amp control digits evident by higher BV ($P<0.0001$) and BL ($P<0.0001$) (Figure 3.5A-C). However, when mice are removed from HU at 12 DPA and allowed to ambulate for four weeks, they regenerate, with a final BV and BL not different from amputation control digits (Figure 3.5A-C). These results suggest that hindlimb unloading temporarily inhibits early regenerative events, but that regeneration can be completed upon re-ambulation. Mice that were allowed to ambulate for the first 12 days before being hindlimb unloaded progress through histolysis similar to

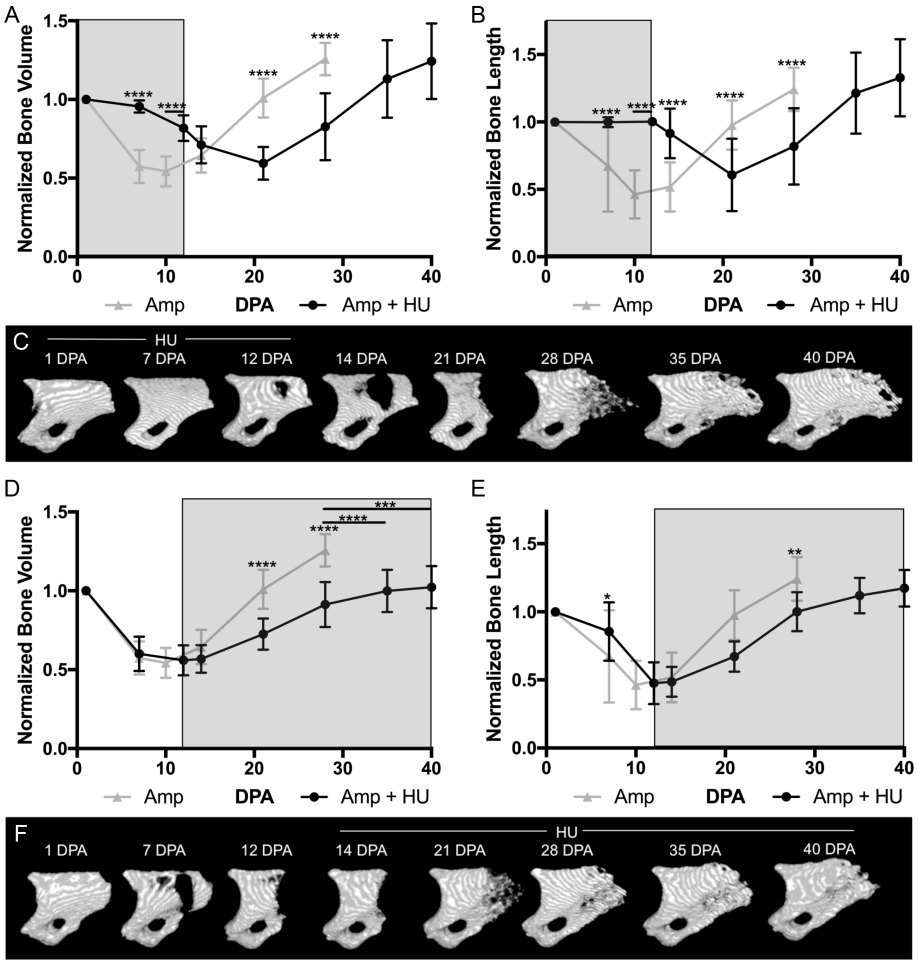

Figure 3.5: Mechanical Loading is Critical for Organizing Early Regenerative Processes (A-C) Mice were hindlimb unloaded (HU) from 0-12 DPA, and then removed from HU and allowed to ambulate normally for four weeks. (A,B) Quantification of bone volume (A) and length (B) measurements normalized to 1 DPA for Amp, and Amp + HU digits. Gray boxes indicate when Amp + HU mice where hindlimb unloaded (n=20 digits/group). (C) µCT renderings of Amp + HU digits from 1-40 DPA. (D-F) Mice were allowed to ambulate from 0-12 DPA, and then hindlimb unloaded for the next four weeks. (D,E) Quantification of bone volume (D) and length (E) measurements normalized to 1 DPA for Amp, and Amp + HU digits. Gray boxes indicate when Amp + HU mice where hindlimb unloaded (n=20 digits/group). (F) µCT renderings of Amp + HU digits from 1-40 DPA. Amp = amputation control; Amp + HU = amputation + hindlimb unloading; DPA = day post amputation; ****=P<0.0001.

amputation control digits (Figure 3.5D-F). After being hindlimb unloaded for four weeks, hindlimb unloaded digits are not statistically different by BL compared to Amp control digits at 28 DPA (P=0.5451), but have regenerated less BV(P<0.0001) (Figure 3.5D-F). These results suggest that hindlimb unloading does not inhibit digit regeneration if wound closure is allowed to occur. Taken together, the data in this chapter show that mechanical loading is required for

organizing the early phases (histolysis, wound closure, blastema formation) of digit regeneration, but not for later regenerative processes (blastema cell differentiation, new bone formation).

Digit denervation does not inhibit digit tip regeneration: Having determined that digit tip regeneration is dependent on mechanical loading, I next sought to investigate the role of peripheral nerves during regeneration while minimizing potentially confounding deficits in mechanical loading resulting from surgical ablation of peripheral nerves. To address the confounding variables associated with sciatic denervation (e.g. reduced mechanical load) I developed a novel surgical procedure in which peripheral nerves were transected within the digit directly (digital denervation) (Figure 3.6A). Digit denervation enables the animal to ambulate and I hypothesize should improve the deficits in mechanical loading of the digits associated with sciatic denervation. The efficacy of the surgery was assessed by quantifying β3T-immunopositive axons and S100β-immunopositive Schwann cells in the digit 21 days post denervation (21 DPDN). I observed that at 21 DPDN, denervated digits had a significant (approximately 95%) reduction in β3T-immunopositive axons ($P<0.0001$) compared to sham control (Figure 3.6B-D). These data demonstrate that the digit denervation surgery successfully denervated the digit (Figure 3.6B-D), although a significant reduction in S100β-immunopositive Schwann cells was not observed ($P=0.1902$; Figure 3.6E-G).

Next, mouse digits were denervated as described above, and the distal tips of their digits were amputated. BV and BL were monitored throughout regeneration using μCT. Digits were collected and sectioned 28 and 42 DPA to ensure that axons had not regenerated. β3T-immunopositive axons in the P3-bone marrow are significantly decreased compared to sham controls at 28 ($P<0.0001$) and 42 DPA($P<0.001$) indicating that denervation surgery was effective in preventing axonal regeneration (Figure 3.6H-J). Moreover, S100β-immunopositive

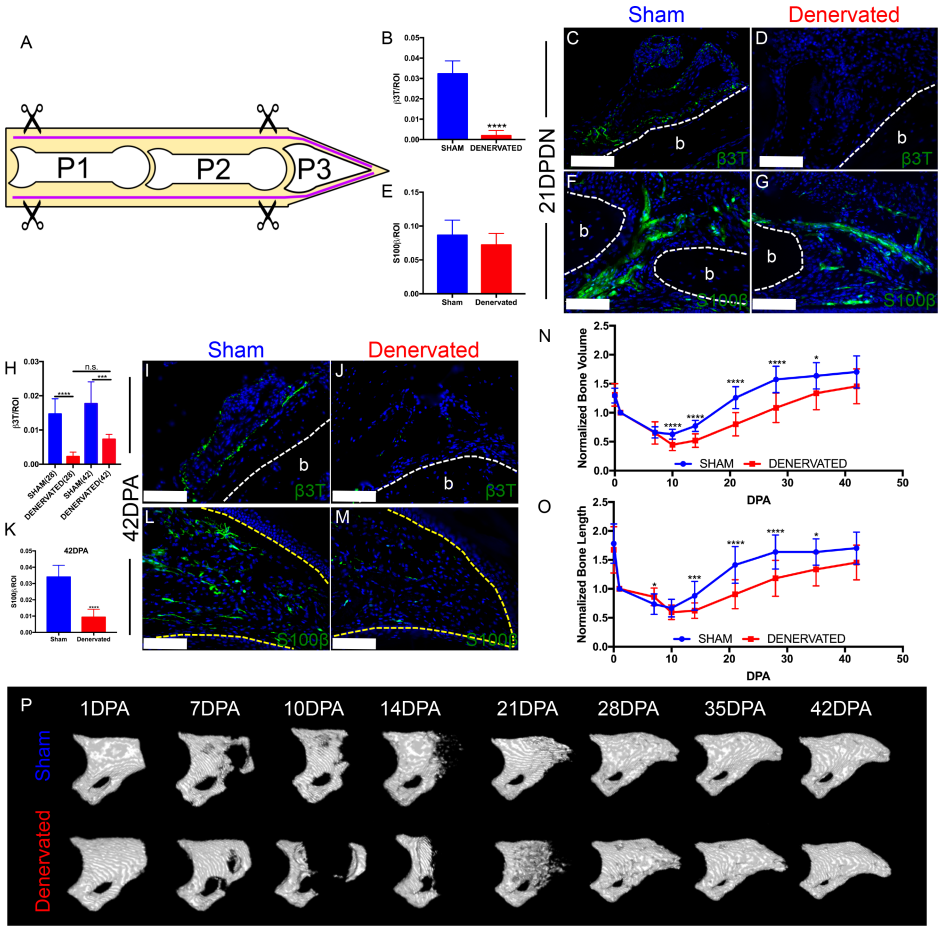

Figure 3.6: Digit Denervation does not Inhibit Digit Tip Regeneration (A) Diagram of the digit denervation surgery. Briefly, an incision is made along the ventral surface of a mouse digit. One of the two digit nerves (purple) is transected at the distal end of the incision near the fat pad, and is then pulled back towards the proximal end of the incision where it is cut for a second time, removing a segment of nerve approximately the size of the initial incision. This procedure is then repeated for the second digital nerve. (B) Quantification of β3T-immunopositive axons in the BM of sham and denervated digits at 21 DPDN (n=8 digits/group). (C,D) β3T-immunopositive axons in the bone marrow of sham (C) and denervated (D) digits at 21 DPDN. White dashed line indicates bone. (E) Quantification of S100β-immunopositive Schwann cells in the connective tissue of sham and denervated digits at 21 DPDN (n=7-8 digits/group). (F,G) S100β-immunopositive Schwann cells in the connective tissue of sham (C) and denervated (D) digits at 21 DPDN. White dashed line indicates bone. (H) Quantification of β3T-immunopositive axons in the bone marrow of sham and denervated digits at 28 and 42 DPA (n=7-8 digits/group). (I,J) β3T-immunopositive axons in the bone marrow of sham (I) and denervated (J) digits at 42 DPA. White dashed line indicates bone. (K) Quantification of S100β-immunopositive Schwann cells in the connective tissue of sham and denervated digits at 42 DPA (n=6-8 digits/group). (L,M) S100β-immunopositive Schwann cells in the connective tissue of sham (L) and denervated (M) digits at 42 DPA. Yellow dashed line indicates epidermis. (N,O) Quantification of bone volume (N) and length (O) measurements normalized to 1 DPA for sham and denervated digits (n=14-22 digits/group). (P) μCT renderings of a sham and denervated digit from 1-42 DPA. C,D,F,G,I,J,L,M: blue = DAPI; scale bars = 100μm. P1 = 1st phalanx; P2 = 2nd phalanx; P3 = terminal phalanx; DPDN = days post denervation; β3T = beta-III-tubulin; b = P3 bone; DPA = day post amputation; μCT = micro-computed tomography. n.s.= not significant; *=P<0.05; ***=P<0.001; ****=P<0.0001.

Schwann cells in the connective tissue of the denervated digit are significantly decreased ($P<0.0001$) at 42 DPA compared to sham control digits (Figure 3.6K-M). Representative 3D-μCT images of a sham operated P3 bone show the endogenous regeneration response of the digit tip (Figure 3.6P). The amputated P3 bone is degraded during the first 10 days after amputation and then new bone is regenerated over the next 4 weeks (Fernando et al., 2011; Dawson et al., 2018). Quantitative measurements of regenerated P3 bone show that denervated digits at 42 DPA are not statistically different from sham controls in either BV ($P=0.1095$) or BL ($P=0.1879$) (Figure 3.6N,O). These data directly demonstrate that digit denervation does not inhibit digit tip regeneration.

Digit denervation delays wound closure: At 28 DPA, when the P3 bone is considered regenerated (Dawson et al., 2018; Fernando et al., 2011), denervated digits have reduced BV and BL compared to sham controls (Figure 3.6N,O) suggesting that denervation delays digit tip regeneration. One explanation for the delayed regeneration response could be that that the rate of new bone formation is slower in denervated digits. To test this idea, I compared the rate of new bone formation in sham control digits relative to denervated digits during the last 4 weeks of regeneration. I observed that sham and denervated digits regenerate BV ($P=0.7793$; Figure 3.7A) and BL ($P=0.7344$; Figure 3.7B) at comparable rates. These data indicate that the observed delayed regeneration response in denervated digits is not a consequence of a slower bone formation rate. These findings provide evidence supporting the idea that the peripheral nerve contribution to regeneration is stage dependent and that the affected stage(s) is (are) prior to new bone formation.

A second potential explanation is that digit denervation impairs blastema formation. Blastema formation is the hallmark of epimorphic regeneration, is part of what distinguishes

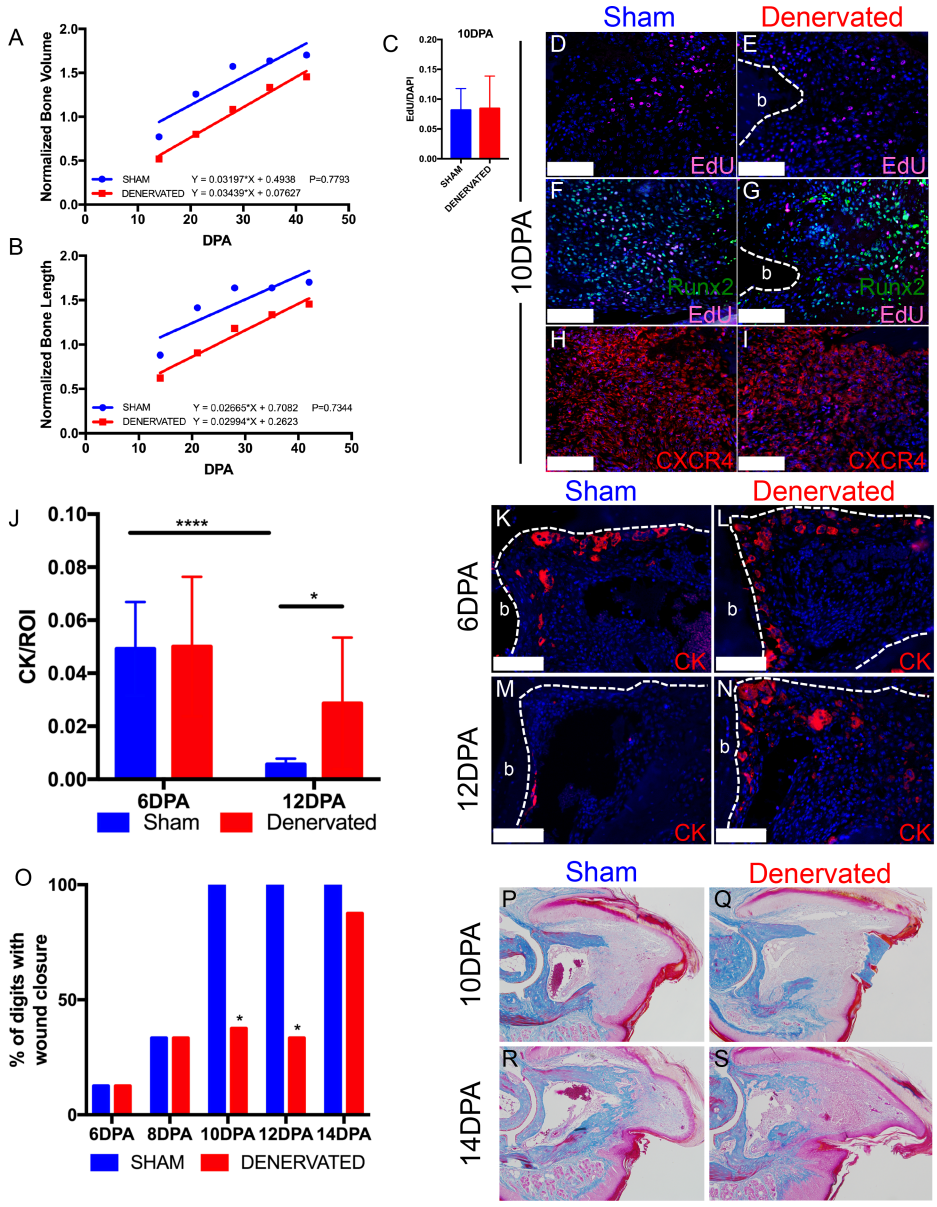

Figure 3.7: Digit Denervation Delays Wound Closure (A,B) Quantification of the rate of new bone volume (A) bone length (B) formation from 14 to 42DPA (n=14-22 digits/group). (C) Quantification of EdU-positive cells in the blastema of sham and denervated digits at 10 DPA (n=8 digits/group). (D,E) EdU-positive cells in the blastema of sham (D) and denervated (E) digits at 10 DPA. White dashed lines indicate bone. (F,G) Runx2-immunopositive osteoblasts in the blastema of sham (F) and denervated (G) digits at 10 DPA, many of which are also EdU-positive. White dashed lines indicate bone. (H,I) CXCR4-immunopositive cells in the blastema of sham (H) and denervated (I) digits at 10 DPA. (J) Quantification of CK-immunopositive multinucleated-osteoclasts at 6 and 12 DPA (n=6-8 digits/group). (K-N) CK- immunopositive multinucleated-osteoclasts at 6 (K,L) and 12 DPA (M,N) in sham and denervated digits. White dashed lines indicate bone. (O) Quantification of the percentage of sham and denervated digits with wound closure from 6-14 DPA. (P-S) Mallory's trichrome staining of sham and denervated digits at 10 (P,Q) and 14DPA (R,S). D,E,F,G,H,I,K,L,M,N: blue = DAPI; scale bars = 100μm. b = P3 bone; DPA = days post amputation; Runx2 = Runt-related transcription factor 2; CXCR4 = C-X-C chemokine receptor type; CK= Cathepsin K; *=P<0.05; ****=P<0.0001.

digit tip regeneration from other mammalian regeneration responses, and is best characterized by high levels of cell proliferation (Fernando et al., 2011). Denervation in urodele amphibians inhibits blastema formation and therefore appendage regeneration. Further, several groups have reported that sciatic denervation decreases blastema cell proliferation (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). To investigate if digit denervation inhibited blastema cell proliferation, I collected sham and denervated digits injected with EdU at 10 DPA and assessed blastema proliferation. The results demonstrated that denervated digit blastema-cells at 10 DPA proliferate at levels similar to sham operated digits ($P=0.902$; Figure 3.7C-E). Moreover, denervated digits are also immunopositive for blastema cells markers such as Runx2 and CXCR4, many of which are also EdU-positive (Figure 3.7F-I). Taken together, these results suggest that blastema formation is not inhibited after digit denervation.

Having ruled out rate of new bone and blastema formation, I next hypothesized that denervation may affect regenerative-event(s) prior to blastema formation. Recall that denervated digits exhibit excessive histolysis compared to sham digits at 10 DPA, but not at 7 DPA (Figure 3.7N). At 6 DPA, similar levels ($P=0.939$) of CK-immunopositive osteoclasts covering the P3 bone stump of sham and denervated digits (Figure 3.7J-L). However, at 12 DPA, CK-immunopositive osteoclasts levels have significantly decreased relative to 6 DPA in sham digits ($P<0.0001$), but not in denervated digits ($P=0.1485$) (Figure 3.7J,M,N) were observed. Further, denervated digits have significantly more ($P<0.0469$) CK-immunopositive osteoclasts compared to sham control digits at 12 DPA (Figure 3.7J,M,N). These data show that the excessive histolysis observed following digit denervation is mediated through prolonged osteoclast-mediated bone resorption.

It has been shown previously that wound closure is, in part, mediated through dynamic changes in oxygen tension, and that delaying wound closure leads to excessive bone histolysis (Sammarco et al., 2015; Sammarco et al., 2014; Simkin et al., 2015). Therefore, I next hypothesized that digit denervation may be delaying wound closure. To test this hypothesis, I repeated the experiment described above and collected digits at 6, 8, 10, 12, and 14 DPA. I observed that wound closure had occurred in 100% of sham control digits by 10 DPA (Figure 3.7O,P), consistent with previous reports (Fernando et al., 2011; Simkin et al., 2015). Conversely, only 33% of amputation wounds of denervated digits were closed by 10 DPA (Figure 3.7O,Q). Wound closure was not observed in the majority of denervated digits until 14 DPA, 4 days after wound closure occurs in sham control digits (Figure 3.7O,R,S). These data clearly show that digit denervation significantly delays wound closure. Taken together, the data are consistent with a model in which the delay in digit regeneration following digit denervation is due to a delayed wound healing response that causes prolonged osteoclast-mediated bone histolysis.

3.5 Discussion

Epimorphic regeneration, defined as the formation of a blastema, is a specialized type of regeneration that is best described during salamander appendage regeneration (Tanaka, 2016), but that also occurs in certain instances of mammalian regeneration, such as after mouse digit tip amputation (Borgens, 1982), ear regeneration after hole punch injury in the MRL/MpJ mice (Clark et al., 1998) and African spiny mice (*Acomys*) (Seifert et al., 2012), and during seasonal antler regeneration (Kierdorf et al., 2007). Salamander limb and mouse digit tip regeneration are comparable and proceed through a similar regenerative sequence including inflammation,

histolysis, wound closure, and blastema formation, and proximal-to-distal blastema differentiation (McCusker et al., 2015). However, while the data in this thesis demonstrate that mouse digit tip regeneration is nerve-independent, salamander limb regeneration is unquestionably nerve-dependent (Pirotte et al., 2016; Stocum, 2011; Todd, 1823). This is evident not only from decades of loss-of-function limb denervation studies where regeneration is completely inhibited, but also from gain-of-function studies where an accessory limb can be induced to form from a simple skin wound if a nerve is re-routed to the wound and a contra-lateral piece of dermis is added to the wound (Endo et al., 2004). Paradoxically, if a portion of the neural tube is removed from the salamander embryo, thereby preventing innervation of the developing limb, the aneurogenic limb can regenerate in the absence of nerves (Steen and Thornton, 1963). Unlike the salamander, I show in studies presented in this thesis that mouse digit tip regeneration is nerve-independent (Figure 3.6; Figure 3.7). This is evident from μCT studies demonstrating that denervated digits progress through all stages of digit tip regeneration, and immunohistochemical studies that show that denervation has no affect on blastema formation (Figure 3.7C-I). Based on previous reports suggesting that mouse digit tip regeneration is nerve-dependent I propose that these conclusions are confounded by the fact that the authors use sciatic denervation, and that regenerative deficits are a consequence of decreased mechanical load during regeneration.

Cells constantly detect and adapt to internal and external mechanical forces (Klein-Nulend et al., 2012). Mechanical loading is essential for the proper maintenance of tissues, and perturbing mechanical load can have severe consequences (Klein-Nulend et al., 2012). This is most obvious when mechanical loading is decreased, such as after spinal cord injury or as for astronauts in space, where there is significant atrophy of skeletal muscle and bone (Giangregorio

and McCartney, 2006; Williams et al., 2009). Conversely, increasing mechanical loading, for instance by exercising, can increase skeletal muscle and bone strength (Bass et al., 2002; Haapasalo et al., 2000; Konopka and Harber, 2014). What is less well understood, is how mechanical loading or unloading affects regeneration. In studies investigating how regeneration occurs in micro-gravity, planarians regenerate normally (Morokuma et al., 2017), while urodele limb and lens regenerates have increased cell proliferation and regenerate at faster rates compared to ground controls, although patterning abnormalities occur, such as cataract formation and enlarged lenses, and limbs with missing or fused phalanges (Mitashov et al., 1996; Mitashov, 1987). During fin regeneration in zebrafish, decreasing mechanical loading by botulinum toxin injection impairs bone ray outgrowth, morphology, and patterning (Recidoro et al., 2014). Curiously, salamander limb regeneration is likely independent of mechanical loading. This conclusion is supported by the fact that aneurogenic limbs, which are intrinsically paralyzed, are able to regenerate (Steen and Thornton, 1963). In this thesis I present data that shows hindlimb unloading completely inhibits mouse digit tip regeneration (Figure 3.2; Figure 3.3). Amputated digits of hindlimb unloaded mice fail to wound heal, have increased osteoclast-mediated bone loss, and fail to regenerate new bone. Impaired wound healing and bone formation have all been reported following prolonged bouts of mechanical unloading (Morey and Baylink, 1978; Radek et al., 2008). These data strongly support the idea that using sciatic denervation influences multiple aspects of regeneration including removing trophic factors (PDGF-AA, oncostatin-M, and FGFs) released by nerves and Schwann cells (Johnston et al., 2016; Takeo et al., 2013) , but also paralyzes the limb thereby reducing mechanical loading (Gross et al., 2010) and hampering skeletal homeostasis.

Another interesting finding is that hindlimb unloading does not permanently inhibit digit regeneration. In mice hindlimb unloaded for 12 days, a robust regeneration response analogous to non-hindlimb unloaded amputation control digits is observed, once they are removed from hindlimb unloading and allowed to ambulate (Figure 3.5A-C). These results indicate that hindlimb unloading stalls regeneration, but that upon re-ambulation, the regeneration process can be re-initiated. In the reciprocal experiment, where mice are allowed to ambulate freely for the first 12 days following amputation and are then hindlimb unloaded, regeneration still occurs but is attenuated (Figure 3.5D-F). In this experiment, histolysis, wound closure, and blastema formation are allowed to occur normally. Taken together with the experiments in the earlier chapters, these data indicate that mechanical loading is critical for organizing the early stages of regeneration.

Interestingly, regenerative inhibition resulting from continuous HU parallels regenerative inhibition following macrophage depletion with Clodronate-liposomes (inhibition of histolysis, wound closure, blastema formation, and new bone formation) (Simkin et al., 2017). Moreover, if digits are treated with Clodronate-liposomes during later stages of regeneration, the regenerate is not affected, similar to the regeneration response observed if mice are hindlimb unloaded after 12 days (Simkin et al., 2017). Collectively, these data suggest that hindlimb unloading inhibits the inflammatory response required for organizing digit tip regeneration. Prolonged hindlimb unloading has been shown to alter the immune system of mice (Gaignier et al., 2014). Immune-system dysfunction is also well-established in astronauts (Cogoli, 1993) and of particular interest, there is a decrease in macrophage progenitor cells collected from the bone marrow of rats flown in space for two weeks (Vacek et al., 1991). Moreover, patients suffering from spinal cord injuries exhibit attenuated macrophage recruitment to sites of cutaneous inflammation

(Marbourg et al., 2017). Therefore, macrophages may be a cell type exquisitely sensitive to changes in mechanical loading, and could be a candidate cell type for regenerative failure following prolonged hindlimb unloading.

While mouse digit regeneration is nerve-independent, regeneration is delayed, and in data presented in this chapter I suggest the delay is due to a delay in wound closure which causes enhanced osteoclast-mediated bone histolysis (Figure 3.7). Wound closure and bone histolysis are intricately linked following digit tip amputation via dynamically changing oxygen tensions (Sammarco et al., 2015; Sammarco et al., 2014). Treating amputated digits with the wound healing agent Dermabond causes rapid wound healing (Simkin et al., 2015), but makes the amputation wound hypoxic, reducing osteoclast levels and the amount of P3 bone degraded (Simkin et al., 2015). Conversely, amputated digits treated with hyperbaric oxygen reduces hypoxia in the amputation wound leading to delayed wound closure, prolonged osteoclast-mediated bone histolysis, and an increase in the amount of bone degraded (Sammarco et al., 2015; Sammarco et al., 2014). Thus, I posit that the denervated digits with attenuated histolysis and delayed wound closure is the result of alternations in oxygen tensions. Delayed wound closure following digit denervation parallel studies from Neufeld's group showing that sciatic denervation delays digit amputation wound closure in rats (Mohammad and Neufeld, 2000). Deficits in wound healing are commonly reported following spinal cord injury (Basson and Burney, 1982). Of particular interest is that Schwann cell-signaling has been shown to promote wound closure (Johnston et al., 2013), and have further been shown to participate in digit tip regeneration via PDGF-AA and oncostatin-M signaling (Johnston et al., 2013; Johnston et al., 2016). Curiously, as shown earlier (Figure 3.6K-M), that S100β-Schwann cells do not regenerate

after digit denervation. Thus, the delay in wound healing following digit tip amputation injury may be due to decreased paracrine signaling from local Schwann cells.

In summary, the observations presented in this chapter support the conclusion that mouse digit regeneration is largely peripheral nerve-independent. Further, I propose that mechanical loading is essential for organizing the early regeneration response, and that determining the precise mechanisms and cell types through which mechanical loading exerts it affects will be a critical future area of study for understanding endogenous and induced mammalian regeneration.

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4. CONCLUSION

4.1 Nerves and Regeneration: A History and Explanation of New Findings

For centuries, man has studied organisms that regenerate for the obvious reason that it is an epic natural phenomena. Reaumer studied crawfish claw regeneration (Reaumur, 1712) Trembley studied hydra body regeneration (Trembley, 1744) and Spallanzani studied tail and limb regeneration in salamander (Spallanzani, 1768). Five decades after Spallanzani, Tweedy John Todd demonstrated that severing nerves from the hindlimbs of newts inhibits regeneration, igniting a brand new subfield of regeneration biology (Todd, 1823). This work was picked up by Marcus Singer in the 1940's, where he demonstrated three important findings that he summarizes in *The influence of the nerve in regeneration of the amphibian extremity* (Singer, 1942, 1945, 1946a, b, 1947a, b, 1952; Singer and Egloff, 1949). First, the nerve requirement is independent of fiber types. This means that it doesn't matter if they are sensory or motor, the amputation wound just requires innervation. Second, the amount of innervation required for regeneration is quantifiable; in essence, this means that having one nerve fiber at the amputation does not necessarily mean regeneration will occur, but rather you need a certain level of innervation to cross a threshold for regeneration. Third, the amount of innervation required for regeneration is amputation level-dependent. For example, an injury through the upper limb requires more innervation then an amputation at the digit level. On the basis of his work, Singer formulates the neurotrophic hypothesis which postulates that nerves produce a "factor" that is required for the formation of a blastema (Singer, 1952).

The neurotrophic hypothesis is provocative, and since its initial proposal, has led to decades of research searching for what this neurotrophic factor (or factors) could be. Many potential candidates have been proposed, such as fibroblast growth factors, anterior gradient

protein, transferrin, and insulin-like growth factor-1 (Pirotte et al., 2016). However, the neurotrophic hypothesis is likely not a single factor, which explains why administration of a single factor rarely is sufficient to stimulate regeneration following denervation.

To take a step back for a minute, the reason why "the" neurotrophic factor has been so readily searched for, and why regeneration is largely studied, is because humans lack the regenerative ability that are innate to organisms like the salamander, planaria and hydra. I think the reason why the neurotrophic hypothesis has stimulated so much research is because if identified, maybe it could be used to stimulate regeneration in humans.

As noted in chapter 1, there have are several reports that humans, children and adults, are able to regenerate their finger tips after injury (Douglas, 1972; Illingworth, 1974; McKim, 1932). Interested in these case studies, in 1982, Borgens demonstrated the mice, like humans, have the ability to regenerate the distal tips of their digits (Borgens, 1982). These findings have since sparked a robust, albeit small, new and exciting field. For the sake of brevity, findings since the initial discovery that mice are intrinsically capable of regenerating their digits tips have shown that this is an epimorphic phenomena, effectively bridging salamander and mammalian regeneration (Dolan et al., 2018).

Given the necessity of peripheral innervation for salamander regeneration, it was not only imperative, but inevitable that someone would investigate if mammalian digit tip regeneration was also nerve-dependent. In 2000, Mohammad and Neufeld did just this, and reported that denervation delays but does not inhibit mammalian digit tip regeneration (Mohammad and Neufeld, 2000). However, reports from several other groups contradict this initial report, and suggest that mammalian digit tip regeneration *is nerve-dependent* (Figure 4.1) (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). The discrepancy between whether or not

mammalian digit regeneration is, or is not, nerve-dependent, is paramount to understanding endogenous regeneration.

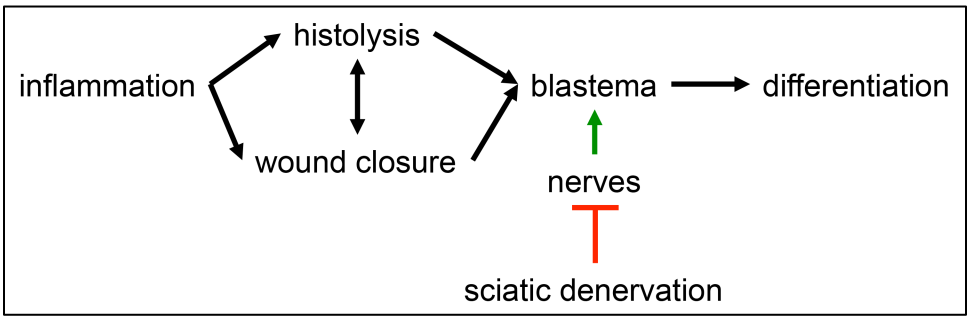

Figure 4.1: Proposed Model 1. Diagram illustrating of how peripheral nerves are thought to contribute to digit tip regeneration.

In reading the relevant publications pertaining to the role of nerves in digit tip regeneration, one of the striking things I noted was that investigators were treating peripheral nerves as a homogenous structure. This is of course an extraordinary over simplification, as the peripheral nervous system is composed of numerous axon subtypes (motor, sensory, autonomic, etc.) and Schwann cells (myelinating, non-myelinating, terminal, etc.) that differ in both form and function (Squire, 2013). Moreover, no studies existed that tested whether peripheral nerves *regenerated* in the context of digit tip regeneration. Thus, as part of this dissertation (chapter 2), I decided to meticulously describe the neurobiology of the digit. In doing so, I hypothesized that the increased understanding of the peripheral nervous system would provide new insights in the regenerative response of the digit.

In Chapter 2, I describe the characterization of the neuroanatomy of the digit. I discovered that the terminal phalanx (P3) of the mouse digit is innervated by two peripheral nerves, each of which bifurcates into two distinct branches: the bone marrow (BM) branch and the connective tissue (CT) branch. The BM branches innervate the P3-bone marrow and are composed of sympathetic axons that are ensheathed by non-myelinating Schwann cells. The CT

branches innervate the connective tissue and epidermal structures of the digit tip, and are composed of sympathetic and sensory axons that are ensheathed by myelinating and non-myelinating Schwann cells (Figure 2.1).

The next step was to describe the neurobiology of the regenerated mouse digit tip-- the hypothesis being that nerves and Schwann cells would regenerate after digit tip amputation. I found that four weeks after amputation, the normal time in which the mouse digit is regenerated in, a dramatic change in the neurobiology of the regenerated digit. First, there is a dramatic decrease the in amount of axons and myelinating-Schwann cells in the connective tissues of the regenerated digit tip. However, no changes in innervation in the P3-bone marrow which is not damaged during digit tip amputation were noted. Moreover, digits were allowed to regenerate for four months, the regeneration of axons or Schwann cells was still absent, demonstrating that peripheral nerve regeneration after digit tip amputation is not delayed, but severely impaired.

I next investigated the neurobiology of the blastema. Given the reported necessity of peripheral innervation to the blastema for salamander limb and mouse digit tip regeneration, I began this investigation under the general hypothesis that the blastema would be heavily innervated and populated by Schwann cells. However, the blastema is actually aneural and devoid of Schwann cells (Figure 2.5). These data are consistent with the observation that peripheral nerve regeneration is impaired, but strikingly differ from results reported by other groups (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). These data led to a simple experiment in chapter 2: would regenerated digits— already suffering from impaired innervation following amputation—be able to regenerate if re-amputated? Re-amputated digits exhibit a robust regeneration response following secondary amputation, and actually regenerate more bone volume compared to the already enlarged regenerate (Figure 2.6).

Thus, thinking about these results—an aneural blastema, impaired peripheral nerve regeneration, and the ability of the digit to regenerate after re-amputation—it begs the question: *is peripheral innervation actually essential for mouse digit tip regeneration?*

As I see it, there are two ways of answering this question. In the first answer, one might suggest that even though peripheral nerves are not regenerating, the nerves and Schwann cells that are not amputated are adequate to stimulate blastemal cells to proliferate and support digit regeneration. The likely and obvious candidate axons and Schwann cells would be those of the BM branches, as they are not damaged by digit amputation. While this answer is plausible, I personally find it unlikely, and moreover difficult to disprove. This would be difficult to disprove because you would have to design an experiment in which you could remove BM branch axons and Schwann cells without damaging the CT branch axons and Schwann cells. In theory, you could test this be severing only the BM branch axons and then waiting for the BM branch Schwann cells to die. However, in practice, it would be exceedingly challenging to develop a surgical procedure this precise.

The second answer is that peripheral nerves are not required for digit tip regeneration. This answer is supported by the data in this dissertation that show that the blastema is aneural, peripheral nerves regeneration is impaired after digit tip amputation, and that the digit maintains the ability to regenerate after re-amputation. However, there are several studies from other groups that show that sciatic denervation inhibits digit tip regeneration (Figure 4.1) (Johnston et al., 2016; Rinkevich et al., 2014; Takeo et al., 2013). This leads to a problem—how to interpret these findings while not dismissing other studies? If you have not skipped to this Chapter, then indeed, you already know the answer. But for the sake of story telling in the context of a PhD dissertation, allow me to retell my thought processes.

One of the things that all of the studies investigating the role of peripheral nerves during digit tip regeneration share is that they all used sciatic denervation to study regeneration in the absence of nerves. I believe the reason they chose this procedure is 1) it is a simple surgery and 2) it is an extremely effective surgery for depleting innervation to tissues downstream of the injury. If I were performing these initial studies, I believe that this is the same approach I would have taken. However, there is a major problem in using sciatic denervation as a method for studying regeneration in the absence of nerves. Sciatic neurectomy generates a myriad of downstream effects that are impossible (or extremely difficult) to control for. The spectrum of potential deficits resulting from sciatic denervation include disruption of arterial tone, muscle atrophy and bone loss (Gross et al., 2010). Muscle atrophy and bone loss are a consequence of decreased mechanical loading, and these losses are well established following spinal cord injury (Giangregorio and McCartney, 2006). Interestingly, there are several studies that suggest that decreased mechanical loading also impairs appendage regeneration (Grinfeld et al., 1996; Recidoro et al., 2014).

This led to a simple question: could the data that suggest digit tip regeneration is a peripheral nerve-dependent be confounded by the fact that sciatic denervation reduces mechanical loading of the denervated limb (Figure 4.2)? To answer this question, I used a model that reduces mechanical loading of the hindlimbs, without damaging hindlimb peripheral nerves, known as hindlimb unloading (Ferreira et al., 2011; Morey-Holton and Globus, 2002).

What was observed when the digits of hindlimb unloaded mice were amputated, was that regeneration is completely inhibited. This surprising result includes complete failure of wound closure and bone formation, and excessive osteoclasts mediated bone histolysis. These data were surprising and completely unexpected. Indeed, in the regenerative literature, interventions that

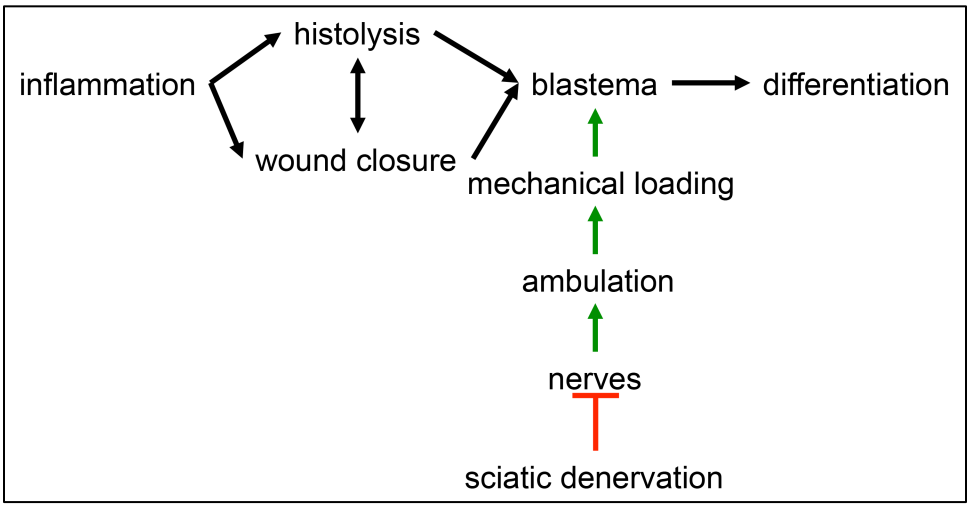

Figure 4.2: Proposed Model 2. Diagram illustrating of how peripheral nerves may be contributing to digit tip regeneration taking into account mechanical loading.

completely inhibit digit regeneration are exceedingly rare. Moreover, regenerative inhibition, without damaging peripheral innervation, suggests that previous reports implicating peripheral nerves in mammalian regeneration are confounded (Figure 4.3).

The findings in this dissertation that mouse digit tip regeneration is mechanical load-dependent merits consideration of the question: is amphibian limb regeneration actually nerve-dependent, or is regenerative failure following denervation a consequence of decreased mechanical load of the limb? It is certainly an enticing question given the similarities between amphibian and mammalian epimorphic regeneration and one that bears consideration. However, I find it highly unlikely, for several reasons, that amphibian limb regeneration is mechanical load-dependent. The first argument against amphibian limb regeneration being mechanical load-dependent is that newts flown in space are able to regenerate their limbs, although many of the regenerated limbs exhibited deficits in pattern formation, such as missing bones and fused phalanges (Mitashov et al., 1996; Mitashov, 1987). While this is the arguably the best experiment to specifically test whether amphibian limb regeneration requires mechanical

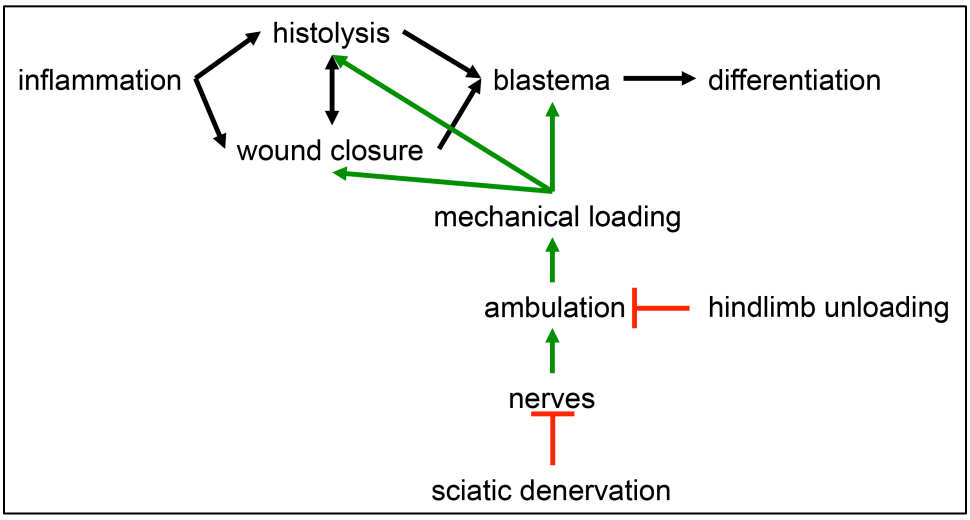

Figure 4.3: Proposed Model 3. Diagram illustrating of how peripheral nerves may be contributing to digit tip regeneration. This model takes into account hindlimb unloading, which inhibits ambulation and decreases mechanical loading of the hindlimb. This inhibits wound closure, histolysis, blastema formation, and new bone formation, ultimately inhibit digit tip regeneration.

loading, the conclusions drawn from these studies are limited given significant flaws experimental design. For instance, newt limbs were amputated one week prior to space flight. This allows for wound closure and other early regenerative events to proceed under normal gravity, and as I demonstrate here with using mice, correct mechanical loading is critical for organizing early regenerative processes. Second, newts flown in space were only flown in a micro-gravitational environment for 2-3 weeks before being brought back to earth where tissues where either collected immediately or several weeks later (Mitashov et al., 1996; Mitashov, 1987). I am sympathetic to the researchers who conducted these studies, as their research was not the primary focus of the endeavor (the Russian's were much more focused on developing rockets for long-term space flight), and were limited by the current technology. In the future, it would be interesting to repeat these studies on the International Space Station, were amputations could be performed in micro-gravity, and regeneration studies could be conducted in 3-6 month intervals.

The accessory limb model provides the second piece of evidence that amphibian appendage regeneration is nerve-dependent and not confounded by deficits in mechanical loading. In this model, a complete limb can be induced to regenerate from a small incision on the limb if a nerve is re-routed in the incision, and if a contra-lateral piece of dermis placed over the initial incision (Endo et al., 2004). The contra-lateral piece of dermis provides positional information to the incision, whereas the re-routed nerves provides mitogenic support (Endo et al., 2004). If the contra-lateral piece of dermis is not placed over the incision, the re-routed nerve is able to induce blastema formation, but it ultimately regresses and a limb is not regenerated. Interestingly, the accessory limb is apparently non-functional, and is thus probably receiving little, if any, direct mechanical usage (personal communications with Dr. Akira Satoh). Curiously though, if the limb is which the accessory limb was regenerated on is amputated, the accessory limb becomes functional (personal communications with Akira Satoh).

The most convincing evidence against amphibian limb regeneration being independent of mechanical loading comes from studies investigating the regenerative potential of the aneurogenic limb. To create an aneurogenic limb, a segment of the neural tube is removed from a salamander embryo preventing peripheral nerves from growing into the developing limb bud. Amazingly, this procedure does not impair limb development, and more importantly, the aneurogenic limb is able to regenerate (Steen and Thornton, 1963). This creates a strange paradox where a normal limb that is innervated, and than denervated and amputated, is unable to regenerate, whereas a limb that is aneurogenic is able to regenerate independent of peripheral nerves. While phenomena is still not fully understood, those who study have reached the conclusion that appendages naïve to innervation possess regenerative potential, but that the regenerative potential of appendages exposed to innervation somehow become peripheral nerve-

dependent. This conclusion is supported by studies demonstrating that if an aneurogenic limb is transplanted onto a host in place of one if its normally innervated limbs, and the nerve is allowed to regenerate into the grafted aneurogenic limb, and then the limb is denervated and amputated, regeneration does not occur (Thornton, 1970).

Regardless, these studies demonstrate that an aneurogenic limb, which is effectively paralyzed, is able to regenerate. Collectively, these studies demonstrate through multiple different approaches that amphibian limb regeneration is peripheral nerve-dependent, and moreover suggest that mechanical loading is unlikely to significantly contribute to appendage regeneration, at least not nearly in as critical of a way as is observed in mouse digit tip regeneration.

This led to the next question: could we study test if digit tip regeneration is peripheral nerve-dependent without decreased mechanical loading (Figure 4.4). To address this question, I

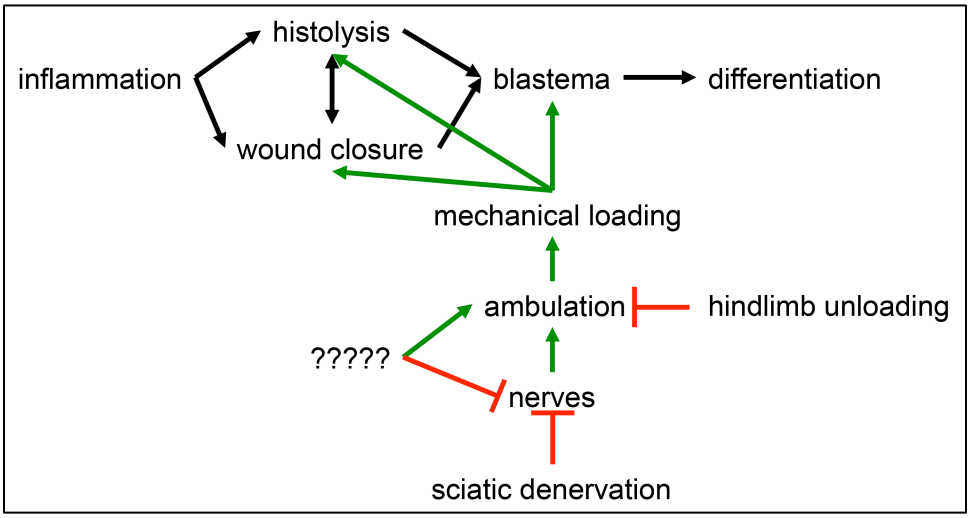

Figure 4.4: Proposed Model 4. Diagram illustrating of how peripheral nerves may be contributing to digit tip regeneration. In this model, we consider how we can denervate the digit while minimizing deficits in mechanical loading.

developed a surgical procedure in which denervation was performed at the digit level (digital denervation). This surgery is extremely effective at depleting peripheral nerves, but does not

alter the animals ability to ambulate, and therefore does not decrease mechanical loading. In the face of this digit denervation procedure, regeneration is delayed, but ultimately, denervated digits fully regenerate. Furthermore, the delay in regeneration is due to a delayed wound closure, and digit denervation has no effect on blastema formation or bone regeneration rate. The finding that digit tip regeneration is peripheral nerve-independent is surprising, and one that is critical for the field of mammalian regeneration (Figure 4.5).

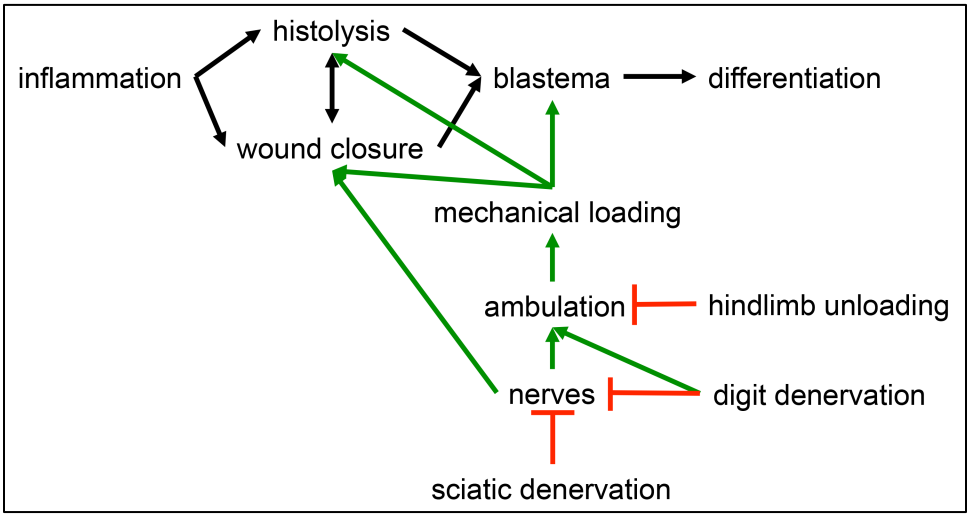

Figure 4.5: Final Model. Diagram illustrating of how peripheral nerves may be contribute to digit tip regeneration. In this model, digit denervation removes from the regenerating digit, but does creates deficits in mechanical loading. In this model, digits are able to mount a robust regeneration response, demonstrating that digit tip regeneration is peripheral nerve-independent. However, peripheral nerves are important for healing the amputation wound.

With this new information on the role of peripheral nerves in endogenous regeneration, let us consider the role of nerves during induced-regeneration. As discussed in Chapter 1, amputations through regions of the digit or limb proximal to the distal digit tip do not regenerate. However, our laboratory has shown that it is possible to induce regeneration following normally non-regenerative amputation injuries with targeted BMP2 treatment (Dawson et al., 2017; Yu et al., 2010; Yu et al., 2012). This includes regeneration of the P2 bone, as well as regeneration of the tibia and fibula, where in some instances, BMP2 treatment allows for the two bones to rejoin.

What remains unknown is if after induced-regeneration, nerves are stimulated to regenerate. The answer is either they do, or they do not, regenerate. Whatever the answer, it is a important question. Indeed, this is an important unanswered questions that should be the focus of continued investigation. You see, if BMP2-treatment does not stimulate nerves to regenerate, then we will be regenerating limbs and digits that are non-functional. In the short-term this is okay, but moving forward, it will critical to consider combining BMP2-treatment with other growth factors (nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3NT-3, glial cell-derived neurotrophic factor, ciliary neurotrophic factor, insulin-like growth factor, etc.) that have been shown to stimulate peripheral nerve regeneration (Apel et al., 2010; Apfel et al., 1998; Geremia et al., 2010; Henderson et al., 1994; Ito et al., 1998; Schecterson and Bothwell, 1992). Alternatively, if nerves are induced to regenerate, then this means that BMP2 is acting either directly or indirectly to stimulate nerve regeneration. If this is the case, BMPs may be a reasonable candidate growth factor to use for treat nerve injuries.

As is the case with most good science, more questions arise with the accumulation of new knowledge. One of the exciting areas of new research interest that arises from the studies in this dissertation is the role of mechanical loading as a factor that regulates regeneration. As I described in chapter 3, hindlimb unloading completely inhibits digit tip regeneration by inhibiting wound closure, preventing new bone formation, and altering bone histolysis (Figure 3.2; Figures 3.3). In future studies, understanding why hindlimb unloading inhibits regeneration will be imperative. Chapter 3 studies began to narrow down potential hypotheses by testing if mechanical loading was required for specific stages of regeneration. I found that if mice are hindlimb unloaded immediately after amputation for 12 days, and then allowed to ambulate for four weeks, their digits regenerate. These data suggest that regenerative inhibition is not

permanent, and that it appears hindlimb unloading "freezes" the regeneration response. In the reciprocal experiment, when mice with amputated digits are allowed to ambulate for 12 days and then hindlimb unloaded for four weeks, amputated digits are able to regenerate. Collectively, these experiments suggest that mechanical loading is essential for organizing early regenerative processes, but it not required at later stages of regeneration.

One of the intriguing observations gleaned from the previous experiment, is that a regeneration response could be initiated during hindlimb unloading if the wound was allowed to heal (the amputation wound heals between 10-12 days post amputation). This is curious since 1) hindlimb unloading inhibits wound healing and 2) regeneration cannot occur in the absence of wound healing To investigate this relationship further, I treated amputated digits with Dermabond, a wound healing agent that we have previously shown as an efficacious treatment for promoting wound closure, and hindlimb unloaded mice for four weeks (Simkin et al., 2015). Curiously, hindlimb unloaded digits treated with Dermabond were able to mount a regeneration response that was equivalent to Dermabond-treated control digits. These results are promising, and suggest an important relationship between mechanical loading and wound healing.

Given that extreme effects that decreased mechanical loading exerts on endogenous regeneration, it is worth investigating if increasing mechanical loading can stimulate endogenous, or perhaps even induced, regeneration. One way to increase mechanical loading is through exercise, and treadmill running exercise is an efficacious method for increasing mechanical loading that has been shown to increase cortical bone density and bone strength as well as having positive effects on bone matrix organization (Huang et al., 2008; Joo et al., 2003; Kodama et al., 2000; Strope et al., 2015). To investigate this, a preliminary experiment in which mice were exercised (treadmill running for 60 minutes/day) either 3 or 5 times a weeks following

digit tip amputation for four weeks. Curiously in both groups of mice, exercise attenuated bone histolysis, and decreased the amount of bone regenerated compared to non-exercised control digits. These data do not support the hypothesis that mechanical loading may enhance endogenous regeneration, but more experiments are certainly required and hopefully in progress. For instance, it would be curious to see if exercising mice after wound closure could enhance bone regeneration.

Future studies should also be conducted to determine whether increasing mechanical loading with exercise, in combination with BMP2, could be a beneficial therapy for non-regenerative injuries. While applying BMP2 to non-regenerative injuries is capable of inducing a substantial regeneration response, it is of course not sufficient to regenerate the entire appendage. As described in chapter 1, the P2 bone stump after amputation is not a static, but dynamic, wound environment, and is analogous to the proximal bone fragment after fracture repair (Dawson et al., 2016). Mechanical loading is a critical component of skeletal health, and increasing mechanical loading post-bone fracture promotes bone regeneration (Claes et al., 1998; Hannouche et al., 2001; Sarmiento et al., 1996). Given the pro-regenerative effects of mechanical loading on the skeleton, it will be interesting to investigate if increased mechanical loading via treadmill running exercise, in combination with targeted BMP2-treatement, could have an enhanced affect on induced regeneration. If the combination of these two therapies has an enhanced affect on regeneration, it could have major implications in a clinical setting.

4.2 Summary

I began this Dissertation with the central hypothesis that peripheral nerves were required for mouse digit tip regeneration (Figure 4.1). However, in chapter 2, I show that axons and

Schwann cells do not regenerate after digit tip amputation—a surprising and unexpected finding. Also in chapter 2, I show that regenerated digits—that is, digits with impaired innervation—are able to regenerate after being re-amputated. Collectively, the data in chapter 2 suggest that peripheral nerves and Schwann cells may not being contributing to mouse digit tip regeneration as was much as was previously believed.

In chapter 3, I begin my reviewing the previous literature suggesting that mouse digit tip regenerating is peripheral nerve-dependent. In reviewing the literature, I made the observation that all of the previous experiments had used a sciatic denervation—a surgical procedure that severs the sciatic nerve but that also causes limb paralysis leading to decreased mechanical loading. Therefore, I reasoned that previous reports may be confounded, and that regenerative failure after sciatic denervation was not due to impaired innervation to the regenerating digit, but due to decreased mechanical loading during regeneration (Figure 4.2). In chapter 3, I test the hypothesis that digit tip regeneration is mechanical load-dependent by hindlimb unloading mice during digit tip regeneration. Indeed, I show that digit tip regeneration is mechanical load-dependent; hindlimb unloading prevents the organization of early regenerative events. What is critical to extract from these findings is that it supports the hypothesis that previous reports demonstrating that digit tip regeneration is peripheral nerve-dependent are confounded (Figure 4.3).

In the remainder of chapter 3, I show that if peripheral nerves are removed so as to minimize deficits in mechanical loading (digital denervation), then amputated digits are able to regenerate (Figure 4.5). Therefore, the major conclusion of this Dissertation is that mouse digit tip regeneration is peripheral nerve-independent—a conclusion that rejects the central hypothesis that I originally began this Dissertation with. The data that I present here shift one of the bedrock

paradigms of Regeneration Biology—that mouse digit tip regeneration is peripheral nerve-dependent—and takes a small step forward in elucidating the ultimate goal of being able to induce and control complex mammalian regenerative responses.

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