INDUCTION OF REGENERATION OF THE MOUSE DIGIT WITHOUT AMPUTATION AND THE EFFECT OF MULTIPLE AMPUTATIONS ON REGENERATION CAPACITY

A Thesis

by

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ABSTRACT

The phenomenon of mammalian digit tip regeneration offers a promising avenue for research in regenerative medicine, as well as the processes of tissue regeneration more generally. Recent developments in gene expression analysis and immunohistochemical studies of the regenerating mouse tissues offer novel insights into the mechanisms by which cells self-organize into complex systems during the growth and development of the organism. While it is generally known within the field of regenerative medicine that the digits of mice are capable of regenerating after amputation, it is less well known that mouse digits may undergo a similar episode of regeneration following injuries which do not directly damage the bone of the distal phalanx. In this work, I will: 1) extensively characterize how the regenerative process can be initiated without amputation, 2) show how changes in the relative bone cell populations correlate with diminished regenerative capacity.

First, I demonstrate to the reader that this capability to initiate regeneration without amputation offers an avenue to gain additional insights into the broader narrative of digit regeneration. To do this, I continue the work of Dr. Tao Li on methods to initiate regeneration without amputation, by creating a non-transecting intrusion with a drill to the lateral face of the nail bed of the digit. This operation initiates a process of histolysis culminating in auto-amputation, and subsequent regeneration to repair the damage. Using micro-computerized tomography (uCT) and associated measurements of bone volume, I offer evidence that the regeneration response in mouse digits is not directly a response to amputation, rather a generalized process to repair the integrity of the digit after injury.

Next, I use quantitative immunohistochemical analysis methods to demonstrate changes in the relative abundance of proliferating cells, osteoclasts, and osteoblasts during the regeneration process in repeatedly amputated digits. I then offer a potential explanation as to how these observed differences may contribute to the changes which we observe in the regenerative capacity of the digit. To further confound the narrative of amputation regeneration, digits which have not experienced prior amputation demonstrate reduced capability for regeneration if the neighboring digits have been repeatedly amputated. While these data are not conclusive, I offer speculation on the cause of these observations and offer potential avenues for future researchers to pursue.

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To Selina, for being both the wind in my sails and my even keel in a stormy sea.

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1. INTRODUCTION

1.1 Summary

The biological study of regeneration offers researchers a window into the complex process of tissue development, wound healing, and cell communication and signaling. Though the field of regeneration is very broad, the quintessential image of regeneration is that of amphibians regenerating an entire limb after amputation. A similar process takes place in the distal portion of the mouse digit. The conservation of this type of wound response in mammals offers the promise of novel treatments for human injury in a burgeoning field known as regenerative medicine. This work discusses a novel instigation of regeneration in the mouse digit, by surgically creating a small hole in the lateral face of the nail. This injury does not create dramatic damage to the underlying bone. Despite this, the procedure can initiate the same regenerative behavior seen after a full amputation. Because of the more limited damage to the digit, and the deep interconnection of the process of wound healing, inflammation, and regeneration, this method may be a useful way to treat the digit with growth factors or other physiologically active compounds while maintaining the structure of the digit in a way that is not feasible in a traditional amputation study. Additionally, studies of the effects of repeated amputations of the mouse digit are considered. These studies found marked difference in the between the repeatedly amputated digit as compared to a digit which has been amputated for the first time. Furthermore, it was found that digits of a mouse which had not been amputated, showed a similar reduction to regeneration as the neighboring digits which had been repeatedly amputated. The reason for this finding remains unknown. It is speculated that systemic cell populations which are needed for regeneration become depleted, or that chronic inflammation may cause some inability for these cells to interact with the regenerating tissue.

1.2 General Introduction

The study of various regeneration phenomena has a long history within biology. Mentions of lizard tail regeneration are to be found in the writings of Aristotle and other philosophers of the ancient world. The earliest scientific study of regeneration was made during the height of the European Enlightenment by Spallanzani in 1768. At this time, the only theoretical frameworks available to explain this ability were that of epigenesis and preformation. The thinkers of the day debated if the replacement appendage arose from "an elongation of the old, or if it takes its origin from some small germ." Ultimately, Sapallanzani committed to ovist preformation as an explanation (Dinsmore 1996).

The epigenetic/preformation debate continued until the improvement of microscope technology in the mid-19th century allowed Rudolph Virchow to famously complete cell theory with the statement, "every cell arises from another cell" (Tan and Brown 2006). This understanding that there was a fundamental unit to life's diversity allowed other researchers to do extensive work during the late 19th through the mid-20th century to characterize the major histological stages in the regeneration of the newt for limb (Singer 1952). However, there existed only vague biochemical explanations as to how the cells involved in regeneration proceeded through a tightly regulated morphogenic process. Work on the inhibition of regeneration by denervation of regenerating amphibian limbs led some researchers to believe, that in amphibian regeneration there are nerves with limb-forming qualities, and others which have specific qualities for the development of other parts of the organism (Singer 1952). The identification of nerves as having a causal link with the healthy regeneration and normal function of the body remains valid, but is a self-evidently incomplete explanation for the process of regeneration.

These researchers and their contemporaries were working before the discovery of DNA (Watson and Crick 1953), and the development of the central dogma of biology (Crick 1970). Once it was established how the cell manifests the information in the genome to form the various proteins necessary for cellular function, new insights into the process of regeneration could be made. Until very recently, it was supposed that the blastema present in all animal models of regeneration consisted of multipotent stem cells and these stem cell likely form the blastema after dedifferentiation from mature tissues at the injury site (Tsonis 2008).

However, other recent research suggests that the reality is more complex. Cells in the blastema are linage restricted, and may trans-differentiate, or phenotypically reprogram from one cell type directly into another. Additionally, it is possible that circulating progenitors of hematopoietic, endothelial, or mesenchymal origins may be recruited to the site of damage and contribute to the reconstitution of the missing tissue (Rinkevich, Maan et al. 2015). The multiple dimensions of this phenomenon make it a profitable avenue of research, and the original research presented in this thesis may help future researchers to expand the scientific knowledge of this problem. A further explanation of the known properties and behavior of the blastema may be read in Chapter 2.

1.2.1 Regenerative Processes

Animals must replace cells and tissues which become damaged during the course of life. While most injuries or worn-out cells are replaced with a typical healing response which includes the development of fibrous scars, some species are able to perfectly replace sophisticated tissues without scar formation. This process of tissue replacement is broadly referred to as regeneration (Krafts 2010). There are several general categorizations of regenerative processes; physiological regeneration, reparative regeneration, hypertrophy, and morphallaxis. Physiologic regeneration

includes the process of atrithropoisis, epidermal replenishment, and molting. Hypertrophy relates to size changes to internal organs due to changes in physiologic demand or compensation after surgical alteration to the body. Morphallaxis refers to the redevelopment of a body after severe damage which is seen in planarians and annelids (Carlson 2007).

Perhaps the most well-known regenerative process is the reparative regenerative process. This category includes processes of tissue regeneration associated with damage to skin, muscles, bones, and heart, as well as the replacement of a missing limb or tail which is famously seen in amphibians. The ability to perfectly replace a complex tissue without scaring, and through a proliferative intermediate structure of undifferentiated cells, known as a blastema, is generally referred to as epimorphic regeneration.

1.2.2 Epimorphic regeneration

The process of epimorphic regeneration specifically involves the development of a regeneration blastema. This structure has traditionally been defined as a mass of undifferentiated cells which proliferates prior to differentiation and arises from epithelial and mesenchymal interactions (Carlson 2007). However, some new research indicates that the blastema is in fact a heterogeneous mix of lineage defined cells (Tweedell 2010). These cells are not fully developed until later in the regeneration process. The blastema contains and expresses morphogenic information necessary to replace the missing structure, as damage to or removal of the blastema results in regenerative failure (Stocum 2017).

The process of epimorphic regeneration is seen to a limited capacity across many vertebrates; including fish, amphibians, reptiles and mammals. Because of its conservation across many philia of vertebrates, there is reason to believe that this capability may remain latent

in many organisms including humans. Additionally, it may be stimulated and enhanced by various morphogenic means, a burgeoning and multi-dispensary field known as regenerative medicine (Dolan, Dawson et al. 2018). While this capability is not yet therapeutically feasible, some recent studies have made progress in the area (Yu, Dawson et al. 2019).

The best characterized example of vertebrate epimorphic regeneration is that of limb regeneration in salamanders. These animals are famously capable of regenerating entire limbs including all of the digits, joints, muscles and nerves of the appendage. The process has been shown to be dependent on positional information which is latent in the cells that make up the tissue. If a piece of epithelium from another region of the body is grafted on to the amputation site, the limb will fail to regenerate (Mescher 1976). It was recently discovered that the salamander Extra Cellular Matrix (ECM) contains position specific information which may inhibit regeneration or produce de novo limb structure. It is believed that this position specific effect is cause by the differential expression of heparin sulfate sulfotransferases. (Phan, Lee et al. 2015). Moreover, it has long been known that if the nerves to the limb are severed, regeneration fails. Furthermore, if the nerve to a given limb is moved to a different region of the body, a limb can be stimulated to originate from this artificially placed nerve (Singer 1952). These results suggest that there is a strong association between neural input and epimorphic regeneration, as well as deterministic effect of the local cells involved in regeneration.

Epimorphic regeneration in mammals is much less common, but a similar instance of epimorphic regeneration is seen in the annual regeneration of antlers in deer (Price, Faucheux et al. 2005), as well as in the closure of ear holes in various rodents, and the regeneration of digits after amputation in mice, rats, monkeys, and humans (Heber-Katz and Messersmith 2018). These animals are capable of regenerating the distal phalanx of their digits after amputation in a series

of well characterized steps. These include: amputation, inflammation, histolysis, wound closure, blastemal formation, and differentiation (Fernando, Leininger et al. 2011). This order of events is somewhat different to the behavior of limb regeneration in urodels, where wound reepithelization and therefor wound closure happen before histolysis (Stocum). This is because the wound epithelium does not cross over the exposed bone in the mouse as it does in salamanders. Instead, the epithelium retracts and leaves a stump of the bone exposed, while a complex histolytic process causes the degradation of the bone and its eventual ejection from the wound. A more detailed examination of wound healing and regeneration in the mouse digit will be discussed in the following section.

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2. NAIL DRILL STUDIES

2.1 Introduction

While not widely known in the general public, there are numerous documented cases of human fingertip regeneration after amputation. First noted by following the healing process after amputation of an infected fingertip with X-Ray images (McKim 1932). Later researchers discovered that conservative treatment of amputations of the most distal phalanx in children, resulted in full regeneration of length (Douglas 1972, Illingworth 1974). However, if the injury is covered with a graft of mature skin, as is the traditional method for treatment of this type of injury, the bone fails to regenerate (Mescher 1976).

The best characterized example of digit regeneration has been shown in embryonic, neonatal, and adult mouse models, but the response is level dependent. An amputation of the terminal phalanx (P3) of the mouse digit, which transects the nail plate, epidermis, dermis including loose connective tissues, blood vessels, nerves, bone and bone marrow possesses the native ability to regenerate following amputation (Simkin, Han et al. 2013). A visual example of this location is indicated in figure 1. Amputations more proximal to the body than the most distal phalanx fail to regenerate, but rather form a stump.

Despite this limitation, the mouse digit blastema has been shown to be remarkably similar to the blastema formed in response to axolotl limb and zebrafish fin amputation. The behavior of the blastema is consistent in all these organisms, in that proliferation and organization proceed through canonical Wnt and Igf pathways, with proximal cells being more differentiated, while more distal cells are less linage defined (Gemberling, Bailey et al. 2013).

Because of the conservation of a blastema mediated epimorphic regenerative phenomena across diverse phyla of vertebrates, there is reason to believe that latent regenerative capability

exists in humans and other vertebrates valued by humans. With proper stimulation, this latent capability might be harnessed to replace missing tissues in a manner which is not seen in natural settings. This is a developing field of research known as regenerative medicine.

2.1.1 The regenerative process in the mouse digit

The process of regeneration in the mouse digit is well characterized, and follows a general pattern of: injury, hemostasis, immune response, progenitor cell recruitment, blastema formation, and morphogenesis (Simkin, Sammarco et al. 2017). However, other researchers define these phases of regeneration as: amputation, inflammation, histolysis, wound closure, blastemal formation, and differentiation (Fernando, Leininger et al. 2011, Dolan, Dawson et al.). The main difference between these stage orders appears to be a subjective delineation of hemostasis and clot formation as being a separate event from the process of inflammation. Additionally, the stage defined as wound closure; that is a migration of epithelial cells across the wound surface, as being distinct from the process of progenitor cell recruitment. These differences of phase description reflect current contentions within the field, but simultaneously remain valid frameworks through which to view the process of wound regeneration.

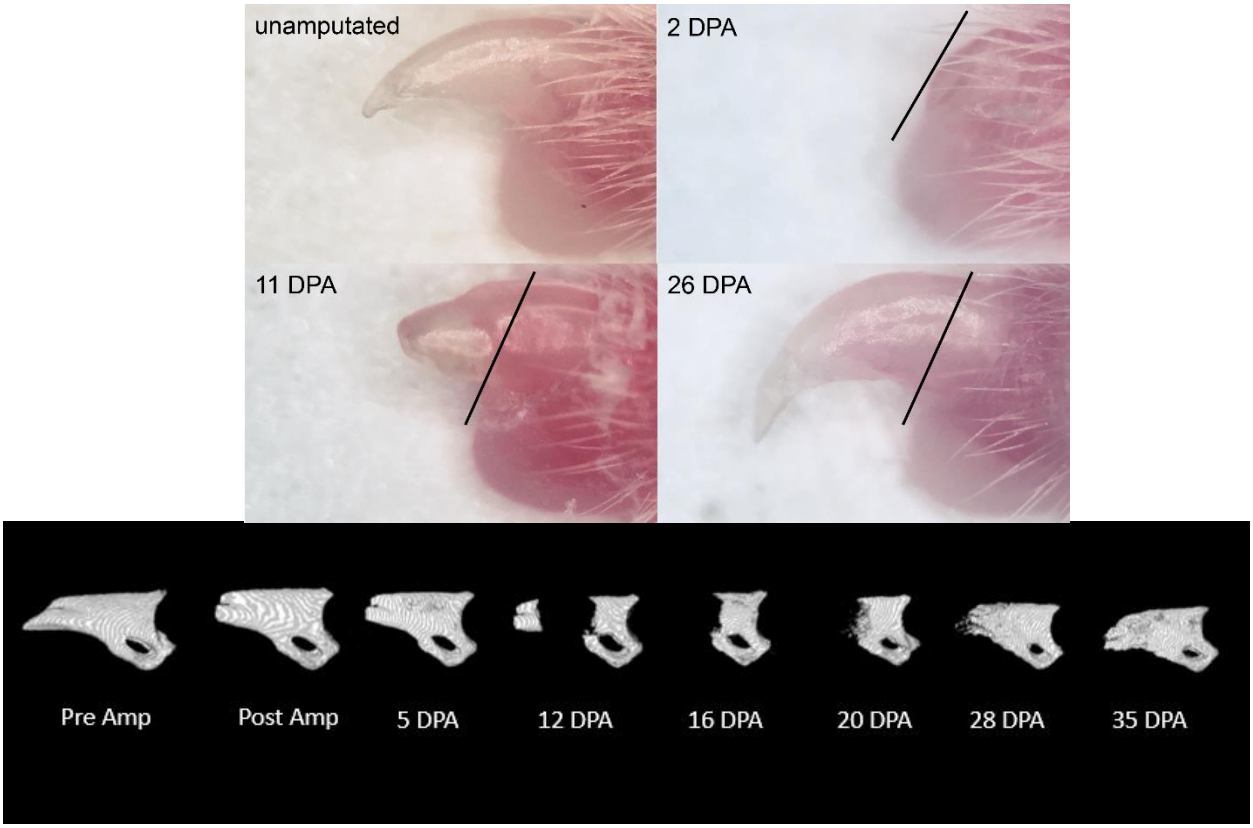

Figure 1: Mouse Digit Tip Regeneration. Black line indicates the amputation plane. DPA = days post amputation. Lower panel shows the process as a series of uCT images showing the histolytic process and subsequent regeneration of P3. Note the large distal piece of bone which has been isolated from the digit at 12 DPA. This bone fragment will be ejected from the distal process of the digit rather than being reincorporated into the regenerate. Additionally, note the disorganized structure of the bone at 35 DPA. This visibly different material is woven bone produced by direct ossification of the extracellular space, rather than lamellar bone.

Immediately following amputation, a typical inflammation and hemostasis wound response is initiated. Damage to the blood vessels causes a release of endothelin and consequent reflex vasoconstriction (Periayah, Halim et al. 2017). When a blood vessel is disrupted, components of the sub-endothelial matrix are exposed to the blood. This exposure activates the main hemostatic process, causing the aggregation of thrombocytes at the wound site by activation of adhesive proteins in the endothelial cells lining the blood vessels. While thrombocyte aggregation is occurring, a cascade of coagulation serine proteases cleave soluble fibrinogen into fibrin, forming a crosslinked mesh at the wound site (Gale 2011). This serves as a temporary shield to protect the wound from the environment. It also serves as a provisional matrix to facilitate cytotaxis during the remainder of the wound healing process (Diaz-Flores,

Gutierrez et al. 2016). Furthermore, the clot is a sources of numerous cytokines and growth factors which provide the biochemical cues for regeneration (Martin 1997).

During clot formation, neutrophils are recruited to the wound site by the degranulation of thrombocytes in the fibrin clot, a process which releases a large number of inflammatory cytokines (Gale). The abundance of these cells is found to peak at 5 DPA, and return to preamputation levels 15 DPA (Simkin, Sammarco et al. 2017). The neutrophils themselves release many highly active antimicrobial and pro-inflammatory chemicals including reactive oxygen species, cationic peptides, and proteases. These are cytotoxic compounds which contribute to destruction of invading agents as well as the histolytic process to prepare the tissue for regeneration (Mittal, Siddiqui et al. 2014). After about 48 hours, monocyte migration from neighboring blood vessels is intensified and these cells differentiate into macrophages. This population of cells assists in recognizing and presenting pathogens to the broader immune system, as well as the active phagocytosis of debris at the site of the injury. Macrophage populations peaks at 7 DPA and returns to pre amputation levels at 21 DPA. These cells localize in different regions of the digit. Macrophages accumulate along the endosteum of the P3 bone, as well as the proximal nail matrix dermis. Neutrophils localize to the P3 bone marrow and associated connective tissue of the bone (Simkin, Sammarco et al. 2017). Studies of the depletion of macrophages in the regeneration process of the spiny mouse ear show that loss of macrophage population delays regeneration until macrophages repopulate the tissue and facilitate blastema formation (Simkin, Sammarco et al.). Additional studies on the digit of the mouse show that loss of macrophage population inhibits re-epithelization and therefor, all subsequent steps in the regenerative process. Rescuing re-epithelization promotes blastema initiation, but the structure then regresses and fails to produce the replacement tissue (Simkin and Seifert). Thus, these

studies suggest a critical role of macrophages in the process of regeneration in adult mammals. Additional studies will need to be conducted to identify the specific role as to how local immunity regulates regeneration.

Closely associated with the inflammatory stage is the histolytic stage. Histolysis is the enzymatic degradation of the extracellular matrix by acid hydrolases and matrix metalloproteases for the release of free cells, resulting in the loss of organization (Yang, Gardiner et al. 1999). Additionally, demolition of cells damaged by the amputation operation, occurs by proteolytic enzymes released from neutrophils and active phagocytosis of the cell debris by macrophages (Tank, Carlson et al. 1976).

Currently, little work on the function of neutrophils in the regeneration process can be found in the literature. It is known that neutrophils but not macrophages infiltrate the blastema (Simkin, Sammarco et al. 2017). However, to the writer's knowledge, no study of the inhibition of neutrophils on the process of tissue regeneration has been conducted. The clearance of apoptotic neutrophils initiates a feed-forward pro-resolution program which is characterized by the release of the cytokines; transforming growth factor beta (TGFB) and interleukin-10 (IL-10). Researchers have identified these growth factors as potential targets for therapies to accelerate tissue repair (Robertson, Holmes et al. 2014), and are thus of interest to the process of mammalian digit regeneration. This leaves a significant gap in the scientific knowledge of the immunological component of regeneration.

Bone degradation conducted by the action of osteoclasts features prominently in the mammalian regeneration response. It is thought that these cells arise from the same population of monocytes which give rise to the macrophages recruited to the cell during the inflammatory phase. They are large, multinucleated cells which bind to the surface of bone and cause

dissolution of the hydroxyapatite via the creation of a highly acidic environment, additionally they produce collagenase which helps to degrade collagen matrix of the bone (Li, Kong et al. 2006). This activity is most clearly observable in real time with micro Computer Tomography (uCT) imaging. Osteoclast numbers peak at 7DPA, but are rapidly depleted by 10 DPA (Dolan, Dawson et al. 2018). During this period, osteoclasts degrade through P3 at a level more proximal than the initial amputation. This process splits the bone into proximal and distal halves. The distal section is not reincorporated, rather it is ejected from the wound during the process of regeneration. This is clearly visualized in the uCT images in figure 1.

It is important to mention that during the regenerative process of the digit, wound closure as defined by Fernado 2011, does not take place until the end of the histolytic phase. This is different from the order of events in other regenerative phenomena, such as ear punch closure in spiny mice (Heber-Katz and Messersmith 2018) and urodle limb regeneration (Scotum 2017), where wound closure takes place before the histolytic phase of regeneration. This delay occurs because the epidermis does not migrate across the bone, but rather retracts and forms connections with the periosteum of the exposed bone. Once the histolytic process has ended, the epidermis migrates beneath the exposed bone and closes off the wound, while the distal fragment is ejected along with the scab as can be seen in figure 1 (Simkin, Sammarco et al. 2015).

The closure of the wound by the migrating epidermis is known as a wound epithelium. It serves to cap the regenerating stump and allow the development of the blastema. If the formation of the wound epithelium is inhibited, or replaced with a graft of mature skin, as is the general treatment of accidental distal amputations in humans, the formation of the blastema is inhibited and regeneration will not proceed (Rinkevich, Maan et al. 2015). This implies that the wound epithelium is essential as signaling center for the elaborate self-ordering of cells which is seen

during the development and maturation of the blastema. Because wound closure is dependent on the completion of the histolytic phase, the timing of wound closure is highly variable. In a study of wound closure, it was found that only 1 of 9 digits observed had completed wound closure. By 10 DPA, 6 of the 9 had completed wound closure. By 12 DPA all of the observed digits had completed wound closure and had developed prominent blastemas (Fernando, Leininger et al.).

As previously discussed, the blastema is a relatively disorganized mass of rapidly proliferating cells which arises from interaction with the wound epithelium and the mesenchyme cells of the wound, resulting in a heterogeneous population of cells from both systemic sources as well as local progenitors. The cells which are more proximal to the digit are more differentiated while the cells more distal are less differentiated but are still likely lineage restricted rather than being fully pluripotent as some have thought (Tweedell 2010). The blastema is characterized by reduced vascularity as compared to mature tissues, (Mescher and Cox 1988) and is associated with reduced vasculogenesis (Said, Parke et al. 2004). Additionally, the structure is hypoxic relative to mature tissue (Sammarco, Simkin et al. 2015), and consequently the cells involved in the process rely more extensively on glycolysis than do cells in mature tissue (Heber-Katz and Messersmith 2018). Once the blastema forms, neutrophils but not macrophages infiltrate the blastema. Between 14 and 21 days post amputation, immune cell levels return to normal (Simkin, Sammarco et al.). It has been found that mice deficient in macrophages are capable of scar free wound healing, but that these mice are not able to form a blastema, and therefore cannot successfully complete digit regeneration (Simkin, Sammarco et al. 2017).

Once the blastema has proliferated sufficiently, the cells undergo a process of differentiation, and become specialized as necessary to replace the digit. This process is highly

coordinated, and progresses in a proximal to distal fashion. The proximal cells differentiate before the more distal cells, and so begin specialized processes earlier (Tweedell). The most visible indication of differentiation is seen in the action of osteoblasts, or bone progenitor cells which fabricate new bone by direct ossification of the extracellular matrix. This is in contrast to the formation of bone during embryonic development, where ossification proceeds by endochondral ossification, and involves an extended period of elongation following birth. It may be inferred that the difference between these two forms of ossification allows for the replacement of the missing bone over a short timeframe, favoring function over structural uniformity. uCT data of the regenerated bone shows significant overshoot of bone volume, while achieving length which is relatively consistent. The bone never reorganizes to form lamellar bone and remains permanently histologically distinct, though it does increase in density with time (Fernando, Leininger et al. 2011).

2.2 Materials and Methods

2.2.1 Drill operation and animal handling

Adult 8-week-old, female CD-1 mice were purchased from Texas Institute for Genomic Medicine. A burr hole was created on the lateral face of the nail of the second and fourth digits of both hind limbs with a drimmle, using a hypodermic insulin needle as a drill bit. The mice were then euthanized and the tissues were harvested for analysis. All animal use and techniques were in compliance with the standard operating procedures of Texas A&M University's Institutiuonal Animal Care and Use Committee.

2.2.2 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 hind limb digits were performed as described in 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.

2.2.3 Antibiotic treatments

After amputation of the 2^{nd} and 4^{th} digits of the left and right hind limb in accordance with the protocol under the section *animals and amputations.* The amputated digits were then treated with either: Silver Sulfadiazine Cream 1% (Asscend), or bacitracin-neomycin-polymyxin (Vetropolycin). The antibiotic was applied generously to the amputation site, once a day, until closure of the epithelium at 12 DPA (Fernando, Leininger et al. 2011).

2.2.4 Tissue collection and histology

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.

2.2.5 µCT and bone volume calculation

µCT images were acquired using a VivaCT 40 (Scanco Medical AG, Brüttisellen, Switzerland) at 1000 projections per 180 degrees with a voxel resolution of 10 µm3 and energy and intensity settings of 55 kV and 145 µA. Integration time for capturing the projections was set to 380 ms using continuous rotation. ImageJ and four of its plugins, BoneJ Optimize Threshold Plugin, BoneJ Volume Fraction Plugin, and 3D viewer Plugin, were used to analyze the µCT data. The BoneJ Optimize Threshold Plugin was used to segment images and the BoneJ Volume Fraction Plugin was used to calculate changes in bone volume. Bone volume data was normalized to the total bone volume directly following amputation. The snapshot feature was used to create 2D images.

2.2.6 Florescent Immunohistochemistry

Antigen retrieval was performed using heat retrieval 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) 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 Olympus BX61 microscope with a Hamamatsu ORCA-ER camera via the Slidebook software (Intelligent Imaging Innovations Inc.).

EdU (5-ethynyl-2’-deoxyuridine) was injected intraperitoneally into mice 3 hours prior to specimen collection at a dose of 10□L/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 were rabbit anti-Osterix(1:400), and mouse anti-PCNA(1:2000). Secondary antibodies were Goat anti-Rabbit 488 (1:500), and Mouse anti-Rabbit 647 (1:500).

2.3 Results and Discussion

2.3.1 Regeneration can be triggered without significant damage to P3 bone

In amputation essentially every injury results in the characteristic regeneration process. In the nail drill procedure, some injures to the nail do not result in regeneration, but rather result in what appears to be a normal healing response. There appears to be some minimal threshold of injury beyond which the organism undergoes a regenerative response but below which a similar wound results in a traditional healing response, careful study of this process may result in novel insights of regeneration. An excellent example of the apparent arbitrary difference between regenerative vs non-regenerative wound healing can be seen in figure 4 below. Notice how the two digits on the same paw receiving injuries which externally look very similar. However, the uCT images show significant damage to the L2 digit. It would appear that damage to the bone may be the cause of digit regeneration in lateral non transecting injury.

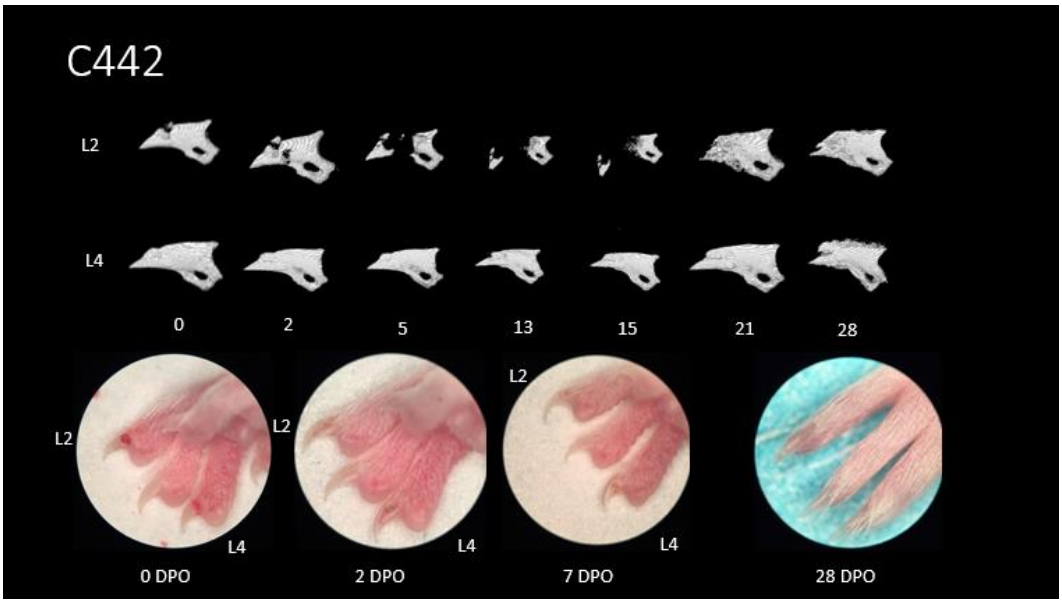

Figure 2: Nail Drill Regeneration Time Serise. Notice the significant damage to L2 digit and the apparent lack of damage to L4 in the uCT images. L2 undergoes a classic regeneration response, while L4 does not, despite the similarity of external injury and inflammation visible through the lateral surface of the nail in the 2DPO photo.

Because the damage to the bone is intuitively associated with regeneration, it was necessary to ensure minimal damage was inflicted to the bone. To do this, a series of operations with much tighter control on the depth and severity of burr hole were conducted. The results of this experiment are summarized in figure 5. The data showed a very reliably controlled regeneration response. The value at -1 day shows the calculated bone volume pre-operation, and the value at 0 days is post operation. The change between the two shows a small but statistically insignificant change in bone volume at 95% confidence. This suggests that damage to the bone itself is not the primary initiator of the regenerative response.

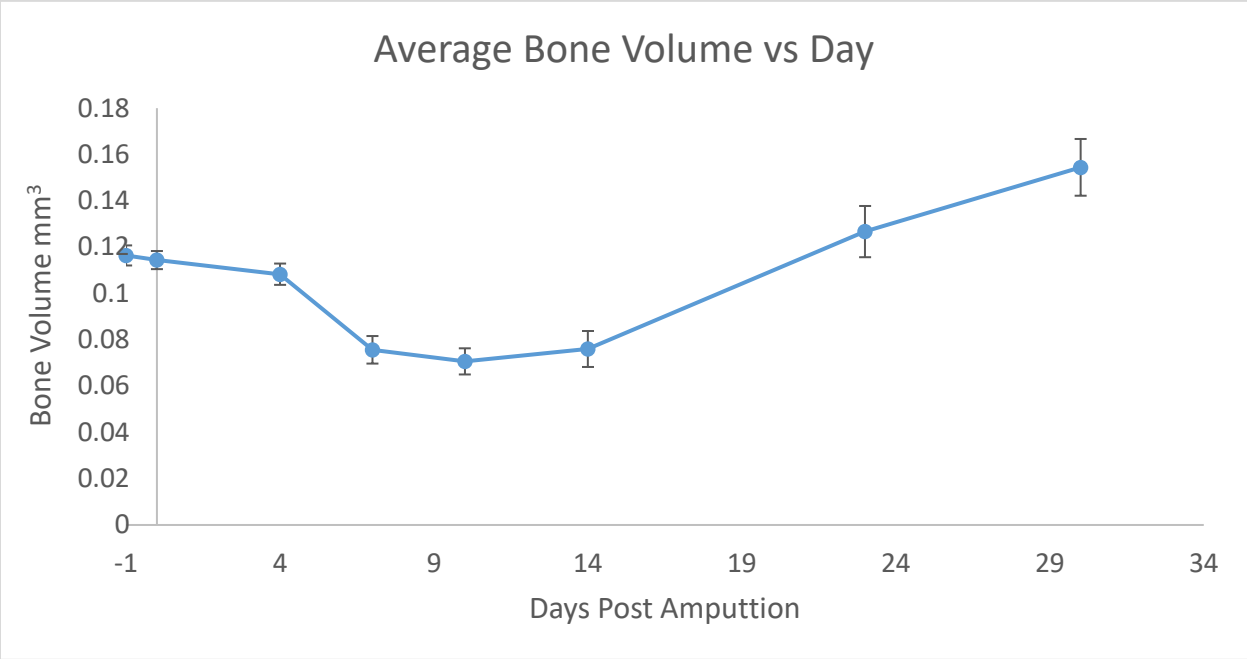

Figure 3: Bone Volume vs Day in Nail Drill Regeneration. Plot of average bone volume calculated by uCT over days post operation. 20 digits were observed. Error bars represent a 95% confidence interval.

Despite the lack of significant changes in regard to bone volume following the creation of the burr hole, this cohort of digits underwent a very typical regeneration process. This suggests that even when minimal damage is infected to the underlying bone, the regenerative response is preferred to a traditional wound healing response.

However, on a subsequent cohort of digits, the needle which was used to create the hole was changed between each animal, rather than being washed in 70% ethanol and changed as necessary when its blade became dull, as was the case in previous case in the initial experiments. This resulted in a greater frequency of digits which did not undergo the regeneration response, despite visible damage to the bone in the uCT images. Only 15 of the 30

digits underwent the regeneration response. Examples of this can be seen in the following images. While more data is required to draw meaningful conclusions about these findings, it is possible that cross contamination of tissue is linked in a more intense immune response, which then contributes to the initiation of the regeneration process.

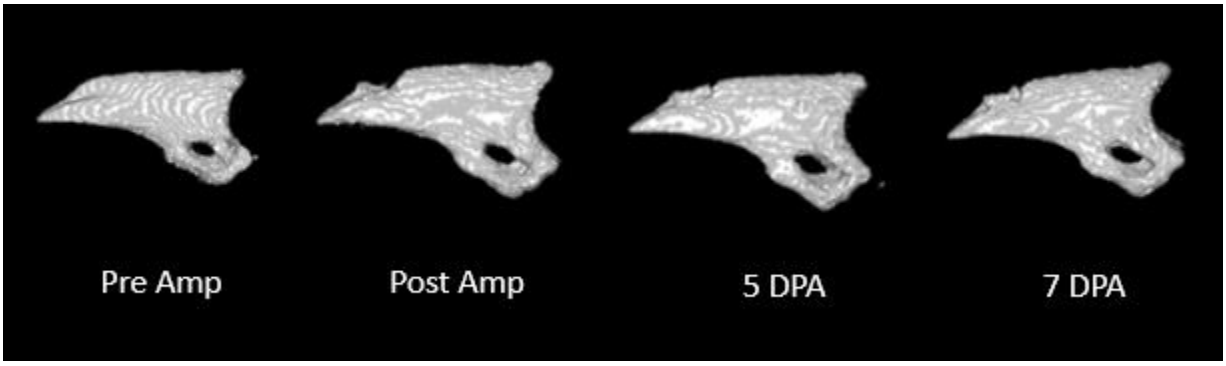

Figure 4: Nail Drill Regeneration Failure. uCT images of significant damage to the bone, which has failed to initiate the regeneration response, and seems to have healed by depositing bone into the void created by the drill. All digits in this study which showed signs of histolysis at 12 DPA, also showed signs of significant bone damage by 7 DPA.

2.3.2 Antibiotic treatment of wound has no effect

The role of immune cells in the regeneration process has been previously discussed, and one of the early examples of regeneration of a human digit was in response to a bacterial infection of the digit tip (McKim 1932). Mice are housed in a condition which allows opportunities for these fresh wounds to come into contact with the animal's waste. Additionally the digits are regularly used for grooming, scratching, and digging. Because these activities provide numerous opportunities for infectious agents to enter into the amputation site, an exploratory study on the effect of topically applied antibiotics to the regenerating digits was conducted. Amputations were performed as is described in the materials and methods section. The antibiotics used are described in the materials and methods section. Animals were the treated daily until 12 DPA, the time of wound closure in all observed digits (Fernando, Leininger et al.). However, no significant difference was observed in the timing of bone degradation, or the completion of regeneration.

2.3.4 The regeneration response to nail drill is mediated by a blastema

While it was known that the nail could be stimulated to regenerate by making a non-transecting hole, it was not known if the process of regeneration proceeded through the formation of a blastema. The blastema is marked by being undifferentiated, rapidly proliferating, avascular, and consequently hypoxic. To test this, immunohistology was conducted on sections of the digits. It was found that after histolysis had been identified in the uCT images, the proximal cells were abundantly positive for Osterix, a marker for mature osteoblasts, while distal cells were positive for PCNA, a marker for cell proliferation and cell migration. These results suggest that the process of regeneration seen after nail drill injury, follows the same pattern as the regeneration process following amputation.

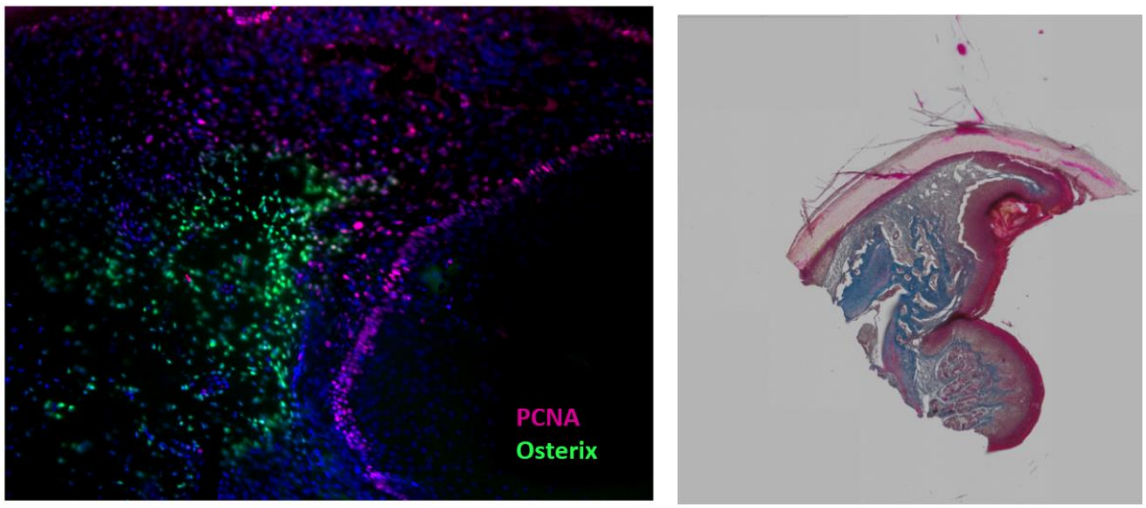

Figure 5: Nail Drill Blastema Immunohistochemistry. Immunohistochemistry of a 12 DPA digit regeneration process, showing the classic regeneration process of proximal differentiation and distal proliferation. Qualitatively, it would appear that the intact structure of the nail causes a physical barrier to the expansion of the blastema. The distal bone fragment and scab are ejected through the ventral surface of the digit while actively proliferating blastema cells infiltrate the dorsal region as regeneration proceeds.

2.4 Conclusion

The induction of regeneration of the digit by creating a non-transecting hole into the nail offers future researchers a novel perspective through which to study this process. The small hole and minimal damage to the underlying bone may be a usefully way to implant beads to test the effect of various growth factors. Because the damage to the digit is less severe than is the case in full amputation, it may be possible to have greater control over the complex cascade of physiological events which take place during digit regeneration.

Additionally, the nail organ remains mostly intact, providing stability and protection for the regenerating digit. It is possible that the structure and cells of the nail organ provide additional direction for the patterning of blastema cells involved in the regeneration process. Because numerous instances of damage to the bone were identified in the uCT data, but the process of regeneration was not initiated, this procedure may also offer future researcher's greater control to observe the specific conditions required to initiate the process of regeneration.

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3. REPEAT AMPUTATION STUDIES AND CONCLUSION

3.1 Introduction

A fundamental question in the regeneration problem is how repeated amputations effect regenerative capacity. It is understood that regenerated digits are not a prefect replacement of the missing tissue, as the replacement bone is of the woven variety produced by direct ossification in the extracellular space which causes bone growth in a relatively a disorganized fashion. This is in contrast to the native, lamellar variety produced by endochondral ossification during the development of the embryo, which results in the ordered architecture seen in vertebrate long bones (Han et al. 2008). Despite the differences between these two forms of bone, the later stages of regeneration are generally considered to be analogous to redevelopment (Simkin, Han et al.).

Though the consensus in the field is that the regeneration may be thought of as a form of redevelopment, it is important to consider the differences between the regeneration of the salamander and the mouse. In the salamander model, the regeneration of missing bone proceeds by ossification of regenerated cartilaginous tissue, bounded by complex mechanisms of pattern formation, directing trans-differentiated cells into specific lineages. This process has been shown to be very similar to the process of limb development in the amphibian embryo. In this process skeletal cells contribute relatively little to the population of blastema cells due to the physical barrier of the ECM (Simkin, Han et al. 2013). This is in contrast with the process of mouse digit regeneration which was discussed in the previous chapter, where the regenerated bone is produced by osteoblast's direct ossification of the ECM to create a structure of woven bone, which is morphologically distinct from the native bone produced during embryonic development.

Recent research on the repeated amputation of the limbs of axolotls shows a reduced regenerative capacity after 5 amputations. This regenerative failure is associated with the deposition of fibrotic tissue, and an upregulation of connective tissue growth factor (ctgf), and amphiregulin (areg). Both of these factors are associated with fibrosis in mammalian contexts (Bryant, Sousounis et al. 2017). This research contradicts previous suggestions that limb regeneration in salamanders is essential unlimited (Yun 2015).

3.2 Materials and Methods

3.2.1 Amputations and animal handling

Adult 8-week-old, female CD-1 mice were purchased from Texas Institute for Genomic Medicine. Digit tip amputations of the 2nd and 4th hind limb digits were performed as described previously (Simkin, Han et al.). Mice were amputated at 28 day intervals, until the fifth time the digits were amputated. The mice were then euthanized and the tissues were harvested for analysis. 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.

3.2.2 Tissue collection and histology

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 section at 4.5um and mounted on microscope slides. For histological staining, slides were incubated at 60^{o}C for 45 minutes, and 37^{o}C for 2 hours. The slides were then deparifanized and stained with Mallory Trichrome. Slides were mounted using Permount Mounting Medium (Thermo Fisher

Scientific). All slides were imaged using the Olympus VS120 microscope with a Pike F-505C camera (Allied Vision) using the DP2-BSW software.

3.2.3 µCT and bone volume calculations

µCT images were acquired using a VivaCT 40 (Scanco Medical AG, Brüttisellen, Switzerland) at 1000 projections per 180 degrees with a voxel resolution of 10 µm3 and energy and intensity settings of 55 kV and 145 µA. Integration time for capturing the projections was set to 380 ms using continuous rotation. ImageJ and four of its plugins, BoneJ Optimize Threshold Plugin, BoneJ Volume Fraction Plugin, and 3D viewer Plugin, were used to analyze the µCT data. The BoneJ Optimize Threshold Plugin was used to segment images and the BoneJ Volume Fraction Plugin was used to calculate changes in bone volume. Bone volume data was normalized to the total bone volume directly following amputation. The snapshot feature was used to create 2D images.

3.2.4 Fluorescent immunohistochemistry

Antigen retrieval was performed using heat retrieval 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) 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 Olympus BX61 microscope with a Hamamatsu ORCA-ER camera via the Slidebook software (Intelligent Imaging Innovations Inc.).

EdU (5-ethynyl-2'-deoxyuridine) was injected intraperitoneally into mice 3 hours prior to specimen collection at a dose of 10□L/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 were rabbit anti-Cathepsin K (1:100), Rabbit anti-Osterix(1:400). Secondary antibodies were Goat anti-Rabbit 488 (1:500), and Goat anti Rabbit 647 (1:500).

Regions of Interest (ROI) were determined after background subtraction of auto florescence in the Slidebook software (Intelligent Imaging Innovations Inc.). Data from these areas was put into Graph Pad software (Prism), and analyzed using; repeated measures one-way ANOVA, Ordinary one way ANOVA, or unpaired T-test as required by the specifics of the data. These data were then displayed as histograms.

3.3 Results and Discussion

3.3.1 Repeat amputations result in diminishing bone volume, but not length

To explore the changes which may accumulate over the course of repeated amputations, a cohort of mice were subjected to a course of amputations at 28 day intervals. As can be seen in figure 6 below, the bone volume of the regenerated digits decreases significantly after each successive amputation, but changes in the length of the regenerated bone remained statistically insignificant until the fifth amputation. It is not known why the length of the digit remains relatively constant until the fifth amputation. It is possible the regeneration of the nail organ provides a structure to support consistent digit length, despite the diminishment of bone volume. This number of cycles is interestingly similar to the number of successful regeneration cycles

observed in axolotls (Bryant, Sousounis et al. 2017). Drawing on this similarity, it may be supposed that regeneration cycle limit is linked to an increase in the amount of fibrotic tissue which accumulates in the digit. Genetic studies of the relative abundance of factors associated with fibrosis in repeatedly amputated digits could reveal more about this observation.

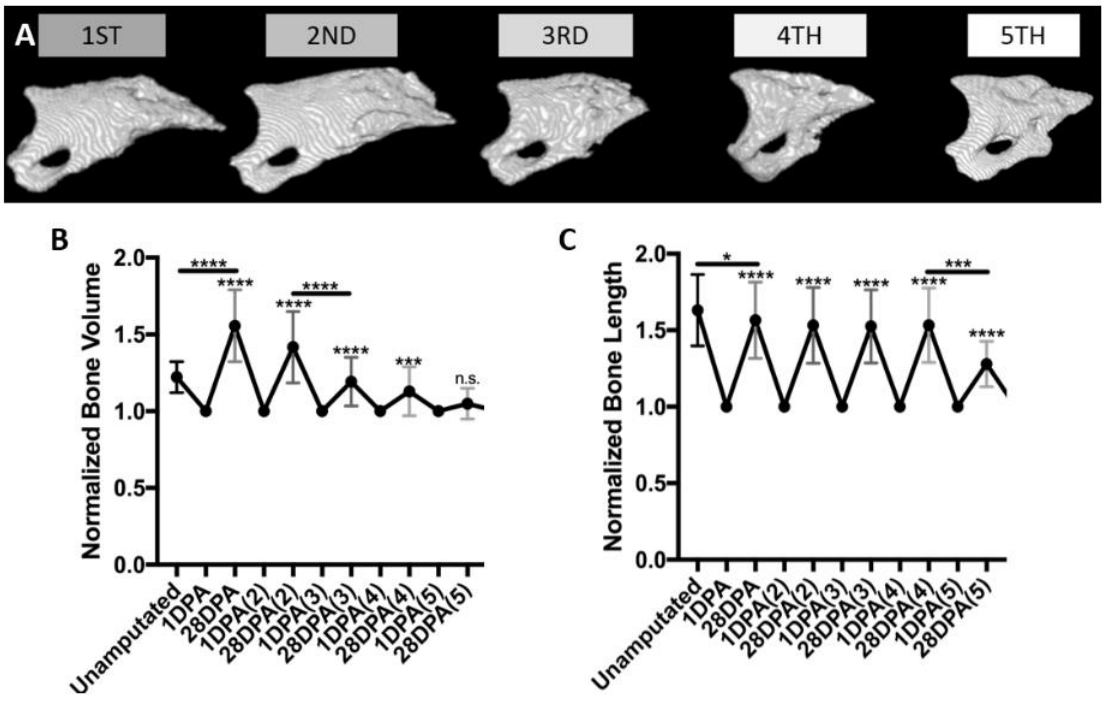

Figure 6: Diminishment of Regeneration with Multiple Amputations. (A) uCT images of repeated amputations at 28 days recovery, just prior to the next amputation in the sequence. (B) Normalized bone volume of showing the diminishing regeneration response of multiple amputations. (C) Normalized regenerated bone length, which shows reasonably consistent regeneration of length until the 5th amputation cycle.

3.3.2 Loss of osteoblasts and EdU+ cells associated with diminished regeneration

Tissue samples from 7, 10, and 14 days post amputation were sectioned and analyzed for osterix positive cells, a marker for mature osteoblasts. It was observed that there was a large decrease in the number of osterix positive cells at 10 DPA in the 5th amputation group as compared to the 1st amputation group, but no significant difference was observed at 7 or 14 DPA. Additionally, the sections were analyzed for EdU positive cells, a marker for cells which were

proliferating at the time of animal sacrifice, and tissue collection. This showed a significant reduction in the number of cells at 10 days post amputation. While the mean number of EdU positive cells appears to be lower at all 3 time points, it was only determined to be statistically significant at 10 DPA. Finally these data were combined to form a composite chart showing the number of cells which were both proliferating, and positive for osterix, indicating a proliferating osteoblast. This number was found to be lower at 7 and 10 days post amputation, but not at 14 days post amputation. This suggests that repeated amputations cause a diminishment in both the number of osteoblasts, and proliferation of all cells involved in the regeneration response including osteoblasts.

The number of osteoblasts in the region of interest remain consistent at 7 and 14 DPA in both the first amputation digit and the fifth amputation digit. This is intriguing, suggesting that the spike of osteoblasts at 10 DPA is necessary for regeneration. However, it is intriguing that the osteoblast population in the fifth amputation digit is consistent with the first amputation at 14DPA. At this time, new bone is being produced at a very rapid rate, as can be seen in the slope of bone volume change in figure 6 panel C. It is possible that the mature osteoblast population in the repeatedly amputated digit is less able to successfully produce new osteoid in the extra cellular space. Additional research will be necessary to answer this question.

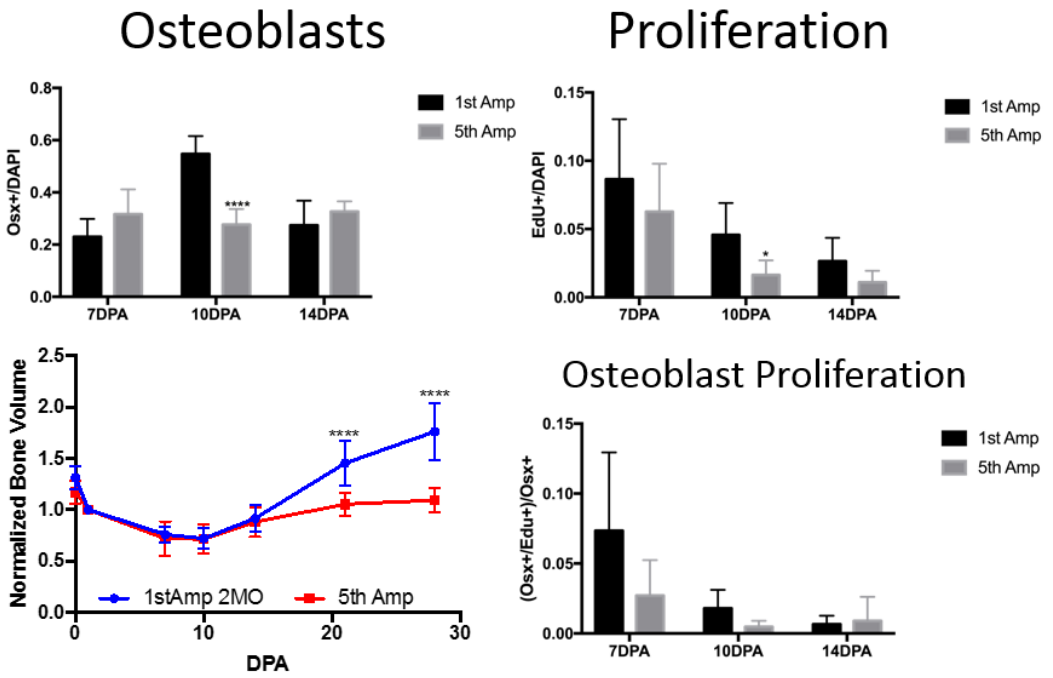

Figure 7: Osteoblasts and Proliferation in Multiple Amputations. Histograms of osterix+ cells, EdU+ cells, and Osterix+/EdU+ cells in relation to normalized bone volume over the course of regeneration between the 5th amputation and the 1st amputation. The reduction in regeneration response is associated with a reduction in osteoblasts and in proliferation at 10 days post amputation.

3.3.3 Decrease of osteoclasts associated with diminished regeneration.

The abundance of Cathepsin K in the 1st amputation digits was dramatically higher than the 5th amputation digits at 7 days, and significantly higher at days 10 and 14. The abundance of EDU positive cells as well as EDU+/osterix+ cells showed similar diminishing patterns to the observed change in Cathepsin K positive cells. Despite this, the abundance of osteoblasts in the fifth amputation digits was not significantly lower than the abundance in the first amputation digits at day 7 or day 14. These results suggest that changes in the population of osteoclasts have a more significant effect on the reduction of regeneration capacity than the change in osteoblasts. The dramatic reduction in the size and abundance of osteoclasts during the histolytic phase suggests that the loss of capacity for bone degradation may contribute to the lack of regeneration. Or, it may be that the lack of degradation inhibits the action of available osteoblasts.

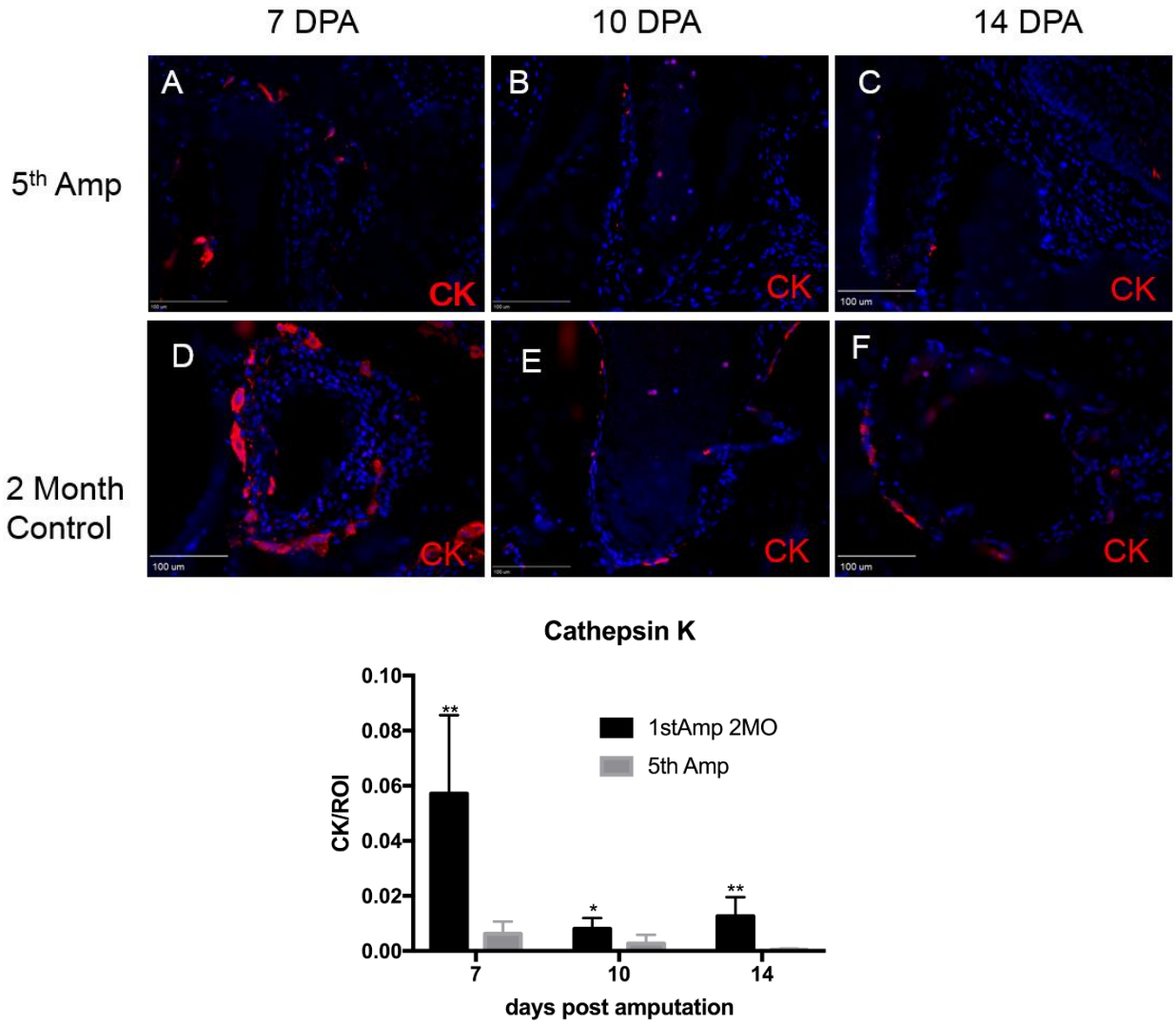

Figure 8: Immunohistochemistry of Multiple Amputations. Immunohistochemistry showing the abundance of Cathepsin K positive cells in the bone marrow of the regenerating digit in the 5th amputation (A-C), and in the 2-month-old first amputation control animal (D-F). Histogram showing the area of Cathepsin K positive cells relative to the Region of Interest (ROI), the bone marrow cavity (G).

3.3.4 Repeat amputations result in diminished regeneration of the middle digit

It is intuitive to believe that repeated amputations may cause a deposition of fibrous scar tissue, which impedes the regeneration process at a local level. To test if there was a broader effect of repeated amputations, the 3rd digit of the hind limb was amputated after the fifth amputation cycle. This digit had not previously been amputated. It was found that, like the repeatedly amputated digits, the middle digit showed diminished regenerative capacity as measured by bone volume from uCT scans. The animals involved were substantially older (8 months old), than the animals typically used for regeneration studies (2 months old). To ensure that the observed difference of regeneration in the middle digit was not caused by age, regeneration bone volumes were compared to 6-month-old mice.

Furthermore, the middle digit amputations showed a similar decrease of Cathepsein K positive cells during the histolytic phase, implying a diminished number of viable osteoclasts. However, osteoblast numbers were relatively constant between the various tests. Once again, these results suggest that there is a causal link between degradation capacity and bone regeneration. Additionally, it may be surmised that an increase in local fibrotic tissue is not the primary reason for reduced regeneration after multiple amputations.

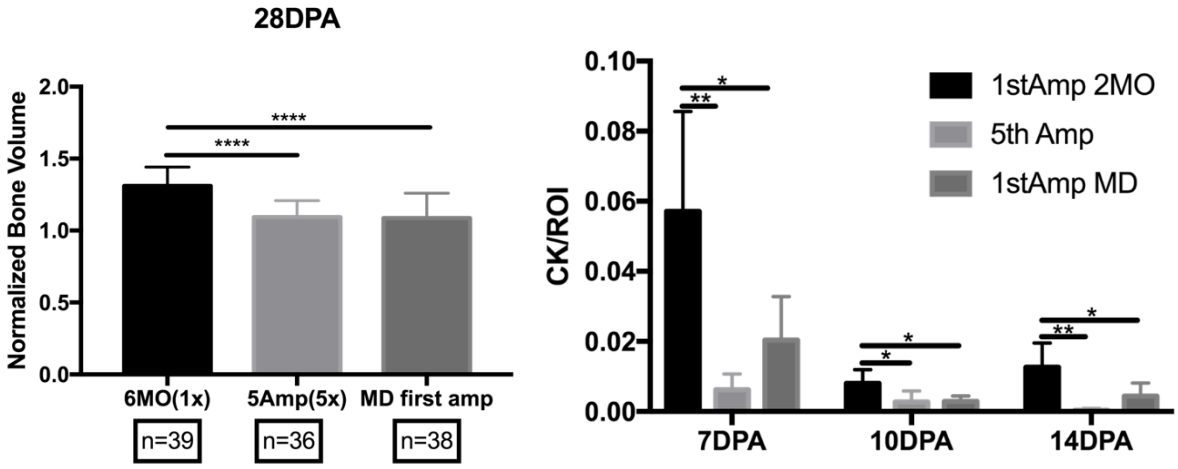

Figure 9: Effect of Multiple Amputations on the Middle Digit. Histogram showing the bone volume of and an aged control animal, 5th amputation animal and the first amputation of the middle digit 28 DPA (A). There is significant difference between the aged control and both the middle digit and the 5th amputation digits. Histogram showing the proportion of the region of interest (ROI), positive for Cathepsin K in the two month control animal, the 5th amputation animal, and the first amputation of the middle digit (B). The first amputation of the middle digit shows a greater proportion of osteoclasts at 7 and 14 DPA than the 5th amputation digit, but both are significantly lower than the control at all time points.

3.4 Final Conclusions and Future Directions

The induction of regeneration of the digit by creating a non-transecting hole into the nail offers future researchers a novel perspective through which to study the phenomenon of digit regeneration. The relatively insignificant damage, and small opening to the underlying bone may be a usefully way to implant beads to test the effect of various growth factors. Because the damage to the digit is less severe than is the case in full amputation, it may be possible to have greater control over the complex cascade of physiological events which take place during digit regeneration.

Additionally, the nail organ remains mostly intact, providing stability and protection for the regenerating digit. It is possible that the structure and cells of the nail organ provide necessary direction for the patterning blastema cells involved in the regeneration process. Because numerous instances of damage to the bone were identified in the uCT data, but the

process of regeneration was not initiated, this procedure may also offer future researcher's greater control to observe the specific conditions required to initiate the process of regeneration.

Multiple amputations were found to reduce the abundance of proliferating cells and osteoclasts in both the repeatedly amputated digit and the first amputation of the middle digit, but not as dramatically affect the number of osteoblasts in either of these cases. Thus, it may be inferred that degradation of the bone is required for the effective formation of new bone. Additionally, the accumulation of scar tissue in the repeatedly amputated digits is not likely to be the primary cause of regenerative failure as was found to be the case in repeated amputation of axolotls (Bryant, Sousounis et al. 2017). However, it would be beneficial to comprehensively study the abundance of fibrous tissue, or the changes to the expression of genes in the repeatedly amputated digits to verify this interpretation.

The lack of regeneration in the middle digit remains puzzling. The reduction in the number of osteoclasts, which corresponds with reduced regenerative capacity does not currently have a satisfactory explanation. To speculate, it is possible that the repeated stress of amputation, and subsequent regeneration may cause depletion of some systemic factor which causes a failure in osteoclast differentiation in the middle digit.

However, as was discussed in chapter 1, the histolytic process is closely associated with the inflammatory and immunological response. It is known that chronic inflammation causes reduced function of the lymphatic system, a major pathway for the migration of macrophages and monocyte progenitors which are believed to give rise to osteoclasts. There is only one major lymphatic vessel which supplies the hind limb of the mouse (Abouelkheir, Upchurch et al. 2017). It may be that repeated amputation and the associated inflammation causes damage to this pathway, and therefor causes diminished ability for macrophages to enter the tissue of the hind

limb. As was previously discussed, macrophages are known to be necessary for successful regeneration (Simkin, Sammarco et al. 2017). Further research on this possible link should be conducted.

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