USING STAGES TO ASSESS THE RELATIONSHIP BETWEEN OSTEOCLASTS AND CELLULAR PROLIFERATION DURING REGENERATION OF THE TERMINAL PHALANX IN CD1 MICE FOLLOWING AMPUTATION

A Thesis

by

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ABSTRACT

Compared to amphibians like the Axolotl, mammals display limited endogenous regenerative ability. However, the digit tips of mice, humans, and other primates possess some regenerative capabilities, providing hope that enhanced mammalian regeneration might be possible. Mouse digit tip regeneration progresses through a series of well-characterized and discrete events: histolysis, blastema formation and redifferentiation. However, the onset of these events varies between digits as well as individual mice. This variation has the potential to dilute meaningful results and hinder the development of future therapies which could improve the lives of many. To address this issue, we used micro-computed tomography (μCT) to develop a novel method for staging digits during regeneration, focusing on the histolytic phase. Features easily identifiable from a μCT scan, i.e. bone pitting and secondary amputation, were used to categorize regenerating digits. When comparing bone volumes, we found that the bone volumes of digits within the same stage were more similar than digits on the same day post amputation. We then used this method of categorization to look at the relationship between osteoclasts, a cell known for its catabolic role in bone, and cellular proliferation, an anabolic event. Although these two, catabolism and anabolism, seem to be opposing events, the role of bone degeneration and bone formation in regeneration is not known. To explore these unknowns, the digit tips of adult CD1 female mice were amputated. The digits were scanned using the μCT and were immunostained for osteoclasts (Cathepsin K) and an indicator of cellular proliferation (EdU). These scans were used to categorize digits based on stage within the degradation event. Osteoclasts and cellular proliferation were quantified by manual cell counting and compared between and across stages. We found that osteoclasts were associated with cellular proliferation,

suggesting that degeneration may be important to the regenerative process. This study provides a method for staging digits and a step towards understanding the role of histolysis during regeneration.

DEDICATION

I would like to dedicate this Thesis to my dog, Obi, who stuck by me every step of the way.

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Ken Muneoka, and my committee members, Dr. Jane Welsh, Dr. Dana Gaddy, for their guidance and support throughout the course of this research.

I would like to give special thanks to my Lab. You all became my family and I will never be able to convey how much you all mean to me.

I would also like to thank the Brazos Runners Club, who helped me understand that limits are self-imposed and that anything is possible.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a wonderful experience.

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Contributors

This work was supported by a thesis committee consisting of Dr. Ken Muneoka [advisor] and Dr. Dana Gaddy of the Department of [Veterinary Integrative Biosciences] and Dr. Jane Welsh of the Department of [Veterinary Integrative Biosciences].

All work conducted for the thesis was completed by the student independently.

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NOMENCLATURE

DPA	Days Post Amputation
P3	Terminal (3^{rd}) Phalanx

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CHAPTER I

GENERAL INTRODUCTION

Introduction

In the United States there are an estimated 2 million people living with limb loss and this number increases by 185,000 people every year [1,2]. Many of the amputees are U.S. soldiers who had a limb amputated due to war related injuries from Iraq or Afghanistan [3,4]. In 2009, the total hospital costs associated with amputations totaled more than 8.3 billion dollars [5]. Although many amputees lose their limbs due to trauma, vascular disease is principally responsible for most cases of limb loss [1]. Vascular disease is currently the leading cause of death in the United States and approximately 84 million people are living with some form of this disease [1, 6]. Taken together, it is a sad likelihood that limb amputation and the associated burdens will only continue to increase. Currently, the treatment options for amputees is almost exclusively limited to prosthetics. Studying limb regeneration in mammals will help aid in expanding and improving therapies. Investigating the trigger(s) of endogenous mammalian regeneration is an essential step to increasing regenerative abilities and providing better therapeutic options for amputees.

Enhancing mammalian digit regeneration using targeted treatments has become a significant and feasible goal in the field of regenerative medicine [7,8**,** 9]. The Axolotl is a well-established amphibian model for regenerative research and has been renowned for its remarkable regenerative abilities. However, unlike the Axolotl, mammals have limited regenerative capabilities. Few structures, such as the digit tip, in humans, mice and other primates possess some regenerative capabilities. As the push for human limb regeneration continues, it is important to study the regenerative capabilities in a mammalian context. Mice are a clinically

relevant model for human limb regeneration because regeneration of the mouse terminal phalanx (P3) resembles clinically documented human fingertip regeneration [10,11,12].

Overview of Amphibian Regeneration

Urodele amphibians, particularly newts and salamanders, are the gold standard of vertebrate regeneration, able to regenerate many complex structures after amputation. This exceptional ability does not seem to be altered by repeat amputations or by age. In a long-term lens regeneration study, the lenses of newts were able to regenerate perfectly after being removed repeatedly over a 16-year period [13]. This astounding regenerative ability has made them a popular model for regenerative research.

After limb amputation, axolotls can regenerate a perfect replacement limb. The key stages of axolotl limb regeneration are the formation and closure of the wound epidermis, the dedifferentiation of cells to form the blastema and the proliferation of the blastema. Shortly after amputation, the wound epidermis is formed by epidermal cells that migrate from the surrounding epidermis**.** Within the wound epidermis, a group of specialized cells form and are collectively called the Apical Ectodermal Cap (AEC) **[**11]. The AEC produces Fgfs which, importantly, maintain the underlying mesenchyme in an undifferentiated and proliferative state [15]. The limb will not regenerate if the AEC is removed [16,17]. Within a few days post amputation, the blastema forms in the area between the bone stump and wound epidermis. The blastema is composed of proliferating mesenchymal stem and lineage committed cells [9]. Once formed, distal outgrowths from the blastema regenerate the lost limb.

Overview of Mammalian Regeneration

The regenerative capacity of mammals is severely limited. This limitation is underscored when compared to Urodele amphibians like the newt and the axolotl. However, the terminal phalanx (P3) of mice have the ability to regenerate after a distal amputation [11,18]. Although P3 can regenerate, the level of amputation plays a critical role in initiating successful regeneration. Proximal amputations initiate a wound healing response culminating in scar formation which results in complete loss of the amputated structure [11, 18]. However, distal amputations undergo complete regeneration and restoration of lost structures. It is not fully understood why the amputation plane has such a deterministic role in regeneration, but some studies have suggested that the conservation of the nail organ is key. Most of the nail organ is usually amputated in standard proximal P3 amputations. When the proximal bone is amputated while leaving the proximal nail organ intact, successful regeneration occurred [19,20] indicating that the nail organ is essential for successful regeneration [21].

After distal amputation, P3 progresses through a series of well-characterized and reproducible events which result in a near-perfect restoration of morphology: Inflammation/histolysis, blastema formation, and redifferentiation [18, 22**]**.

Initially, there is an inflammatory response that is characterized by an influx of macrophages, monocytes and neutrophils [23]. Macrophages have been shown to be important for regeneration. When clodronate encapsulated liposomes were used to remove macrophages, the digits failed to regenerate compared to controls [23]. Although osteoclasts and macrophages share a monocyte lineage, removing osteoclasts did not inhibit regeneration but did result in less regenerated bone volume [23]. In normal histolysis, activated osteoclasts degrades through the bone stump to form a secondary amputation which exposes the marrow cavity [18, 23].

After histolysis, the blastema forms between the bone stump and the wound epidermis [18, 24, 25]. The blastemal is composed of undifferentiated, lineage committed mesenchymal cells. Currently, the contributing sources to the undifferentiated cells of the blastema are still unknown.

During redifferentiation, the amputated bone is regenerated by osteoblasts which are derived from the undifferentiated mesenchymal cells of the blastema [11, 18]. Bone is laid down in a proximal to distal direction. Bone initially forms as bone islands which then coalesce to form the regenerate.

Together, these events result in an epimorphic regeneration response to rebuild bone, the surrounding soft connective tissue, blood vessels, epidermis, nerves and nails. While the mechanisms that control the regeneration response are not fully known, studies have suggested the wound epidermis [16,17], blood vessels [26], nail [21], and nerves [26, 27] as promising candidates. Although the events of regeneration are well characterized, how each event effects the other is not understood. Understanding the controlling mechanisms as well as the effect of one event on the other is essential to developing new therapies.

CHAPTER II

STAGING DIGITS REDUCES VARIATION IN BONE VOLUME

Introduction

People who have renovated or built a house know that timing is an inadequate measure of completeness. Initial timing estimates are usually inaccurate due to an infinite number of factors that can often be unpredictable. Referring to certain milestones, such as the completion of the foundation or the installation of the drywall, gives a more inclusive picture. This idea of staging by milestones instead of time is also a common infrastructure used in medicine. A prominent example is the use of staging for cancer. In cancer, staging is used to describe the amount of cancer a patient has in addition to the location of the cancer. Cancers of the same stage, despite the differences between patients, often have similar outlooks and can be treated similarly [28]. Therefore, cancer stage is an important consideration when a doctor decides which treatment options are appropriate. Staging is also important in basic and clinical research because it allows researchers to study cells which are similar enough to draw relevant comparisons [28].

Currently, regenerative research in P3 uses days post amputation (DPA) to group digits for comparison. However, we observed that different digits on the same DPA can have markedly different phenotypes (Figure 1). Since different phenotypes are associated with different cellular and molecular events, it is logical to presume that treating these digits the same might result in different and potentially misleading outcomes**.** In a study looking at the effects of hyperbaric oxygen on regenerating P3 digits in CD1 mice, the results showed that if hyperbaric oxygen treatment would either be effective, or refractory based the stage of P3 during application [29]. To ask quantitative questions in a more precise way, we applied the idea of staging regenerating

P3 digits in mice by using phenotypic landmarks that are easily identifiable from a µCT scan. Stages were based on the following criteria: bone smoothness, pitting, connection of the external cavity to the bone marrow, and separation of the bone fragment from the bone stump (Table 1, Figure 1).

Table 1.

Stage 1	After Amputation, smooth bone surface
Stage 2	Pitting clearly present on the bone surface, no connection of the marrow and external cavities
Stage 3	The marrow and external cavities are connected
Stage 4	Separation of the distal bone fragment from the bone stump has occurred

Table 1: Stages of P3 During Histolysis. Each stage is determined by a set of anatomical features identifiable from µCT scans. Stage 1 is characterized by a smooth bone surface with no evidence of pitting. Stage 2 is characterized by clear evidence of pitting but no connection of the bone marrow to the external cavities. Stage 3 is characterized by a connection of the bone marrow to the external cavity surrounding P3. Finally, stage 4 is characterized by a complete separation of the distal bone fragment from the bone stump. The bone fragment does not have to be ejected in order for the digit to enter stage 4.

Figure 1.

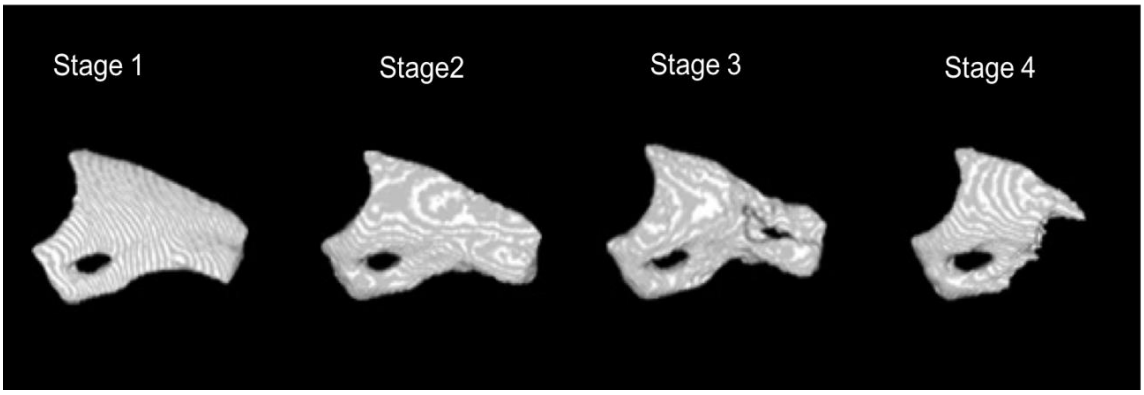

Figure 1. Stages of the Regenerating P3 During Histolysis. Digits imaged using μCT. Stage 1 is identified after amputation by a smooth bone surface. Stage 2 is identified by the presence of pitting on the exterior bone surface. Stage 3 is characterized by severe pitting and connection of the marrow cavity to the external cavities. Stage 4 is characterized by a separation of the distal bone fragment from the bone stump.

Digits were categorized as stage 1 if the bone was smooth and there was no pitting on the bone surface. The digit enters stage 2 once pitting becomes evident on the surface of the bone but the marrow cavity is not exposed. Pitting was chosen as a stage indicator because the pits are associated with activated osteoclasts. When the marrow cavity is exposed the digit is in stage 3. This was selected as a stage indicator because it links the internal marrow cavity to the external environment surrounding the bone and is easily observable on a μCT scan. Stage 4 occurs when the distal bone fragment is disconnected from the bone stump. Bone fragment ejection was chosen as a stage indicator because this is associated with blastema formation [29]. We hypothesized that staging digits would reduce the variation in bone volume compared to DPA.

Results and Discussion

We observed that the terminal phalanx of digits on the same day post amputation (DPA) could have markedly different phenotypes (Figure 2). Figure 2 shows an example of two digits taken from the same mouse on DPA 7. At the time these digits were harvested, Digit A had already

undergone a secondary amputation while Digit B was still undergoing histolysis. Since secondary amputations are associated with blastema formation, a predominantly anabolic event, it is likely that these two digits were also molecularly different as well. Therefore, it is unlikely that treating Digit A, predominately anabolic, and Digit B, predominantly catabolic, would result in a meaningful comparison. This inconsistency between phenotype and time was not unique to this mouse and was seen across the histolytic event.

Figure 2.

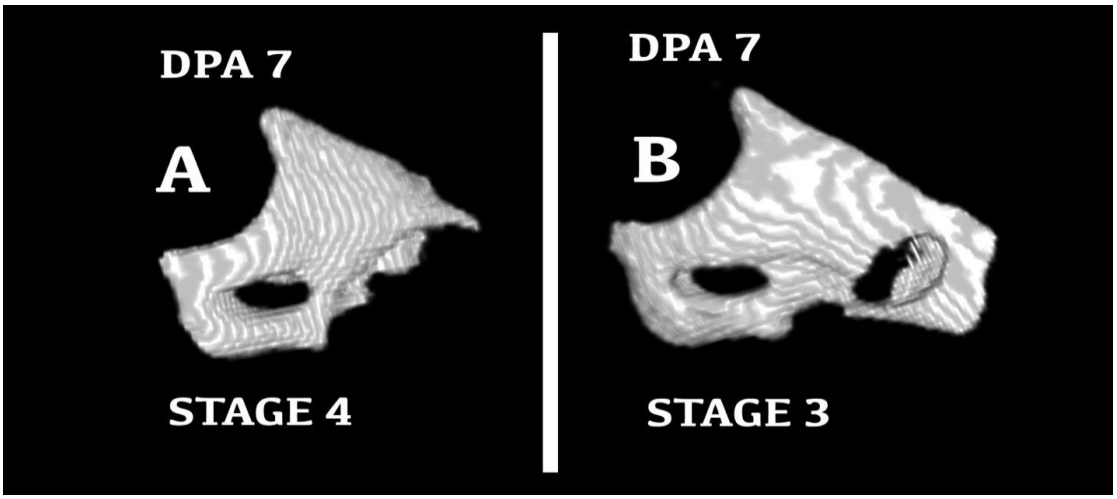

Figure 2. Comparison of Two Digits at 7 DPA. These digits were obtained from the same CD1 mouse at 7DPA and scanned using a μCT. (A) The distal bone fragment has separated from the bone stump causing a secondary amputation. (B) The distal bone fragment is still attached to the bone stump which means the digit is still in the histolytic phase and has not yet not entered the blastema phase.

Different phenotypes are commonly observed on the same DPA (Figure 3) and correlate with an increased variation in bone volume (Figure 4). Based on this variation of phenotype and bone volume, DPA it is an inadequate metric to compare regenerating P3 digits in the mouse. The variation that DPA can inject into data sets make it harder to ask precise quantitative questions that yield meaningful and replicable results.

Figure 3.

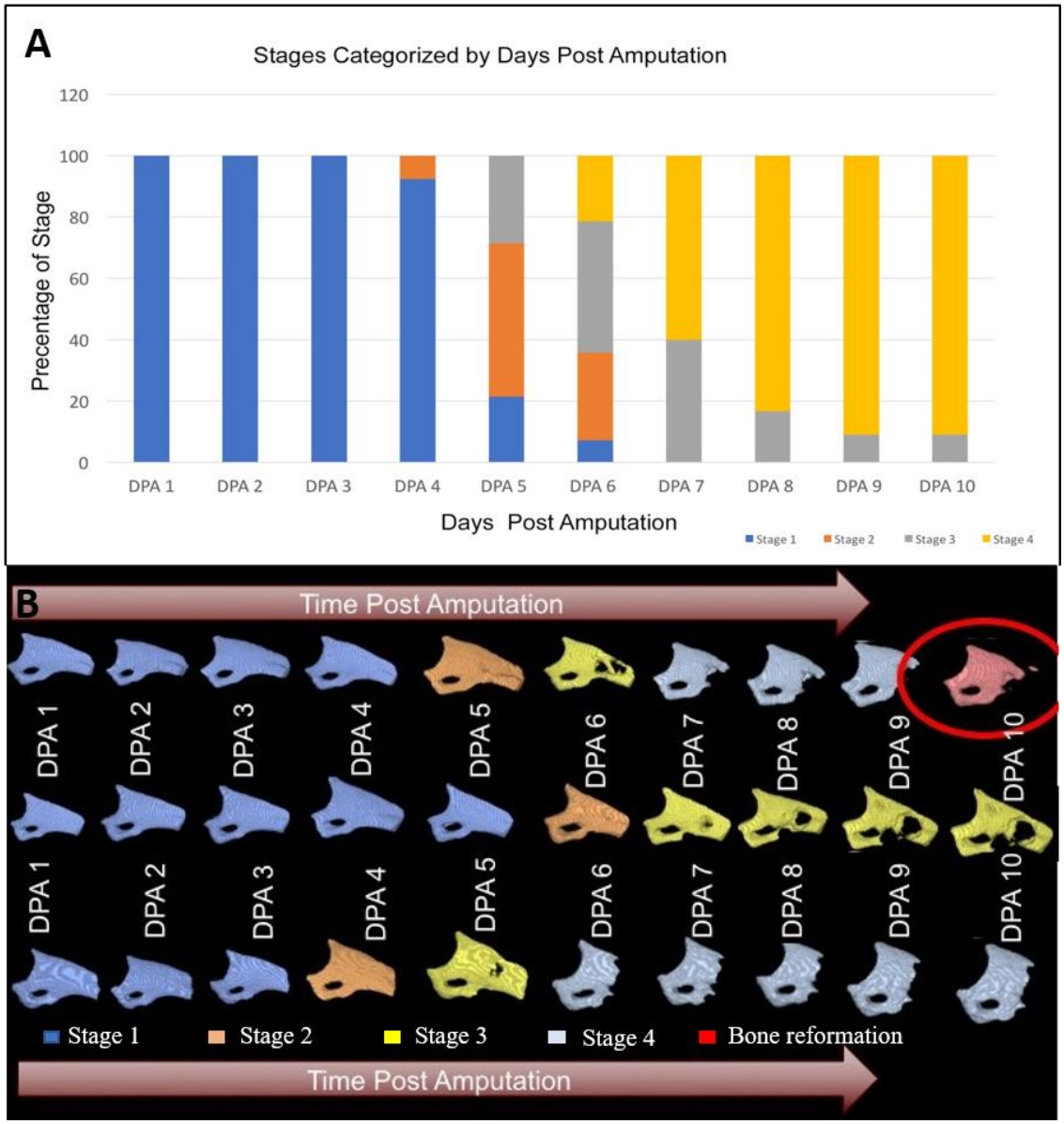

Figure 3. Distribution of Stages from DPA1-DPA10. (A) n=8 per DPA. Blue represents stage 1 and is the prominent stage for the first 4 days post amputation. Stage 2 is represented in orange and appeared between DPA4-DPA6. Stage 3 is represented in gray and appears on DPA 5 but persists through DPA10. Stage 4 appears around DPA6 and continues to appear through DPA10. There was stage overlap. DPA1-3 had the lowest overlap. DPA5-7 had the highest overlap. (B) 3 digits tracked over the first 10 DPAs of regeneration. This is a visual representation of how digits can progress through the stages of regenerations at different rates.

Figure 4.

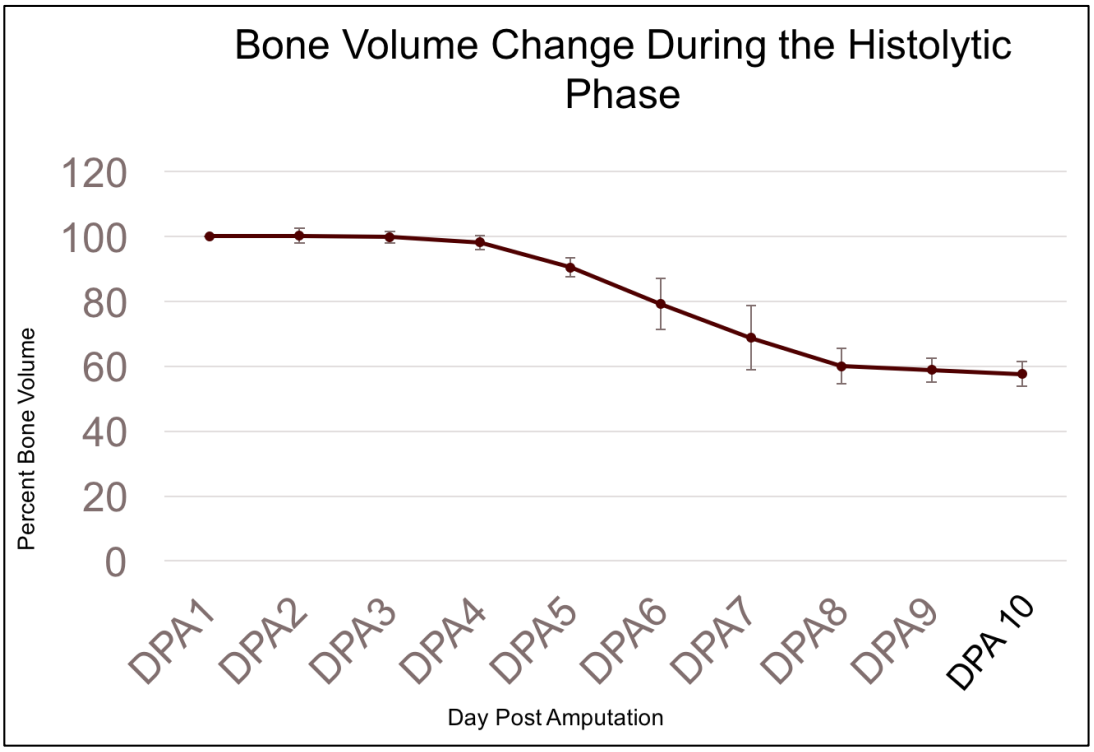

Figure 4. Bone Volume of P3 by DPA, n = 8 per DPA. 95% confidence intervals are represented for each DPA. Bone volume decreased during the first 10 days of regeneration. Bone volumes varied most during DPA 5-8. Variations of bone volumes was smallest during DPA 1-3.

Categorizing regenerating digits using our novel staging criteria minimized the variation in bone volume by DPA (Figure 5*).* This is demonstrated by a narrower 95% confidence interval, which shows that bone volume can be more accurately predicted using stage opposed to DPA. In addition to bone volume, we observed that osteoclast location was more similar based on stage than when based on DPA (Figure 6). The similar location of osteoclasts gives justification to the premise that staged digits are more similar in terms of bone volume and cellular location compared to DPA digits. Based on the results, staging digits has the potential to increase our ability to ask specific quantitative questions by providing a way to group similar digits together in a non-time dependent way.

Figure 5.

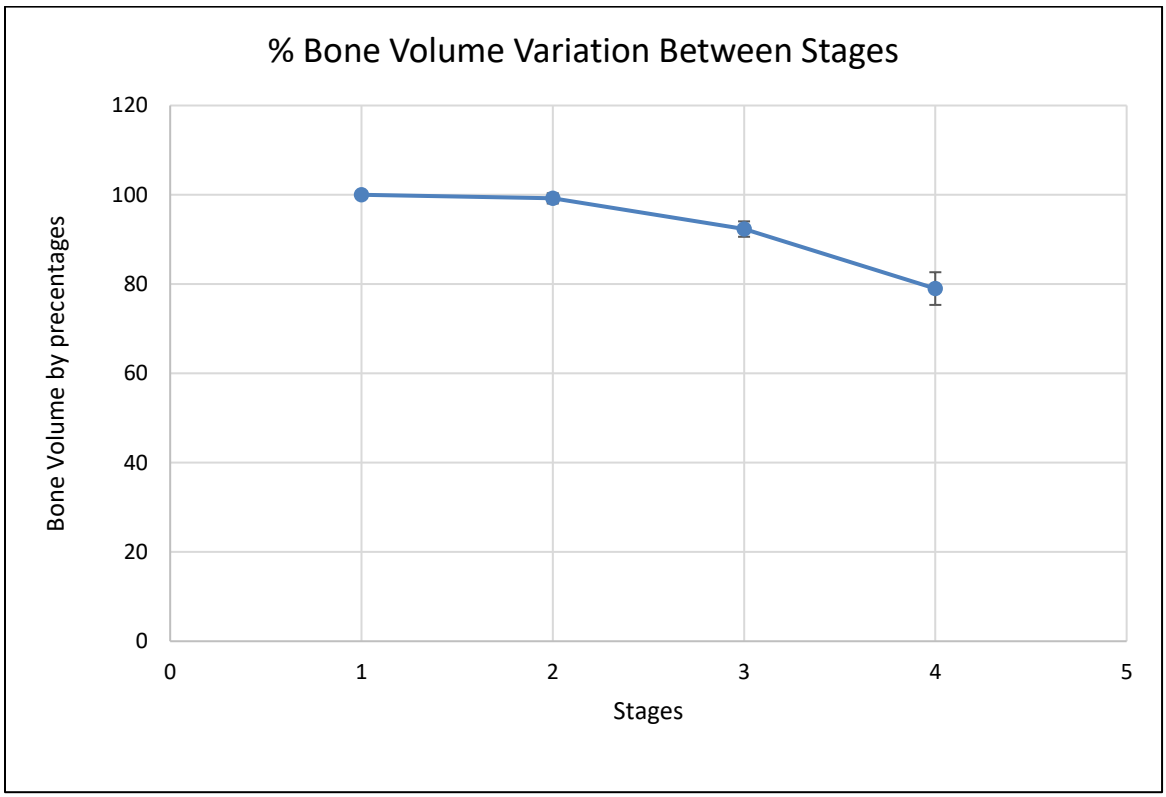

Figure 5. Bone Volume of P3 by Stage. 95% confidence intervals are represented for each stage. Bone volumes between stages are all significantly different from one another ($P<0.05$). Digits were categorized based on the established criteria. Bone volume decreased during all stages of histolysis.

Figure 6.

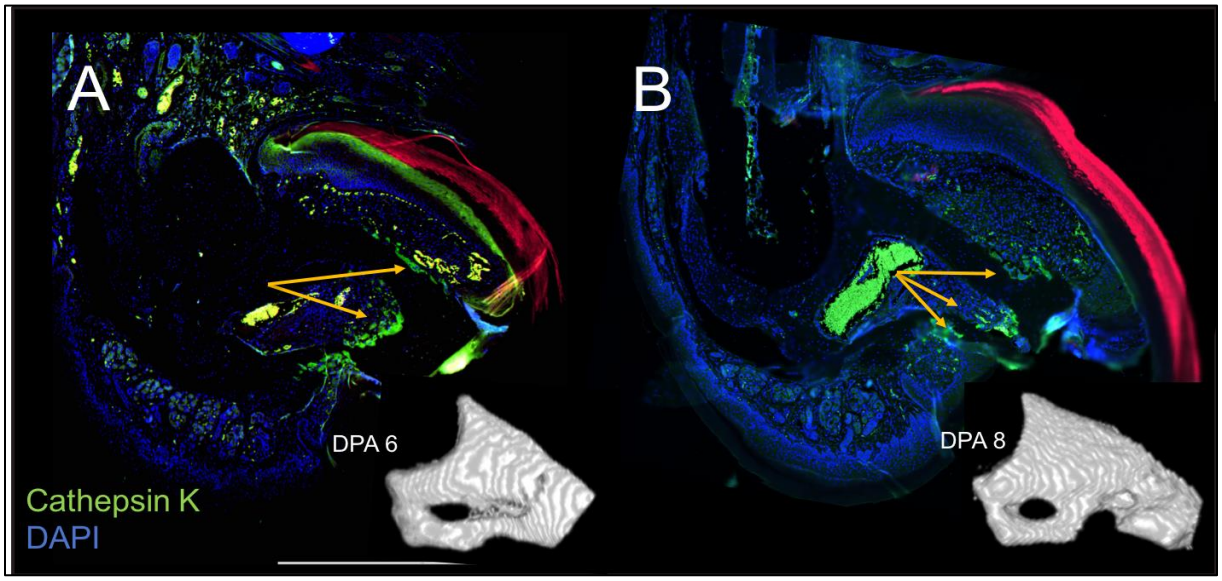

Figure 6. Osteoclast Location at Stage 3. Image shows two digits at the same stage but at two different days: DPA 6 (A) and DPA 8 (B). Arrows point to Cathepsin K + cells that are localized to the distal aspect of the amputated bone.

Materials and Methods

Amputations and Animal Handling

Adult 8-week-old female CD1 mice were obtained from the Texas Institute of Genomic Medicine (College Station, TX, USA) and were anesthetized with isoflurane inhalant anesthesia. Amputations were performed at the distal level of the second and fourth digits of the hind limbs as previously described [18]. Regenerating digits were collected daily between 1 and 10 days post amputation to encompass the degeneration phase of regeneration. All experiments were performed in accordance with the standard operating procedures approved by the Institutional Animal Care and Use Committee of Texas A&M University.

µCT

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 μm^3 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, Trabecular Thickness 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 Trabecular Thickness Plugin was used to track changes in bone thickness and the 3D viewer Plugin was used to create 3D renderings of the µCT scans and the bone thickness maps. The snapshot feature was used to create 2D images.

CHAPTER III

OSTEOCLASTS AND CELL PROLIFERATION

Osteoclasts in Regeneration

The use of staging, discussed in Chapter II, was implemented to address the relationship between osteoclast-mediated bone catabolism and bone anabolism by looking at osteoclasts and cell proliferation over the course of histolysis.

Osteoclasts are the hallmark cell of the histolytic phase. These highly specialized cells are responsible for catabolic bone degradation, removing bone matrices and initiating bone remodeling **[**18**].** Osteoclasts are responsible for degrading the distal portion of the bone stump, exposing the marrow cavity to the wound site which results in a secondary amputation [18]. Although osteoclasts are well known for their catabolic role, they are also involved in bone formation. Osteoclasts activity is intrinsically linked to increased osteoblast activity [18]. Osteoblasts are a bone anabolic cell within the mammalian P3 blastema that is responsible for regenerating the amputated bone [24, 25]. This connection between osteoclast and osteoblast activity suggests that osteoclasts could have an important anabolic role during regeneration.

Osteoclasts and osteoblasts are continuously working together to remodel mammalian bone. This remodeling is vital to biomechanical stability and mineral homeostasis [30]. The control and coordination of activity is important for normal bone physiology. Skeletal diseases characterized by a decreased bone density, such as osteoporosis, can occur when there is an imbalance of osteoclast and osteoblast activity. However, this imbalance is also transiently present during bone repair after injury [31] and bone regeneration after amputation [18]. Whether the initial imbalance that favors osteoclast activity and results in bone histolysis is important to

successful proliferation is yet to be fully explored. Exploring this relationship between osteoclasts and cell proliferation could reveal that osteoclasts have a larger role in bone anabolism than previously thought.

Cell Proliferation in Regeneration

Like the axolotl, regeneration of P3 is blastema-mediated and involves the coordination and proliferation of multiple tissues. The blastema is a heterogeneous population of undifferentiated mesenchymal cells and lineage-committed progenitor cells that regenerate the lost structures [18]. In mice, the blastema forms in the region between the open marrow cavity and the wound epidermis [18, 25]. The osteoprogenitor cells within the blastema differentiate directly into osteoblasts, forming bone by intramembranous ossification. Once differentiated, osteoblasts regenerate the digit tip by laying down woven bone in a proximal-distal direction [11, 18]. Proximal amputations, which are regeneration incompetent, do not result in a blastema. Cell proliferation is an important aspect of regeneration and the ability to control cell proliferation will be vital to manipulating the regenerative process.

Results and Discussion

Osteoclasts Spike at Stage 2 and Persist Through Histolysis

Osteoclasts are the primary cells that digest bone and are required for optimal bone health. During regeneration, osteoclasts digest the bone by secreting hydrogen ions into the resorption cavity which results in a secondary amputation [18]. Although it was found that inhibiting osteoclasts did not inhibit regeneration, it did result in a smaller regenerate and smaller blastemas [22, 23]. This suggests that although osteoclasts do not have a rate limiting role in regeneration, they may have a larger contribution to bone formation than previously thought.

Cathepsin K is a lysosomal cysteine proteinase involved in bone remodeling and resorption and is predominantly expressed in osteoclasts. The antibody to cathepsin K was used to identify osteoclasts and positive cells were counted manually. Cathepsin K+ osteoclasts were more abundant at stage 2 than at stage 1 ($P<0.05$), however, the abundance of cathepsin K+ osteoclasts did not differ between stage 2, 3 or 4 (Figure 5). These results are consistent with what is known about regeneration because degradation is occurring continuously through histolysis. Morphologically, we observed osteoclasts at the distal end of the marrow cavity before spreading to more proximal areas of the bone marrow (Figure 6).

Figure 7.

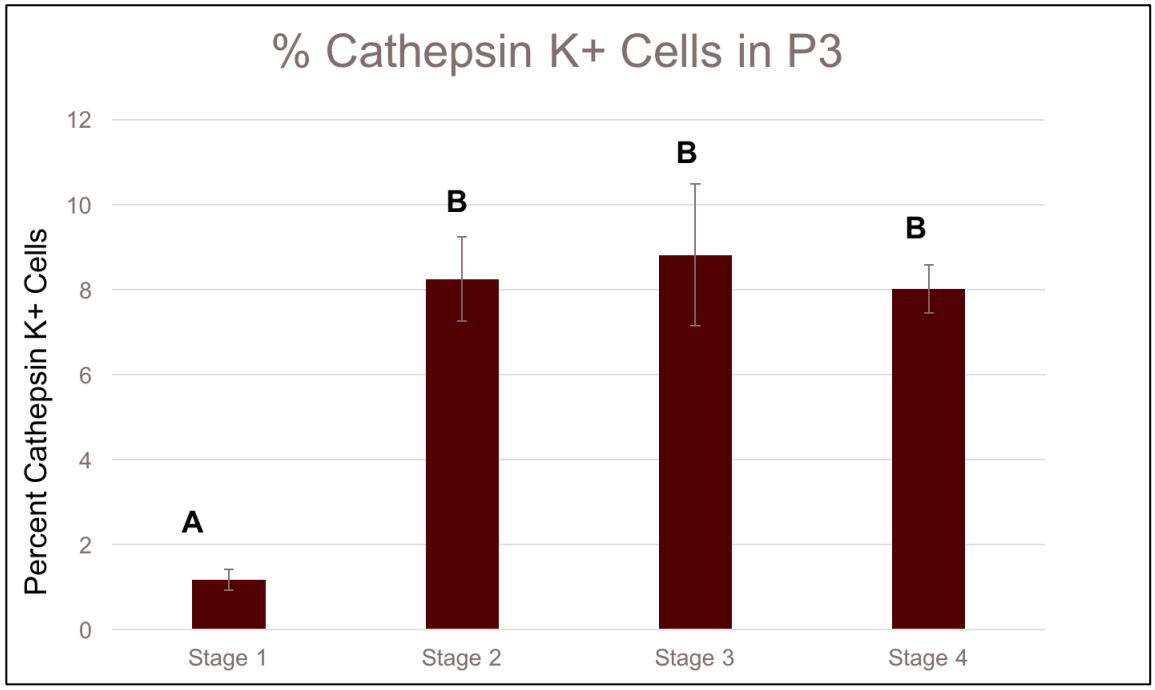

Figure 7. Percentage of Cathepsin K+ Cells. % Cathepsin K positive cells in P3 during different stages of regeneration. Cathepsin K + osteoclasts significantly increased between stages 1 and 2 ($P< 0.05$) but did not significantly change between stages 2, 3, and 4 ($P>0.05$). Standard error bars are indicated for each stage.

Figure 8.

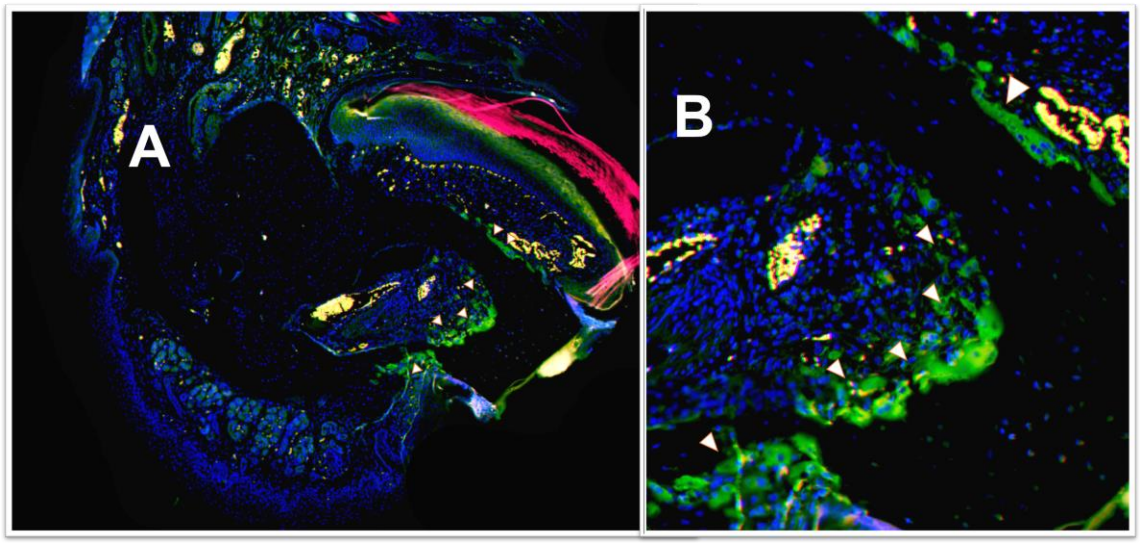

Figure 8. Cathepsin K+ Cells at Stage 3. Arrows point to the Cathepsin K+ cells observed in a regenerating digit at stage 3. Cathepsin K+ cells are observed predominantly in the distal portion of P3. Autoflouresence can be seen within the vasculature and nail bed (seen as yellow) in addition to the nail (seen as red). Image B is zoomed into a distal aspect of image A.

Cellular Proliferation Spikes at Stage 2 and Persists Through Histolysis

P3 bone growth in the mouse initiates from the blastema [18]. EdU was used to quantify cellular proliferation in the mouse P3 model. EdU incorporates into the DNA of dividing cells and is used to directly measure DNA synthesis or S-phase synthesis of the cell cycle. Positive cells were counted manually. The number of cells undergoing EdU+ Cellular proliferation was peaked at stage 2 and was sustained for the remainder of histolysis ($P<0.5$). There was no significant difference between stages 2, 3, and 4 (Figure 7). Morphologically, cells undergoing EdU+ proliferation were predominantly observed within the bone marrow (Figure 8).

Figure 9.

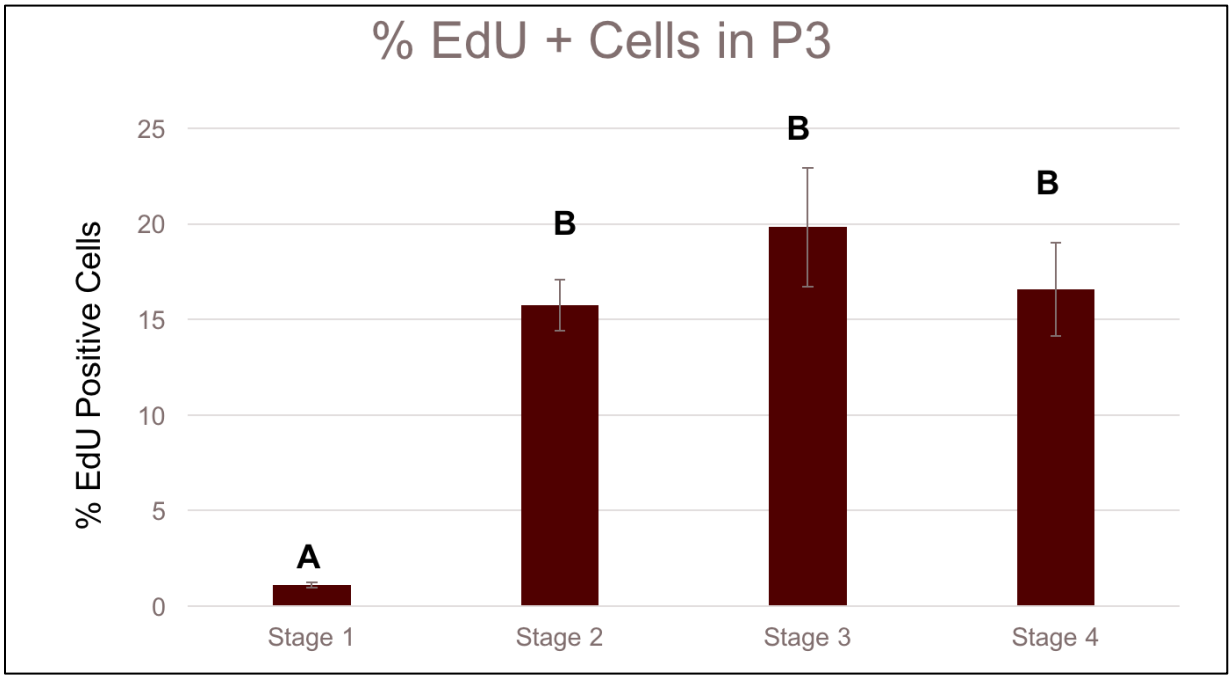

Figure 9. Percentage of EdU+ Cells. This graph shows cell proliferastion, as signaled by EdU, over the 4 stags of histolysis. EdU+ cells spike in stage 2 and are maintained through regeneration ($P < 0.05$). Stage 2, 3 and 4 are not different from one another ($P > 0.05$).

Figure 10.

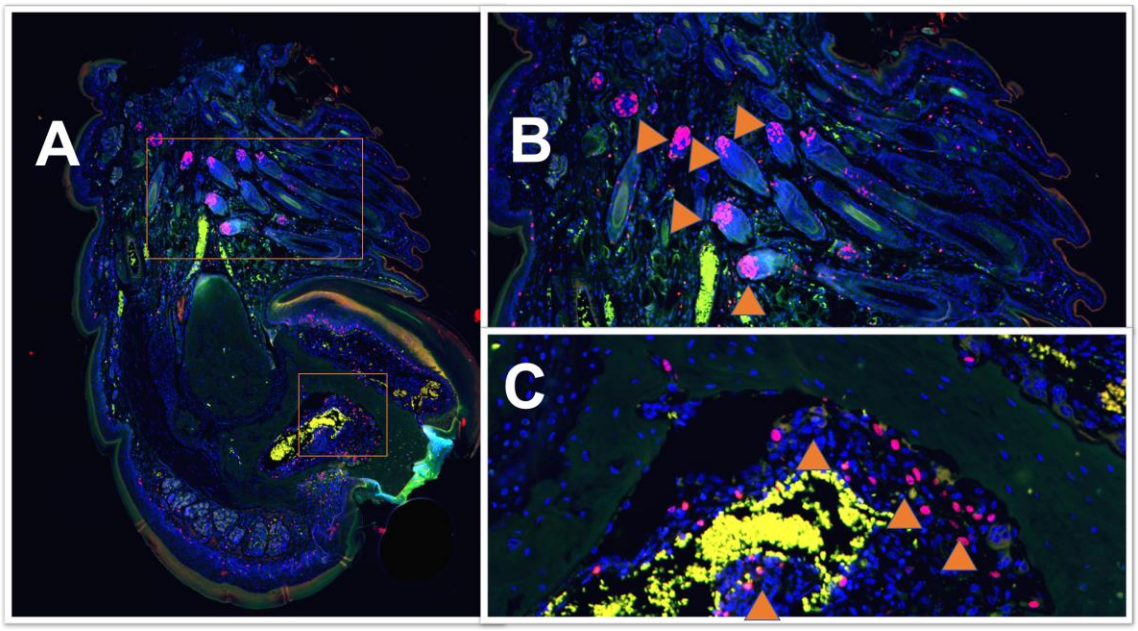

Figure 10. EdU+ Cells at Stage 3. EdU+ cells around the amputated bone are observed predominantly in the marrow region of P3. (A) Shows the full image of the amputated digit with insets shown in images B and C. (B) EdU+ cells are clearly observed in the hair follicles of the digit. (C) Arrows point to EdU+ cells within the bone marrow of P3. Autofluorescence can be seen in the bone marrow vasculature as well as the nail and nail bed.

Osteoclasts are Associated with Cell Proliferation

Both Cathepsin K + cells and EdU + cells spikes at stage 2 and are maintained throughout the duration of histolysis (Figure 11). To further examine the association between osteoclasts and cell proliferation, samples were double labeled with Cathepsin K+ cells and EdU + Cells. We observed that there was an association between Cathepsin K + cells and Edu + cells (Figure 12). The similar signal trend in addition to the signal association in samples suggests that there is a relationship between osteoclasts and cellular proliferation during regeneration.

Figure 11.

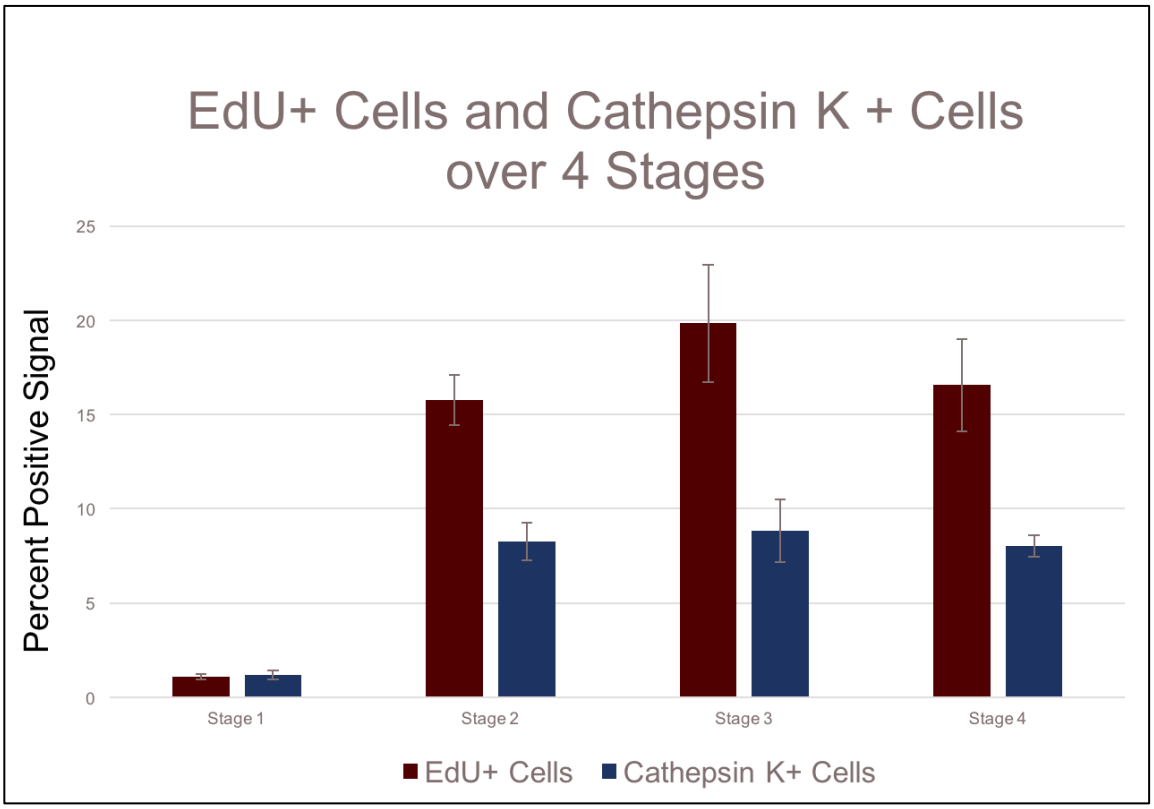

Figure 11: EdU+ Cells and Cathepsin K+ Cells Over Stages of Histolysis. This graph highlights the similar pattern of observed signal from EdU and Cathepsin K. Both EdU and Cathepsin K+ cells spike at stage 2 and are maintained throughout histolysis. Stages 2, 3, and 4 for both EdU and Cathepsin K are different from stage 1 ($P<0.05$).

Figure 12.

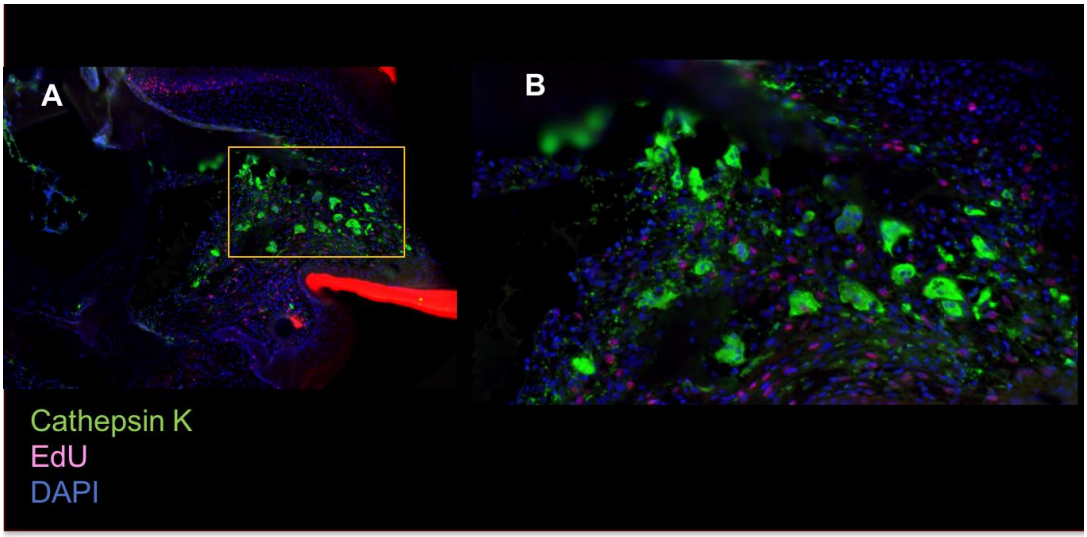

Figure 12: Cathepsin K+ Cells are Associated with EdU + Cells. (A) shows the Cathepsin K+ cell signal and associated EdU + cell signal within the mouse P3. (B) Is a closer examination of the association between Cathepsin K+ cell signal and EdU+ cell signal.

Final Conclusions and Future Directions

The terminal phalanx of the mouse is an established model for studying epimorphic regeneration in mammals [22]. The endogenous regenerative ability of this model provides a unique opportunity to study the cellular and molecular mechanisms controlling mammalian regeneration [11,12]. After an amputation injury, P3 regeneration progresses through a series of well-characterized and reproducible events: histolysis, blastema formation, and redifferentiation. Histolysis is closely linked to enhanced osteoclast activity which results in a secondary amputation by cleavage of the distal bone fragment from the bone stump [18, 32]. Degradation typically occurs in the first 10 days post amputation but can vary greatly between digits. This variation is pervasive even among digits of the same mouse. Therefore, categorizing digits by DPA ignores the intricacies of this process and can have a large effect on treatment success and resulting conclusions. Although not addressed directly, examples of this can be found in the

literature. For example, oxygen is a highly dynamic and important aspect of regeneration which has been shown to have a considerable influence on osteoclast activity and wound healing [32]. However, digits at different stages treated with hyperbaric oxygen on the same DPA resulted in the digit either responding or being refractory to treatment [32]. To improve the way digits are categorized and compared, we developed a *in vivo* staging protocol for regenerating P3 digits using phenotypic markers easily identifiable from a µCT scan. Although the idea of staging is not novel, this is the first time that a *in vivo* staging protocol has been developed for the mouse digit regeneration model. We found that staged digits had similar bone volumes and we observed that osteoclast location was similar among digits from the same stage. Based on our results, we believe staging digits based on µCT scans offers an *in vivo* solution to minimize potentially misleading data. Going forward, staging digits has the potential to make a huge impact because it will allow researchers to ask and answer targeted quantitative questions more effectively.

We applied this staging principle to examine the association between osteoclasts and cellular proliferation during the histolytic phase. We found that osteoclast activity, indicated by Capthepsin K+ staining, and cell proliferation, indicated by EdU staining, followed a similar pattern of activity. Both osteoclasts and cell proliferation spiked at stage 2 and were maintained for the duration of histolysis. In addition, we observed an association between osteoclasts signal and cell proliferation signal. The similar pattern and association of signals suggests that osteoclasts could have an anabolic role in P3 digit regeneration. Although pairing the roles of bone catabolism and bone anabolism seems counterintuitive, this could explain past studies that found osteoclast inhibition resulted in smaller blastemas [22].

In bone development and fracture repair, blood vessels transport progenitor cells to the site of injury [11,12]. However, the blastema in the regenerating P3 is avascular [18, 37], suggesting that progenitor cells likely come from local sources. This supports the notion that osteoclasts-mediated degradation releases local progenitors, potentially from the periosteum, which contribute to the blastema.

Osteoclast activity initiates activation of some osteoprogenitors within the mammalian model [18]. Osteoprogenitors contribute to the mammalian P3 blastema and the regenerated bone [25, 24]. Several osteoprogenitors have been shown to reside in the periosteum [31, 33,34]. When the periosteum is removed from the second phalange in the mouse, the bone fails to mount a regeneration attempt [31]. Similar results were found when the periosteum was removed from P3 [Dawson, et al., unpublished]. Therefore, osteoclast-mediated bone degradation during histolysis could play an anabolic regeneration by freeing osteoprogenitors from the bone matrix, which could allow for larger blastemas and increased proliferation. This is supported by our observations that Cathepsin K+ signal was associated with EdU and by studies that have shown that attenuated osteoclast activity is associated with the formation of smaller blastemas and smaller bone volumes [22,23]. These conclusions are also consistent with other injury models showing that osteoclastogenesis and osteoclast activation triggers proliferation and mobilization of stem cells associated with the endosteal niche of the bone marrow [35,36].

The bone is a reservoir of calcium, collagen and growth factors. As osteoclasts break down bone, these components are released into the surrounding environment. Ionic calcium has become a strong candidate for amplified bone regeneration due to its positive relationship with BMP-2. In the critical-size defect model, Ionic calcium has been shown to increase the phosphorylation of SMAD1, which correlates with the induction of BMP-2 and

BMP-4 gene expression [38]. The amplification of the BMP-2 signal by ionic calcium increases bone formation from marrow-derived mesenchymal stem cells *in vivo* [38]. In addition to enhanced BMP-2 induction within the critical size defect model, ionic calcium has also been shown to enhance the effects of Osteocalcin, RunX2, Osterix to promote overall bone regeneration.

In mice, BMP-2 is known increase osteogenesis, even in regeneration incompetent structures. BMP-2 is required for the ignition of periosteal proliferation after bone injury and is upstream of the BMP4 and BMP7 feedback loop [40] and all three of these growth factors have been shown to induce digit regeneration [8,41,7,42]. BMP-2 can increase cellular proliferation and bone growth of in the second phalangeal element and proximal amputation of P3 [8]. In addition, BMP-2 can stimulate a new endochondral ossification center which suggests the ability of BMP-2 to relay positional information [8]. Therefore, increasing ionic calcium in the might function to increase bone regeneration through enhancing the induction of molecules such as BMP-2.

The catabolic role of osteoclasts could have an anabolic consequence in P3 regeneration because of their role in releasing osteogenic components. As osteoclasts degrade P3, mesenchymal-derived stem cells from the bone marrow, osteoprogenitors from the periosteum, growth factors and components such as ionic calcium are released. As ionic calcium is released, it could increase osteogenesis of the mesenchymal-derived stem cells by amplifying the BMP-2 signal via the SMAD1 pathway. The release of osteoprogenitors from the periosteum would also increase proliferation and bone growth. In addition, osteoclasts are known to release RANKL which stimulates osteoblast proliferation. This would explain why we see an association of Cathepsin K+ and EdU+ cells. This could explain why attenuating osteoclasts stunts but does not

halt the regeneration attempt and why removing the periosteum halts the P3 regeneration attempt.

Limb regeneration is a process of essential transient events that direct the regrowth of bone and the surrounding soft tissue. As human regeneration becomes the ultimate quest for many in the field of regeneration, it is important to understand these events *in vivo*. Staging digits during the degenerative phase is an *in vivo* method that allows us to reduce the variation in bone volume. We applied this staging principle and saw a delayed association between cellular proliferation and osteoclasts during degeneration. Taken together, this suggests that degradation might be a contributor to increased cell proliferation during digit tip regeneration.

Future experiments should continue to validate and expand upon the mouse digit regeneration stages. This includes doing looking at how accurately stage predicts cell location and quantity compared to DPA. Re-analyzing past experiments using the staging method will also be important. Continuing to stage regenerating P3 during the the blastema and redifferentiation events will also be an essential future step to complete the staging method.

Materials and Methods

Amputations and Animal Handling

Please refer to "Amputations and Animal Handling" in Chapter 2.

Tissue Collection and Histology

Mice were euthanized via cervical dislocation on the designated day post amputation after anesthesia with isoflurane inhalant. The terminal phalanx of the second and the fourth digits

were removed from the hind limb and fixed in zinc-buffered formalin (Z-fix; Anatech, Battle Creek, MI, USA) for 24 hours. Decalcification of bone was performed using a formic-acid-based decalcifier (Decal I; Surgipath, Richmond, IL, USA) for 24 hours. After decalcification, all samples were processed for paraffin embedding using a Leica TP 1020 Processor (Leica, Buffalo Grove, IL, USA). Cells undergoing proliferation were identified using a Click-iT EdU cell proliferation assay (Invitrogen, Carlsbad, CA, USA), based on an analogue of bromodeoxyuridine: EdU (5-ethynyl-2′-deoxyuridine) in accordance with the manufacturer's protocol. Fluorescent images were captured using an Olympus VS120 Virtual Slide Microscope (Olympus, Center Valley, PA, USA). Adobe Photoshop CS6 (Adobe Inc, San Jose, CA, USA) was used to generate the final figures.

μCT

Please refer to "μCT" under the Materials and Methods section of Chapter 2.

2-D Videos

3-D bone renderings of µCT files were created using the BoneJ Optimize Threshold Plugin for ImageJ. Snapshots of the rendering were captured and saved in a Tag Image File Format(TIF), a format used for exchanging raster graphics between application programs. The TIF files were transferred to Adobe Photoshop CS6 (Adobe Inc., San Jose, CA, USA) for editing. Each TIF file was opened as a new layer chronologically. Then the orientation and size of each layer was matched. Each layer was saved as a new image and opened as a stack in ImageJ. The stack was saved as an Audio Video Interleave (AVI) file, a file type used to store video and audio information in a single file.

Fluorescent Immunohistochemistry

Immunofluorescent staining was performed on deparaffinized and rehydrated sections with specific primary antibodies: Cathepsin K (Abcam, San Francisco, CA, USA) and EdU (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. The primary antibody was visualized using Alexa Fluor secondary antibodies (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. Sections were stained with the nuclear dye, DAPI (Fisher Scientific, Waltham, MA, USA), per manufacturer's instructions. Positive signal was quantified by manually counting cells within the marrow cavity and immediately adjacent to the bone surface. The percentage of positive signal was calculated using the ratio of total Cathepsin K, or EDU+ area per total DAPI+ area.

Statistics

For statistical analysis, digits were categorized based on stage. Statistically significant differences were determined using unpaired *t*-tests with two-tailed distributions using Excel. A value of $p < 0.05$ was deemed statistically significant.

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