REGENERATIVE CAPACITY IN A MOUSE MODEL FOR ACCELERATED AGING

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

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Advanced age is the major underlying risk factor for many chronic and debilitating diseases, such as cardiovascular disease, metabolic syndrome, cancer, and osteoporosis. One driving factor of the aging process is the loss of regenerative capacity. However, the effects of aging on regenerative power are mostly studied using isolated stem cells, or single tissue types such as the intestinal lining. Therefore, very little information is available about how different cell types interact during regeneration, and how regenerative events are timed.

Similar to the axolotl or salamander limb, the mouse and human digit tip are capable of epimorphic regeneration, which is the complete and near-perfect replacement of an amputated multicellular structure. To date, the effects of aging on this response are unknown. In order to study this age-related decline of regenerative power, the digit tips of a mouse model of Hutchinson-Gilford progeria syndrome (HGPS), a disease of accelerated aging, were analyzed at different time points in the regenerative process.

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NOMENCLATURE

HGPS	Hutchinson-Gilford Progeria Syndrome
BL6	C57BL/ 6 Mice
wt	Wild Type
DPA	Days Post Amputation
µCT	Computer-assisted microtomography
BMP9	Bone morphogenic protein 9
BMP2	Bone morphogenic protein 2
DAPI	4′,6-diamidino-2-phenylindole
CatK	Cathepsin K
Osx	Osterix
P3	Third/tertiary/terminal digit phalanx
ROS	Reactive oxygen species

CHAPTER I

INTRODUCTION

Regeneration

All animals possess the capability to build all the necessary systems and structures of a multicellular organism from a single cell. Early in development, a mammalian fetus can rebuild almost any damaged tissue structures without scar formation, but these regenerative capabilities are generally lost by the end of sexual development.[1] This absence of regenerative ability does not imply that the cellular mechanisms for rebuilding damaged body systems are irreversibly eliminated; rather, it is thought that regeneration is superseded by other wound healing processes such as fibrotic healing and scar formation.[2] For example, a non-regenerating species of Planarians has been induced to regenerate by altering only one signaling pathway.[3]

Epimorphic Regeneration

Epimorphic regeneration is the process of rebuilding damaged complex body systems and tissues in which a blastema, a heterogenous mass of undifferentiated proliferative cells containing multipotent stem cells, forms at the site of injury.[4] This process most famously occurs in urodele amphibians such as the salamander[5] and axolotl[6] after amputation of the limb. Although mammals do not possess the same regenerative capabilities as salamanders, the mouse digit tip exhibits promising similarities to urodelean epimorphic regeneration, including the formation of a blastema.[7] Since clinical reports have documented cases of regenerative potential in the digit tips of children and young adults, the regenerating mouse digit is a useful platform to

discover mechanisms of regeneration in both mice and humans, with the ultimate goal to induce regeneration at non-regenerative sites.[8i]

Regenerative Medicine

One of the main goals of regenerative medicine is to enhance or activate wound healing and regeneration[9] through the combination of engineering and life science principles.[10] Current approaches involve injecting stem cells into nonregenerative wound sites,[11] modulating the individual's immune system to create a more regeneration-permissive environment,[12] and implanting biocompatible tissue scaffolds mimicking native extracellular matrix to direct cell behavior and contribute to the mechanical structure and function of nearby tissue.[13] Introduction of such sophisticated and extensive procedures into the patient's body may prove to be an expensive, invasive, and surgical skill-dependent endeavor which could elicit immune rejection of implanted foreign cells and materials.

A novel approach derived from the field of regeneration biology aims to stimulate endogenous multicellular regeneration mechanisms with a simple and minimally invasive procedure to fully regrow damaged or missing body parts.[14] This idea stems from the fact that the digit tips of both humans and mice are capable of completely regenerating after amputation without any medical intervention.[15] The replacement structure is nearly identical to the lost digit tip, indicating some retention of regenerative power from embryonic development. Building on regenerative mechanisms in the mouse digit tip, it has been found that BMP2 treatment of non-regenerative, proximal digit amputation wounds stimulates bone regeneration. Furthermore, another member of the BMP family, BMP9, stimulates the regeneration of a synovial joint, and a carefully timed combination of BMP2 and BMP9 treatment resulted in the regeneration of bone

and a joint. This indicates that it is possible to induce a regeneration response from a non-regenerating amputation wound, at least in young animals.[16] It is likely that aged tissues will require different regimens for successful induced regeneration.

Aging

The term "aging" most generally refers to the changes that take place during an organism's life, the rate of which vary widely.[17] These changes may be visual, such as wrinkling skin or graying hair,[18] and they lead to an increased risk of mortality,[19] such as changing blood pressure,[20] respiratory cycles,[21] vision, [22], auditory system, function of the central nervous system,[23] and the musculoskeletal system, leading to frailty.[24] Many theories for the causes of aging exist (such as the free radical theory of aging discussed below), but unified theories encompassing genetic and environmental determinants and stochastic elements are becoming increasingly accepted, highlighting that there is not one single cause for aging, but that aging of is driven by a complex network of events that vary widely between species and individuals and culminate in ultimate decay.[25]

Reactive Oxygen Species

One of the oldest and most popular theories of aging, the free radical theory of aging, is taught in most university level introductory biology or physiology classes. Reactive oxygen species (ROS), or free radicals, are highly reactive molecules which attack and damage the cell's DNA or other important complex molecules such as proteins or lipids. In the case of DNA, oxidative damage leads to replication errors within the genome and thus drive the aging process via accumulation of somatic mutations, especially in mitochondria, which are the source of

reactive oxygen species, and aberrant tertiary protein structure.[26] The prediction of the radical theory of aging is that neutralization of ROS would decelerate the aging process. However, an increasing wealth of data pointing to the trend that inhibiting the formation or neutralizing these free radicals with antioxidants has little to no effect on slowing the aging process.[27] Therefore, other mechanisms must exist which more directly control aging, with parameters that may be modified to change the rate at which aging proceeds. One such mechanism that has recently gained momentum is cellular senescence.

Senescent Cells

Prior to around 1961, there was a sort of scientific consensus that individual cells were immortal and could multiply indefinitely in vitro. However, based on L. Hayflick's work, mammalian cells were demonstrated to have a finite capacity for cell division, known as the "Hayflick Limit".[28] After reaching the Hayflick limit, cells assume a state that is called "replicative senescence". Senescent cells (SNCs) are unable to contribute to tissue repair through proliferation, but are resistant to apoptosis and highly metabolically active, secreting factors that recruit inflammatory cells, so-called SASP factors.[29] Accumulation of SNCs in tissues affect function by driving fibrosis, tumorigenesis, and inhibition of stem cell function,[30] which can be directly linked to characteristics of chronological aging.[31] While critics considered cellular senescence a cell culture artifact that is irrelevant in vivo, it has recently been shown that selective elimination of SNCs from laboratory animals extends the time period which they live without chronic diseases of aging (also called the "healthspan") such as atherosclerosis, osteoarthritis, renal dysfunction, and sarcopenia. These findings have given way to the development of so-called senolytics, drugs that selectively remove senescent cells and therefore

prevent the onset of abovementioned age-associated diseases, and therefore may extend healthspan in humans in the near future.[32]

Aging Biomarkers

Within a sample of individuals with similar chronological age, there still is a considerable variation in the extent of disease and functional impairment risk. Since the progression of aging is characterized by the accumulation of degenerative processes in biological pathways rather than just the passage of time, identification of a specific set of biomarkers could more accurately describe an organism's progression through the process of aging, and help with evaluation of future anti-aging interventions.[33] The definition of an "aging biomarker", according to the American Federation of Aging Research, has four components: it must predict the rate of aging, monitor a basic process that underlies the aging process, be able to be tested repeatedly without harming the person, and be applicable to humans and laboratory animals.[34]

Considering the numerous intertwined biological pathways which could each have some measurable effect on an organism's functional age, one novel way of identifying key, easily attainable biomarkers is by using a deep learning neural network analyzing trends present in large phenotypic datasets.[35] This technique has been employed to find biomarkers for aging present in a complete blood cell measurement, a well-established, relatively easily attainable laboratory analysis protocol used in clinical and biomedical research applications.

Reviewing empirical data without a deep neural network, studies in the past have shown numerous other biomarkers of aging, some of which are even easier to gather than a blood sample. Functional parameters such as walking speed, chair stand, standing balance, grip

strength, body mass index, and muscle mass have been determined to be adequate biomarkers of aging which predict patient mortality, risk for cardiovascular disease, or cognitive decline.

Progeria

Initiating multicellular repair mechanisms may prove more challenging as the individual gets older, since aging is associated with higher levels of tissue disrepair and dysfunction. Such differences in physiology must be accounted for before biological principles can be applied clinically. One useful way to model advanced age is with Hutchinson-Gilford progeria syndrome (HGPS). This syndrome is caused by a point mutation in the LMNA gene, specifically, in the codon for amino acid 609, which causes afflicted individuals to age at an accelerated rate.[36]

In the study of how aging impacts the regenerative ability of mammals, analyzing how fast amputated digit bone structures grow back provides an overview of how the regenerative process proceeds. For mouse digit regeneration, if the bone does not completely regenerate, or does not regenerate at all, then it can be assumed that the process of aging would also inhibit induced epimorphic regeneration if not accounted for, and regenerative strategies will have to be adjusted for elderly patients.

CHAPTER II

METHODS

This project entailed raising C57BL/6 LmnaG609G mice to 2 months of age[37] before amputating about 20-30% of the distal phalanx, analyzing differences in bone formation using longitudinal, in vivo computer-assisted microtomography (μCT), collecting digit samples at various timepoints after amputation, and immunohistochemical staining (IHC).

Mice Preparation

Raising and weaning

Two groups of mice were raised concurrently: C57BL/6 wildtype mice to establish the control group, and C57BL/6 LmnaG609G mice to establish the experimental group. Both groups were given equal access to sterile food and water while being housed as a litter in the same cages as their parents. At one month of age, each litter was weaned and organized so males were housed with other males of their corresponding litter and females were housed with other females of their corresponding litter.

Distal digit tip amputation

At two months of age, all mice in the control group and experimental group had about 20-30% of their second and forth hind digit terminal phalanx amputated. This was done by hand using a sterile scalpel after the mice were anesthetized using inhaled isoflurane.

Computer-assisted microtomography (μCT)

For μCT analysis, the second and fourth hind digit tip on each paw of each mouse was scanned to set a normal starting bone volume. After amputation, the same digit tips were scanned at 3-7-day intervals to track regeneration, until 56 days post amputation (DPA). These time points were 1, 7, 10, 14, 17, 21, 28, 35, 42, 49, and 56 DPA. Each digit scan was then processed using ImageJ to yield a measurement for bone volume at each time point.

Immunohistology analysis

For IHC analysis, the second and fourth amputated hind digits were retrieved at 7, 10, and 14 DPA and analyzed for quantities of osteoclasts and osteoblasts. Retrieved tissue samples were fixed in zinc-buffered formalin, decalcified, mounted in paraffin, and serial sectioned to a thickness of 4μm per section. These sections were attached to microscope slides and immunohistochemically stained for cell nuclei (marker: DAPI), osteoclasts (marker: cathepsin K) or osteoblasts (marker: osterix)[38], and proliferating cells (marker: incorporation of the uridine analog EdU).[39]

Quantification of results

In order to generate numerical data, images of the sections were taken at 20x magnification using a Virtual Scanning microscope (Olympus). The obtained images were then quantified using ImageJ by assessing the amount of fluorescence attached to cell nuclei, osteoclasts, osteoblasts, and proliferating cells. For sections stained with DAPI, osterix, and EdU, the number of cells fluorescing osterix positive and EdU positive were recorded. These numbers were then normalized by the number of DAPI positive cells present, resulting in data

showing the percentage of cells which were osteoblasts and the percentage of cells which were proliferating. For sections stained with DAPI, cathepsin K, and EdU, the number of cells fluorescing cathepsin K positive were recorded. These numbers were then normalized by the bone perimeter of the third phalanx, resulting in data showing the fraction of bone perimeter covered by osteoclasts.

CHAPTER III

RESULTS

Computer-assisted microtomography (µCT) data

Data generated through µCT of the second and fourth hind digits found a delay in both the bone resorption and bone formation regenerative events of the P3 bone in mice with HGPS mutation as compared to wildtype mice. This data is displayed in figure 1 below. The point of lowest absolute bone volume occurs at 10 DPA for wildtype mice while occurring at 14 DPA for mutant mice. After this time point, absolute bone volume begins to increase, representing the bone formation stage of the regeneration process.

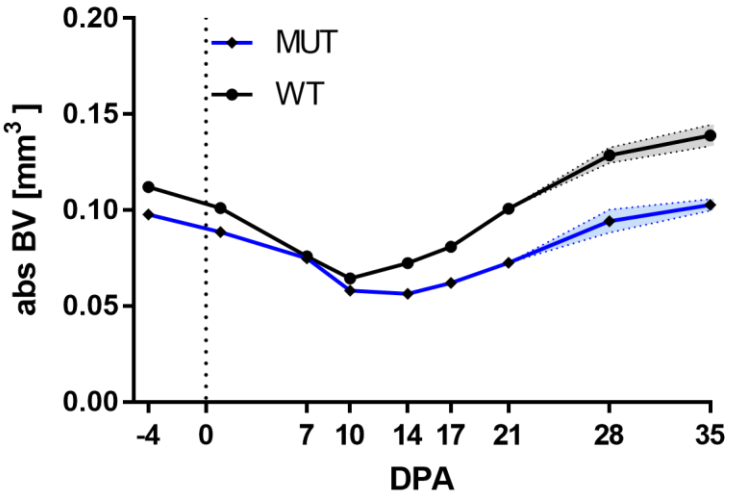

Figure 1 above displays the relationship between absolute bone volume measured in mm^3 and time after amputation measured in days post amputation for both the control and experimental group of mice. The shaded area around the plot outlined with dotted lines indicates the range of a 95% confidence interval at each time point.

Treating each time point as its own separate data set, table 1 below is a table displaying statistical measures of absolute bone volume data obtained from wildtype and HGPS mice. Similar to figure 1, there appears to be a significant difference in absolute bone volume at all time points except for 7 dpa.

Table 1 Statistical Measures of HGPS and Wildtype Mice absolute bone volume data

DPA	Wildtype Mean	Wildtype Standard Deviation	Wildtype Number of Data Points Sampled	HGPS Mean	HGPS Standard Deviation	HGPS Number of Data Points Sampled	P-value
-4	0.1121	0.0103	76	0.09769	0.0078	80	<0.0001
1	0.1011	0.0102	76	0.08851	0.0083	80	<0.0001
7	0.7600	0.0118	76	0.07498	0.0087	80	0.5367
10	0.0644	0.0086	60	0.05803	0.0113	72	0.0009
14	0.0724	0.0110	64	0.05638	0.0115	64	<0.0001
17	0.0810	0.0095	56	0.06204	0.0085	52	<0.0001
21	0.1008	0.0089	48	0.07254	0.0093	48	<0.0001
28	0.1286	0.0131	44	0.09427	0.0206	48	<0.0001
35	0.1389	0.0154	32	0.1028	0.0097	40	<0.0001

Note: P-value is calculated using the Student's t-test with Holm-Sidak correction, assuming that the standard deviation is not consistent.

Taking the first derivative of the data shown in figure 1 above yields figure 2 below, depicting the rate of bone resorption and formation between each of the time points.

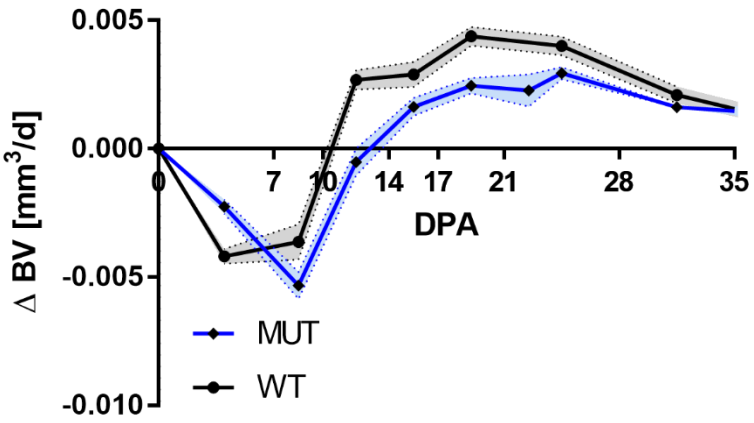

Figure 2 above displays the relationship between rate of bone volume change measured in mm^3/day and time after amputation measured in days post amputation for both the control and experimental group of mice.

Treating each time point as its own separate data set, table 2 below is a table displaying statistical measures of rate of bone volume change data obtained from wildtype and HGPS mice. Similar to figure 2, there appears to be a significant difference in rate of bone volume change at all time points except for 38.5dpa.

Table 2 Statistical Measures of HGPS and Wildtype Mice rate of bone volume change data

DPA	Wildtype Mean	Wildtype Standard Deviation	Wildtype Number of Data Points Sampled	HGPS Mean	HGPS Standard Deviation	HGPS Number of Data Points Sampled	P-value
4	-0.0042	0.0013	76	-0.0023	0.0012	80	<0.0001
8.5	-0.0036	0.0029	68	-0.0053	0.0022	72	0.0007
12	0.0027	0.0015	64	-0.0005	0.0021	64	<0.0001
15.5	0.0029	0.0017	48	0.0016	0.0013	52	0.0004
19	0.0044	0.0013	48	0.0025	0.0011	48	<0.0001
24.5	0.0040	0.0012	44	0.0029	0.0008	44	<0.0001
31.5	0.0021	0.0009	32	0.0016	0.0005	40	0.0297
38.5	0.0010	0.0008	32	0.0013	0.0010	32	0.2587

Note: P-value is calculated using the Student's t-test with Holm-Sidak correction, assuming that the standard deviation is not consistent.

Immunohistolochemistry analysis

Osterix Staining

Immunohistochemical staining using a primary antibody for the protein osterix (Osx) was employed to identify the presence, prevalence, and possible contribution of osteoblasts to the regenerative process. Figure 3 below shows the results of the histology on representative images for each time point, suggesting that osteoblastic quantity sharply increases in wildtype mice around 14dpa while no such increase exists for the HGPS mice at 14dpa.

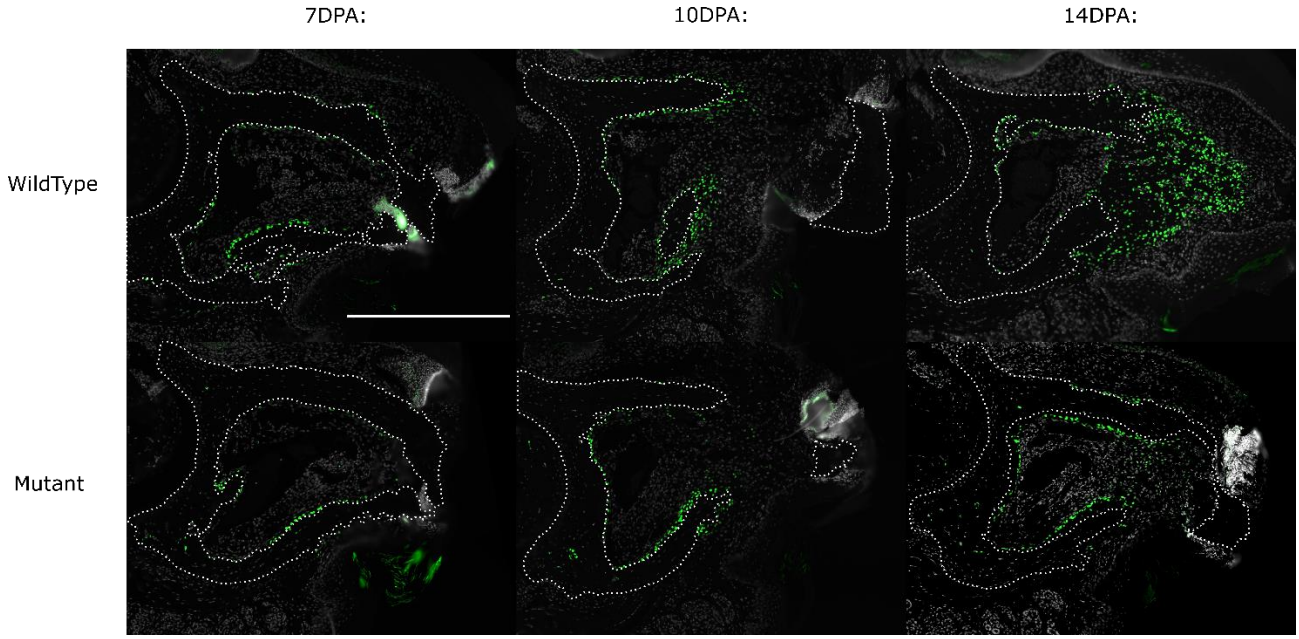

Figure 3 above shows 20x serial sectioned digit tips stained using an Osx primary antibody fluorescing in green and DAPI fluorescing in grey. The white scale bar in the top left-hand section has been set to 500 µm, and the P3 bone has been outlined in a white dotted line in each section image.

Cathepsin K Staining

Furthermore, immunohistochemical staining using a primary antibody for the protein cathepsin K (CatK) was employed to identify the presence, prevalence, and possible contribution of osteoclasts to the regenerative process. Figure 4 below shows the results of the histology on representative images for each time point, suggesting that osteoclastic quantity sharply decreases in wildtype mice around 10 dpa while no such decreases exists for the HGPS mice at any time point accounted for in this investigation.

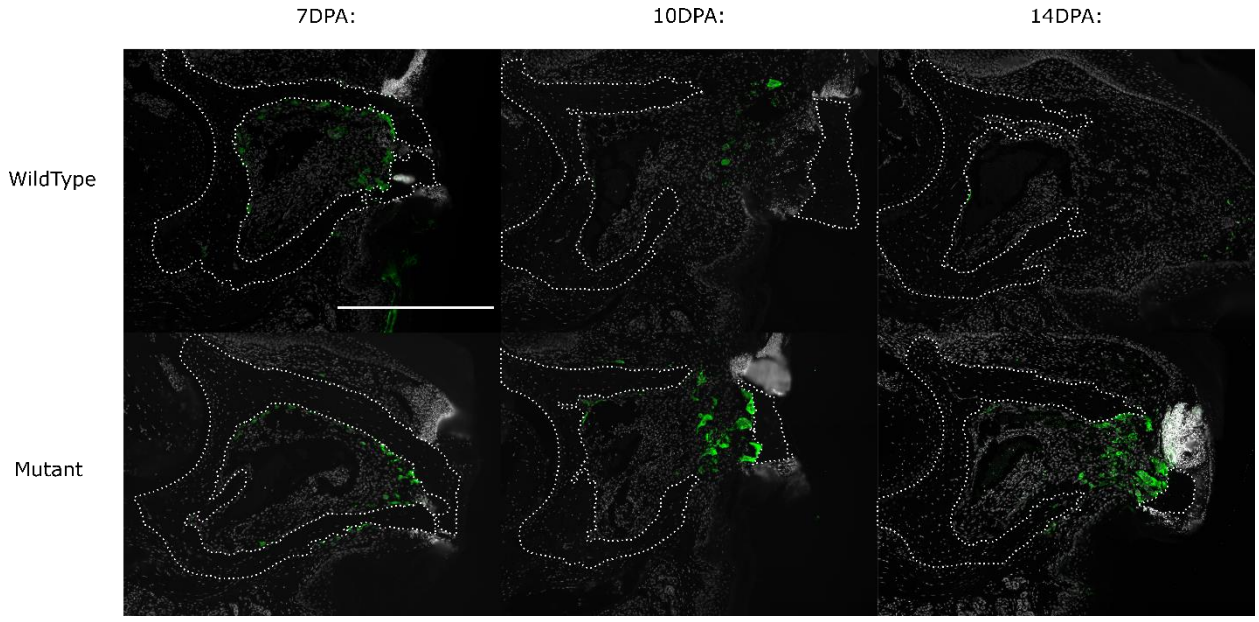

Figure 4 above shows 20x serial sectioned digit tips stained using a CatK primary antibody fluorescing in green and DAPI fluorescing in grey. The white scale bar in the top left-hand section has been set to 500 µm, and the P3 bone has been outlined in a white dotted line in each section image.

Quantification of Immunohistolochemistry Data

Quantification using ImageJ is currently underway for both sets of immunohistochemistry data using cathepsin K and osterix to stain for osteoclasts and osteoblasts.

CHAPTER IV

DISCUSSION

Here, we found that in a mouse model for accelerated aging, the HGPS mouse, digit tip regeneration is attenuated by i) a delay and exacerbation of the bone resorption phase, accompanied by an extended presence of osteoclasts, and ii) a delay and attenuation of the bone formation phase, which was accompanied by decreased osteoblast numbers during this phase.

Progeria as a Model for Aging

Although it is widely used in biological research as a time-efficient model for chronological aging, scientific consensus on the similarities between HGPS and natural aging still is not prevalent. Some argue that the general process of aging has not been understood well enough to determine if any disease of early death could be an adequate substitute.[40] This has led to the adoption of the term 'segmental aging', which refers to the phenomena where only specific segments or subsystems of the organism seem to be undergoing accelerated aging through progeria.[41] In these cases, aging is defined as an accumulation of damage due to dysfunctional cell-maintenance and survival stress-response systems, which can be modeled by the accelerated accumulation of cellular damage present in HGPS.[42]

Genetic mapping of HGPS reveals that it is a sporadic, autosomal dominant disease caused by a point mutation on the LMNA gene[43] which codes for a structural inner nuclear membrane protein. This nuclear protein, lamin A, becomes misshapen progerin, which dramatically changes the shape and morphology of affected cell nuclei.[44] Accumulation of progerin in cell nuclei induce disease symptoms such as hair loss, joint contractures, and

cardiovascular disease resembling atherosclerosis through mechanisms yet-to-be discovered.[45] The exact mechanisms by which HGPS elicits symptoms of accelerated aging may not be similar to the actual mechanisms behind natural aging.. However, we found that the mouse model of HGPS still exhibited a significant decline in regeneration potential, which follows the established expectation that regenerative power decreases with age.[46]

Bone Regeneration Mechanics

Bone remodeling and regeneration mainly occur through the activities of two types of cells: osteoclasts, which are primary bone-resorbing cells,[47] and osteoblasts, which are bone forming cells that regulate osteoclast activation.[48] μCT data showed a significant delay in the bone resorption and bone formation events along with an increase in the magnitude of bone resorption and decrease in bone formation for mice with progeria. This suggests that there was either an increased quantity of osteoclasts and decreased quantity of osteoblasts or an increased function of osteoclasts and decreased function of osteoblasts. Our immunohistochemistry results suggest that osteoclast and osteoblast recruitment are not affected in mice with progeria, since both cell types are equally present at early time points; however, during later stages, osteoblast numbers do not increase in progeroid digits, as they do in the wild type controls, and osteoclasts are not cleared. This suggests that the timing of cell behavior is affected in progeria.

One possible mechanism for the altered timing of events in progeria could be the rate of wound closure. Hyperbaric wound treatment of amputated digit tips caused the wound to stay open and the P3 bone to degrade all the way down to the joint before bone formation was initiated.[49] Since osteoblasts regulate osteoclast activation, a decreased amount of osteoblasts in the hyperbaric wound environment would explain the increased bone degradation, as more

osteoclasts may be recruited with a lower osteoblastic regulating presence. Similarly, another study found treatment with etidronate, a drug in the class of bisphosphonates which typically treat osteoporosis by inhibiting osteoclastic resorption of bone, in rat calvaria bone wounds promoted both osteoblast differentiation and wound closure,[50] suggesting that the two events are somehow linked. Progeria results in a similar decrease of regenerative power as natural aging, which could lead to slower wound closure, decreased osteoblast differentiation, and dysfunctional termination of osteoclast activity.

CHAPTER V

CONCLUSION

According to Dr. Ken Muneoka, a regeneration biologist at the vet school of Texas A&M University, “with adequate funding, human-finger regeneration in children will be possible within 20 years”.[51] Looking further into the future, human regenerative therapies will have to be made effective for all age groups, especially considering that older patients would form the larger demographic in need of regenerative treatment. With advanced age comes a litany of new variables to account for, such as changing blood pressure, respiratory cycles, vision, and most importantly, overall loss of regenerative capacity. Therefore, exploring the effects of aging on regeneration of the distal mouse digit bone gives clues about how regenerative induction will have to be optimized for older patients.

The digit tips of mice and humans are capable of undergoing epimorphic regeneration, a blastema mediated regenerative process which can replace complex multicellular structures. How aging affects epimorphic regeneration in mammals is not known to date, and establishing and maintaining an aged mouse colony is time-intensive. Therefore, the digit tips of a mouse model of Hutchinson-Gilford progeria syndrome, a disease of accelerated aging, were analyzed with regard to their ability to undergo epimorphic regeneration, using immunohistochemistry and microcomputed tomography at different time points during the regenerative process. Osteoclastic and osteoblastic quantity mediated delays found in the bone resorption and bone formation processes of mice with progeria may be used to adjust the timing of regenerative therapies in elderly patients for optimal treatment.

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