Osteoclast Precursor Variability from Mandible and Femur-Derived Bone Marrow Cells in Older Mice: A Descriptive Study

A THESIS

SUBMITTED TO THE FACULTY OF THE

UNIVERSITY OF MINNESOTA

BY

Emilyn Dolores S. Asinas

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

Kim Mansky, PhD, Amy Tasca, DDS, PhD

JUNE 2024

Acknowledgements

I would like to express heartfelt gratitude to my supervisor, Dr. Kim Mansky, for her unwavering support and guidance throughout the entirety of this research endeavor. Her passion for research in bone biology is inspiring and groundbreaking. I am grateful to have been a part of her research lab during my time in residency.

To my committee members: Dr. Amy Tasca, and Dr. Rajaram Gopalakrishnan. Thank you for dedicating your time to serve as valued members on my committee. Your insightful feedback and valuable suggestions have been appreciated in developing my thesis.

I would also like to thank everyone in the Mansky Lab, including Dr. Rachel Clark, for all of their assistance in the data collection for this project. I have enjoyed working alongside such bright and intelligent individuals.

Finally, I would like to give thanks to my family and significant other. Their never-ending love and support have carried me through the toughest of times and have been a constant reminder of why I keep going.

Dedication

This thesis is dedicated to my parents, Reuel Asinas and Emerita Santos. The sacrifices you both have made to start a life in the United States and resilience you have demonstrated in the toughest of times motivate me to never settle for less. I am grateful to have your support no matter what distance is between us. This accomplishment is as much yours as it is mine. Thank you for everything.

Abstract

Aim: To determine if there are differences between osteoclasts from the mandible and femur of 1-year-old mice by evaluating gene expression and differentiation potential.

Methods: Monocytes were collected from male and female C57BL/6 mice aged to 1-year-old. Osteoclast differentiation of the monocytes collected was performed by plating the cells in osteoclast media supplemented with 1.5% CMG 14-12 culture supernatant and 5 ng/mL RANKL in preparation for differentiation experiments. TRAP staining was done to evaluate the quantity and size of the osteoclasts at each skeletal site. qRT-PCR analyzed osteoclast precursor gene expression in the mandible and femur. Bulk RNA-Seq allowed for an unbiased look at the overall gene expression signature of the monocytes collected from the two skeletal sites.

Results: Osteoclasts from the mandible of 1-year-old mice were larger in size compared to those in the femur, but significantly less in number. The differentially regulated genes with increased expression in the mandible are associated with osteoclast lineage commitment, proliferation, and inflammation; similar to those in the 2-month old mice. The femur only showed increased expression of inflammatory modulators. Bulk RNA sequencing demonstrated that the mandible had more upregulated genes compared to the femur, and the pathways enriched in the mandible were distinct from those in the femur.

Conclusions:

- The osteoclast precursors from the mandible of 1-year-old mice demonstrate differences in gene expression and differentiation capabilities compared to the femur.
- Craniofacial and appendicular regions of the skeleton may be regulated by different mechanisms.
- The femoral data collected from the 1-year-old mice exhibits a more inflammatory environment compared to the data collected from 2-month-old mice.
- The differences seen the in osteoclasts between young and old mice requires further investigation, with emphasis in understanding how these difference impact orthodontic tooth movement and bone remodeling in older patients.

Table of Contents

List of Figures

Tables

Introduction

Over the last several years, there has been a considerable increase in the proportion of adult patients seeking orthodontic treatment. This movement has evolved from increased awareness of what treatment entails, technological advancement in the field, and cosmetic concerns. It is a general presumption that orthodontic treatment in the older population requires more careful treatment planning due to periodontal risks and the difficulty of achieving tooth movements in skeletally mature patients, but there appears to be limited publications explaining why this is so. In 1972, Dr. Weiss stated that adults require a longer hyalinization period in addition to a longer mobilization period in order to prepare the tissues for orthodontic changes[1]. In 2020, Schubert et al. sought to determine the age effect on orthodontic tooth movement rate and concluded that the effect of aging as an influencing factor is rather unclear[2]. Previous studies lack certainty and data on the cellular processes within the craniofacial region in older populations to explain this concept. To understand this process, foundational concepts regarding osteoclast physiology need to be understood.

Review of the Literature

The skeleton serves as the structural foundation of the body that supports mobility, bodily functions, and protection of vital organs. In addition to its mechanical role, it also regulates hematopoiesis, mineral homeostasis, and endocrine function. The bones of the skeleton are composed of a mineralized extracellular matrix containing osteoblasts, osteocytes, osteoclasts, and collagen[3]. Bone is further differentiated at the tissue level into cortical and trabecular layers, with the lamina dura of alveolar bone containing bundle bone. This type of bone is considered an anatomical entity that responds to both pathological and physiological stresses[3]. This intricate network of bones, joints, and connective tissues undergoes continuous adaptation and remodeling throughout life, reflecting the dynamic reciprocity between genetic factors, environmental influences, and physiological demands. Bone mass and shape continuously adapt to variations in load caused by physical activity, mechanical force, hormones, nutrients, and several additional osteogenic signaling molecules[4]. This cyclic remodeling is essential for the health and maintenance of this highly specialized organ.

Osteoclasts

Osteoclasts are multinucleated cells of hematopoietic origin that play a pivotal role in the bone remodeling process, resorbing old or damaged bone tissue to facilitate skeletal adaptation and repair. These cells result from the fusion of mononuclear osteoclast precursors derived from the monocyte/macrophage lineage[5].

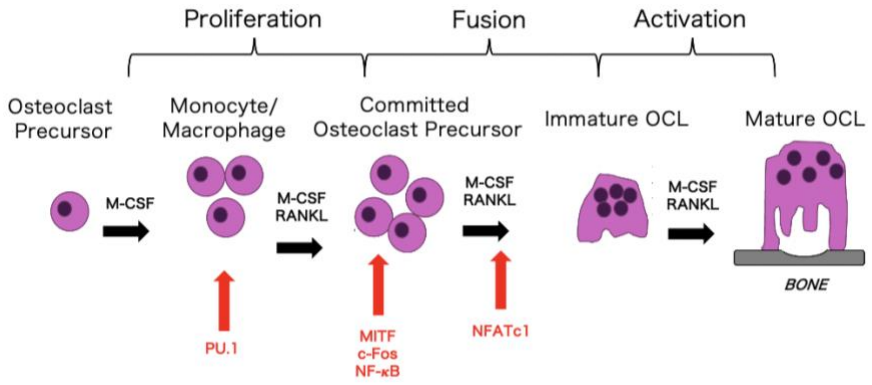

Figure 1. Schematic illustrating transcriptional regulation of osteoclasts.

Both mouse and human hematopoietic monocytes form osteoclasts when cultured in the presence of macrophage colony-stimulating factor (M-CSF) and receptor activator of NF-κB ligand (RANKL), released by osteoblasts[6]. Osteoclast precursors circulate in the blood, like other leukocytes, and are attracted to bone remodeling units in response to cytokines, chemokines, and other factors elaborated at sites either destined for or currently undergoing resorption[7]. When differentiation is complete, mature osteoclasts tightly adhere to bone and develop ruffled borders, multiple plasma membrane folds resembling microvilli, sealing zones that isolate the resorptive compartment, and a circular adhesive structure at their interface with bone[8],[9]. Chloride channels and proton pumps lie within the ruffled borders to facilitate the transport of transcellular acid, which acidifies the resorptive compartment[10]. Osteoclasts secrete hydrochloric acid and proteolytic enzymes, including TRAP and cathepsin K, to dissolve minerals and degrade organic compounds within the sealing zone[11]. However, they can only resorb mineralized bone and do not resorb unmineralized or demineralized bone, as bone minerals are required for their sealing zone formation[12],[13]. The processes of osteoclast formation and bone resorption have been

viewed as energy-intensive steps that require active metabolic reprogramming[10]. Bone resorption makes up only one side of the bone remodeling process, with osteoblasts responsible for the other process of bone deposition. It is with the precise coordination of activities between osteoclasts and osteoblasts that bone remodeling may be accomplished and set up the delicate balance essential for skeletal homeostasis[14].

BMP and WNT Signaling

To maintain the coordination between osteoclasts and osteoblasts, various pathways coordinate the cross talk that occurs between osteoblasts and osteoclasts in the bone microenvironment. BMP and WNT signaling pathways are only two of the many that regulate the cross talk between osteoclasts and osteoblasts. Osteoblasts and osteoclasts actively recruit and stimulate each other through these pathways to repair any bone damage or maintain bone homeostasis[15]. It is through either paracrine signaling or contact with membrane-bound ligands that they coordinate bone remodeling[16]. Bone morphogenic proteins (BMPs) are one of cytokines secreted by osteoblasts that regulate RANKL-mediated osteoclast differentiation, proliferation, fusion and survival[17]. Inversely, osteoclast activity may release a reservoir of growth factors such as BMP2, BMP4 and BMP7 stored in bone, eventually stimulating osteoblasts to promote mineralization[18]. Once osteoblasts are stimulated, they may then release factors that regulate osteoclast function and differentiation, which is required to drive hematopoietic stem cells into the osteoclast lineage[19].

As BMP signaling stimulates osteoblast differentiation, mineralization and survival to maintain healthy bone density and to sustain a complex supporting microstructure[20], WNT signaling works to promote osteoblast proliferation and differentiation, enhancement of

osteoblast and osteocyte survival, and osteoblast commitment from osteo/chondro-progenitors[21]. WNTs, similar to BMPs, have been implicated in regulating osteoblast differentiation[22]. These glycoproteins can be upregulated by BMPs and are secreted for development and homeostatic renewal of bone and many other tissues[22]. The intricate signaling that occurs between these pathways work with one another towards a similar goal, but there are factors that may interfere with this activity.

Effects of Aging on Bone Loss

Aging is one of the several factors that impact the differentiation and activity of osteoclasts which may be manifested as bone loss. It is known that both men and women exhibit bone loss with advancing age, but it is unclear what happens at the molecular level to cause this or what constitutes normal bone loss rates throughout life. After skeletal maturity at approximately the age of 20 years, the amount of cortical bone in many different bones has been reported to decrease as a normal response of the aging process[23]. In women, annual rates of bone loss were found to increase by 0.5 – 0.9% in the perimenopausal period[24], accelerate in the first years after menopausal transition to mean annual loss rates above 1%[25], then remain around 1% into old age[26]. In men, small bone mineral density loss was detected in the fourth decade of life, increasing with increasing age to mean annual loss rates of approximately 0.8% in old age[27]. Additionally, altered systemic conditions can affect the homeostatic process of bone remodeling. Osteoporosis is one of those systemic conditions which should not be overlooked. Osteoporosis occurs in approximately one-third of women over 60 years of age, with 26% of women having osteoporotic fractures by 60 years of age and 50% by 75 years of age[28]. This disease may manifest in the oral cavity as excessive alveolar ridge resorption, chronic destructive

periodontal disease, tooth loss, and referred maxillary sinus pain or fracture[29]. Kribbs et al. found a significant correlation between skeletal osteopenia and residual ridge and alveolar bone density, particularly in the edentulous mandible[30]. Improper mineral cell activity resulting in loss of calcium due to various mechanisms may also result in increased bone loss. Calcium metabolism and nutrition are essential for the transport of nutrients within the body and if their balance is tampered with, homeostasis would decline and be followed by apoptosis[28]. Women lose calcium in different ways: pregnancy, loss of estrogen and changes in hormonal balances at menopause, as well as reducing diets that result in decreased ingestion of all nutrients. Any loss of calcium results in loss of bone density, thus resulting in an osteoporotic state[28].

Bone Analytic Techniques in Regards to Aging

To understand what is happening at these skeletal sites, different types of analyses and techniques have been performed. Radiographs is one example of a method, but are only able to detect diseased states like osteopenia where a minimum bone mass loss of 30% or even 50-60% is needed[31]. Total bone mass (body calcium) can be accurately measured by neutron activation analysis, showing a significant correlation with regional bone mineral content as measured by photon absorptiometry[31]. In a study done by Kribbs et al. in 1983, intraoral microdensitometry demonstrated a high degree of precision within different scans of the step wedge and bone of the mandible on the same radiograph as well as between scans of wedge of bone and different radiographs[31]. This was also shown to compare quite favorably with techniques used determine the bone mass in other bodily regions with high precision. Benson et al. utilized panoramic mandibular index, which was defined as a radiomorphometric index of mandibular cortical bone mass, to acquire a

preliminary estimation[29]. In addition to analyzing bone mineral content, these skeletal sites can be further studied at the molecular level with techniques such as quantitative RT-PCR. Quantitative PCR allows analysis of gene expression levels in cells of interest. Clark et al. utilized this method to determine changes in gene expression in monocytes isolated from the bone marrow from different skeletal sites in young mice to understand mechanisms regulating changes in osteoclast differentiation[32]. Single cell RNA sequencing is another useful method that provides a comprehensive transcriptional environment for analyzing tissue heterogeneity at the individual cell level, allowing exploration of the contribution of various cell subtypes to physiological function[33]. Utilizing advanced techniques to analyze transcriptomic profile in cells as well as imaging techniques to visualize changes in the bone at different skeletal regions may help us correlate transcriptional changes with changes in bone architecture.

Literature Supporting Similarities between the Mandible and Femur

With the various techniques available to study different properties and processes within bone, the question on the impact of aging on bone loss can be further narrowed down to its effects on types and density of bone. In the mandible, Epker et al. have documented a progressive thinning of cortical bone after adulthood is attained, stemming from enlargement of the marrow cavity due to a negative bone balance at the cortical-endosteal surface in excess of a positive balance at the periosteal surface[34]. The difference in bone density seen with aging in the mandible can be further distinguished between cortical and trabecular portions in the mandible, where the cortical bone mass appears to diminish considerably over the years while the trabecular bone demonstrates individual variation amongst all ages[35]. Benson et al. reported that the general trend toward bone loss persists

throughout the skeleton although some bones, such as those with a large proportion of trabecular bone, are affected earlier and more severely than others[29]. Liu et al. assessed alveolar bone samples obtained at autopsy and during third molar extractions and found that a significant decrease in pore volume, decrease in lacunar-canalicular pore diameter and an increase in density were found with increasing age[36]. With information collected on the craniofacial site of the mandible, researchers sought to compare the findings of different skeletal sites to each other such as the bones in the craniofacial region and those in the appendicular skeleton.

Two distinct stances on the matter exist with one group of researchers indicating that both mandible and femoral sites lose bone with aging while the other group indicates that bone is primarily lost in the femur with aging[30, 33, 37, 38]. Henrikson et al. provides support for the first viewpoint, reporting that the mandible and long bones both experience age-related changes that are affected similarly despite their difference in morphology[37]. Ham et al. addressed the assumption that the jaw bones are somehow different from other bones due to their difference in ossification (intramembranous in the jaws compared to endochondral in the appendicular bones)[39]. Although the terminology tends to suggest that the bone tissue developing in these two skeletal sites is different, some investigators believed they were in fact the same[39]. Another study by Kribbs et. al. reported findings of similarity between sites under the systemic effects of the condition of skeletal osteopenia, stating that residual ridge and alveolar bone density does show a significant relationship to total body calcium and regional bone mass[30]. More support for similarity between the craniofacial and appendicular sites came from a study done by Klemmeti et al., where their study indicated that particularly the bone mineral density (BMD) of the buccal part of the mandibular cortex correlated remarkably well with that of the femoral column, reflecting the general

osteoporotic status of the human body[40]. The comparison of bone loss activity between skeletal sites under various conditions continues to be investigated.

Literature Supporting Differences between the Mandible and Femur

The studies published in the late 1900's were generally supportive of the theory that the craniofacial and appendicular skeletal sites exhibit similar cellular activity with aging. As more research has been conducted, studies thereafter have shown that osteoclasts, including osteoclastogenesis dynamics, exhibit different characteristics between the mandible and long bones[41]. The alveolar bone found in the craniofacial region of the skeleton has been reported to differ morphologically and functionally from other bones. Huja et al. stated that the remodeling process of alveolar bone occurs more rapidly than that of other skeletal bones[42]. This distinction in bone turnover is likely due to the difference in germ layer origin, with alveolar bone arising from the neural crest cells of the neuroectoderm germ layer and the femur from the mesoderm[43]. Difference in osteogenic potential have also been observed, where alveolar-derived bone marrow stromal cells or mesenchymal stem cells exhibit higher osteogenic potential compared to other skeletal bones[44-48]. In examining the distinct population found in murine mandibular bone marrow, myeloid populations were found to consistently make up a lower percentage of the total bone marrow population compared to the long bone marrow myeloid populations[33].

These skeletal sites are also subjected to different forms of stimulation and exhibit distinct inflammatory processes in response. For example, occlusal stress stimulation and tooth-derived inflammatory responses, which exist only in the alveolar bone, affects metabolism as well as remodeling[42],[49-51]. Kwack et al. found that craniofacial bone immune homeostasis is more likely to be directly affected by microorganisms than any other

osseous tissue due to its intimate contact with pathogenic microorganisms that may penetrate the periodontal barrier during diseased states[33]. The difference of immune microenvironments between alveolar bone and long bone were observed as the result of occlusal forces, where mechanical stimulation is seen to promote activation of myeloid-derived monocytes in the femur[52]. The environment of the mandibular alveolar bone has also been shown to exhibit higher osteoblastic capacity in order to maintain homeostasis in its microbial laden microenvironment[53]. Furthermore, the bone in the mandible and long bones differ in type of ossification, where the alveolar process proceeds by intramembranous rather than endochondral ossification[54]. Skeletal diseases that have a greater predilection for the alveolar bone, such as cherubism, hyperparathyroid jaw tumor syndrome and osteonecrosis associated with anti-resorptive therapeutics, including bisphosphonates, demonstrate the clinical significance of these differences[38, 55, 56]. Therefore, the different physiological reactions, pathological responses and clinical presentations between mandibular bone and long bones reveal the fundamental differences that exist. Because of the specificity of the mandibular bone and the ease of access to cells from long bone compared to mandibular bone, there is a relatively scarce data focused on the cellular differences between them[33]. Many of the recent publications supporting the distinct population of osteoclasts between skeletal sites were done in a younger population of mice with lack of comparison to its effects in an older population. Despite the amount of literature previously mentioned, a gap in knowledge remains about what processes occur within the cellular populations found at these skeletal sites and how they may change with age. Our study begins to address that question with comparison of the transcriptome between mandibular and femoral monocytes (i.e. osteoclast precursors) in 1-year-old mice.

Osteoclastogenic Potential Between Mandibular- and Femoral-Derived Bone Marrow Cells in 2-Month-Old Mice

The Mansky lab conducted studies in mice looking at mandible and femur-derived osteoclasts precursors in young (2-month-old) mice[32]. From those experiments they determined differences in osteoclast size from different skeletal sites i.e. that mandibular-derived osteoclasts are larger than femoral-derived osteoclasts[32]. Flow cytometry of mandibular- and femoral-derived bone marrow precursor populations was an additional experiment performed to determine if they had an impact on the differences found between skeletal sites[32]. Osteoclastic differentiation potential of both bone marrow macrophage and monocyte cultures was observed after separating the beads with CD11b microbeads for positive selection, then further stained with anti-Ly6C antibody[32]. This was done to select for the cells that had the most osteogenic potential[32]. From this experiment, they found that the bone marrow cells from the mandible exhibited a higher percentage of Ly6CHigh+ cells and the femur–derived CD11b+ cells contained significantly more Ly6CHigh cells compared with mandible–derived cells[32]. Gene expression was also evaluated and demonstrated increased levels of lineage transcription factors and osteoclast genes in mandible-derived osteoclasts. Bone marrow-derived macrophages from the mandible also exhibited the trend of increased osteoclast differentiation potential in the mandible compared to the femur[32].The original study by the Mansky lab was done on 2-month-old mice, being comparable to an adolescent human. However, the question remains if these trends in gene expression and osteoclast differentiation persist or change with increasing age.

Aims and Hypotheses

The aim of this study is to determine differences in mandible- and femur-derived osteoclast precursors from 1-year-old mice by examining gene expression and differentiation capacity. I hypothesize that osteoclast precursors derived from the bone marrow of the mandible of 1-year-old mice will have differences in gene expression and increased differentiation capabilities compared to monocytes derived from the femur. The results of these studies will contribute to the understanding of differences between osteoclasts at different skeletal sites and how their gene expression and differentiation potential may change with age. If a deeper understanding of the properties of osteoclasts derived from the mandible versus the femur in different age populations is understood, then we can anticipate that we will be able to find applicability to tooth movement and/or capacity of bone healing.

Materials and Methods

Ethics. The use and care of mice used in these study procedures were reviewed and approved by the University of Minnesota Institutional Animal Care and Use Committee, IACUC protocol number 2104-39006A. Euthanasia was performed by CO_2 inhalation.

Experimental Preparation of Osteoclasts. Male and female C57BL/6 mice aged to 1-year-old were utilized. Primary bone marrow macrophages were harvested from the mandible and femurs of 1-year-old wild type mice. Once the mandibles and femurs were dissected and adherent tissue was removed, the marrow was flushed from the inner compartments. Bone marrow monocyte selection was performed on total bone marrow. Monocytes were selected following the protocol of the mouse monocyte isolation kit (Miltenyibiotec, catalog #130-100-629). Briefly, flushed cells were pelleted and resuspended in MACS buffer (phosphate-buffered saline (PBS), pH7.2, 0.5% bovine serum albumin (BSA), 2mM EDTA). The cells then were incubated with FCR Blocking Reagent and Monocyte Biotin-Antibody Cocktail. After five minutes the cells were rinsed with MACS buffer, pelleted, and resuspended in MACS buffer. The cells were incubated with Anti-Biotin Microbeads for ten minutes. After this incubation period the cells were applied to the MACS columns in a magnetic field. Flow through was collected as isolated monocytes. The columns were rinsed three times to collect unbound monocyte cells. The monocyte cells were plated in 24-well plates (TPP, MidSci) at 2 $x10^4$ cells/cm^2 in osteoclast media supplemented with 1.5% CMG 14–12 culture supernatant overnight. The next day cells were re-fed with 1.5% CMG 14–12 culture supernatant and 5 ng/mL RANKL (R and D Systems, catalog #462-TEC-010) to stimulate osteoclast differentiation. Cultures were fed every other day for up to 4 days for differentiation experiments.

Quantitation of Osteoclast Gene Expression. RNA was isolated from cells plated in duplicate using Trizol reagent (Ambion, Life Technologies) and quantified using UV spectroscopy. cDNA was prepared from 1 µg RNA using the iScript cDNA Synthesis Kit (Bio-Rad) as per the manufacturer's protocol. qPCR samples were prepared with 1 µl of the cDNA of monocyte RNA in combination with 19 microliters of a master mixcontaining 1 µl of the reverse and forward primers of an osteoclast marker, 8.8 microliters of water, and10 µl of iTaq Universal Sybr Green. HPRT used as the control gene. The PCR conditions were the following: 95°C for 3 minutes, and the 40 cycles of 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 30 seconds, followed by melting curve analysis (95°C for 5 sec, 65°C for 5 sec and then 65°C to 95°C with 0.5°C increase every 5 seconds).

Table 1. Sequence of forward and reverse primers used for real-time PCR.

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
Junb	TCA CGA CGA CTC TTA CGC AG	CCT TGA GAC CCC GAT AGG GA
Klf4	CGA TGA ACT GAC CAG GCA CTA C	CCT CTT AT GTG TAA GGC AAG GTG
cJun	TCC CCT ATC GAC ATG GAG TC	TGA GTT GGC ACC CAC TGT TA
cFos	CCA AGC GGA GAC AGA TCA ACT T	TCC AGT TTT TCC TTC TCT TTC AGC AGA
Atf3	GAG GAT TTT GCT AAC CTG ACA CC	TTG ACG GTA ACT GAC TCC AGC
Grk3	AGG AGG GTT TGG GGA AGT TTA	CAT GAT CCT CTC GTT CAA AGC C
Birc5	CCG ATG ACA ACC CGA TAG AGG	GGC TTC TGG GGG CAG AAT TT
Ccr2	ACA CCC TGT TTC GCT GTA GG	TGC ATG GCC TGG TCT AAG TG
Irf4	AAT CCC CAT TGA GCC AAG CA	CTC GTC GTG GTC AGC TCT TT
Cd74	CCG CCT AGA CAA GCT GAC C	ACA GGT TTG GCA GAT TTC GGA
S100A8	AAA TCA CCA TGC CCT CTA CAA G	CCC ACT TTT ATC ACC ATC GCA A
S100A9	ATA CTC TAG GAA GGA AGG ACA CC	TCC ATG ATG TCA TTT ATG AGG GC
Ccl2	TTA AAA ACC TGG ATC GGA ACC AA	GCA TTA GCT TCA GAT TTA CGG GT
Ccl3	TTC TCT GTA CCA TGA CAC TCT GC	CGT GGA ATC TTC CGG CTG TAG

TRAP staining of osteoclast cultures. Monocytes were cultured to the desired time point and fixed in 4% paraformaldehyde for 20 minutes at 4°C and then washed in PBS. Cells were stained with the TRAP buffer containing 5 µL 0.1 M Acetate Buffer, 1 mL 0.3 M tartrate, 100 µL 10 mg/mL Nupthol, 20 µL Triton X-100, 3.9 mL water, and 3 mg Fast Red Violet. The TRAP-stained multinuclear osteoclasts were photographed with light microscopy and size and number of total nuclei were quantified by NIH ImageJ.

Bulk RNA Seq. Monocytes were collected from 1-year old mice. Cells were lysed and total RNA collected using RNA Plus Mini Kit (Qiagen, catalog #74134) following manufacturer's instructions. High throughput RNA sequencing was performed by the UMN Genomics Center. 2 x 50bp FastQ paired end reads for 6 samples (n=62.1 million average reads per sample) were trimmed using Trimmomatic (v 0.33) enabled with the optional "-q" option; 3bp sliding-window trimming from 3' end requiring minimum Q30. Quality control on raw sequence data for each sample were performed with FastQC. Read mapping was performed via Hisat2 (v2.1.0) using the mouse genome (GRCm38 v94) as reference. Gene quantification was done via Feature Counts for raw read counts. Differentially expressed genes were identified using the edgeR (negative binomial) feature in CLCGWB (Qiagen, Redwood City, CA) using raw read counts. The generated list was filtered based on a minimum 2x Absolute Fold Change and FDR corrected $p < 0.05$. Data is available upon request. Differential pathway analysis was performed using DAVID analysis [57, 58]. Heatmap was plotted by http://www.bioinformatics.com.cn/srplot, an online platform for data analysis and visualization.

Results

Mandible-derived osteoclasts from 1-year-old mice are larger than femur-derived osteoclasts. The Mansky lab has previously demonstrated that osteoclasts differentiated from the bone marrow of the mandible are larger in size compared to osteoclasts differentiated from the bone marrow of the femur[32]. However, these experiments were done with 2-month-old mice. To determine if the same was true of aged mice, I isolated bone marrow from the mandible and femur of 1-year-old mice, isolated monocytes and differentiated the cells into osteoclasts in the presence of macrophage colony stimulating factor (M-CSF) and receptor activator of NF-kB ligand (RANKL). Mandible-derived osteoclasts from 1-year-old mice were significantly less in number (Fig 2B, p=0.0022) compared to the femur, but approximately twice as large in size (Fig 2C, p=0.0013).

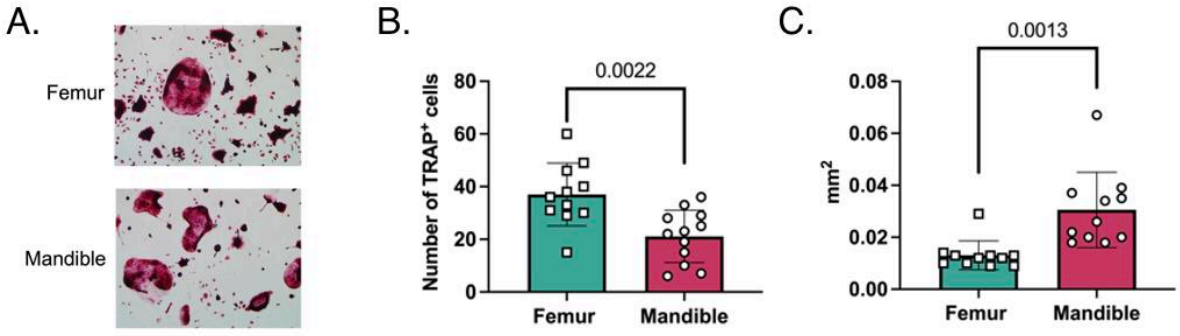

Figure 2. Mandible-derived osteoclasts are larger in size compared to femur-derived osteoclasts. Monocytes were isolated from the bone marrow of femur and mandible. Monocytes were cultured in M-CSF and RANKL to stimulate osteoclast differentiation. (A) Representative images of TRAP-stained osteoclasts. (B) number and (C) average size of TRAP-positive multinuclear cells.

Mandible differentially regulated genes are conserved in both young and aged mice.

The osteoclast markers used in qRT-PCR were the genes that were upregulated in the mandible of the 2-month old mice indicated by single cell sequencing of CD11b+ cells from the mandible and femur. Results from that experiment indicated that genes upregulated in the mandible of 2-month-old animals included genes involved in osteoclast lineage commitment, proliferation and inflammation (Mansky lab, personal communication).

To determine if these same differentially regulated genes were changed in the 1-year-old animals, I isolated RNA from monocytes from the femur and mandible bone marrow and performed qRT-PCR. Mandibular-derived monocytes demonstrated increased expression of the majority of genes tested with similar trends as was seen in the 2-month-old animals. I saw a significant increase in transcription factors involved in osteoclast lineage commitment: *JunB*, *Klf4*, *c-Jun and c-Fos* (Fig 3). I also determined increases in genes involved in proliferation (*Atf3*, *Grk3* and *Birc5)* (Fig 4A-C), as well as genes involved in inflammation (*Ccr2*, *Irf4*, *Cd74*, *Ccl2*, and *Ccl3)* in mandible-derived monocytes (Fig 5A-C,F,G), I determined a significant increase of *S100A8* and *S100A9* in femur-derived monocytes (Fig 5D,E).

The genes with increased expression in the mandible have shown association with the process of osteoclastogenesis, whereas the genes highly expressed in the femur play a role in the regulation of inflammation and immune responses. Overall, the data supports our observation of a difference in gene expression between osteoclasts in the craniofacial and appendicular sites. Additionally, my data demonstrates that there are differentially regulated genes that are conserved between “young” (2-month-old) and “aged” (1-year-old) mice.

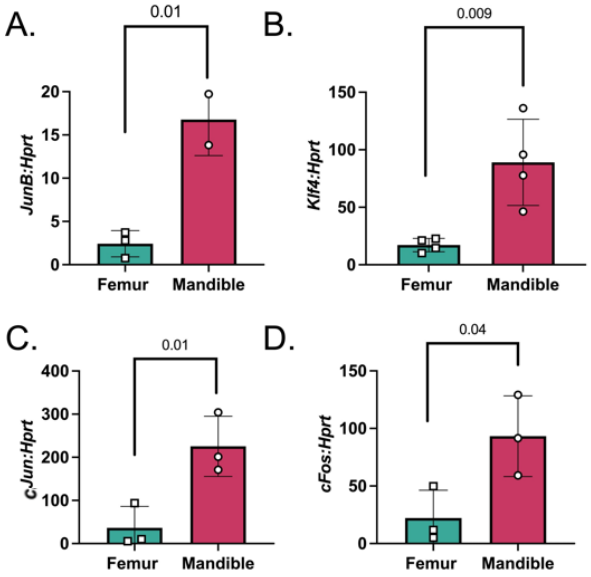

Figure 3. Mandible-derived monocytes express higher levels of osteoclast transcription factors. Monocytes were isolated from bone marrow of the mandible and femur. RNA was isolated and qRT-PCR was done to measure gene expression. Relative expression of (A) *JunB*, (B) *Klf4,* (C) *Jun* and (D) *cFos.*

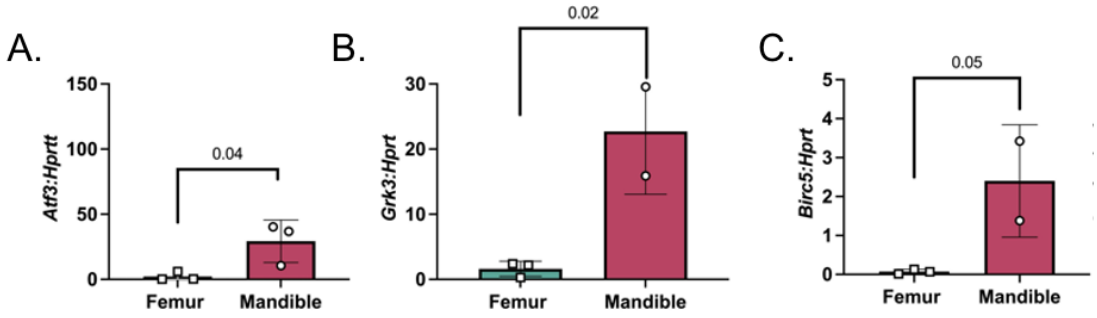

Figure 4. Mandible-derived monocytes express higher levels of genes that regulate proliferation. Monocytes were isolated from bone marrow of the mandible and femur. RNA was isolated and qRT-PCR was done to measure gene expression. Relative expression of (A) *Atf3*, (B) *Grk3* and (C) *Birc5*.

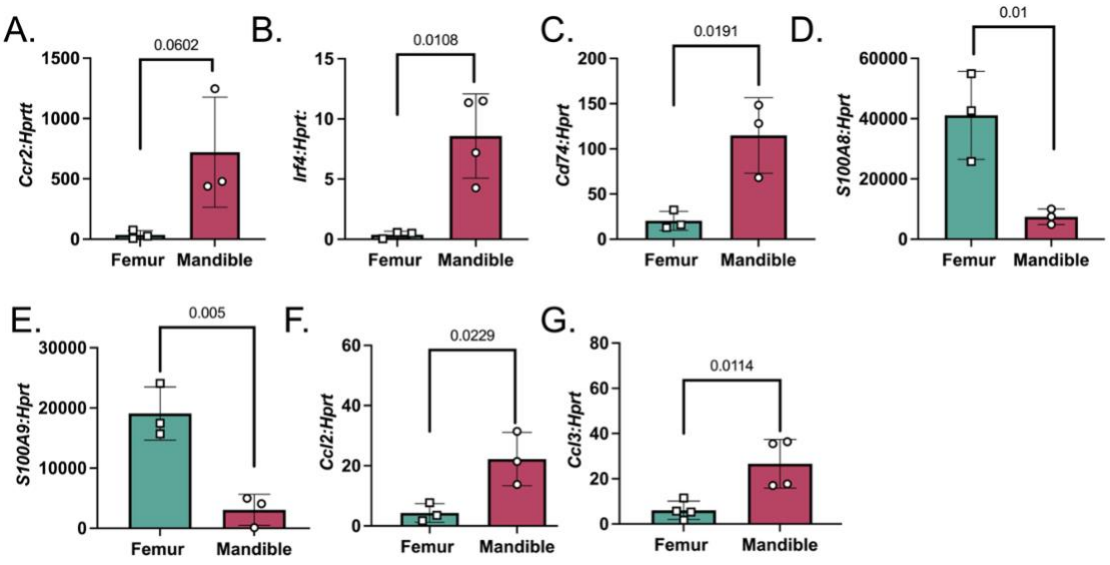

Figure 5. Mandible-derived monocytes express higher levels of inflammation genes. Monocytes were isolated from bone marrow the mandible and femur. RNA was isolated and qRT-PCR was done to measure gene expression. Relative expression of (A) *Ccr2*, (B) *Irf4*, (C) *Cd74*, (D) *S100A8*, (E) *S100A9*, (F) *Ccl2* and (G) *Ccl3.*

Expression profiles and differential pathway analysis of RNA collected from mice.

To obtain an unbiased understanding of the differences of mandible and femur-derived monocytes (i.e. osteoclast precursors), I performed bulk RNA sequencing of monocytes isolated from the bone marrow of the mandible and femur of 1-year-old mice. From the 948 genes differentially expressed, 689 were upregulated in the mandible compared to the femur and 259 were downregulated in the mandible compared to the femur as show in the volcano plot (Fig 6A). Upregulated genes are represented as red dots and downregulated genes are shown as blue dots. The most enriched pathways upregulated in mandible population included the WNT signaling, biomineralization, and cell adhesion pathways (Fig 6B). When looking at the downregulated pathways, metabolism (electron transport and respiratory chain), cell adhesion, chromatin remodeling, and chemotaxis appeared to be the

most enriched pathways in the femur-derived monocytes (Fig 6C). Heat maps in Figure 7 demonstrate the individual genes that are changed in the 1-year-old mandible monocytes compared to femur monocytes and include osteogenic signaling (i.e. WNT and BMP signaling (Fig 7A), calcium and PI3K signaling (Fig 7B) and other signaling pathways (Hippo, Hedgehog and TGF-b, Fig 7C).

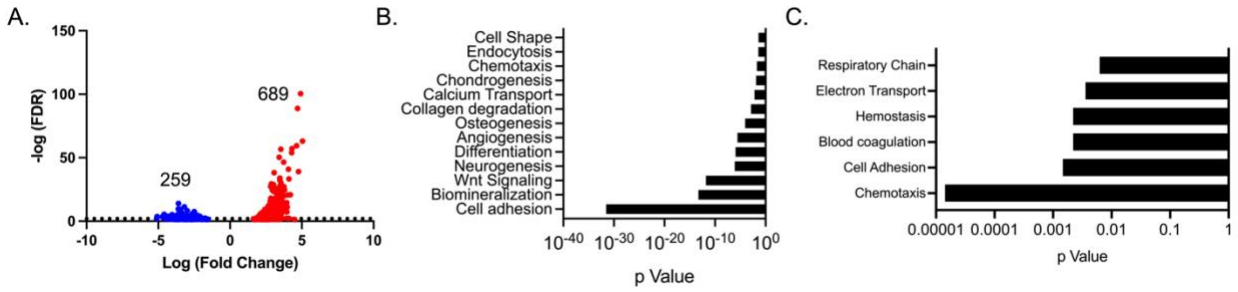

Figure 6. Analysis of bulk RNA-SEQ data from monocytes of 1-year-old mice. (A) Volcano plot (B) Upregulated pathways and (C) Downregulated pathways in mandibles.

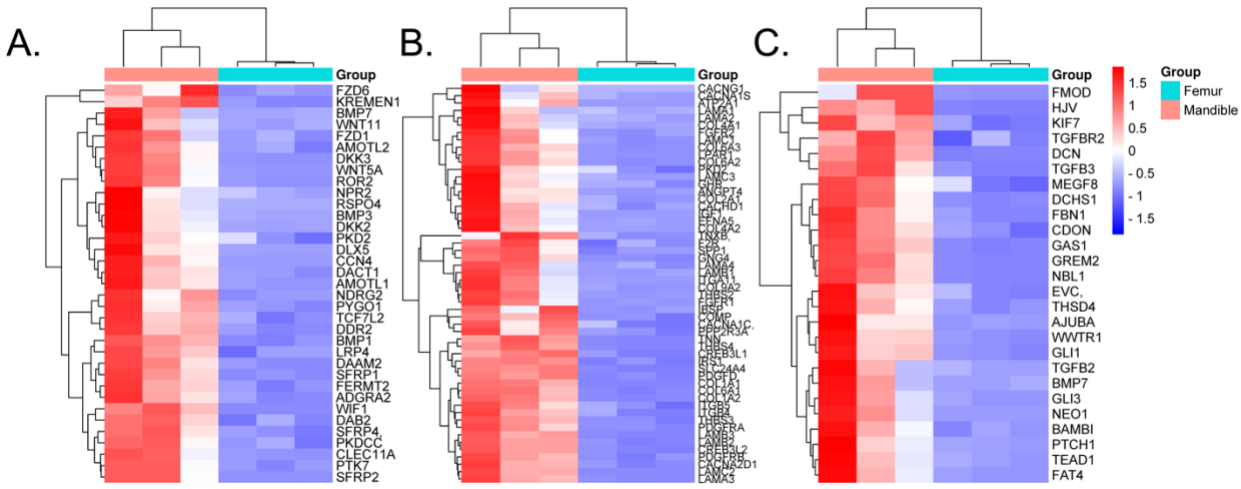

Figure 7. Heat maps of upregulated genes in mandible-derived monocytes. (A) Osteogenic (B) Calcium and PI3 kinase signaling genes and (C) Hedgehog, Hippo and TGF-β signaling pathways.

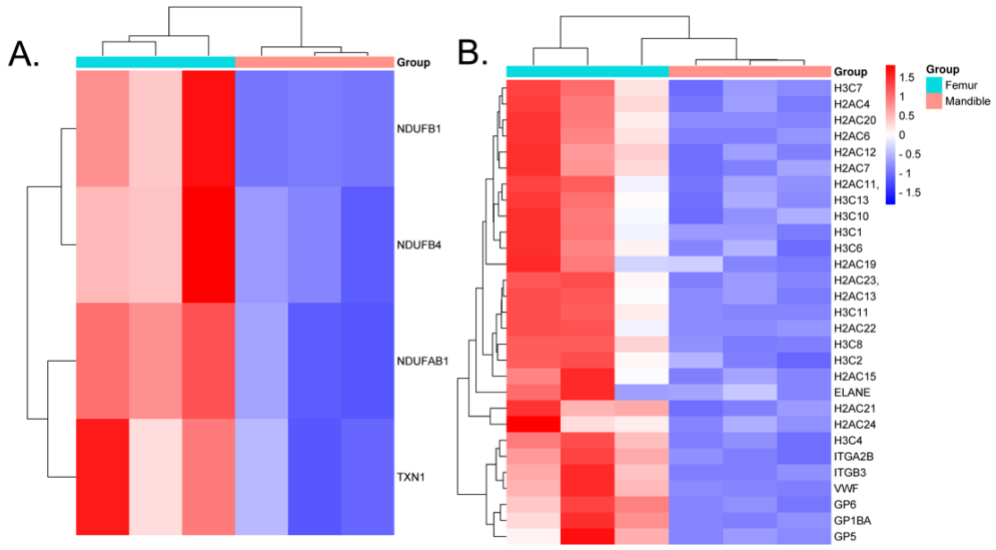

Figure 8. Heat maps of genes downregulated in mandible-derived monocytes. (A) Genes involved in electron transport and respiratory chain and (B) genes involved in chromatin remodeling.

Discussion

In a previous study done by Clark et al., the osteoclast population in the mandible was found to be distinct in comparison to osteoclasts located in the femur of 2-month-old mice[32]. Those general trends were also observed in the 1-year-old mice population in this study. Mandible-derived monocytes of this mice population appeared larger in size and reduced in number compared to femur-derived monocytes, similar to what was seen in 2-month-old mice[32]. In a study investigating the resorptive efficiency of small and large osteoclasts, it was reported that large osteoclasts resorb 2.5 times more per cell than small osteoclasts, but the amount resorbed per nucleus was the same between the two categories[59]. Lees et al. also determined that small osteoclasts exhibited a lower capacity for degradation of bone matrix[59]. A resorption pit assay was not used in this study but should be performed to determine a correlation to this previous finding. The genes that were upregulated in the 1-year-old mice are associated with transcription factors involved in the same processes as those upregulated in the 2-month old mice, suggesting that genes involved in osteoclast lineage commitment, proliferation, and inflammation are unaffected by the aging process in the mandible. Looking into the differences between the craniofacial and appendicular site in the 1-year-old mice, the femur only showed increased expression of inflammatory modulators (S100A8 and S100A9). Elevated S100A8/9 protein levels have been noted as a hallmark of numerous pathological conditions associated with inflammation[60]. This data suggests that there is a more active state of osteoclastogenesis in the mandible, driven by lineage commitment factors and local inflammation. Inflammatory transcription factors in the mandible may reflect local inflammatory responses potentially due to localized tissue damage, periodontal disease, or tooth loss. These findings denote different regulatory environments between skeletal sites.

Bulk RNA sequencing data from monocytes isolated from the bone marrow of 1-year-old mice revealed differentially regulated genes in the mandible and femur. The information on transcriptome complexity unveiled that WNT signaling, biomineralization, and cell adhesion pathways were highly enriched in the mandible while electron transport, respiratory chain, cell adhesion and chemotaxis were upregulated in the femur. In addition to the number of pathways being upregulated between the two skeletal sites, the number of genes upregulated in the 1-year-old mice was more than that seen in the 2-month-old mice. With this, aging could be viewed as having a greater effect on changes in gene expression compared to normal physiology. In the mandible, the prominent presence of those pathways could suggest that there could be a relatively lower level of osteoclast activity.

In the mandibular monocytes, there was an upregulation of *Wnt5a* and *Bmp7*. It has been shown that deletion of *Wnt5a* from mature osteoclasts decreases bone formation, which is likely responsible for the osteopenic phenotype observed in *Wnt5a* knockout (KO) mice[61]. Kresnoadi et al. found an increased BMP7 expression with the administration of ethanol extract of propolis and bovine bone graft, suggesting that an increase in BMP7 signifies an intensification of the osteogenesis process[62]. This could suggest that during aging, osteoclast precursors secrete molecules promoting bone homeostasis. Additional studies are necessary to support this finding.

In femoral monocytes, *Elane* and *Itgb3* were some of the many upregulated genes present. Fang et al. found that *Elane* was one of the few differentially expressed genes in RNA-sequencing analysis of RNA collected from the osteoclasts of CYP27A1 knockout (KO) mice[63]. CYP27A1 is a member of the cytochrome p450 family of proteins that catalyzes many reactions involved with synthesis of cholesterols, steroids, other lipids, and drug metabolism[63]. Through proteolysis of collagen-IV and elastin, *Elane* plays a key role

in degenerative and inflammatory diseases, with implications in osteoarthritis[63]. With this gene differentially expressed, it suggested that CYP27A1 KO potentially facilitates osteoclast differentiation via proteolysis of collagen-IV and elastin[63].

In a study done by McHugh et al., mice lacking *Itgb3* generated osteoclasts that were unable to properly polarize upon bone, exhibiting a defect in bone resorption. *Integrin beta 3 (Itgb3)* is a critical mediator of the interaction of differentiating osteoclasts with bone substrate[64, 65]. In differentiating and mature osteoclasts, *Itgb3* is highly induced and forms a complex with integrin alpha v on the surface of osteoclasts that binds to the extracellular matrix components present in bone[65]. These findings accentuate the critical role of *Itgb3*-mediated adhesion to bone in osteoclast differentiation and activation[65]. Two properties yet to be determined in mandible-derived osteoclasts from aged mice are differences in adherence and/or changes in proliferation. Clark et al. demonstrated that mandible-derived osteoclasts precursors proliferate less compared to femur-derived osteoclasts, however, all their experiments were performed in young mice[32].

The cell adhesion pathway could denote that monocyte attachment is modified through aging and could have effects on their ability to resorb. Sakai et al. studied osteoclasts in suspension culture of inverted Terasaki plates and found that the rate of apoptosis increased more than twofold due to complete deprivation of cell adhesion, demonstrating that osteoclasts require adhesion for their survival and this characteristic may contribute to limited localization of osteoclasts on proper substrates such as bone surfaces in the body[66]. The adhesion pathway was also upregulated in the femur, but by different integrins. It is known that cell adhesion is important for osteoclast resorption, but the significance of this pathway in these two skeletal sites is unclear at the moment.

Experimental tests on genes involved in these pathways may be done in future studies to validate their roles at these skeletal sites.

In the femur, the presence of those pathways may suggest that increase in activity of aged monocytes is the result of ATP generation of metabolic genes. In a study done by Taubmann et al., they noted a simultaneous increase in both glycolytic activity and mitochondrial respiration when comparing activated osteoclasts to resting osteoclasts, suggesting that bone resorption considerably increases osteoclast metabolic and energy requirements[67]. This adaption between the osteoclasts was thought to be linked to an increased demand in energy, that may also be supported by the presence of metabolic pathways in the femoral monocytes of 1-year-old mice. The upregulation of the chemotaxis pathway in the femur-derived monocytes may denote recruitment of immune cells to the site, including osteoclast precursors, to support repair or bone remodeling[68]. Based on these findings, it is possible that there could be more osteoclast activity in the femur compared to the mandible. Additional experiments looking more into these enriched pathways and genes will be included in future studies.

With the data collected from the studies on 2-month-old mice and 1-year-old mice, we can see that not only are the animals getting older but the cells within them are changing as well. Understanding the changes in cellular processes with aging in mice contributes to our understanding of the molecular changes at these skeletal sites in humans. 1-year-old mice were used in this study to correlate to humans 38-47 years of age, but perhaps more significant findings could be seen in additional studies with mice 18-24 months, being comparable to humans of 56-69 years of age (Jackson Laboratory). The data collected from these experiments was compared to a study done by Clark et al. who studied the osteoclasts of 2-month-old mice. The experiments on 1-year-old mice primarily utilized

osteoclast precursors, which are seen earlier on in the osteoclast lineage. Since non-adherent cells were more suitable for the experiments performed, monocytes were used in this study. Although they exist within the same family, there could be discrepancies when inferring trends between the two data sets. Future studies may also include a bone resorption assay to look at osteoclast activity between the skeletal sites. As this study sought to answer the question of variability between osteoclast precursors at different skeletal sites, information is lacking on the amount and rate of resorption found at those sites. An *in vitro* bone pit resorption assay would serve as a valuable investigational tool in this study, as it can evaluate the major parameters controlling bone remodeling (new sites initiated and rate of resorption by osteoclasts) in normal and pathologic states[69]. Since this study rid mice of any pathological factors to solely study the effect of aging, the effect of estrogen changes on the osteoclast activity at these skeletal sites could be advised. Estrogen is known to protect bone loss in men and women by suppressing bone resorption and inversely, deficiency in estrogen predisposes an individual to osteoporosis[70]. Even though estrogen has mainly been thought to affect cells of osteoclast lineage, in rodent studies there is evidence suggesting that it stimulates bone formation[71] and in humans they have found evidence to infer that high doses could stimulate bone formation[72]. Aging could potentially be driven more by osteoblasts than osteoclasts. The mechanisms underlying estrogen deficiency and its effects on bone cell activity are not completely understood, but implementing this type of experiment utilizing ovariectomized mice may contribute.

From previous research, it was unclear as to what changes we would have expected to see in these experiments. Two schools of thought exist to say that the craniofacial region and appendicular skeleton are similar or that these skeletal sites differ. Much of the earlier research publications claimed that there were no differences between the craniofacial

region and appendicular skeleton. In studying variation in mineral density of the mandible and of the radius in both sexes, Henrikson et al. claimed that the changes seen in these skeletal sites with age were qualitatively the same[37]. Hildebolt et al. explained that cross-sectional QCT data of selective measures of vertebral cortical and trabecular bone demonstrated similar rate of cortical demineralization between the skeletal sites[73]. These studies were done on individuals in diseased states such as osteoporosis or nutritional deficiencies and lacked descriptions of the cellular processes within these skeletal regions. More recent research publications have shifted views with more diverse experiments to support the idea that these sites represent drastically different populations. Aghaloo et al. looked at osteogenic potential of bone marrow stromal cells in the mandible and long bones and found significantly increased alkaline phosphatase and osteocalcin mRNA expression in the mandible compared to the femur, demonstrating that mandible bone marrow stromal cells possess higher osteogenic potential compared to their long bone counterparts[46]. A similar finding was found in our study, where the highly enriched pathways in the mandible and femur suggest an osteoblast-mediated environment in the mandible and higher osteoclast activity in the femur. With analysis of the expression of osteoclast- and osteoclastogenesis-related genes, de Souza Faloni et al. confirmed that kinetic differences in osteoclast-forming capacity between the mandible and long bones exist[41]. These recent studies supporting the claim of the mandible and femur representing distinct entities were performed on younger mice (8 weeks or 1-month-old). There is limited amount of information on what happens to the cellular composition and activity at these skeletal sites as an individual ages, but our study begins to fill that gap of knowledge. Our research is supported by more recent publications, stating that the mandible and femur represent two distinct environments regulated by different mechanisms.

With more evidence revealing and contributing to our understanding of changes that occur between skeletal sites with aging, strategies have been proposed to help mitigate its effects. Kribbs et al. found a significant relationship between residual ridge and alveolar bone density with total body calcium and regional bone mass, therefore concluding that therapeutic measures targeting osteoporosis could exert an effect on jaw residual ridge density[30]. Tissue engineering technologies have also been studied to offer clinically practical solutions to bolster insufficient bone formation in the elderly, osteoporotic patient[74]. Methylsulfonylmethane, a potent anti-inflammatory, can be used as a therapeutic agent to improve bone loss-associated diseases, including periodontitis, post-menopausal osteoporosis, and rheumatoid arthritis[75]. Measurement analyses such as microdensitometry could be helpful in depicting the efficacy of different therapeutic strategies to address bone loss[31]. Considering various treatment strategies to address patients' particular needs will be an integral part in all stages of treatment.

We have seen a substantial increase in the number of adult patients seeking orthodontic care over the past years. That being said, it is imperative that we understand the differences of the craniofacial bone composition that occur with aging. We cannot simply presume that the conventional treatment that our younger patients undergo can be applied to those of older age without any risks, particularly those of periodontal concern. The differences in growth potential between these two populations is an important distinguishing factor for particular orthodontic treatment modalities, but does not omit patients of older age to seek care for their particular needs. Limited studies have been published on this matter, but Dyer et al. studied 30 female patients with a mean initial records age of 12.5 and 26 female patients with a mean staring age of 27.6 presenting with a class II sagittal molar relationship[23]. This study found that adult treatment did not necessarily equate to a longer

duration of treatment and treatment times were comparable to both groups at 2.5 years. This study also revealed that adult patients exhibited dental sequelae of class II elastic force more than the younger patients[23]. In 2018, Schubert et al. performed a systematic search of prospective clinical trials investigating the rate of orthodontic tooth movement and/or studies assessing age-related changes shown in the composition of gingival crevicular fluid[2]. Patients were healthy individuals who ranged between the ages 16 and 43. It was found that more pronounced cytokine levels and faster orthodontic tooth movement were shown by the younger patients, and a delayed reaction to orthodontic forces was seen in the older patients[2]. Both studies mentioned the lack of clarity on whether aging affects the rate of orthodontic tooth movement, and it appears that interindividual variations play a more significant role in which biological processes are triggered by orthodontic forces[2]. These studies lack data about the bone remodeling activity with respect to osteoclasts and osteoblasts within the mandible. In our study, data from RNA-seq inferred that aging may be an osteoblast-driven process, with increase in biomineralization, and could be reflected in Shubert et al.'s study as the reason in which a delay in reaction to orthodontic forces was observed. Additional studies are needed to draw connections between the study populations.

In summary, this study provides insight of the osteoclast precursor population at mandibular and femoral sites, including gene expression profile and differential pathway analysis. The results supported my hypothesis of differences in gene expression and increased differentiation capabilities of osteoclast precursors derived from the bone marrow of the mandible compared to the femur. The larger size and decreased number of osteoclasts found in the mandible when compared to the femur was seen in both 2-month-old and 1-year-old mice. Through bulk RNA-sequencing, it was shown that different

pathways affect these two skeletal sites in an aging population. This may suggest that there may be different mechanisms regulating the craniofacial and appendicular regions. This study provides a foundational understanding of how a healthy state presents in comparison to a diseased state. Additional experiments can be performed to determine the resorption activity at both skeletal sites for further understanding. The mandible and femur of 1-year-old mice exhibit distinct characteristics from each other and with 2-month-old mice in regards to femoral data. Through appreciation of the differences observed, a better understanding of what osteoclastic changes occur with aging may be noted and utilized within treatment planning for older patients seeking orthodontic care.

Conclusions

- Differences in gene expression and differentiation capabilities exist between the mandible and femur of 1-year-old mice.
- Distinct pathways may regulate the craniofacial and appendicular regions.
- Similarities between 2-month-old and 1-year-old mice exist:
    - Trends in size and amount of osteoclasts
- The differences seen the in osteoclasts between young and old mice requires further investigation, with emphasis in understanding how these differences impact orthodontic tooth movement and bone remodeling in older patients.

References

1. Weiss, R.C., *Physiology of adult tooth movement.* Dent Clin North Am, 1972. **16**(3): p. 449-57.
2. Schubert, A., et al., *Age effect on orthodontic tooth movement rate and the composition of gingival crevicular fluid : A literature review.* J Orofac Orthop, 2020. **81**(2): p. 113-125.
3. Barr, C., et al., *Noninflammatory Stress-Induced Remodeling of Mandibular Bone: Impact of Age and Pregnancy.* J Oral Maxillofac Surg, 2021. **79**(5): p. 1147-1155.
4. Hadjidakis, D.J. and Androulakis, II, *Bone remodeling.* Ann N Y Acad Sci, 2006. **1092**: p. 385-96.
5. Boyle, W.J., W.S. Simonet, and D.L. Lacey, *Osteoclast differentiation and activation.* Nature, 2003. **423**(6937): p. 337-42.
6. Quinn, J.M., et al., *A combination of osteoclast differentiation factor and macrophage-colony stimulating factor is sufficient for both human and mouse osteoclast formation in vitro.* Endocrinology, 1998. **139**(10): p. 4424-7.
7. Muto, A., et al., *Lineage-committed osteoclast precursors circulate in blood and settle down into bone.* J Bone Miner Res, 2011. **26**(12): p. 2978-90.
8. Pierce, A.M., S. Lindskog, and L. Hammarström, *Osteoclasts: structure and function.* Electron Microsc Rev, 1991. **4**(1): p. 1-45.
9. Väänänen, H.K., et al., *The cell biology of osteoclast function.* J Cell Sci, 2000. **113 (Pt 3)**: p. 377-81.
10. Park-Min, K.H., *Metabolic reprogramming in osteoclasts.* Semin Immunopathol, 2019. **41**(5): p. 565-572.
11. Nakamura, M., et al., *Expression of tartrate-resistant acid phosphatase and cathepsin K during osteoclast differentiation in developing mouse mandibles.* Biomed Res, 2021. **42**(1): p. 13-21.
12. Chambers, T.J., et al., *Resorption of bone by isolated rabbit osteoclasts.* J Cell Sci, 1984. **66**: p. 383-99.
13. Saltel, F., et al., *Apatite-mediated actin dynamics in resorbing osteoclasts.* Mol Biol Cell, 2004. **15**(12): p. 5231-41.
14. Ramasamy, S.K., et al., *Endothelial Notch activity promotes angiogenesis and osteogenesis in bone.* Nature, 2014. **507**(7492): p. 376-380.
15. Heubel, B. and A. Nohe, *The Role of BMP Signaling in Osteoclast Regulation.* J Dev Biol, 2021. **9**(3).
16. Shi, C., et al., *BMP Signaling Mediated by BMPR1A in Osteoclasts Negatively Regulates Osteoblast Mineralization Through Suppression of Cx43.* J Cell Biochem, 2017. **118**(3): p. 605-614.
17. Pinho, S. and P.S. Frenette, *Haematopoietic stem cell activity and interactions with the niche.* Nat Rev Mol Cell Biol, 2019. **20**(5): p. 303-320.
18. Aluganti Narasimhulu, C. and D.K. Singla, *The Role of Bone Morphogenetic Protein 7 (BMP-7) in Inflammation in Heart Diseases.* Cells, 2020. **9**(2).
19. Mandal, C.C., G. Ghosh Choudhury, and N. Ghosh-Choudhury, *Phosphatidylinositol 3 kinase/Akt signal relay cooperates with smad in bone*

morphogenetic protein-2-induced colony stimulating factor-1 (CSF-1) expression and osteoclast differentiation. Endocrinology, 2009. **150**(11): p. 4989-98.
20. Wang, C.-Y., *Bone Morphogenic Proteins, Osteoblast Differentiation, and Cell Survival During Osteogenesis*. Vol. *The Skeleton: Biochemical, Genetic, and Molecular Interactions in Development and Homeostasis* . 2004: Humana Press.
21. Khosla, S., J.J. Westendorf, and M.J. Oursler, *Building bone to reverse osteoporosis and repair fractures.* J Clin Invest, 2008. **118**(2): p. 421-8.
22. Pederson, L., et al., *Regulation of bone formation by osteoclasts involves Wnt/BMP signaling and the chemokine sphingosine-1-phosphate.* Proc Natl Acad Sci U S A, 2008. **105**(52): p. 20764-9.
23. Dyer, G.S., E.F. Harris, and J.L. Vaden, *Age effects on orthodontic treatment: adolescents contrasted with adults.* Am J Orthod Dentofacial Orthop, 1991. **100**(6): p. 523-30.
24. Bainbridge, K.E., et al., *Risk factors for low bone mineral density and the 6-year rate of bone loss among premenopausal and perimenopausal women.* Osteoporos Int, 2004. **15**(6): p. 439-46.
25. Guthrie, J.R., et al., *A prospective study of bone loss in menopausal Australian-born women.* Osteoporos Int, 1998. **8**(3): p. 282-90.
26. Burger, H., et al., *Risk factors for increased bone loss in an elderly population: the Rotterdam Study.* Am J Epidemiol, 1998. **147**(9): p. 871-9.
27. Wilsgaard, T., et al., *Lifestyle impact on lifetime bone loss in women and men: the Tromsø Study.* Am J Epidemiol, 2009. **169**(7): p. 877-86.
28. Baxter, J.C., *Relationship of osteoporosis to excessive residual ridge resorption.* J Prosthet Dent, 1981. **46**(2): p. 123-5.
29. Benson, B.W., T.J. Prihoda, and B.J. Glass, *Variations in adult cortical bone mass as measured by a panoramic mandibular index.* Oral Surg Oral Med Oral Pathol, 1991. **71**(3): p. 349-56.
30. Kribbs, P.J., D.E. Smith, and C.H. Chesnut, 3rd, *Oral findings in osteoporosis. Part II: Relationship between residual ridge and alveolar bone resorption and generalized skeletal osteopenia.* J Prosthet Dent, 1983. **50**(5): p. 719-24.
31. Kribbs, P.J., D.E. Smith, and C.H. Chesnut, 3rd, *Oral findings in osteoporosis. Part I: Measurement of mandibular bone density.* J Prosthet Dent, 1983. **50**(4): p. 576-9.
32. Clark, R., et al., *Mouse mandibular-derived osteoclast progenitors have differences in intrinsic properties compared with femoral-derived progenitors.* JBMR Plus, 2024. **8**(5): p. ziae029.
33. Kwack, K.H., et al., *Discovering Myeloid Cell Heterogeneity in Mandibular Bone - Cell by Cell Analysis.* Front Physiol, 2021. **12**: p. 731549.
34. Epker, B.N., M. Kelin, and H.M. Frost, *Magnitude and location of cortical bone loss in human rib with aging.* Clin Orthop Relat Res, 1965. **41**: p. 198-203.
35. von Wowern, N. and K. Stoltze, *Sex and age differences in bone morphology of mandibles.* Scand J Dent Res, 1978. **86**(6): p. 478-85.
36. Liu, C.C., et al., *Pore size measurements and some age-related changes in human alveolar bone and rat femur.* J Dent Res, 1977. **56**(2): p. 143-50.

37. Henrikson, P.A. and K. Wallenius, *The mandible and osteoporosis (1). A qualitative comparison between the mandible and the radius.* J Oral Rehabil, 1974. **1**(1): p. 67-74.
38. Ruggiero, S.L., et al., *Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases.* J Oral Maxillofac Surg, 2004. **62**(5): p. 527-34.
39. AW, H. and C. DH, *Histology*. 8th ed. 1979.
40. Klemetti, E., et al., *Cortical bone mineral density in the mandible and osteoporosis status in postmenopausal women.* Scand J Dent Res, 1993. **101**(4): p. 219-23.
41. de Souza Faloni, A.P., et al., *Jaw and long bone marrows have a different osteoclastogenic potential.* Calcif Tissue Int, 2011. **88**(1): p. 63-74.
42. Huja, S.S., et al., *Remodeling dynamics in the alveolar process in skeletally mature dogs.* Anat Rec A Discov Mol Cell Evol Biol, 2006. **288**(12): p. 1243-9.
43. Chai, Y. and R.E. Maxson, Jr., *Recent advances in craniofacial morphogenesis.* Dev Dyn, 2006. **235**(9): p. 2353-75.
44. Matsubara, T., et al., *Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells.* J Bone Miner Res, 2005. **20**(3): p. 399-409.
45. Akintoye, S.O., et al., *Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals.* Bone, 2006. **38**(6): p. 758-68.
46. Aghaloo, T.L., et al., *Osteogenic potential of mandibular vs. long-bone marrow stromal cells.* J Dent Res, 2010. **89**(11): p. 1293-8.
47. Damek-Poprawa, M., et al., *Human bone marrow stromal cells display variable anatomic site-dependent response and recovery from irradiation.* Arch Oral Biol, 2010. **55**(5): p. 358-64.
48. Yamaza, T., et al., *Mouse mandible contains distinctive mesenchymal stem cells.* J Dent Res, 2011. **90**(3): p. 317-24.
49. Gruber, R., *Osteoimmunology: Inflammatory osteolysis and regeneration of the alveolar bone.* J Clin Periodontol, 2019. **46 Suppl 21**: p. 52-69.
50. Lerner, U.H., E. Kindstedt, and P. Lundberg, *The critical interplay between bone resorbing and bone forming cells.* J Clin Periodontol, 2019. **46 Suppl 21**: p. 33-51.
51. Connizzo, B.K., et al., *Nonuniformity in Periodontal Ligament: Mechanics and Matrix Composition.* J Dent Res, 2021. **100**(2): p. 179-186.
52. Lin, W., et al., *Mapping the immune microenvironment for mandibular alveolar bone homeostasis at single-cell resolution.* Bone Res, 2021. **9**(1): p. 17.
53. Li, Z., et al., *Mouse embryonic head as a site for hematopoietic stem cell development.* Cell Stem Cell, 2012. **11**(5): p. 663-75.
54. Karaplis, A.C., *Principles of Bone Biology*. 33-IV ed. Vol. Embryonic development of bone and the molecular regulation of intramembranous and endochondral bone formation. 2002.
55. Ueki, Y., et al., *Mutations in the gene encoding c-Abl-binding protein SH3BP2 cause cherubism.* Nat Genet, 2001. **28**(2): p. 125-6.

56. Simonds, W.F., et al., *Familial isolated hyperparathyroidism: clinical and genetic characteristics of 36 kindreds.* Medicine (Baltimore), 2002. **81**(1): p. 1-26.
57. Huang da, W., B.T. Sherman, and R.A. Lempicki, *Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources.* Nat Protoc, 2009. **4**(1): p. 44-57.
58. Sherman, B.T., et al., *DAVID: a web server for functional enrichment analysis and functional annotation of gene lists (2021 update).* Nucleic Acids Res, 2022. **50**(W1): p. W216-W221.
59. Lees, R.L., V.K. Sabharwal, and J.N. Heersche, *Resorptive state and cell size influence intracellular pH regulation in rabbit osteoclasts cultured on collagen-hydroxyapatite films.* Bone, 2001. **28**(2): p. 187-94.
60. Gebhardt, C., et al., *S100A8 and S100A9 in inflammation and cancer.* Biochem Pharmacol, 2006. **72**(11): p. 1622-31.
61. Roberts, J.L., et al., *Deletion of Wnt5a in osteoclasts results in bone loss through decreased bone formation.* Ann N Y Acad Sci, 2020. **1463**(1): p. 45-59.
62. Kresnoadi, U., J. Widjaja, and H. Laksono, *Ethanol extract of propolis-bovine bone graft combination as a prospective candidate for socket preservation: Enhancing BMP7 and decreasing NFATc1.* Saudi Dent J, 2021. **33**(8): p. 1055-1062.
63. Fang, Z., et al., *CYP27A1 deficiency promoted osteoclast differentiation.* PeerJ, 2023. **11**: p. e15041.
64. Ross, F.P. and S.L. Teitelbaum, *alphavbeta3 and macrophage colony-stimulating factor: partners in osteoclast biology.* Immunol Rev, 2005. **208**: p. 88-105.
65. McHugh, K.P., et al., *Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts.* J Clin Invest, 2000. **105**(4): p. 433-40.
66. Sakai, H., et al., *Cell adhesion is a prerequisite for osteoclast survival.* Biochem Biophys Res Commun, 2000. **270**(2): p. 550-6.
67. Taubmann, J., et al., *Metabolic reprogramming of osteoclasts represents a therapeutic target during the treatment of osteoporosis.* Sci Rep, 2020. **10**(1): p. 21020.
68. Sanchez-Fernandez, M.A., et al., *Osteoclasts control osteoblast chemotaxis via PDGF-BB/PDGF receptor beta signaling.* PLoS One, 2008. **3**(10): p. e3537.
69. Helfrich, M.H. and Stuart H. Ralston, *Bone Research Protocols*. Vol. 80. 2008: Springer Science & Business Media.
70. Khosla, S., L.J. Melton, 3rd, and B.L. Riggs, *Estrogens and bone health in men.* Calcif Tissue Int, 2001. **69**(4): p. 189-92.
71. Samuels, A., M.J. Perry, and J.H. Tobias, *High‐dose estrogen induces de novo medullary bone formation in female mice. . Journal of Bone and Mineral Research* ,, 1999: p. 178-186.
72. Tobias, J.H. and J.E. Compston, *Does estrogen stimulate osteoblast function in postmenopausal women?* 1999, *Bone*. p. 121-124.
73. Hildebolt, C.F., *Osteoporosis and oral bone loss.* Dentomaxillofac Radiol, 1997. **26**(1): p. 3-15.
74. Hollinger, J.O., S. Winn, and J. Bonadio, *Options for tissue engineering to address challenges of the aging skeleton.* Tissue Eng, 2000. **6**(4): p. 341-50.

75. Aljohani, H., et al., *Methylsulfonylmethane Increases the Alveolar Bone Density of Mandibles in Aging Female Mice.* Front Physiol, 2021. **12**: p. 708905.