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# Contribution of genetic and environmental factors on osteocyte mineralization

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# CONTRIBUTION OF GENETIC AND ENVIRONMENTAL FACTORS ON OSTEOCYTE MINERALIZATION

by

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**HANNAH COOPER**

ABSTRACT

As the rates of osteoporosis continue to rise, it is necessary to gain a better understanding of the genetic and environmental factors contributing to disease development and progression. Innate factors such as age, sex, and race significantly impact the likelihood of developing osteoporosis. Acquired factors such as BMI, smoking status, and renal disease have also been shown to impact bone growth and stability. The goal of this study was to evaluate the effects these factors have on osteogenic cell growth. It was hypothesized that primary cultures of human osteogenic cells will be more affected by comorbidities present at the time of harvest. As the cultures expand and homogenize the impact of the innate and acquired factors should dissipate. To examine this hypothesis primary marrow stromal cultures grown under osteogenic conditions were compared to secondary cultures grown under the same conditions. Specific biochemical features of the cultures that were compared, were the overall growth of the cultures based on their DNA contents, and DNA normalized values of Alkaline Phosphate (ALP) activity, protein, calcium, and hydroxyproline. Osteogenic phenotypic properties were further assessed, by assaying the specific mRNA expression of osteogenic lineage stage specific genes. Although there were no significant differences between the primary and secondary cultures, there were trends of increased calcium and hydroxyproline in secondary samples. Several moderately strong positive correlations were found between

biochemical markers, as well as a moderately strong negative correlation between calcium and hydroxyproline in secondary cultures. To determine significance between these differences and relationships, increased sample size and further analyses are needed.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ALP | Alkaline phosphatase |
| $\alpha$-MEM | Minimum essential media - alpha |
| Anti-anti | Antibiotic-antimitotic |
| ATP | Adenosine triphosphate |
| BMD | Bone mineral density |
| BMI | Body Mass Index |
| cDNA | Complementary DNA |
| Col1 | Collagen type 1 |
| DMP1 | Dentin Protein Matrix 1 |
| DXA | Dual x-ray absorptiometry |
| ERT | Estrogen replacement therapy |
| FBS | Fetal bovine serum |
| PPi | Inorganic pyrophosphate |
| MSC | mesenchymal stem cell |
| OC | Osteocalcin |
| OPG | Osteoprotegrin |
| PTH | Parathyroid hormone |
| pNPP | P-nitrophenol phosphate |
| RANKL | Receptor activator of nuclear factor kappa-$\beta$ ligand |
| RT-qPCR | Reverse transcriptase quantitative polymerase chain reaction |
| SOST | Sclerostin |

| | |
|---|---|
| ucOC | Undercarboxylated osteocalcin |

# INTRODUCTION

## Bone Physiology

### *Structure*

The skeletal system has many critical roles such as mechanical support, protection, and hematopoiesis. Bone is a connective tissue composed of organic and inorganic compounds. The primary organic element is type I collagen. Embedded within the collagen matrix are hydroxyapatite crystals, which constitute the inorganic component (Toosi and Behravan, 2020). Structurally, bone is categorized by age and structure (Figure 1). Primary bone, also known as woven bone, is characterized by poorly organized type I collagen. Woven bone is highly cellular and low in mineral content. Eventually, woven bone is replaced by secondary, or lamellar. Lamellar bone is further broken down into osteons, trabecular, circumferential, and interstitial bone. Osteons are the primary structural unit. Concentric lamellae surround a central Haversion canal, which houses blood vessels, nerves, and lymphatics. Osteons in trabecular, or cancellous bone are arranged differently. This results in sinusoids, which allow for communication between marrow and mineralized bone (Bartold et al., 2019). The increased surface area provides increased cellular and vascular content. The outer surface of bone is known as compact, or cortical bone. Densely packed osteons provide high resistance to bending and torsion. The outer surface of cortical bone is surrounded by periosteum. This thick collagenous layer serves as an attachment point for ligaments and provides blood supply (Bartold et al., 2019).

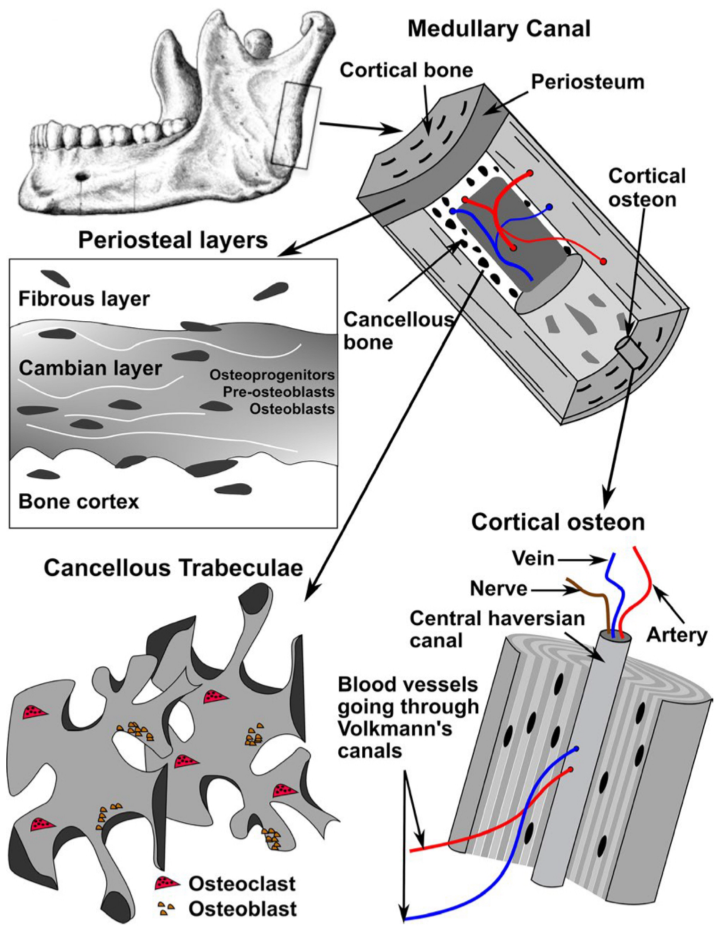



**Figure 1 – Periosteal, cortical, and cancellous components of bone (taken from Bartold et al., 2019).**

*Embryology*

Connective tissues such as bone and cartilage of the axial and appendicular skeleton are derived from the mesoderm. During embryogenesis, bone forms via intramembranous or endochondral ossification. In endochondral formation, cartilage models are replaced with mineralized bone (Berendsen and Olsen, 2015). Several local factors progress the chondrocytes into a non-proliferative hypertrophic state while simultaneously inducing differentiation of osteoblast progenitors. Concurrently, osteoclasts, blood vessel endothelial cells, and hematopoietic cells enter the cartilage matrix (Berendsen and Olsen, 2015). These cells work with the osteoblasts to form the trabecular bone of the marrow cavity. Additional osteoblasts from the perichondrium form the cortical bone that surrounds the trabecular bone and marrow cavity. Most of the skeleton is formed by endochondral ossification: skull base, posterior skull, axial skeleton, and appendicular skeleton.

Intramembranous ossification is the formation of bone without a cartilage scaffold. Mesenchymal stem cells (MSCs) different directly into osteoblasts and osteoid is deposited directly within a connective tissue space (Bartold et al., 2019). The collagenous matrix calcifies, leaving mineralized bone. Additional MSCs surround the new bone, eventually forming the periosteum. Only the neuro- and viscerocranium and clavicle are formed via intramembranous ossification (Berendsen and Olsen, 2015). Following embryonic formation, endochondral and intramembranous bone undergo continual cycles of resorption and formation.

### *Bone Remodeling*

The coordinated actions of osteoblasts and osteoclasts results in continuous bone remodeling (Figure 2). Osteoblasts are responsible for bone formation and mineralization, while osteoclasts are responsible for bone resorption. A third cell type, osteocytes, are responsible for signal transduction of mechanical stimuli (Toosi and Behraven, 2020). Osteoblasts are derived from MSCs. Their differentiation is triggered by several local and systemic factors, such as Indian hedgehog and parathyroid hormone-related peptide

(Berendsen and Olsen, 2015). Commitment to the osteoblastic lineage is regulated by Wnt/B-catenin signaling (Boyce and Xing, 2007). This pathway directly regulates OPG expression and plays a central role in determining the amount of bone deposited by osteoblasts. Mature osteoblasts secrete a collagenous protein called osteoid. Osteogenic activity is halted once osteoblasts embed themselves in the osteoid and become osteocytes.

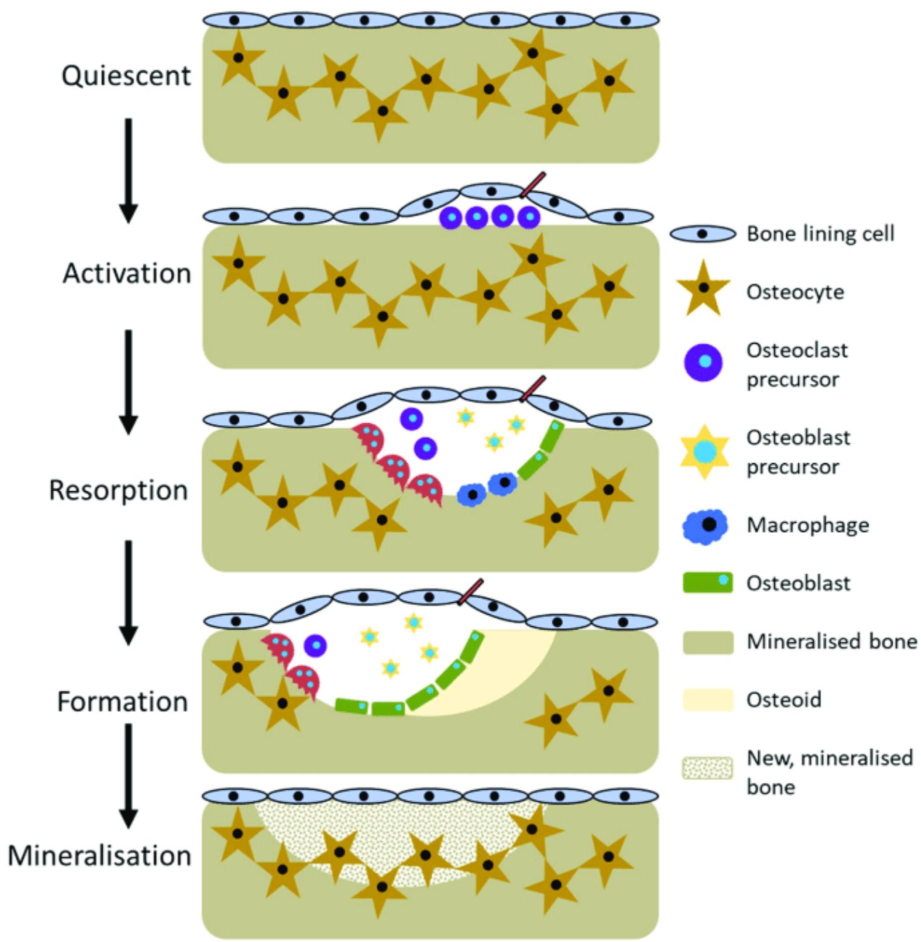



**Figure 2 – Coupled remodeling of bone (taken from Owen, 2018).**

Osteoblasts secrete osteoprotegrin (OPG), which inhibits osteoclast differentiation. OPG works as a decoy receptor and prevents the binding of RANKL to the RANK receptor (Sandhu and Hampson, 2011). Increased RANK mediated signaling results in increased osteoclast differentiation and activity. The OPG/RANKL ratio is critical to maintain normal bone turnover and bone mass (Sandhu and Hampson, 2011). Many factors responsible for RANKL expression, such as parathyroid hormone, also regulate OPG. Thus, RANKL and OPG work on a negative feedback loop. Macrophage colony-stimulating factor (M-CSF) is also released by osteoblasts and is critical to osteoclast differentiation (Boyce and Xing, 2007).

Osteoclasts are multinucleated cells from the myeloid lineage of hematopoietic stem cells. Bone resorbing ability often correlates positively with the number of nuclei (Boyce and Xing, 2007). Differentiation of myeloid progenitor cells is mediated by the secretion of RANKL from osteoblasts. Once bound to the RANK receptor, several intracellular pathways are activated, leading to the induction of osteoclastogenic genes (Sandhu and Hampson, 2011). Expression of one such transcription factor, c-Fos, is required for differentiation of osteoclast precursors to osteoclasts (Boyce and Xing, 2007). Mature osteoclasts resorb bone by binding to the bone's surface, where they release hydrochloric acid and cathespin K (Boyce and Xing, 2007). The bone mineral is dissolved, and the collagen is degraded.

### *Pathophysiology*

Osteoporosis is characterized by a failure to achieve peak bone mass due to impaired bone remodeling. This is typically due to a combination of excessive bone resorption and/or decreased bone formation (Sandhu and Hampson, 2011). Osteoporosis presents with biochemical and morphological evidence of high bone turnover, specifically in cancellous bone. Degradation of spicules decreases the available surface area of templates used for new bone formation (Prestwood et al., 1995). Genetics, hormonal status, and environmental factors all contribute to reaching peak bone mass (Sandhu and Hampson, 2011).

Bone relies on the coordinated action of osteoblasts and osteoclasts to maintain its structural integrity. Disruption of OPG/RANKL/RANK and Wnt/B-catenin signaling pathways has a significant impact on bone mass (Boyce and Xing, 2007). Wnt/B-catenin directly affects gene expression in osteoblasts, while OPG/RANKL/RANK expression impacts osteoclast activity. There are several genetic variants that regulate bone mass, such as low-density lipoprotein, receptor-related protein 5 (LRP5), sclerostin (SOST), OPG, estrogen receptor 1, and RANK pathway genes (Sandhu and Hampson, 2011).

Hormonal status plays a significant role, especially for postmenopausal women. Decreased estrogen leads to increased bone resorption and decreased bone formation (Prestwood et al., 1995). Aging is also associated with decreased bone formation. MSCs differentiate preferentially into adipocytes rather than osteoblasts (Sandhu and Hampson, 2011). Decreased osteoblast presence, and therefore activity, results in decreased bone formation. Decreased bone formation coupled with increased bone resorption significantly improves fracture risk.

### *Diagnosis*

Most cases of osteoporosis are diagnosed only after a fracture has occurred. Fractures most associated with osteoporosis are fracture of the vertebral bodies, ribs, proximal femur, humerus, and distal radius (Glaser and Kaplan, 1997). The diagnostic criteria established the World Health Organization is based on bone mineral density (BMD) value obtained from dual-energy x-ray absorptiometry (DXA). As the x-ray beams pass through bone, energy is absorbed, and the detected on the other side. Denser bone will absorb more energy, therefore resulting in less energy detected (Berger, 2002). Measurements are typically taken from the femoral neck and vertebral column (Lane, 2006). A reference population is used to determine the patient's T-score. A normal T-score is between +1 and -1. T-scores between -1 and -2.5 indicate low bone mass, while a T-score of -2.5 or below is indicative of osteoporosis (Glaser and Kaplan, 1997). BMD is the most used diagnostic tool; however, a patient's fracture history and additional risk factors must be considered.

For women who are premenopausal and men below the age of 50, the International Society for Clinical Densitometry recommends using the Z-score. A Z-score less than or equal to -2.0 indicates BMD below the expected range for age (Sandhu and Hampson, 2011). A diagnosis of osteoporosis considers BMD, fracture history, and hormonal status. Lab values for serum calcium, inorganic phosphorus, and alkaline phosphatase (ALP) typically fall within the normal range (Glaser and Kaplan, 1997).

### *Biochemical Markers*

BMD is the basic phenotypic property that is measured as feature of normal bone health and as feature related to the risk of fracture. BMD is the best diagnostic tool; however, it is most useful for monitoring changes that have occurred over long periods of time. Rapid changes due to therapeutic interventions cannot be easily identified. Instead, biochemical markers of bone formation, resorption, and turnover can be used as a diagnostic aid and to identify rapid therapeutic changes.

Alkaline phosphatase (ALP) is a plasma membrane enzyme produced by osteoblasts during bone formation. ALP provides inorganic phosphate for synthesis of bone's mineral component, hydroxyapetite. Additionally, ALP hydrolyzes inorganic pyrophosphate, which is an inhibitor of bone mineralization. Measurements of ALP correlate with rates of bone mineralization and indicate metabolic activity (Swaminathan, 2001).

Osteocalcin (OC) is the most abundant non-collagenous protein produced by osteoblasts. Structurally, there are three gamma carboxyl glutamic acid residues that facilitate OC binding to hydroxyapatite (Atalay et al., 2012). Increased serum OC is indicative of high bone turnover and decreased bone mineral density. Imperfect gamma carboxylation results in undercarboxylated osteocalcin (uOC), which is also a marker of bone turnover and is associated with increased hip fracture risk (Vergnaud et al., 1997). In cases of postmenopausal osteoporosis, serum OC, uOC, and ALP are highest 1-5 years after the onset of menopause (Atalay et al., 2012).

Procollagen I is secreted by osteoblasts. Prior to incorporation into the organic bone matrix, extension peptides are cleaved and released into circulation (Swaminathan, 2001). The resultant cleaved procollagen I carboxyterminal propeptide and procollagen I aminoterminal propeptide are markers of bone formation (Christenson, 1997).

Hydroxyproline is a post-translational product of proline, an amino acid found in type I collagen (Christenson, 1997). Because hydroxyproline is not reusable, it serves as an indicator of collagen breakdown and therefore a marker of bone resorption. Type I collagen degradation by osteoclasts also produces N-telopeptide and C-telopeptide. N-telopeptide is measured through urine assay, while serum or urine assays are used for C-telopeptide (Christenson, 1997).

*Types*

It is important to identify the type of osteoporosis, as this informs treatment protocols. Primary osteoporosis is the most common form and is further classified as Type I or Type II. Type I, also known as postmenopausal osteoporosis, is caused by estrogen deficiency (Glaser and Kaplan, 1997). Estrogen controls osteoblast differentiation and has anti-apoptotic effects (Marie and Kassem, 2011). Low estrogen results in excessive bone resorption. The rate of bone loss in postmenopausal women is greatest within the first five years of onset (Atalay et a., 2012).

Type II is age-related and occurs in both men and women. Aging is associated with decreased proliferative and differentiation abilities of MSCs (Marie and Kassem, 2011). Consequently, limited osteoblast availability leads to decreased bone formation and imbalanced bone remodeling. Additionally, MSCs preferentially differentiate into adipocytes rather than osteoblasts (Sandhu and Hampson, 2011).

Secondary osteoporosis is caused by several different diseases and medications. Hypercalcemia, as seen in hyperparathyroidism, is a common cause of osteoporosis (Prestwood et al., 1995). Increased parathyroid hormone (PTH) results in increased calcium release from bone. Gastrointestinal diseases also frequently result in osteoporosis. Release of inflammatory cytokines stimulates osteoclast activity, netting increased bone resorption (Ganesan et al., 2022). Diseases such as celiac decrease calcium and vitamin D absorption. Medications can also affect bone remodeling. For example, long term treatment with glucocorticoids is the most common cause of secondary osteoporosis (Marie and Kassem, 2011). Recent studies have shown decreased Wnt/B-catenin signaling, which is critical to osteoblast differentiation. Glucocorticoids also have an inhibitory effect on osteoblast proliferation, and lead to increased osteoblast apoptosis (Marie and Kassem, 2011).

### *External Factors*

Genetics, hormonal status, and environmental factors all contribute to peak bone mass (Sandhu and Hampson, 2011) (Figure 3). Behavioral factors such as physical activity, alcohol intake, and tobacco use can significantly impact BMD (Marie and Kassem, 2011). Age-related reduction in physical activity leads to progressive loss of muscle quantity and strength. Less mechanical force is placed on the skeletal system. Consequently, osteoblast proliferation and differentiation are impaired, and bone formation is reduced.

Nutritional status also plays a significant role in bone health. Nutritional components such as calcium, protein, and vitamin D are critical to bone maintenance. Vitamin D deficiency can lead to secondary hyperparathyroidism, bone loss, and increased fracture risk (Prestwood et al., 1995). Calcium and vitamin D supplementation have been found to decrease bone loss and fracture risk. Adequate protein intake is critical for preservation of bone formation and mass (Marie and Kassem, 2011).

Chronic alcohol consumption significantly impacts osteoblast activity and increases adipogenic differentiation. Oxidative stress due to alcohol may also inhibit Wnt/B-catenin signaling, thereby reducing differentiation of MSCs to osteoblasts (Marie and Kassem, 2011). Cigarette smoke also inhibits osteoblast proliferation and differentiation and is closely related to decreased bone mass and increased fracture risk (Pouresmaeili et al., 2018).

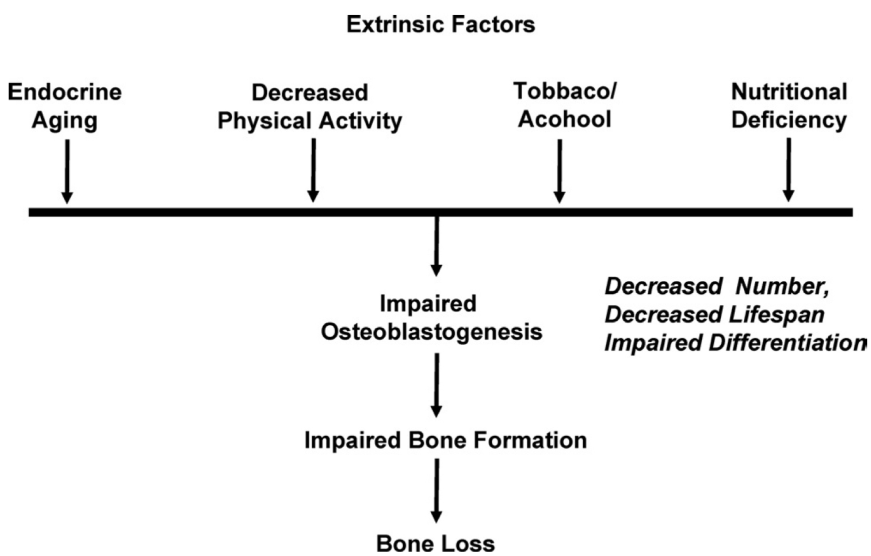



**Figure 3 – Extrinsic factors associated with decreased bone formation (taken from Marie and Kassem, 2011).**

*Drug Therapies*

Dietary and behavioral modifications include increased calcium and vitamin D intake, increased physical activity, decreased alcohol intake, and smoking cessation. For elderly patients, measures should be taken to decrease fall risk.

The most common drug therapy is bisphosphonates, which are nonhydrolyzable analogs of inorganic pyrophosphate (PPi) (Prestwood et al., 1995). PPi is a byproduct released in many tissues and inhibits calcification by binding to hydroxyapatite crystals.

By regulating PPi, bone mineralization can also be regulated. Bisphosphonates, which mimic the structure of PPi, have a high affinity for hydroxyapatite crystals (Drake et al., 2008). Bisphosphonates are preferentially incorporated into sites of bone remodeling and work to suppress bone resorption, induce osteoclast apoptosis, and limit osteoblast and osteocyte apoptosis (Fuggle et al., 2020).

Structurally, bisphosphonates contain a central nonhydrolyzable carbon surrounded by phosphate groups and variable side chains. There is a hydroxyl group on R1, which increases calcium binding ability (Drake et al., 2008). There are three generations of bisphosphonates. First generation bisphosphonates, such as etidronate, clondronate, and tiludronate, are non-nitrogen containing. Non-nitrogen containing bisphosphonates are structurally like PPi. Consequently, after osteoclast mediated uptake from the bone surface, they are incorporated into new adenosine triphosphate (ATP) (Drake et al., 2008). Accumulation of the bisphosphonate containing ATP is cytotoxic, resulting in inhibition of ATP dependent processes and osteoclast apoptosis (Sandhu and Hampson, 2011**)**.

Second and third generation bisphosphonates (alendronate, riserdronate, pamidronate, zoledronic acid) have nitrogen containing R2 side chains. Once taken up by osteoclasts, the nitrogen containing side chain allows the bisphosphonates to bind and inhibit the activity of farnesyl pyrophosphate synthase, a regulatory enzyme of the mevalonic acid pathway (Drake et al., 2008). This leads to inhibition of posttranslational protein modifications, resulting in osteoclast apoptosis. Although farnesyl pyrophosphate synthase is present in all cells, apoptosis is induced exclusively in osteoclasts. Due to the selective adherence to and retention within bone, the nitrogen-containing bisphosphonates are endocytosed only within osteoclasts (Drake et al., 2008).

Despite their efficacy, bisphosphonates can have negative side effects such as atypical femur fractures and osteonecrosis of the jaw (Tsourdi et al., 2017). Alternative biologic and anabolic agents have been the subject of alternative pharmacologic interventions. Denosumab is a human monoclonal antibody that binds to and inhibits RANKL, thereby inhibiting activation by RANK. The resultant decrease in osteoclast formation, function, and survival leads to decreased bone resorption (Tu et al., 2018). Trials of denosumab have shown significant reduction in new vertebral fractures, non-vertebral fractures, and hip fractures in women with osteoporosis (Cummings et al., 2009).

Teriparatide, an analog of PTH, has been found to reduce the risk of vertebral and non-vertebral fractures. Although chronic excess PTH leads to increased bone resorption, intermittent PTH has been shown to have a net anabolic effect. The slight increase in bone resorption is outweighed by the significant increase in bone formation. Due to the possibility of an increase in osteosarcoma risk, dose and treatment duration is limited.

Another alternative to bisphosphonates is estrogen replacement therapy (ERT). ERT has shown significant improvement in BMD, however it poses an increased risk of endometrial and breast cancer, as well as adverse cardiovascular events (Khosla and Hofbauer, 2017). Vitamin D and calcium supplementation also help to reduce fracture risk (Sunyecz, 2008). Therapeutic interventions result in rapid and dynamic changes that cannot be assessed with BMD (Atalay et al., 2012). Consequently, biochemical markers of bone remodeling are helpful for monitoring response to treatment.

### *Present Study*

The primary objective of this study is twofold: to evaluate the effect of innate versus acquired factors on marrow stromal osteogenic cells, and to evaluate the biochemical differences between primary and secondary cultures. Factors such as age, sex, race, BMI, smoking status, and renal disease have been shown to impact osteogenic differentiation and mineralization. Thus, as cell cultures expand and homogenize, the impacts of these innate and acquired factors should dissipate. Secondary cultures are likely to show decreased variability

## PROCEDURES

### MSC Isolation

Human research was done under a Boston University School of Medicine Institutional Research Board Approval: IRB Number: H-35199 "Bone Tissue Repository". Reamings obtained from coring of the acetabulum during total hip arthroplasty were placed in sterile 100ml capped specimen cups. The specimen cups were placed on ice and transported to the Orthopedics Laboratory at Boston University School of Medicine. The following procedures were performed in a sterile tissue culture hood. The bone marrow was transferred into a sterile 500ml Pyrex Wide Mouth Jar (Corning 1397-500) along with 100ml of Dulbecco's phosphate-buffered saline (DPBS) (GE Healthcare SH30028.LS) and 10ml 100X antibiotic-antimycotic (anti-anti) (Gibco Life Sciences #15240062). The jar was sealed and shaken vigorously for 30 seconds. A stainless-steel cell dissociation sieve was placed over a clean 500ml Pyrex jar. The wash was poured through the sieve and into the new jar while the marrow was kept in the original jar. An additional 100ml of DPBS and 10ml of anti-anti were added to the original jar with the bone marrow. The jar was sealed and shaken, and the second cell-containing wash was passed through the sieve into the second jar.

### MSC Processing

The final 200ml of wash was evenly divided among 6 x 50ml falcon tubes (Corning 430828). The tubes were centrifuged at 1150rpm for five minutes. 5ml of the top fat containing layer was removed from each tube. Each tube was shaken to resuspend the pellet. The wash from each tube was passed through a 70µm (Fisherbrand 22-363-

548) and then a 40µm (Fisherbrand 22-363-547) cell strainer, into a new falcon tube. The tubes were centrifuged at 1150rpm for five minutes. After aspirating the supernatant, 35ml of DPBS was added to each tube, the pellet was resuspended, and the tubes were spun down at 1150rpm for five minutes. The process was repeated two times, with cells being resuspended in 10ml of DPBS. The wash was combined into one falcon tube and centrifuged one last time at 1150rpm for five minutes. The supernatant was aspirated, and the final pellet was resuspended in 10ml of DPBS. After the final wash a 1:1000 dilution of cell suspension was prepared with DPBS. Cells were counted with a hemocytometer.

**Preparation of Frozen Stock**

12 5ml cryotubes (Greiner Bio-One 124276) were prepared at 20 million cells per vial. The calculated volume of cell suspension was added to 90% fetal bovine serum (FBS), 10% DMSO solution (Fisher Chemicals D128-500) and mixed thoroughly. 5ml of cell/FBS/DMSO solution were added to each vial and placed in a CoolCell 5ml LX Cell Freezing Container (Corning 432005). The freezing container was then stored at -80°C.

**MSC Plating**

Cells were plated at a density of $2.66 \times 10^6$ cells/cm$^2$. This equated to 24 million cells/well for 6-well plates (Corning 3516) and 5.32 million/well for 12-well plates (Corning 3513). 100mm dishes (Thermo Fisher #130182) were plated at a density of 80 million cells spread evenly across four dishes.

Prior to seeding, plates were treated with Animal Component-Free Cell Attachment Substate (Stem Cell Technologies #07130). A 1:150 dilution of attachment substrate and DPBS was made. Each 100mm dish was coated with 6.5ml of dilute attachment substrate. 24-well plates were prepared with 0.5ml per well and 6-well plated were prepared with 1ml per well. Plates were incubated for at least two hours. Immediately prior to plating, all culture ware was washed once with DPBS.

## MSC Growth

All cultures were initially plated in artificial basal media. The basal media consisted of 500ml Mesencult-ACF Plus Medium (Stem Cell Technologies #05445), 1ml Mesencult-ACF Plus 500X Supplement (Stem Cell Technologies #05447), 5ml 200mM L-Glutamine (2mM final concentration) (Stem Cell Technologies #07100), and 5ml 100X anti-anti (1X final concentration). Four days after plating a half media change was performed by removing half the volume of media and replacing it with fresh basal media. On day seven a full basal media change was performed. Thereafter the basal media was replaced in full every two days for two weeks.

### *Primary Cultures*

After two weeks in basal media, primary cultures in 6-well and 12-well dishes were switched to osteoinductive media. The osteoinductive media consisted of 500ml a-MEM Nucleosides (Gibco #12571063), 50ml FBS, 5ml anti-anti, 4ml 1M B-glycerophosphate (8mM final concentration), and 500ul $1x10^{-5}$ Dexamethasone ($1x10^{-8}$ final concentration). 1 µl/ml media of L-Ascorbate (12.5ug/ml final concentration) was added fresh on the day of feeding. Osteoinductive media was changed every two days. Biochemical assays and RNA extraction were performed after 21 days in osteoinductive media.

*Secondary Cultures*

After 14 days cultures in 100mm dishes were switched to FBS basal media consisting of a-MEM, 10% FBS, and 1% anti-anti. FBS basal media was changed every two days for an additional 14 days. After two weeks, cells were trypsinized and replated. On the day of trypsinization, media was aspirated, and each plate was washed with DPBS three times. After adding 5ml of trypsin-EDTA (0.05%) (Gibco #25300054) to each dish, plates were incubated for five minutes at 37 °C. Plates were assessed under microscope to ensure greater than 90% of cells had detached. To neutralize the trypsin 1ml of FBS was added to each dish. Cells were transferred to a 15ml tube and spun down at 1150rpm for five minutes. The supernatant was aspirated off and cells were resuspended in 1ml FBS basal media. Cell count was performed manually with a hemocytometer.

Cells were replated in one 12-well dish at 50,000/well density and one 6-well dish at 100,000/well density. FBS basal media was replaced every two days. After one week, cultures were switched to osteoinductive media. Osteoinductive media was changed every two days. Biochemical assays and RNA extraction took place 21 days after osteoinductive media.

**RNA Extraction**

RNA extraction was performed on the 6-well plates for primary and secondary cultures. The media was aspirated, and each well was washed once in DPBS. Two wells at a time, the DPBS was aspirated and 500µl of Qiazol was added to each well. A sterile cell scraper (Fisherbrand 08-100-241) was used to remove the cells from the bottom of each well. The full contents of two wells were added into one sterile 2ml Eppendorf tube.

After adding a 5mm stainless steel bead (Qiagen 5mm Cat. #69989) to each tube, samples were lysed for two minutes at 30Hz. Eppendorf tubes were placed directly on ice after removal from the tissue lyser. The lysed cell-qiazol mixture was transferred to three new 2ml eppendorf tubes, each containing an additional 500µl of qiazol. After two minutes on ice, 200µl of bromo-3-chloropropane (Sigma #MKCH2273) were added to each tube. Samples were vortexed and placed on ice for two minutes. The samples were then centrifuged at 14,000rpm at 4 °C for 15 minutes. The aqueous phase was transferred to a new 2ml eppendorf tube and an equal volume (~500ul) of cold isopropranolol was added to each tube. Samples were inverted to mix and centrifuged at 14,000rpm at 4 °C for 30 minutes. The supernatant was poured out and 500µl of cold 100% ethanol was added to each tube. Samples were centrifuged for five minutes at 14,000 at 4 C. A final 500µl of cold 100% ethanol were added to each table and samples were centrifuged for a final time at 14,000rpm at 4 °C for five minutes. The ethanol was removed, and the tubes were left open on ice to allow the pellet to dry. Pellets were resuspended in 10µl of cold RNAse-free water and combined into one tube. 4µl were removed for gel electrophoresis, and the remaining extracted RNA was stored at -80 °C.

## Gel Electrophoresis

Gel electrophoresis was performed to evaluate the integrity of the RNA. The NanoDrop 2000/2000c spectrophotometer was used to determine the concentration of RNA obtained from each sample. Based on the RNA concentration, the amount of RNAse free dH2O needed to prepare a 1µg/10ul sample was calculated. 2µl of loading dye was added to each sample.

An agarose gel was prepared by mixing 1.0g agarose with 100ml TAE. The mixture was microwaved until clear then poured into a gel mold. Combs wee added and the gel was left to cool until hardened. After removing the combs, the gel was fully submerged in 1X TAE. 10ul of gel star was added to 100ml of 1X TAE and poured over the gel. The gel was loaded with 10µl of each sample and 10µl of RNA ladder. The power until was turned on and set to 80V and ran for 45-60 minutes. The gel was viewed and photographed under a UV lamp.

**Complementary DNA Synthesis**

Complementary DNA (cDNA) was prepared from the previously extracted and frozen RNA. RNA concentration was used to determine the amount needed to prepare a 1µg sample. The amount of RNAse free dH2O was calculated using the following equation: 16ul - RNAul = RNAse free dH2Oul. 4.0ul of qScript cDNA SuperMix (Quantabio 95048) were added to each sample, bringing the total volume to 20µl. PCR machine (Applied Biosystems 4375786) was set to quantum cDNA. After the cycle was completed, samples were removed and stored at -80 °C.

**Reverse Transcriptase Quantitative Polymerase Chain Reaction**

The previously prepared cDNA was diluted 1:50. Primer mix was prepared for each well by combining 10ul Universal Master Mix (Applied Biosystems Cat#4304437) and 1µl of the primer set. 9µl of diluted cDNA and 11µl of the primer/master mix were added to each well of the 96-well qPCR plate. For negative controls, wells were loaded with 9µl DNase-free water and 11µl of the primer/master mix. The plate was covered with clear film (Applied Biosystems Cat#4306311) and spun down in a centrifuge at

1500rpm for 2 minutes. The qPCR reaction was set up as follows: initial denaturation at 95 °C for 10 minutes; 40 repeats at 95 °C for 15 seconds, 50 °C for 2 minutes, 60 °C for 1 minute.

## Biochemical Assays

Biochemical assays were performed in the following order: alkaline phosphatase, DNA, protein, calcium, hydroxyproline.

### *Alkaline Phosphatase Assay*

A stock of ALP assay buffer consisting of 0.1M glycine (0.75g per 100ml DI H2O) and 1mM MgCl2 (9.5mg per 100ml DI H2O) made to pH 10.5 with NaOH was made and stored at 4 °C. ALP substrate was prepared the day of by dissolving 20mg p-nitrophenol disodium salt (Sigma #4876-1gm) per ml DI H20. After aspirating the media and washing with DPBS three times, 450µl of buffer and 50ul of substrate were added to each well. The plates were incubated in the dark for 30 minutes at room temperature. While the plates incubated, a standard curve from 1mM stock of 4-nitrophenol and reaction buffer was prepared (Table 1). 1mM stock was prepared by diluting 100µl of 10mM stock with 900ul of DI H20. After incubation, 500µl of 0.2M NaOH were added to each well to stop the reaction. Samples from each well were then added to a 96-well plate and read at an absorbency of 410nm.

**Table 1. ALP Standards.**

| 1mM stock (μl) | APase Buffer (μl) | NaOH | Concentration (nM/ml) |
|---|---|---|---|
| **5** | 495 | 500 | 5 |
| **10** | 490 | 500 | 10 |
| **20** | 480 | 500 | 20 |
| **30** | 470 | 500 | 30 |
| **40** | 460 | 500 | 40 |
| **50** | 450 | 500 | 50 |

*Cell Extraction*

After removal of the p-nitrophenyl phosphate, disodium salt (pNPP) solution, plates were washed with DPBS. Extraction buffer was made by dissolving 38.21g 4M Guanidine-HCL, 1ml 1% Triton X-100 lysis buffer, and 1X TE buffer 7.4 (10X solution) in 100ml of DI H2O. 200μl of extraction buffer were added to each well and plates were stored at -80 °C until assays were performed. On the day of assay, plates were placed on a shaker for 30 minutes. Contents of each well were scraped up and added to a 1.5ml eppendorf tube with 100μl of ultrapure H2O. The insoluble matrix was spun out at 12,000 rpm for 10 minutes at 4 °C.

*DNA Assay*

25μl of soluble sample extract from each well of the 24-well plate were transferred to a 96-well plate. Sample volume was brought up to 100μl by adding 75μl 1X TE per well. PicoGreen (Molecular probes catalog #P-11496) reagent was diluted 1:200 in 1X TE. The amount of PicoGreen reagent needed was calculated by multiplying the number of samples by 100μl. Prior to adding PicoGreen, DNA standard was added to each plate. The standard curve was made via serial dilution of DNA standard and 1X TE to produce varying concentrations of DNA (Table 2). 100μl of each standard dilution were added to the 96-well plate. 100µl of dilute PicoGreen reagent were added to each well. Plates were covered in foil and placed on the shaker for 2-5 minutes. Plates were read on a fluorescent plate reader at excitation/emission: 485/20, 530/25nm.

**Table 2. DNA Standards.**

| Vial | Concentration (ng/ml) | DNA (µl) | 1X TE (µl) |
|---|---|---|---|
| 1 | 1000 | 10 stock | 990 |
| 2 | 500 | 500 vial 1 | 500 |
| 3 | 200 | 400 vial 2 | 600 |
| 4 | 100 | 400 vial 3 | 400 |
| 5 | 50 | 250 vial 4 | 250 |
| 6 | 5 | 50 vial 5 | 450 |
| | 0 | 0 | 500 |

*Protein Assay*

The Protein Assay Kit (Thermo Scientific #23225) was used to prepare the MicroBCA Working Reagent (WR) in a 25:24:1 ratio of reagents MA:MB:MC. The total volume of WR required was calculated with the following formula: (# of standards + # of unknowns + 2) x (150ul WR). 125µl of 1X TE was added to each well, followed by 150µl of WR. The plate was mixed thoroughly on a shaker for 30 seconds. 25µl of soluble extract was transferred from each eppendorf tube and into the prepared wells. Diluted albumin (BSA) standards were prepared via a serial dilution of BSA and 1X TE (Table 3). After adding the standards to the 96-well plate, the plate was covered with sealing tape and incubated at 37 °C for 2 hours. The plate was cooled to room temperature and measured at an absorbance of 562 nm.

**Table 3. Protein Standards.**

| Vial | Vol 1X TE (µl) | Vol/Source of BSA (µl) | BSA Concentration (µg/ml) |
|---|---|---|---|
| **A** | 900 | 100 stock | 200 |
| **B** | 100 | 300 vial A | 150 |
| **C** | 200 | 200 vial A | 100 |
| **D** | 200 | 100 vial B | 50 |
| **E** | 600 | 100 vial A | 40 |
| **F** | 300 | 300 vial E | 20 |
| **G** | 300 | 300 vial F | 10 |
| **H** | 300 | 300 vial G | 5 |
| **I** | 300 | 300 vial H | 2.5 |
| **J** | 300 | 200 vial I | 1 |
| **K** | 200 | 200 vial J | 0.5 |
| **L** | 400 | N/A | blank |

*Acid Hydrolysis*

After adding 200µl of 6N HCl, samples were transferred to a pressure-tight polypropylene vial with PTFE-lined cap and placed in a heating block to incubate at 120 °C for 3 hours. After cooling to room temperature, an aliquot of 55µl was set aside for the calcium assay. The remainder was centrifuged at 12,000 rpm for 5 minutes at 18 °C. 200µl of each sample were transferred to a 96-well plate and left to dry in a vacuum hood at 60 °C for 24 hours.

*Calcium Assay*

The calcium assay was performed with the Calcium Colorimetric Assay Kit (Sigma-Aldrich No. MAK022). 55µl of each sample were added to each well of a clear 96-well plate. The calcium standard curve was prepared by combining 10µl of 500nM calcium standard stock with 990ul of DI H20 to reach a final 5nM stock (0.2 µg/µl). Varying volumes of 5nM calcium stock and extraction buffer were used to create a standard curve (Table 4), and 50µl of each standard were added to the wells. 90µl of chromogen and 60µl of calcium assay buffer were added to the sample and standard wells. The plate was covered and incubated at room temperature for 10 minutes, and then read at an absorbance of 575nm.

**Table 4. Calcium Standards.**

| Volume Extraction Buffer (µl) | Volume Ca Stock (µl) | Final Ca concentration (µg) |
|---|---|---|
| **50** | 0 | Blank |
| **48** | 2 | 0 |
| **46** | 4 | 0.4 |
| **44** | 6 | 0.8 |
| **42** | 8 | 1.2 |
| **40** | 10 | 1.6 |
| **30** | 20 | 2.0 |
| **20** | 30 | 3.0 |
| **0** | 50 | 5.0 |

*Hydroxyproline Assay*

The assay was performed using a Sigma-Aldrich Hydroxyproline Assay kit and a clear 96-well plate. Hydroxyproline standards were prepared by diluting 10µl of 1mg/ml standard solution with 90µl of ultra-pure water, yielding a 0.1mg/ml standard solution. Varying amounts of the diluted standard solution were placed in individual wells (Table 5).

100ul of Chloramine T/Oxidation Buffer (6µl Chloramine T concentrate with 94ul of oxidation buffer) were added to all sample and standard wells, and the plate was incubated at room temperature for 5 minutes. 100µl of dilute DMAB reagent (50µl DMAB concentrate and 50µl of Perchloric Acid/Isopropanol Solution) were added to each sample and standard well. After covering and incubating for 90 minutes at 60 °C, absorbance was measured at 560nm.

**Table 5. Hydroxyproline Standards.**

| Volume 0.1mg/ml hydroxyproline standard (μl) | Concentration (μg) |
|---|---|
| **0** | blank |
| **2** | 0.2 |
| **4** | 0.4 |
| **6** | 0.6 |
| **8** | 0.8 |
| **10** | 1.0 |

## Participants

All participants underwent a total hip arthroplasty at Boston Medical Center. Verbal and signed consent were obtained prior to the procedure. Patient age ranged from 36-77 years old, with a mean age of 54.58 years. 50% of patients (N = 6) identified as male, while 50% of patients (N = 6) identified as female. Patients self-identified their race as Hispanic (N = 2, 16.67%), White (N = 8, 66.67%), and African American (N = 2, 16.67%). The mean Body Mass Index (BMI) was 31.92 kg/m2. For this study, a BMI greater or equal to 30 kg/m2 was classified as overweight (N = 5), while those who fell below were classified as healthy (N = 7). Three patients were diagnosed with renal disease (25%), two with type II diabetes mellitus (16.67%), and four were identified as pre-diabetic (33.33%). Smoking status was also assessed, with two patients identifying as current smokers (20%) and 6 as former smokers (50%). Patients were also categorized by vitamin D status. Patients with vitamin D levels of 19.9 or below were categorized as deficient (N = 2, 16.67%), while those above 20 fell within normal range (N = 10, 83.33%). Exclusion criteria included chemotherapy recipients, patients with sickle cell disease or rheumatoid arthritis, and patients receiving bone metabolic modifying drugs.

## Quantitative Assessments and Statistical Treatments for Biochemical Assays

For the analysis of the biochemical assays, the optical density of the blank was subtracted from the values produced by all sample and standard wells. The adjusted standard values were then graphed and fit with a linear model. After applying the appropriate dilution factor, sample concentrations were determined by plugging the adjusted sample values into the line of best fit. ALP, protein, calcium, and hydroxyproline were all normalized against DNA. The calcium and hydroxyproline values were then converted to nanograms per ml. All comparisons were made by taking the average of all wells across each sample. A one-way ANOVA with $p<0.5$ was used to determine significance between the normalized DNA, ALP, protein, calcium, and hydroxyproline concentrations of primary and secondary cultures. After applying the blank correction to patient 274, all DNA values fell below zero. All statistics were performed with a 95% confidence interval, and a p-value of significance was less than 0.05. Means were calculated with all data, but significance was only tested within matched pairs. Since all data was normalized against DNA, patient 274 was not included in any analyses of the biochemical assays

# RESULTS

## Biochemical Assessment

### *DNA Assay*

The average DNA concentration across all primary cultures was 100.88 ng/ml, while the average across all secondary cultures was 97.52 ng/ml (Figure 4). When only paired cultures were considered, the primary average was 100.25 ng/ml and the secondary average 99.62 ng/ml. An ANOVA was run on the standardized values of the paired primary and secondary cultures. This produced an f-ratio = 0.0004, and p-value = 0.98, indicating no significant difference between DNA concentration in the primary and secondary cultures.

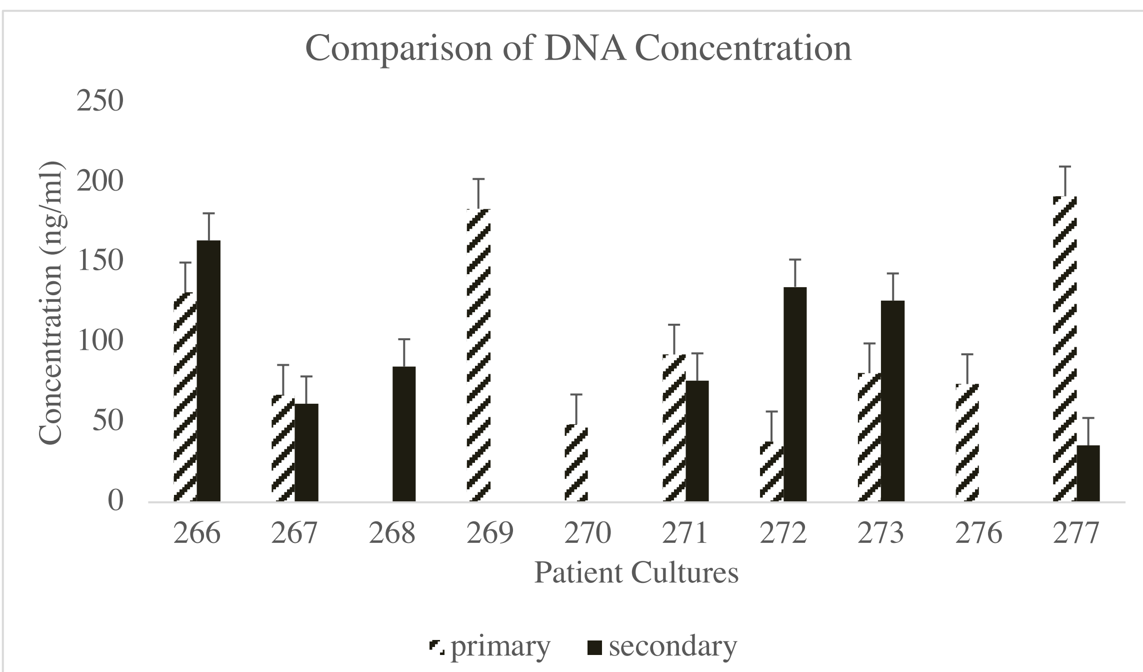
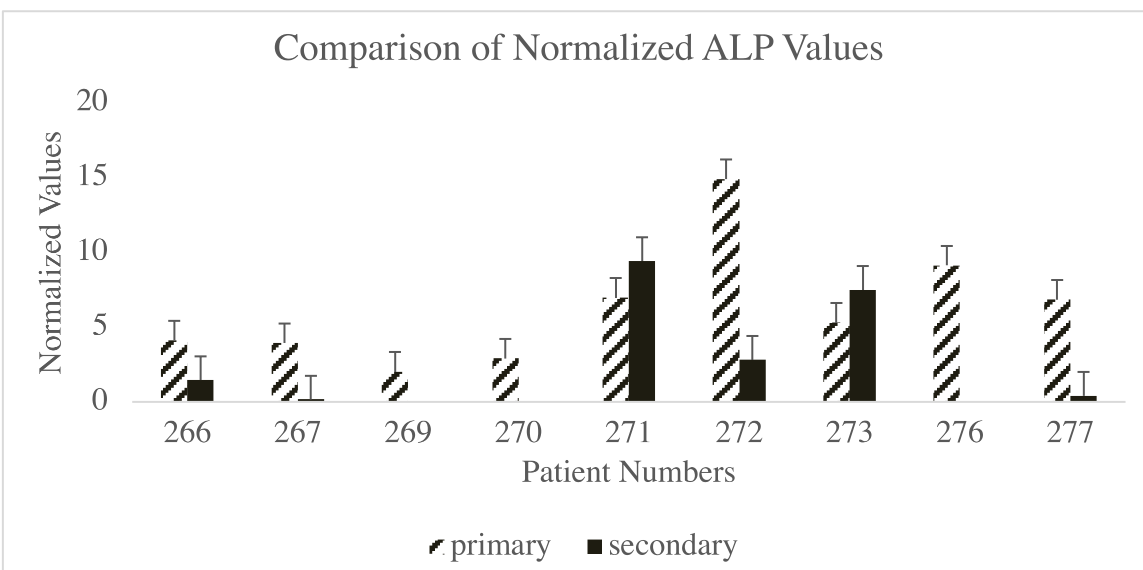



**Figure 4 – Primary and Secondary Culture Comparisons of DNA.**

*Alkaline Phosphatase Assay*

When taking all samples into account, the mean normalized ALP values were 6.23 for primary cultures and 3.62 for secondary cultures (Figure 5A). After removing non-paired samples, the normalized mean of primary cultures increased to 7.0, while the normalized mean of secondary cultures remained 3.62. An ANOVA revealed no significant difference between the normalized ALP values of paired samples (f-ratio = 2.16; p-value = 0.17). Mean ALP concentration for all primary cultures was 557.42 nM/ml, and 358.82 nM/ml for all secondary cultures (Figure 5B). Mean ALP concentrations for paired primary and secondary cultures were 638.28 nM/ml and 358.82 nM/ml respectively. An ANOVA of paired samples showed an f-ratio = 1.81 and p-value = 0.21, indicating no significant difference between overall ALP concentration of primary and secondary cultures. Patient 268 did not produce a readable amount of ALP and was thus excluded from all analysis of ALP.

**A)**



**B).**

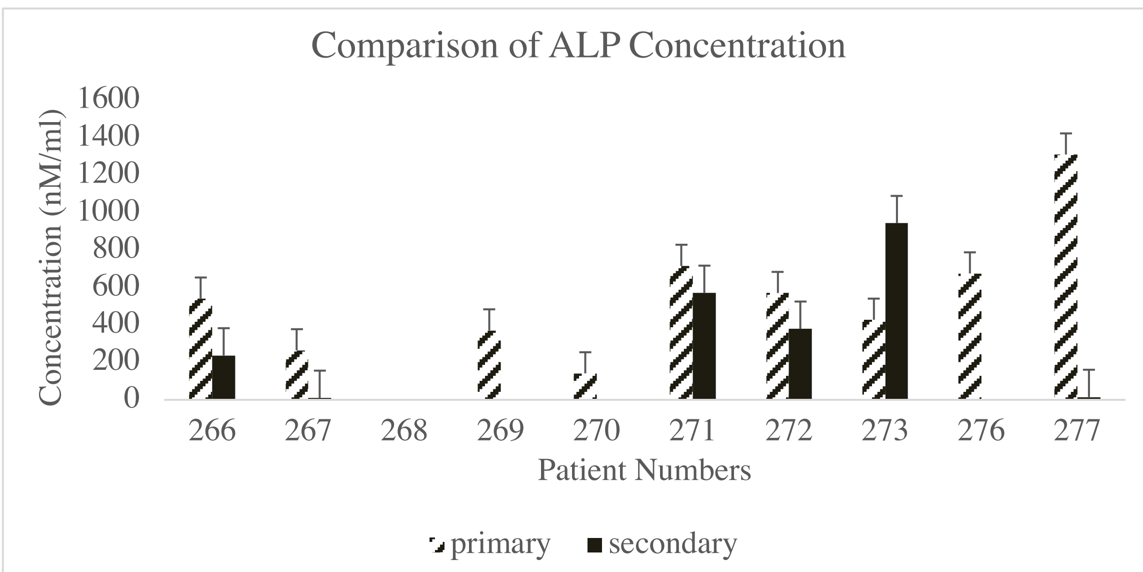
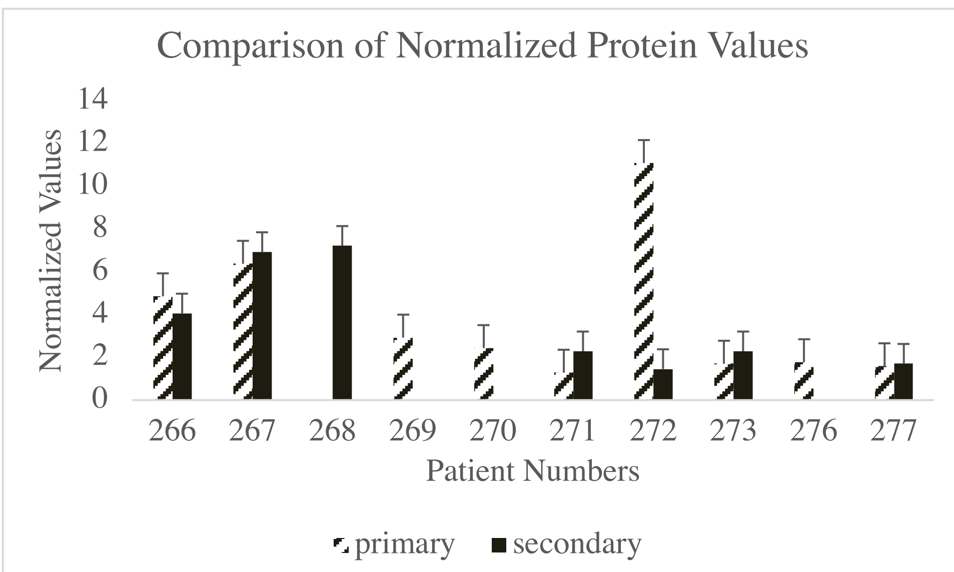



**Figure 5 - Primary and Secondary Culture Comparisons of ALP.** A) Comparison of normalized values. B) Comparison of concentration.

*Protein Assay*

The mean across all normalized protein values was 3.8 for primary cultures and 3.7 for secondary cultures. Across paired sampled, the mean normalized protein value was 4.48 for primary cultures and 3.11 for secondary cultures. Despite the difference between the matched samples, an f-value = 0.56 and p-value = 0.46 showed no significance between the primary and secondary cultures. The mean concentration for all primary cultures was 314.11 ug/ml, while the mean concentration for all secondary cultures was 345.25 ug/ml. For the paired samples, the mean concentration of primary samples was 340.25 ug/ml, and the mean concentration of secondary cultures was 300.80 ug/ml. An ANOVA showed an F-value = 0.12 and p-value = 0.75, indicating no significant differences between the protein concentration in primary and secondary cultures.

**A)**




B)

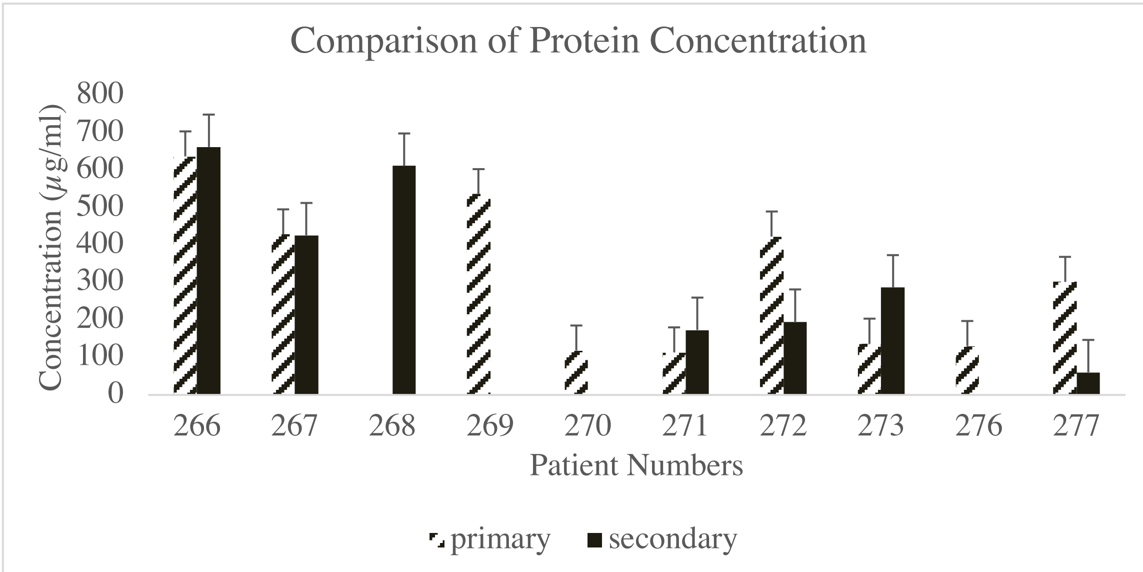
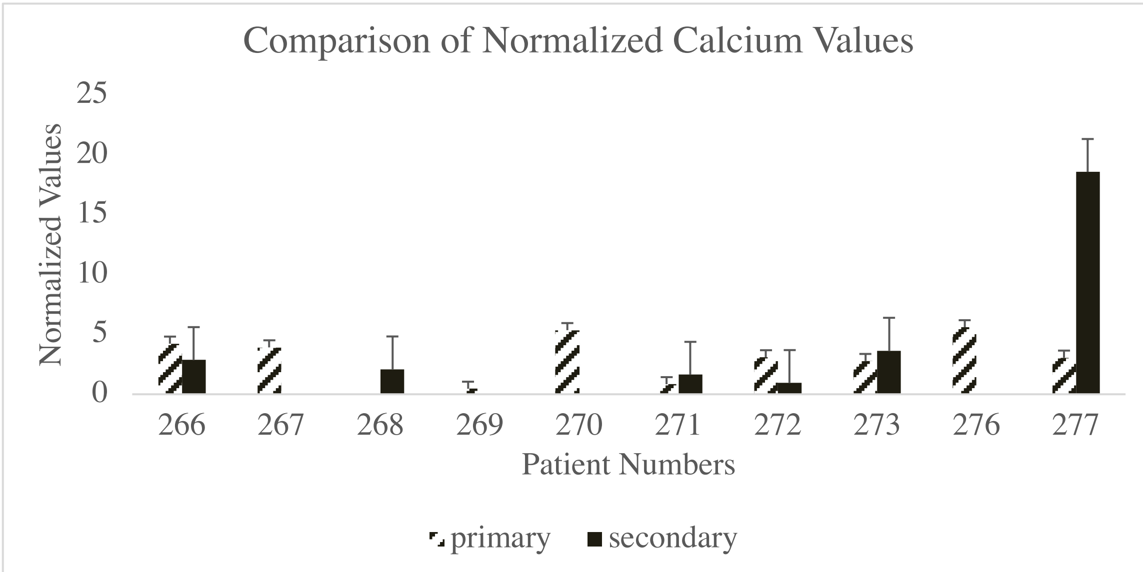



**Figure 6 – Primary and Secondary Culture Comparisons of Protein.** A) Comparison of normalized values. B) Comparison of concentration.

*Calcium Assay*

Despite secondary samples having a higher normalized calcium value in both paired and unpaired models, this an f-value = 0.67 and p-value = 0.43 showed that this difference was not significant. Mean normalized calcium values of all samples were 3.26 for primary cultures and 4.96 for secondary cultures. The mean for paired primary samples was 2.8, while the mean for paired secondary samples was 5.53. Secondary cultures also showed higher mean calcium concentration. However, f-value = 0.0004 and p-value = 0.95 showed that this difference was not significant. The mean of all primary samples was 292.58 ng/ml and the mean of all secondary samples was 299.47 ng/ml. The mean for primary paired samples was 311.70 ng/ml, and the mean for paired secondary samples was 320.06. Despite the insignificance, there is a clear trend towards increased calcium in secondary samples.

**A)**




B)

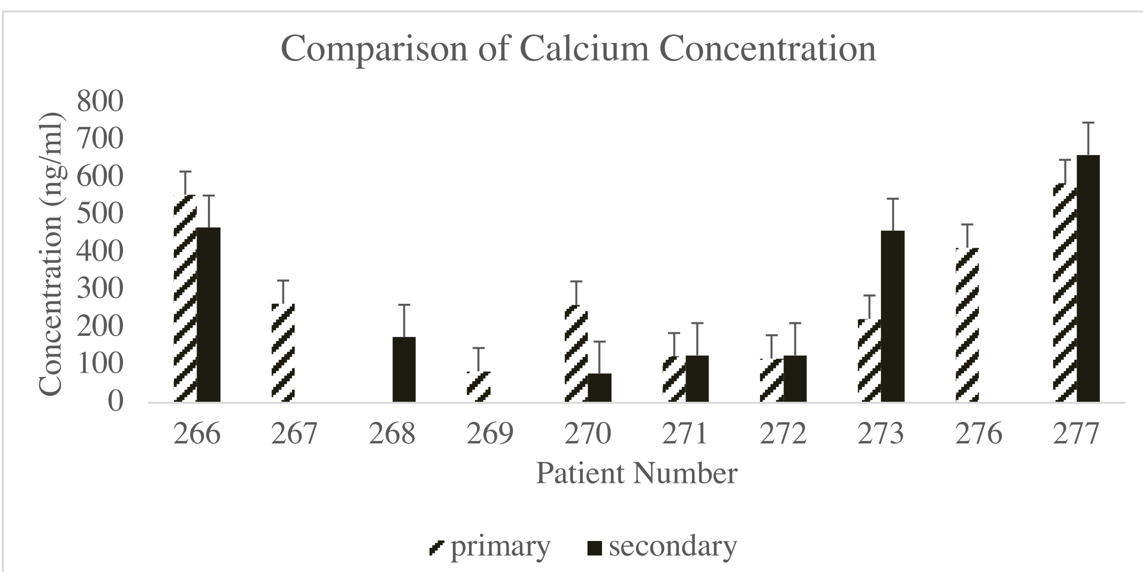
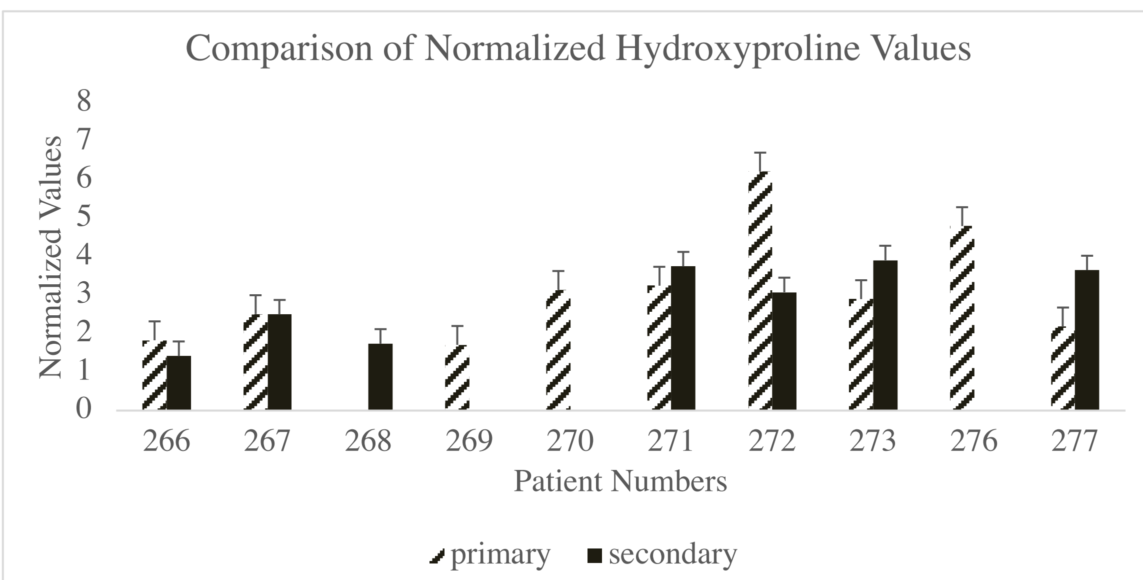



**Figure 7 – Primary and Secondary Culture Comparisons of Calcium.** A) Comparison of normalized values. B) Comparison of concentration.

*Hydroxyproline Assay*

The mean normalized hydroxyproline values was 3.17 for all primary cultures and 2.86 for all secondary cultures. For the paired samples, the mean normalized hydroxyproline values were 3.15 for primary cultures and 3.05 for secondary cultures. In both cases, the mean normalized value of primary samples was greater than that of the secondary samples. However, f-value = 0.02 and p-value = 0.90 showed that this difference was not significant. The mean concentration for all primary samples was 270.2 ng/well and 265.19 ng/well for all secondary cultures. Matched pairs had a mean of 268.28 ng/well for primary cultures and 284.83 ng/well for secondary cultures. F-value = 0.06 and p-value = 0.81 showed that this difference was not significant.

**A)**




B)

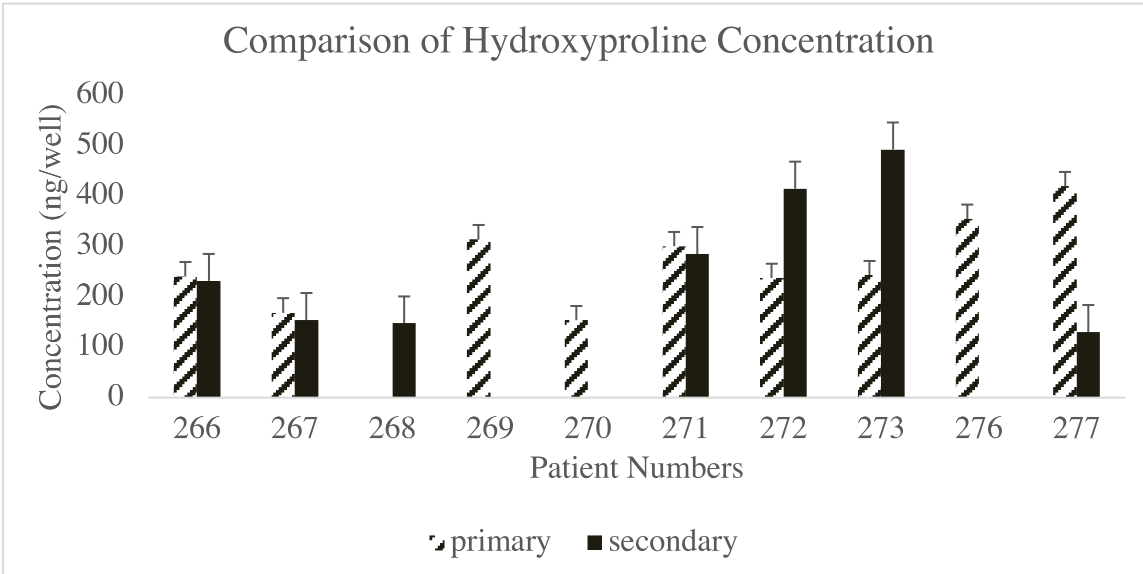



**Figure 8 – Primary and Secondary Culture Comparisons of Hydroxyproline.** A) Comparison of normalized values. B) Comparison of concentration.

*Correlation Between Biochemical Markers*

To determine the relationship within biochemical markers, a Pearson correlation coefficient was used to compare all DNA, ALP, calcium, and hydroxyproline concentrations. Comparison between DNA and ALP concentration showed moderate positive correlation ($r = 0.466$) that was not significant ($p = 0.080$). The relationship between DNA and protein was also moderately positive ($r = 0.421$) but not significant ($p = 0.104$). DNA and hydroxyproline also had a strong positive ($r = 0.601$) and significant correlation ($p = 0.014$). When comparing ALP to hydroxyproline, there was a strong, significant positive correlation ($r = 0.791$; $p < 0.001$). Calcium also showed a weak positive correlation to ALP ($r = 0.115$), protein ($r = 0.036$), and DNA ($r = 0.156$). Protein showed a weak negative correlation with both ALP ($r = -0.077$) and hydroxyproline ($r = -0.251$).

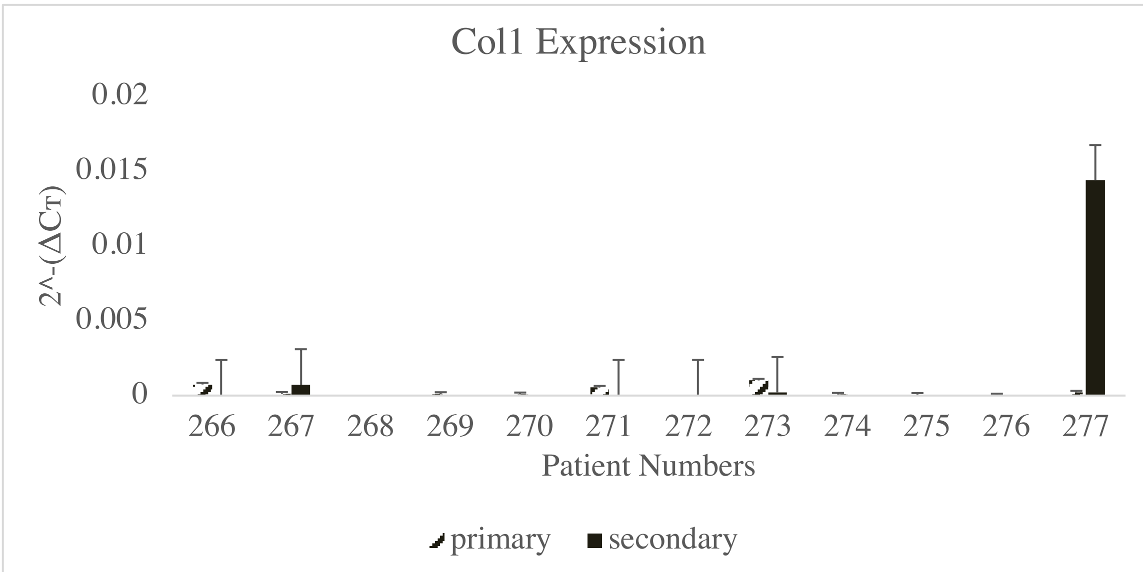
**Table 6. Pearson Correlation Coefficient of Biochemical Markers.** * correlation is significant at the 0.05 level. ** correlation is significant at the 0.01 level.

| | | DNA (ng/ml) | ALP (nM/ml) | Protein (ug/ml) | Calcium (ng/ml) | Hydroxyproline (ng/well) |
|---|---|---|---|---|---|---|
| **DNA (ng/ml)** | **r value** | 1 | 0.466 | 0.421 | 0.156 | 0.601* |
| | **Significance (2-tailed)** | | 0.080 | 0.104 | 0.579 | 0.014 |
| **ALP (nM/ml)** | **r value** | 0.466 | 1 | -0.077 | 0.115 | 0.791** |
| | **Significance (2-tailed)** | 0.080 | | 0.784 | 0.695 | <0.001 |
| **Protein (ug/ml)** | **r value** | 0.421 | -0.077 | 1 | 0.036 | -0.186 |
| | **Significance (2-tailed)** | 0.104 | 0.784 | | 0.899 | 0.491 |
| **Calcium (ng/ml)** | **r value** | 0.156 | 0.115 | 0.036 | 1 | 0.033 |
| | **Significance (2-tailed)** | 0.579 | 0.695 | 0.899 | | 0.907 |
| **Hydroxyproline (ng/well)** | **r value** | 0.601* | 0.791** | -0.186 | 0.033 | 1 |
| | **Significance (2-tailed)** | 0.014 | <0.001 | 0.491 | 0.907 | |

## RT-qPCR

There are several proteins specific to connective tissue and exclusive to osteocytes. Collagen type 1 (Col1) is also used to confirm differentiation from MSCs to connective tissue. Dentin Matrix Protein 1 (DMP1) and Sclerostin (SOST) are osteocyte specific and are used to confirm differentiation into osteocytes. Every sample tested expressed the housekeeping gene, 18s. The $C_T$ values ranged from 10.87 to 32.56. Sample 268 had the highest $C_T$ value for 18s, and did not express any Col1, DMP1, or SOST. Therefore, the conclusion can be drawn that sample 268 did not successfully differentiate from MSCs to osteocytes. The secondary sample for patient 272 did not express 18s, Col1, or SOST, but did express DMP1. The remainder of the samples all expressed 18s and Col1, and/or DMP1 or SOST. Primary samples from patients 266, 267, 271, 275, and 277 expressed all three target genes. Secondary samples from 273 and 277 expressed all three target genes. The only patient to express all target genes in both primary and secondary samples was patient 277.

A)




B)

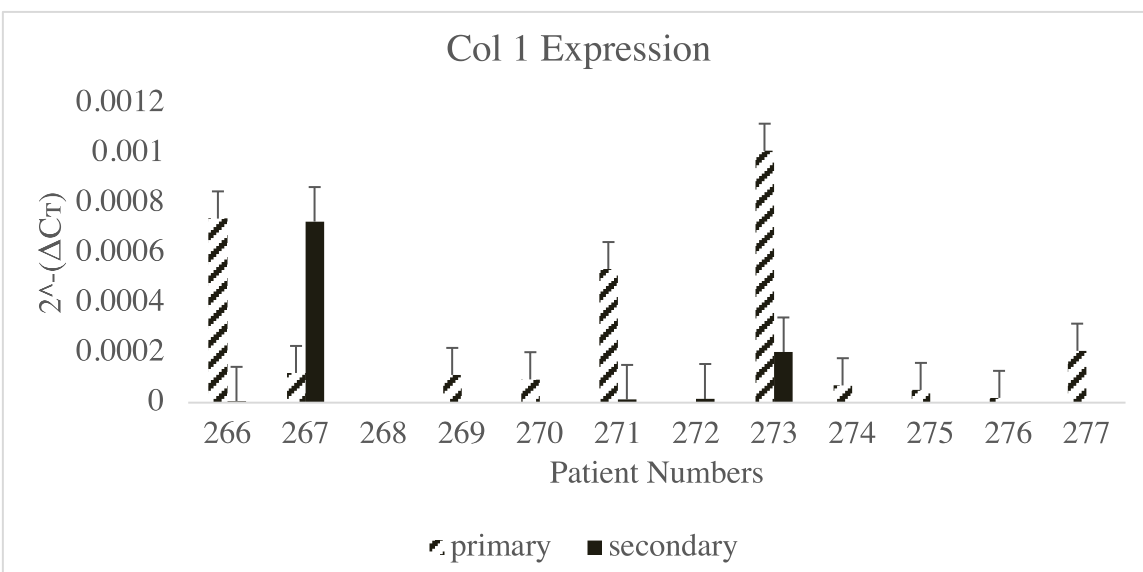
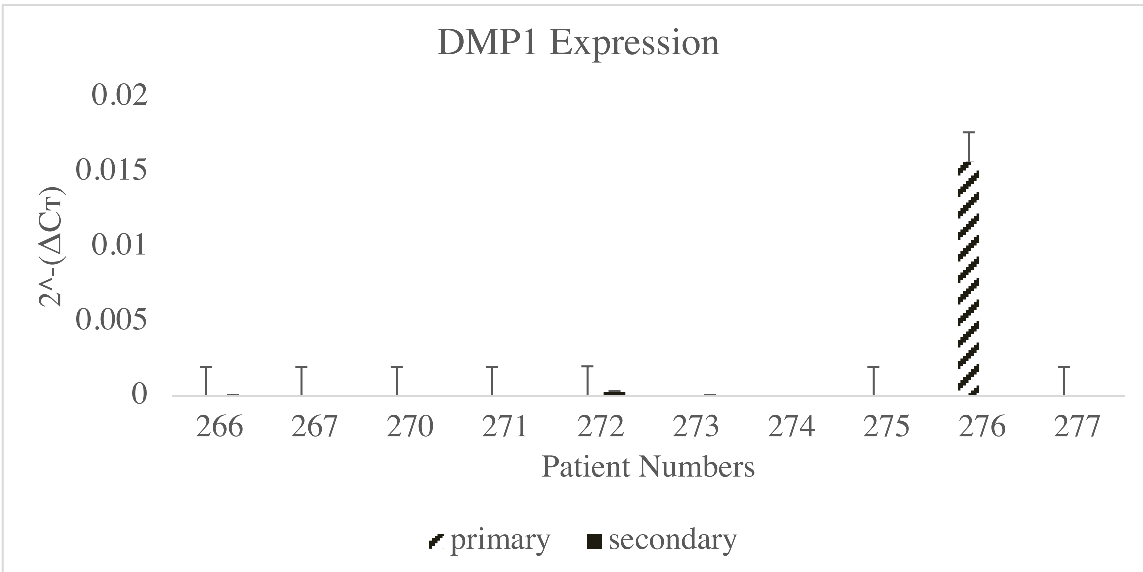


Figure 9 – Collagen 1 Expression. A) Col1 across all samples. B) Co11 with outlier (277 secondary) removed.

A)




B)

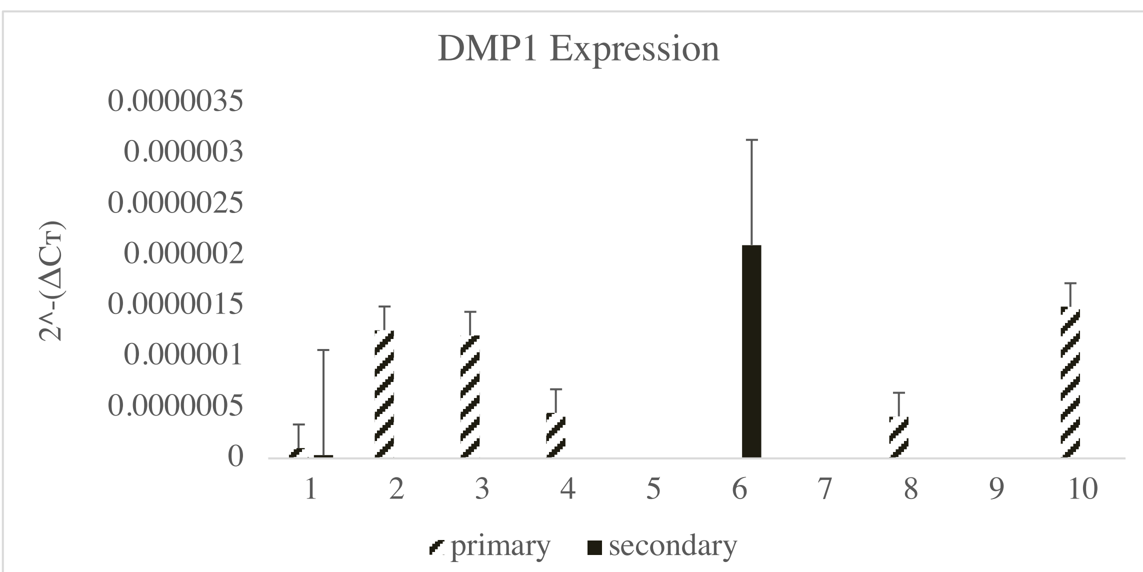
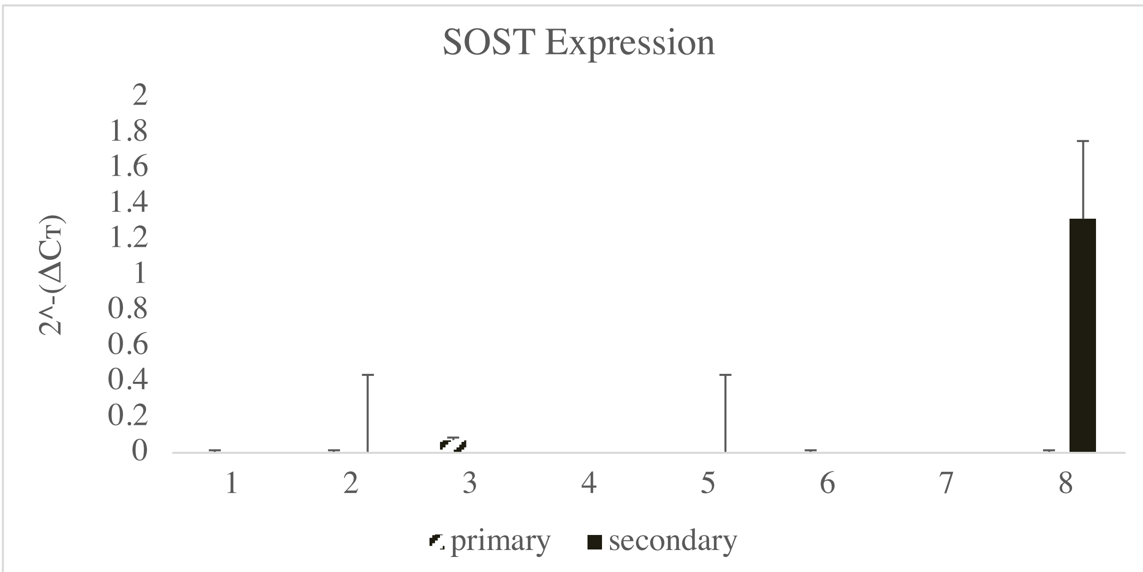


Figure 10 – DMP 1 Expression. A) DMP1 across all samples. B) DMP1 with outliers (272 primary and secondary; 276 primary) removed.

A)




B)

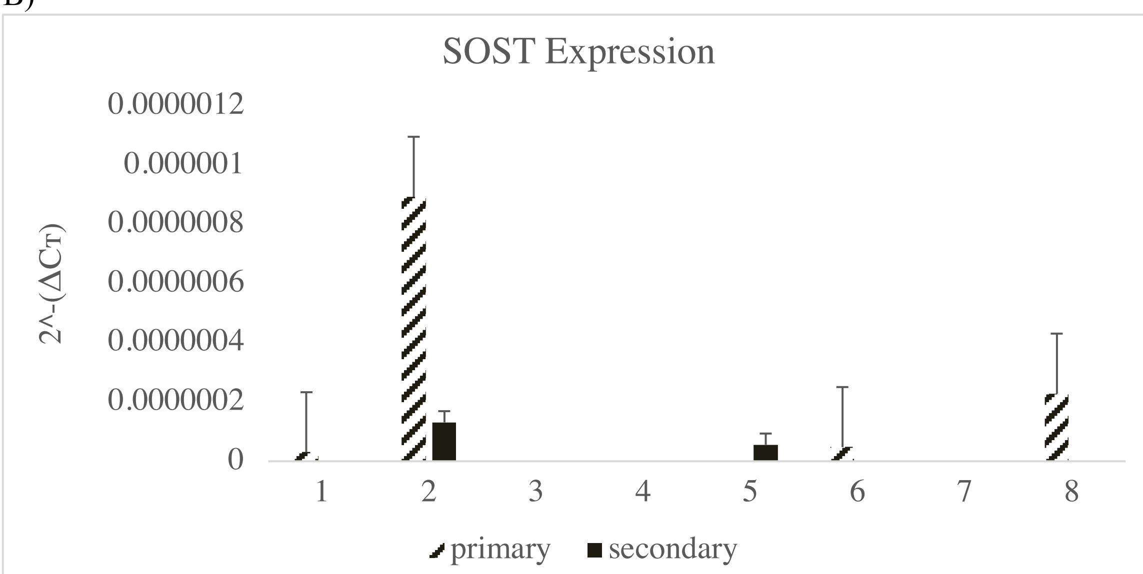


Figure 11 – SOST Expression. A) SOST across all samples. B) SOST with outlier (271 primary and 27 secondary) removed.

## Clinical Factors

The effect of innate factors (sex, age, race) and acquired factors (BMI, smoking status, renal disease, vitamin D) on primary culture assay concentrations were evaluated using a MANOVA. There was a significant interaction between race and ALP concentration ($p = 0.033$). Unfortunately, there were not enough groups to perform post hoc tests, so the significance cannot be further evaluated at this time. Females had higher concentrations of DNA, protein, hydroxyproline, and calcium, while males had higher a concentration of ALP. However, none of these differences were significant. The relationship between age and ALP was significant ($p = 0.037$). Patients greater than 50 ($n = 7$) had a mean ALP concentration of 270.14 nM/ml, while patients less than 50 ($n = 2$) had an average of 254 nM/ml. There was no significant difference between healthy (BMI < 30) and overweight (BMI > 30) patients. Patients who did not smoke had higher concentrations of DNA, ALP, hydroxyproline, and protein. Patients who were current of former smokers had increased concentration of calcium. None of these differences were significant.

# DISCUSSION

## *Biochemical Markers*

No significant differences were found between normalized values or concentration when comparing primary to secondary cultures. The lack of significance difference between DNA and normalized protein values indicates similar overall metabolic activity of the primary and secondary cultures. When looking at osteocyte markers such as ALP, hydroxyproline, and calcium, there were no significant differences between normalized values or concentration of primary and secondary cultures.

When comparing biochemical markers to each other, several relationships were revealed. Significant positive correlations were found between hydroxyproline and DNA, and hydroxyproline and ALP. Moderately strong positive correlations were found between DNA and ALP, DNA and protein. A weak negative correlation was found between protein and hydroxyproline. A larger sample size would provide greater insight into the strength and significance of these relationships.

## *Comparison Between Primary and Secondary Cultures*

There were no significant differences between the normalized values of ALP, protein, calcium, and hydroxyproline of primary and secondary cultures. However, trends were seen when comparing concentration. There is a clear trend towards increased calcium and hydroxyproline concentrations in secondary samples. Although this was hypothesized to be the case, the difference was insignificant. The difference may be insignificant due to small sample size but could be significant if the sample size is increased. Primary cultures showed much greater variability in concentrations. This is consistent with the hypothesis that primary cultures are more greatly impacted by the different innate and acquired factors present at the time of cell collection. The increased homogeny of secondary cultures should produce more consistent results.

### *Clinical Factors*

To get the full picture, only patients with complete primary data sets were included in the analyses of clinical factors. This resulted in a small sample size of only 9 patients. Thus, it is difficult to draw any conclusions from the data, as many times there were only one or two patients in a category. There was a significant relationship between race and ALP, and age and ALP. Patients aged 50 or greater had a higher mean concentration of ALP. Although the relationship between race and ALP was significant, there was only one patient who identified as Hispanic, and only two who identified as African American. The unequal distribution of race in this sample set can lead to skewed results. Age, sex, race, gender, BMI, smoking status, renal disease, and vitamin D all impact BMD and contribute to osteoporosis. With a larger sample size, the impact of these innate and acquired factors should show significance.

### *Limitations and Future Directions*

The primary limiting factors for this study were sample size and time. Due to COVID, there was a significant decrease in the number of elective procedures, including total hip arthroplasty. Paired with the length of time it takes for cultures to grow out, there were many unmatched samples. Additionally, several samples were contaminated at different points during the expansion process. This also contributed to the lack of sufficient paired primary and secondary cultures. Some cultures were grown out from frozen stock, while others were plated immediately after they were harvested. This may also influence cells' ability to expand and mineralize sufficiently.

To better understand the relationship between biochemical markers of primary and secondary cultures, the sample size must continue to grow. Although there were no significant differences found between primary and secondary cultures, there were several trends consistent with prior research, and they should be evaluated more thoroughly.

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**CURRICULUM VITAE**

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