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SCHOOL OF MEDICINE

Thesis

A COMPARISON OF OSTEOBLAST AND OSTEOCYTE EXPRESSION IN HUMAN BONE AND CULTURED MESENCHYMAL STEM CELLS

by

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B.S., University of California, Davis 2016

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

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A COMPARISON OF OSTEOBLAST AND OSTEOCYTE EXPRESSION IN HUMAN BONE AND CULTURED MESENCHYMAL STEM CELLS

JESSICA ROSE CASHEN

ABSTRACT

Osteoporosis is an immense and growing health and economic burden of the aging population. Its prevalence in both men and women makes it an important target for biochemical research and development. A comparative study of the skeletal gene expression in native trabecular bone and cartilage tissues to marrow mesenchymal stem cells (MSCs) was carried out on tissues and cells obtained from acetabular reaming from total hip replacement patients (n=10). The intent of these comparisons was to examine the differentiation potential of MSCs grown from the bone marrow that was cultured for 21 days in three types of media: control media supplemented with fetal bovine serum with (CM) and without dexamethasone (CM-D), and an artificial (AFM) devoid of any animal product supplementation. RNA was extracted from these samples, and the bone and cartilage, and qRT-PCR was carried out to measure the expression of five genes of interest. The genes of interest (COL1A1, RUNX2, SP7, DMP1, and SOST) were chosen to respectively assess the progression of cellular and tissue differentiation from MSCs into osteocytes.

There were significant differences in gene expression of RUNX2, SP7, DMP1, and SOST between CM, AFM, and bone samples suggesting that each of the culture conditions promoted differing amounts of osteogenic differentiation. CM samples were relatively undifferentiated compared to the CM-D and AFM cultured cells, and likely

contained little or no osteocytes but likely had some amount of osteoblasts. AFM cells appeared to be the most differentiated based on comparisons of their expression of mRNAs found in osteocytes. Bone and cartilage samples were also tested for the same genes of interest. AFM, CM, and CM-D had higher gene expression in markers for osteoblasts that are in the process of differentiating, while bone and cartilage showed a higher expression of the terminal osteocytic marker. Overall, this study showed that artificial media cultured MSCs differentiate at a faster rate than cells grown in the animal supplemented media.

TABLE OF CONTENTS

TITLE……………………………………………………………………………...i

COPYRIGHT PAGE…………………………………………………………………...ii

READER APPROVAL PAGE……………………………………………………..iii

ACKNOWLEDGMENTS iv

ABSTRACT.. v

TABLE OF CONTENTS... vii

LIST OF TABLES viii

LIST OF FIGURES ix

LIST OF ABBREVIATIONS.. x

INTRODUCTION 1

METHODS 14

RESULTS 22

DISCUSSION 34

REFERENCES 43

CURRICULUM VITAE... 52

LIST OF TABLES

Table	Title	Page
1	Study Subject Demographics	14
2	DNA Primers Used in qRT-PCR and Expression IDs	20
3	Mean ΔCt Values for All Samples	29
4	Mean Log Transformed ΔCt values for All Samples	29
5	Mean ΔΔCt values for All Samples	33
6	Statistical Summary	33

LIST OF FIGURES

Figure	Title	Page
1	Cells of Bone	4
2	Effects of Osteoporosis on the Human Body	7
3	Percent of United States Population over 65 1950-2050	8
4	Sample Prep Set-up	16
5	Bone and Cartilage Sample	16
6	RNA Samples on Agarose Gel Under UV Light	18
7	COL1A1 Expression Relative to 18S RNA	23
8	RUNX2 Expression Relative to 18S RNA	24
9	SP7 Expression Relative to 18S RNA	25
10	DMP1 Expression Relative to 18S RNA	27
11	SOST Expression Relative to 18S RNA	28
12	Gene Fold Expression of CM-D Samples Compared to CM Samples	31
13	Gene Fold Expression of AFM Samples Compared to CM Samples	32

LIST OF ABBREVIATIONS

AC .... Articular Cartilage

AFM .... Artificial Media

ANOVA .... Analysis of Variance

BMD .... Bone Mineral Density

cDNA .... complimentary DNA

CM .... Control Media

CM-D .... Control Media without Dexamethasone

Ct .... Cycle Threshold

DEX .... Dexamethasone

DEXA .... Dual Energy X Ray Absorptiometry

DMP1 .... Dentin Matrix Protein 1

HIPPA .... Health Insurance Portability and Accountability Act of 1996

MSC .... Mesenchymal Stem Cell

OSX .... Osterix

PTH .... Parathyroid Hormone

PBS .... Phosphate Buffered Saline

qRT-PCR .... Quantitative Real Time Polymerase Chain Reaction

RT-PCR .... Real Time Polymerase Chain Reaction

SERM .... Selective Estrogen Receptor Modulator

INTRODUCTION

Bone Physiology

Bone is a connective tissue that is composed of a calcified extracellular matrix and three main types of cells: osteoblasts, osteocytes, and osteoclasts. These three types of cells work together to create and maintain the structure of bone, as well as perform many other important functions necessary to maintain body homeostasis, including the regulation and storage of calcium (Mescher & Junqueira, n.d.).

Osteoblasts are derived from mesenchymal stem cells (MSCs) and have a number of roles in formation and maintenance of the bone (Mescher & Junqueira, n.d.). MSCs are important cells throughout the body because of their ability to self-renew, and because they are able to differentiate into many kinds of cell throughout the body depending on nearby growth factors, these include adipose, cartilage, bone, muscle, tendon, and ligament tissues (Almalki & Agrawal, 2016). MSCs that differentiate into osteoblasts in vitro are usually influenced by the growth factors, hormones [dexamethasone] and biochemical additives [β-glycerophosphate and ascorbic acid] (Ding, Shyu, & Lin, 2011). Once differentiated from MSCs, osteoblasts begin producing the proteins that will form the bone matrix, including type I collagen, fibronectin, laminins. As they progress, osteoblasts will make more specialized extracellular matrix proteins related to the mineralization of their matrix. Additionally, osteoblasts produce both proteases and osteoclastogenic factors in order promote osteoclastogenesis. This process is important in the homeostatic remodeling of the bones, which repairs and maintains the optimal bone

structure (Raggatt & Partridge, 2010). Both MSCs and osteoblasts can be found in individuals of any age in the periosteum and endosteum, and on specific trabecular surfaces of the bone, meaning that bone cells are turned over and renew throughout an individual's life (De Bari et al., 2006).

The second prominent cell of bone is the osteocyte. Osteocytes are osteoblasts that have been embedded within calcified bone matrix produced during bone formation. Osteocytes can communicate to each other long dendritic processes that pass though channels in the mineralized matrix called canaliculi. These dendritic process terminate with cell to cell contacts through gap junctions (Mescher & Junqueira, n.d.). Osteocytes are the most common cells in bone, making up to 90% of the cell population in the tissue. They are crucial in monitoring bone homeostasis by regulating both the formation and resorption of bone tissue (Bellido, 2014). The presence of viable osteocytes is necessary to maintain the integrity of bone. Osteocytes possess mechanosensory mechanisms enabling them to sense and respond to loads applied to the bone. They can also detect damage to the bone through these mechanosensory mechanisms, and initiate remodeling in response to the damage (Bonewald, 2011; Raggatt & Partridge, 2010) .

The third and final type of bone cell is the osteoclast. Osteoclasts, unlike osteoblasts, are derived from the hematopoietic lineage and more specifically come from the monocytic arm of the myeloid lineage. Osteoclasts are characteristically large cells with multiple nuclei. These cells are the main cell involved in bone resorption and remove old, calcified bone matrix (Mescher & Junqueira, n.d.). They are able to do this by creating an acidic environment between themselves and the bone which mobilizes the

local mineralized matrix. They also produce proteases that degrade matrix proteins that become exposed once the mineral has been mobilized (Teitelbaum, 2007). Osteoblasts and other cells then take over the space and produce new bone tissue through their own mechanisms. This coupled turnover of bone tissue is essential to maintain bone homeostasis, and it occurs throughout an individual's lifespan (Mescher & Junqueira, n.d.; Siddiqui & Partridge, 2016). It has been shown that an imbalance in the processes of formation and resorption, with an increase in resorption, or a decrease in formation leads to an increase in bone fractures due to weakened tissue (Fisher, Fisher, Srikusalanukul, & Smith, 2018).

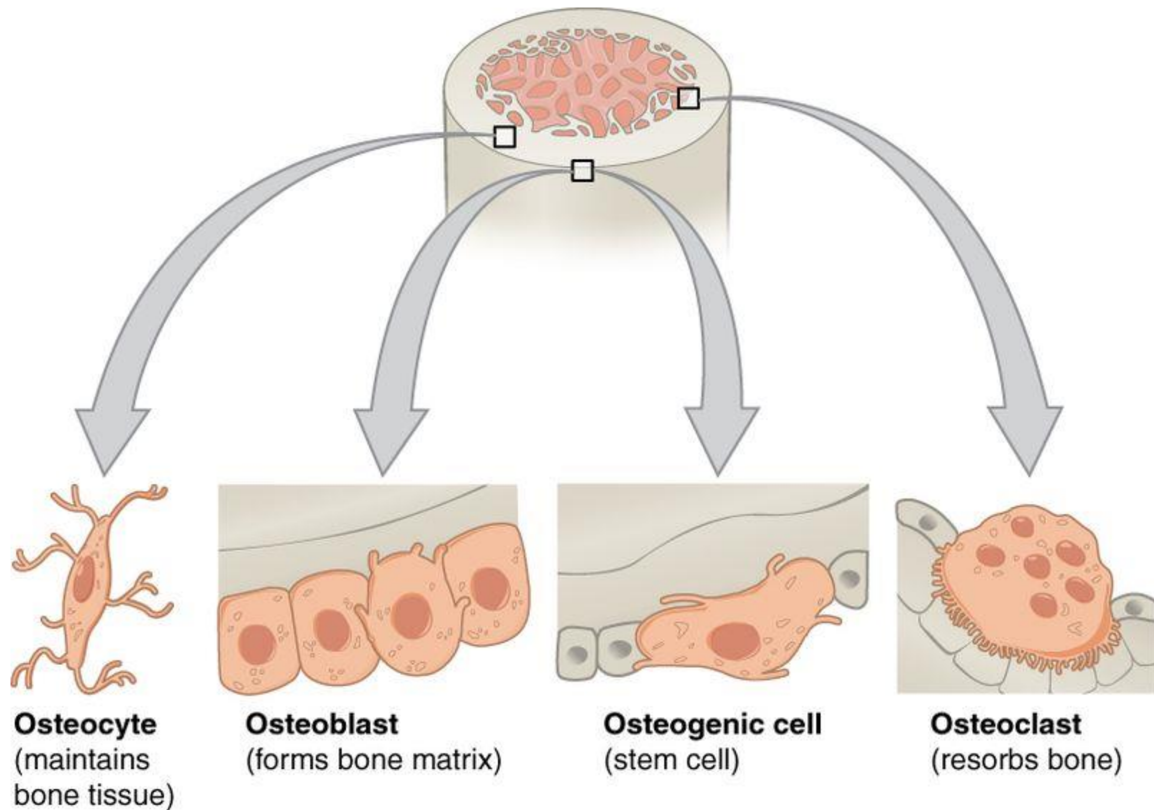


Figure 1. Cells of Bone. There are three main cells of bone, osteoblasts, osteocytes, and osteoclasts. An example of each is depicted in this image. Balance between each type of bone cell is necessary to maintain healthy bone tissue and quality of life (OpenStax College, 2013).

There are two ways that the bones of the human body are initially formed during fetal development. The first is intramembranous ossification, in which osteoblastic cells are formed directly from fetal mesenchymal tissue and begin the process of producing the bone matrix and the subsequent formation and calcification of bone tissue. This is the way that many of the flat bones of the cranium and facial skeleton are formed. The second type of bone formation is endochondral ossification. This is the process which forms most of the bones in the body (Mescher & Junqueira, n.d.). This type of formation begins with mesenchymal condensation, but differs in that the next step is the

differentiation of cells into chondrocytes, which secrete a matrix that will become cartilage. This forms a growth plate where the chondrocytes will eventually be replaced with bone cells and the bone continues formation from the growth plate (Long & Ornitz, 2013).

Articular Cartilage

There are several types of cartilage in the human body. The cartilage that covers the joints of the body is called articular cartilage (AC). AC is avascular and aneural, and therefore depends on simple diffusion of oxygen and nutrients to maintain its function. This trait allows AC to function to transmit loads, smooth joint articulation, provide wear resistance, and facilitate movement, but at the cost of a limited repair function when the tissue is damaged. AC is mostly composed of water, but also contains important proteins such as type II collagen, proteoglycans, and glycoproteins (S. Chen, Fu, Wu, & Pei, 2017).

The Hip Joint

The hip joint is a ball and socket joint that handles a lot of wear and tear over the course of a person's life. It is designed to support the weight of the human body, which means the mobility of the leg is compromised in favor of body stability (Drake, Vogl, & Mitchell, 2012). The joint is further stabilized by layers of surrounding muscles and

ligaments. The spherical head of the femur and the acetabulum of the pelvis articulate to form this joint and are covered with AC, and these two surfaces are especially prone to damage over time (Navarro-Zarza et al., 2012).

Osteoporosis

Osteoporosis is a disease which affects the density of bone and its microstructure, which leads to an increased risk of bone fracture (Black & Rosen, 2016). In addition to an increased risk of serious injury, osteoporosis also leads to an increased risk of death (Gass & Dawson-Hughes, 2006). This can cause changes in quality of life and overall health, and makes osteoporosis a serious life-threatening condition (see Figure 2). Further insights into this disease could benefit patients diagnosed with osteoporosis for years to come. Examples in animal studies have shown that as one ages, there is an imbalance between the processes of bone formation and resorption, which leads to a net bone loss (Galea et al., 2017). Bone loss is something that can hopefully be prevented in the future, especially in those who are genetically predisposed to develop osteoporosis.

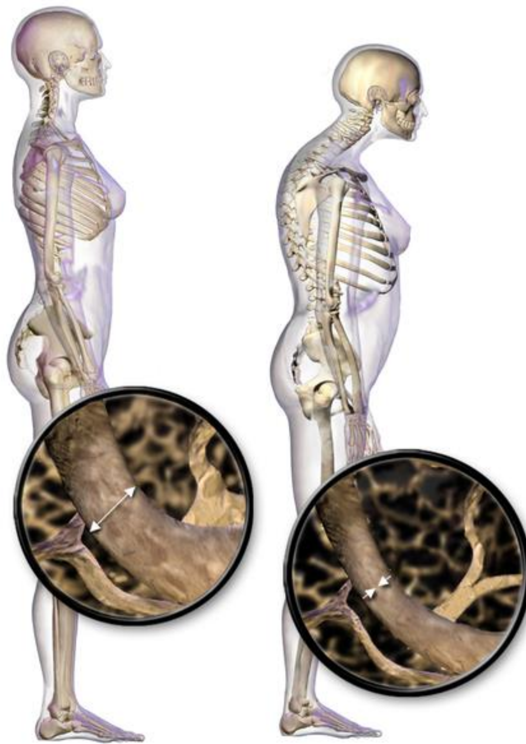


Figure 2. Effects of Osteoporosis on the Human Body. This image shows the decrease in bone mass due to osteoporosis at the level of bone, as well as the effects in posture due to such bone loss (“Medical gallery of Blausen Medical 2014,” 2014).

Public Health and Epidemiology

Osteoporosis is a huge public health concern in around the world. It poses a large financial burden, in the United States alone the disease cost patients $19 billion annually, not including extra costs including for travel and assisted living. In the European Union, it is estimated that the disease costs € 37 billion a year (Curtis, Moon, Harvey, & Cooper, 2017). This disease is often found in older adults and the population of the United States is rapidly aging (Day, 1992). This will lead to a larger population that is prone to osteoporosis, and therefore increased risk of fractures and possible death. Currently in the United States, 10 million people are living with osteoporosis, and 44 million have low

bone density and are at risk for the disease (*Elevating Osteoporosis on America's Healthcare Agenda 2 0 1 7 A N N U A L R E P O R T*, n.d.). In addition to the personal health risks and financial burden patients are also at an increased risk of job loss and changes to their working and family lives (Barker, Toye, & Lowe, 2016). These reasons make osteoporosis a concern for the public health of the country. If specific genes were known to increase risk to osteoporosis, patients with these genes could be identified and they could be treated preventively for osteoporosis and could be aware of risks.

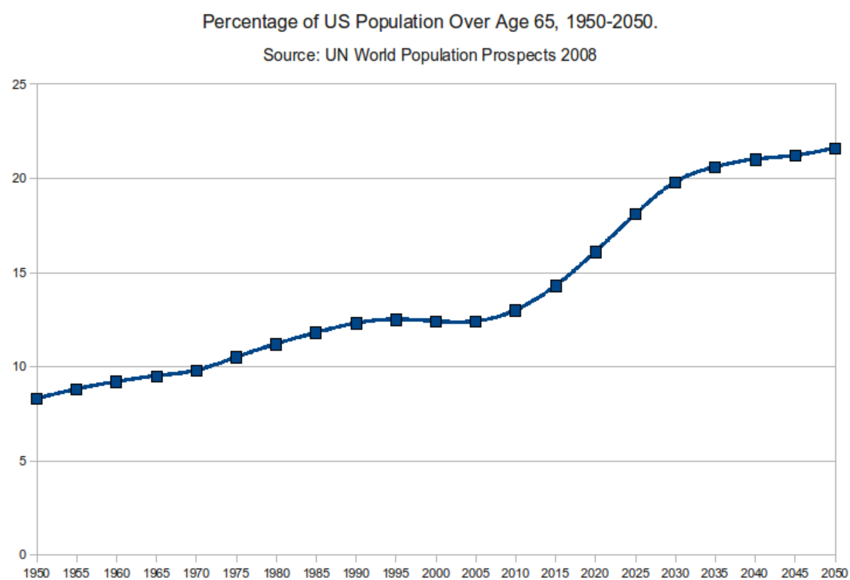


Figure 3. Percent of United States Population over 65 1950-2050. This graph shows the rapidly increasing senior population of the US. Information from UN World Population Prospects 2008 (Rcragun, 2009).

In patients over the age of 50, one in three women have been found to develop osteoporosis and one in five men will develop the disease (Barker et al., 2016). There have been some differences shown between races in addition to the difference between sexes. Black women have the highest bone mineral density (BMD), while Asian women

have the lowest, and this has been shown in patients of all ages. Generally, most BMD differences can be explained by weight differences, this has not been the case for African American women, who show no difference in BMD across different weights. When it comes to fracture risk, white women and Hispanic women are at the highest risk (Barrett-Connor et al., 2004) .These demographic differences could be useful in an initial diagnosis of osteoporosis.

Diagnosis and Treatment

There are several stages of severity of osteoporosis. The main factor in determining whether someone has the disease is the patient's bone mineral density (BMD) and whether the patient has experienced a fragility fracture. BMD is measured using dual energy X-ray absorptiometry (DEXA). There are also web based tools such as FRAX ® that can predict risk of osteoporosis by utilizing information from DEXA tests as well as demographic information. The FRAX ® algorithm can predict the probability that a patient will develop osteoporosis or have a fracture in the next ten years. FRAX ® does not require a BMD input, which makes it easy to use and readily available even if a patient does not have a DEXA scan (Curtis et al., 2017). Low BMD will lead to a diagnosis of osteoporosis (Kanis, Melton, Christiansen, Johnston, & Khaltaev, 2009). There are a number of treatment options available for those diagnosed with osteoporosis. There are different pharmacological interventions and these are mostly aimed at decreasing bone turnover, but can come with unwanted side effects (K. D. Grant, Busse, Park, & Baker, 2018). Current antiresorptive drug options include estrogen, selective

estrogen receptor modulators (SERMs), calcitonin, bisphosphates, and RANKL antibodies. These drugs act to inhibit the disease process of bone resorption. Another pharmacological option are anabolic drugs. These include parathyroid hormone (PTH) and its analogs. These drugs increase bone formation while keeping bone resorption levels low. New directions in drug treatment include sclerostin inhibitors and cathepsin K inhibitors. Both of these drugs aim to increase bone mass by inhibiting a crucial signaling pathway or protein degradation in bone (Khosla & Hofbauer, 2017).

Risk Factors

Development of osteoporosis is strongly linked to family history. BMD, which is the most important factor in bone fracture and the diagnosis of osteoporosis, as well as other factors that influence bone maintenance have been shown to be heritable traits. Due to the amount of different traits that can affect the presence of osteoporosis in an individual, it is believed that the heritability is polygenic, and no causal genes have been identified (Ralston & de Crombrugghe, 2006). The development of osteoporosis is also separately influenced by an individual's age, sex, lifestyle, and pre- or post-menopausal state. Lifestyle risk factors include an inadequate intake of calcium and vitamin D, physical inactivity, smoking, excessive alcohol consumption, and length of use of cortical steroid use (Hendrickx, Boudin, & Van Hul, 2015).

Genetics of Osteoporosis

A number of different of studies, including association studies, linkage analyses, and studies in model organisms, have led to the conclusion that genetics strongly

influence the development of osteoporosis (Ralston & de Crombrugghe, 2006). Family studies have also shown the significance of genetics, and that the environment less likely to play a role in whether someone will be diagnosed with osteoporosis in their lifetime, and additionally that multiple genes influence the development of the disease (Guéguen et al., 2009).

The key factor in diagnosing osteoporosis, BMD, has been separately shown to have significant genetic influence, which further emphasizes that the genetic component of osteoporosis is of great significance (Smith et al., 1973). Twin studies have strengthened the evidence for BMD as a heritable trait, and have lead to interest in further research into specific DNA studies (Pocock et al., 1987). BMD has been shown to be the strongest predictor of risk of fractures, and that men and women with equally low BMDs will be effected, so insight into the specific genes which control BMD could give more information than the effects of sex or environment or other lifestyle risk factors (Estrada et al., 2012; Johnell et al., 2005).

Genetic Regulation

Transcription of genes is a highly regulated process, and this regulation is specific to different tissues within the body. It is very likely that transcription in these different tissues is regulated by multiple genes, and identifying these different transcriptional regulators could provide specific and valuable insight into etiological causes of tissue specific pathologies (Melé et al., 2015). One way to look into the genes that are transcribed in a tissue is through the use of RNA-seq. This method can provide insights

into what genes are transcribed and can also provide insights about genetic changes from disease processes or aging. Sequencing RNA from the tissue of interest provides a window into the specific regulation and gene expression of that tissue and this information can be used in order to learn more about a disease process and can even be used in diagnosis (Cummings et al., 2017).

RT-PCR

Using the technology of Real Time Polymerase Chain Reaction (RT-PCR) allows researchers to easily and accurately assess changes in gene expression. RT-PCR technology amplifies complimentary DNA (cDNA) up to 1 million times, which allows the study of a small amount of DNA, and in the case of this study, RNA, to be possible. (Valasek & Repa, 2005). These reactions are performed in a thermacycler which can detect fluorescence levels, and is highly sensitive to the levels of gene amplification. This method provides a very measurement of gene expression levels by using a cycle threshold (Ct). This threshold is the level at which the fluorescence reaches a detectable intensity. This value is also key for understanding the data and reporting gene expression levels (Jensen, 2012).

Use of RT-PCR in this study is crucial and will allow investigation into target genes of osteoporosis. RT-PCR has previously been used to study osteoporosis. A study used tissues collected during hip replacements to compare the fragility of the bone between osteoporotic and osteoarthritic patients, which showed the patients with osteoporosis have lower osteoblastic differentiation and activity and is something that

will be investigated in this study as well (Giner, Montoya, Vázquez, Miranda, & Pérez-Cano, 2013).

New Challenges and the Purpose of this Study

The challenges that we face now in learning more about osteoporosis are identifying genes that affect the development of the disease, as well as the mechanisms through which they work. One of the aims of this study is to obtain insight into what these might be and how widely they are expressed in patients with osteoporosis. This study will compare the expression of genes that are known be expressed in osteoblasts between samples of human bone and cartilage tissue against bone cells grown in various media. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) will be used to measure gene expression from RNA samples of both human tissue and the cultured MSCs.

This is a preliminary study that also aims to compare three different types of cell culture media conditions. The main aim of comparing these conditions is to see if one type of media is better at producing viable osteocytes from MSCs. Once the ideal media is identified, the study can continue using that media to culture all future MSCs.

METHODS

Sample Collection

All human research was done under a Boston University School of Medicine Institutional Research Board Approved protocol: "Bone Tissues Repository", IRB Number: H-35199.

Femoral heads and acetabular reamings were collected from Boston Medical Center patients undergoing total hip replacements. A total of ten patients were included in this study, both men and women were included and ranged in age from 51-68 years old. Exclusion criteria for subjects in the study include: receiving chemotherapy, sickle cell, rheumatoid arthritis, osteoporosis drugs that affect bone metabolism, including Forteo (PTH), SERMS, Prolia (Denosumab), and bisphosphonates, and other therapies that can modifying bone including anti- TNF and cortical steroids. Before each surgery and sample collection, subjects were asked to consent to the study according to HIPAA guidelines. Once the femoral head and acetabulum samples were retrieved from the patient by the orthopedic surgeon, they were immediately brought back to the lab for processing on ice to ensure minimal degradation of tissue.

Table 1. Study Subject Demographics.

Patient #	Sex	Age
203	F	59
204	F	60
205	F	51

206	M	58
207	F	61
208	M	64
209	M	68
210	M	53
211	F	54
212	F	55

Sample Preparation

The sample reamings were obtained from the second and third coring of the patient's acetabulum in order to maximize the amount of bone and cartilage in the sample. Once the samples reached the lab they were weighed, and then transferred to a new, sterile container using a lab spatula. The reamings were then rinsed with 120ml of sterile phosphate buffered saline (PBS). The media was filtered through sterile steel mesh into a different sterile container and the remaining tissue sample was washed again with 100 ml of PBS and then strained. The reamings were rinsed with PBS again to remove all non-adherent cells, fat tissues, and blood cells and then was placed into a large petri dish on ice. Bone and cartilage fragments were separated from the remaining material using forceps. Once the bone and cartilage chips were isolated, they were placed into a 70µm filter on a 50ml tube and rinsed with 25 ml of PBS. They were then placed into a sterile 15ml tube and quickly frozen with liquid nitrogen. Once the sample was frozen, the labeled tube was placed into an -80°C degree freezer until further processing.

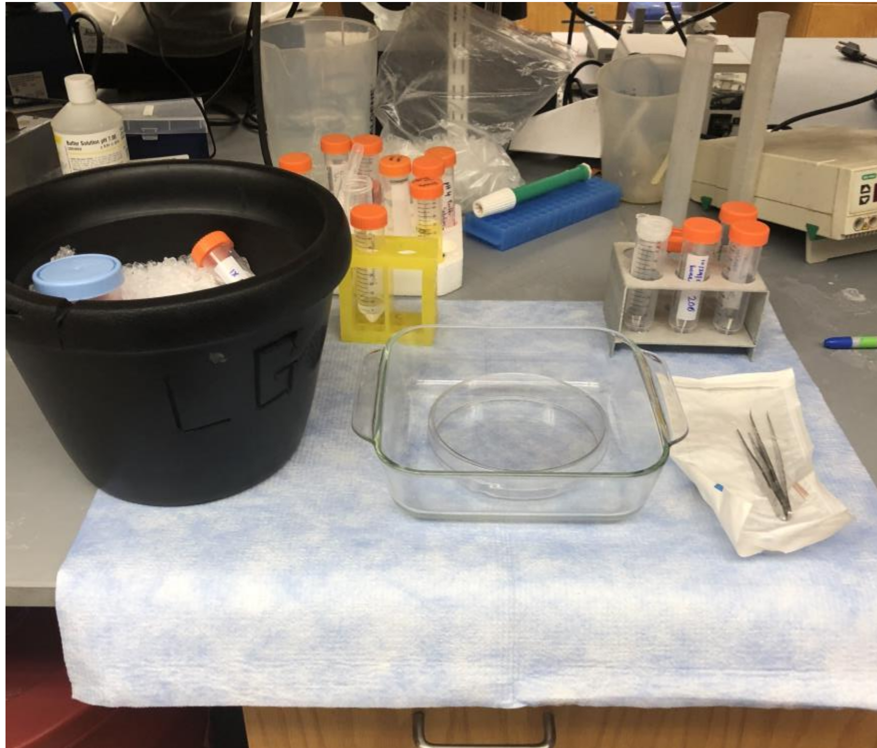


Figure 4. Sample Prep Set-up. Lab bench set up for bone and cartilage extraction from acetabular reamings collected from hip replacement surgery.

Figure 5. Bone and Cartilage Sample. Example of a typical reaming sample after processing for MSCs. The sample is a mixture of bone, cartilage, adipose, and connective tissues that need to be separated before further investigation. All sample preparation takes place on ice to minimize RNA degradation.

Cells from the marrow were plated and grown in 3 types of media: control media containing fetal bovine serum with (CM) and without dexamethasone (CM-D), and artificial media (AFM) containing no serum or animal derived components. The cells were seeded at 12 x10^6 cells/3mm diameter using 6-well cell culture plates (Corning Inc., Corning, NY) and grown in a humidified incubator (37° C, 5% CO_2). Cells were fixed 21 days after plating for an RNA assay that was used for RNA extraction.

RNA Extraction

In order to extract RNA from frozen tissue samples, a mortar and pestle was used to crush the sample into a fine powder. The sample was kept frozen during this process with liquid nitrogen. For cell culture samples, this first step was skipped and samples were placed directly into tubes for subsequent tissue dissociation using a Qiagen tissue lyser. The powder was then placed into a 2ml tube that contained 0.75ml of Qiazol (Qiagen, Cat#79306). A stainless-steel bead was added to the tubes to further lyse the bone tissue (Qiagen 5mm Cat#69989). The tubes were placed into pre-cooled metal tissue sample holders that were locked into place in the tissue lyser machine (QIAgen Tissue Lyser II®). The tissues were lysed for 2 minutes at 30Hz. The sample was then transferred to a new tube and 1ml of Qiazol was added. This stood on ice for 2 minutes and then 100ul of BCP (Sigma Cat #B9673) was added to the tube. The mixture was vortexed, rested on ice, and then was vortexed again. The sample was centrifuged at 14,000 RPM at 4°C for 15 minutes, then the aqueous phase of the sample was transferred to a new tube and an equal volume of isopropanol was added. The sample tube was

inverted and then centrifuged again at 14,000 RPM at 4°C for 30 minutes. The supernatant was removed and washed with 0.5ml of 100% ethanol and centrifuged at 14,000 RPM at 4°C for 5 minutes. The ethanol was then removed, and the last step was repeated. The ethanol was removed and the remaining pellet of RNA in the tube was left to dry. Once dry, the RNA pellet was resuspended in RNAse free water according to the size of the pellet and stored at -80°C.

Gel Electrophoresis

To assess the success of the RNA extraction, a small amount of the sample was run on a 1% agarose gel. 2µl of the RNA sample, 2µl of loading buffer containing glycerol, xylene cyanol, and bromophenol (Boston BioProducts), and 8µl of RNAse free water was mixed and then pipetted onto the gel. The gel was run at 80V for an hour and was then assessed under ultraviolet light for bands indicating 28S and 18S RNA were intact in the sample.

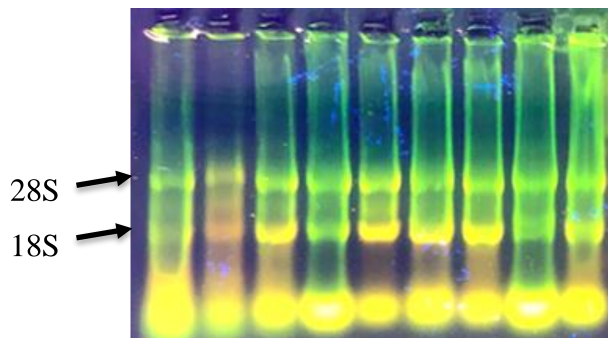


Figure 6. RNA Samples on Agarose Gel Under UV Light. This figure shows an example of the bands that intact RNA samples produce when run on an agarose gel.

RNA Spectrophotometry

The concentration of RNA in each sample was determined using a ND-1000

Spectrophotometer. 1µl of each sample was pipetted onto the pedestal of the Nanodrop machine and was measured for sample concentration (ng/ul) and purity ratios (260/280nm).

Reverse Transcription

The RNA extracted from the human bone, cartilage, and cell culture samples was reverse transcribed. 2µg of the RNA sample was added to a microcentrifuge tube and diluted with RNase free water to achieve a total volume of 10.4µl. A combination of MgCl2 (6.61µl), dNTP mix (6.0µl), 10X RT buffer (3.0µl), random hexamers (1.5µl), RNase Inhibitor (0.6µl), and Taqman Reverse Transcriptase (1.89µl) was added to each sample tube. The tubes were placed in a thermal cycler (Eppendorf Mastercycler® Personal). The cycles were as follows: 25° C for 10 minutes, 37° C for 60 minutes, 95° C for 5 minutes. The resulting complementary DNA (cDNA) was diluted at a ratio of 1:25 and stored in a freezer at -20C until needed for qRT-PCR.

qRT-PCR

The cDNA was used in quantitative Real Time Polymerase Chain Reaction (qRT-PCR) in order to gain further insight into gene expression in the tissue of interest. 96 well plates were loaded with 9 µl of diluted cDNA, 10 µl Universal PCR Master Mix (Applied Biosystems®), and 1 µl of a primer set. The wells were then covered with lids and spun in a centrifuge at 1,500 RPM for 2 minutes to ensure any bubbles in the samples were eliminated before the experiment was run. An Applied Biosystems 7300 Real-Time PCR

System was used to perform the qRT-PCR reaction. The reaction cycle ran as follows: 50° C for 2 minutes, 95° C for 10 minutes, 95° C for 15 seconds, 60° C for 1 minutes for a total of 40 cycles. 2 wells were run for each sample and the average of the two wells were normalized to 18S RNA to produce a ΔCt value. If a sample had an undetectable level of the gene of interest in qRT-PCR measurements, a value of 40 was assigned. This value indicates that the level of the gene is very low and allowed the calculation of a ΔCt value when it otherwise would not produce one. The ΔCt of the experimental media was compared to the ΔCt of the control media to produce a ΔΔCt value. This value was then log transformed to find the gene fold expression of the experimental media compared to the control media.

Table 2. DNA Primers Used in qRT-PCR and Expression IDs. The primers of six genes of interest and their corresponding assay IDs are listed. All primers were purchased from Thermo Fisher Scientific, Waltham, MA.

Genes	Taqman Expression ID
18S RNA	Hs03928990
COL1A1	Hs01076777
RUNX2	Hs00231692
SP7	Hs00541729
DMP1	Hs00189368
SOST	Hs00228830

Statistical Analysis

The normalized ΔCt values were used to find mean gene expression in relation to 18S RNA. To determine whether mean expression varied significantly for each gene across different media, 2-way analysis of variance (ANOVA) tests were used to compare ΔCt values between different patients and media. Two tailed t tests were used to compare the means between media and bone and cartilage samples. A p-value less than 0.05 was considered significant for all statistical analyses.

RESULTS

Patient Demographics

Data was collected from a group of ten hip replacement patients at Boston Medical Center (Table X). The sample group was made up of mostly female patients (F=6, M=4). The patients ranged in age from 51-68 years old (mean=58.3, standard deviation=5.25). Patients are indicated by numbers 203 through 212.

COL1A1

COL1A1 is a widely expressed gene that codes for collagen and is expected to have a high expression in most samples. The results of a 2-way ANOVA show that there is no significant difference between all media and no significant difference between patients for COL1A1 gene expression ($p= 0.15$, $F= 1.79$; $p= 0.26$, $F=2.49$). Results of two tailed T tests for differences between CM and CM-D, CM and AFM, and CM and human bone samples were not significant ($p=0.22$, $t=1.27$; $p=0.16$, $t=1.48$; $p=0.70$, $t=-0.39$). There was no difference in a T test between bone and cartilage expression of COL1A1 ($p=0.37$, $t=-0.94$). ΔCt values were log transformed and plotted in Figure 8. Each dot in the graph represents a sample, this method will be used to represent gene expression for all genes of interest. Figure 8 shows that AFM had the highest relative COL1A1 expression compared to other samples.

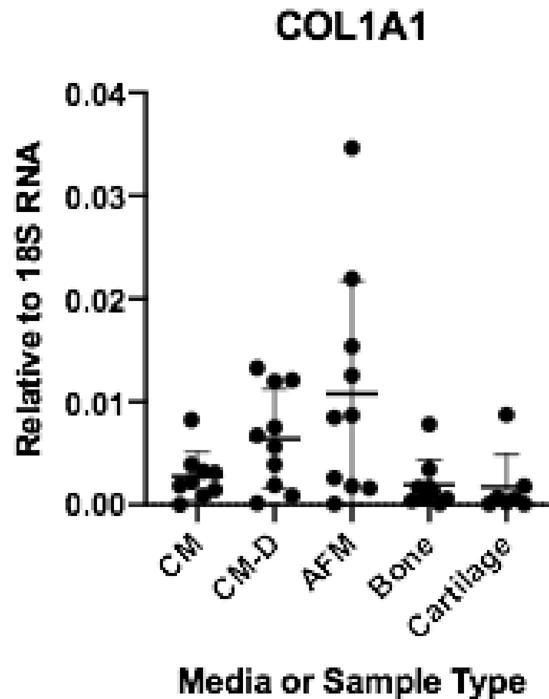


Figure 7. COL1A1 Expression Relative to 18S RNA. This graph shows the distribution of COL1A1 expression across various media and sample types measured with qRT-PCR. Expression values were normalized to 18S RNA expression values to achieve a ΔCt value which was then log transformed to produce comparable data points.

RUNX2

RUNX2 is the next gene of interest that was studied. The results of a 2-way ANOVA show that there is no significant difference between all media and no significant difference between patients for RUNX2 gene expression measured by qRT-PCR (p=0.76, F=0.27; p=0.21, F=1.59). Results of two tailed T tests for differences between CM and CM-D and CM and AFM were not significant (p=0.44, t=-0.79; p=0.64, t=-0.49). There was a significant difference in RUNX2 expression between CM samples and human bone tissue samples (p<0.01, t=-3.84). There was no difference between RUNX2 expression in human bone and cartilage samples (p=0.69, t=-0.42). AFM and cartilage samples showed

the highest and lowest relative RUNX2 expression, respectively. Figure 9 plots the distribution of RUNX2 expression for all samples, and shows the relatively higher expression in cultured cells over human bone and cartilage tissues.

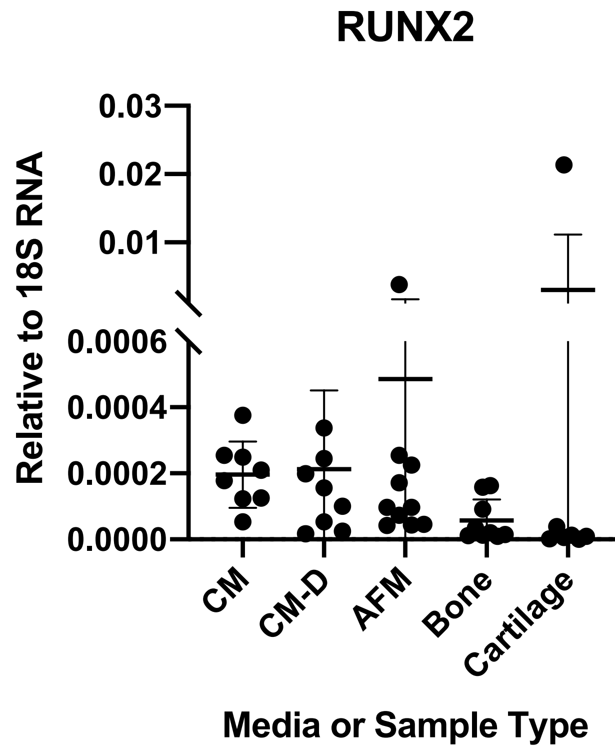


Figure 8. RUNX2 Expression Relative to 18S RNA. This graph shows the distribution of RUNX2 expression across various media and sample types measured with qRT-PCR. Expression values were normalized to 18S RNA expression values to achieve a ΔCt value which was then log transformed to produce comparable data points.

SP7

The next gene of interest in the lineage is SP7, expression levels of this gene will start to give a picture of how many samples have MSCs differentiating into osteoblasts. The results of a 2-way ANOVA show that there is a significant difference between all media conditions for SP7 expression ($p<0.01$, $F=7.09$), and that there is no difference

between all patients ($p=0.15$, $F=2.54$). Results of two tailed T tests for differences between CM and CM-D were not significant ($p=0.44$, $t=0.79$). However, there was a significant difference between CM and AFM SP7 expression and a difference between CM and bone SP7 expression ($p<0.05$, $t=2.6$; $p<0.01$; $t=3.57$). There was no difference between SP7 expression in human bone and cartilage samples ($p=0.52$, $t=-0.66$). These results show that CM-D and AFM plated cells seem to be differentiating into osteoblasts. Figure 10 shows more CM-D and AFM samples with higher expression, whereas CM samples remain relatively low in their relative SP7 expression.

Figure 9. SP7 Expression Relative to 18S RNA. This graph shows the distribution of SP7 expression across various media and sample types measured with qRT-PCR. Expression values were normalized to 18S RNA expression values to achieve a ΔCt value which was then log transformed to produce comparable data points.

DMP1

DMP1 is a protein that will indicate if samples have reached the osteocyte stage. A 2-way ANOVA showed that there was a significant difference between all media conditions for DMP1 expression ($p<0.01$, $F=9.68$), and that there is no difference between patients ($p=0.43$, $F=1.06$). Results of two tailed T tests for differences of DMP1 expression between CM and CM-D were not significant ($p=0.57$, $t=0.58$). There was a significant difference between CM and AFM DMP1 expression, and between CM and bone expression ($p<0.01$, $t=3.27$; $p<0.05$, $t=2.55$). There was no difference between SP7 expression in human bone and cartilage samples ($p=0.61$, $t=-0.52$). AFM had the highest mean level of DMP1 expression, and likely has some osteoblasts differentiating into osteocytes. As Figure 11 shows, AFM samples are expressing higher relative levels of DMP1 compared to the other media. It also shows that bone and cartilage samples are expressing higher levels than some media samples.

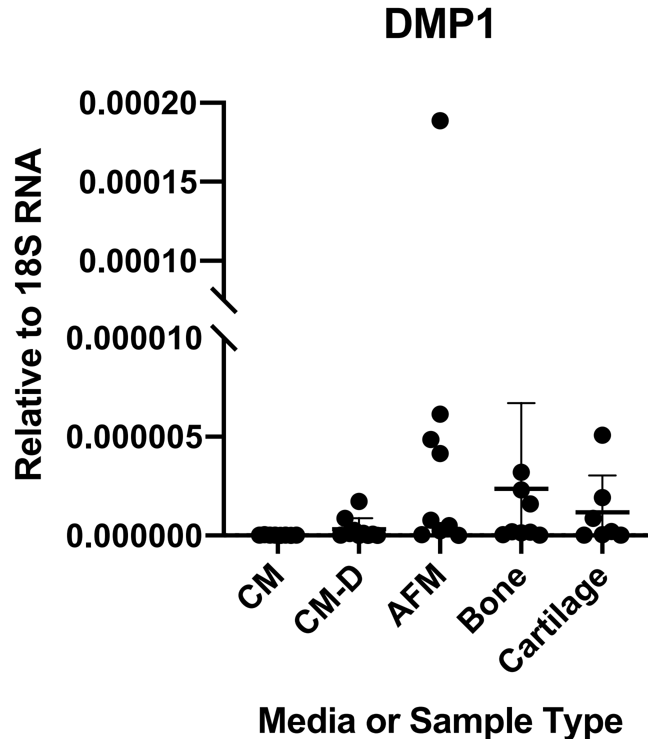


Figure 10. DMP1 Expression Relative to 18S RNA. This graph shows the distribution of DMP1 expression across various media and sample types measured with qRT-PCR. Expression values were normalized to 18S RNA expression values to achieve a ΔCt value which was then log transformed to produce comparable data points.

SOST

SOST is the final gene of interest that was tested and will indicate which samples, if any, are the most differentiated in the osteocyte lineage. A 2-way ANOVA showed that there was a significant difference between media for SOST expression ($p<0.05$, $F=4.02$), and that there was no difference between patients ($p=0.26$, $F=1.46$). Results of two tailed T tests for differences in SOST expression between CM and CM-D and differences between CM and AFM were not significant ($p=0.22$, $t=1.27$; $p=0.89$, $t=0.14$). There was a difference in SOST relative expression for CM and human bone samples ($p<0.05$, $t=2.74$). There was no difference between SP7 expression in human bone and cartilage

samples (p=0.83, t=-0.22). This is the only gene where AFM does not have the highest expression, suggesting that AFM has a lower level of osteocytes. Figure 12 illustrates the overall very low levels of SOST in all samples, and highlights the similar levels of SOST across the three media types. The distribution of bone and cartilage samples, with a slightly higher level of expression, is also emphasized by Figure 12.

Overall, the general trend for most samples' relative mean expression was a decrease in the level of the gene of interest, shown in Table 4. AFM generally had the greatest relative expression, while CM generally had the lowest.

Figure 11. SOST Expression Relative to 18S. This graph shows the distribution of SOST expression across various media and sample types measured with qRT-PCR. Expression values were normalized to 18S RNA expression values to achieve a ΔCt value which was then log transformed to produce comparable data points

Table 3. Mean ΔCt Values for All Samples. Mean ΔCt values for all sample groups and genes of interest with standard deviations.

Genes	CM	CM-D	AFM	Bone	Cartilage
COL1A1	9.46±2.7	8.06±2.1	7.69±2.5	9.87±1.8	10.89±2.4
RUNX2	12.5±0.9	13.05±1.8	12.8±1.9	14.9±1.7	15.8±4.6
SP7	22.9±1.5	21.4±2.3	18.6±3.7	18.2±3.0	19.13±2.8
DMP1	25.32±0.7	23.79±2.9	19.44±4.1	21.10±3.1	21.9±3.0
SOST	25.34±0.9	23.02±2.5	24.38±1.6	21.19±2.5	21.46±2.3

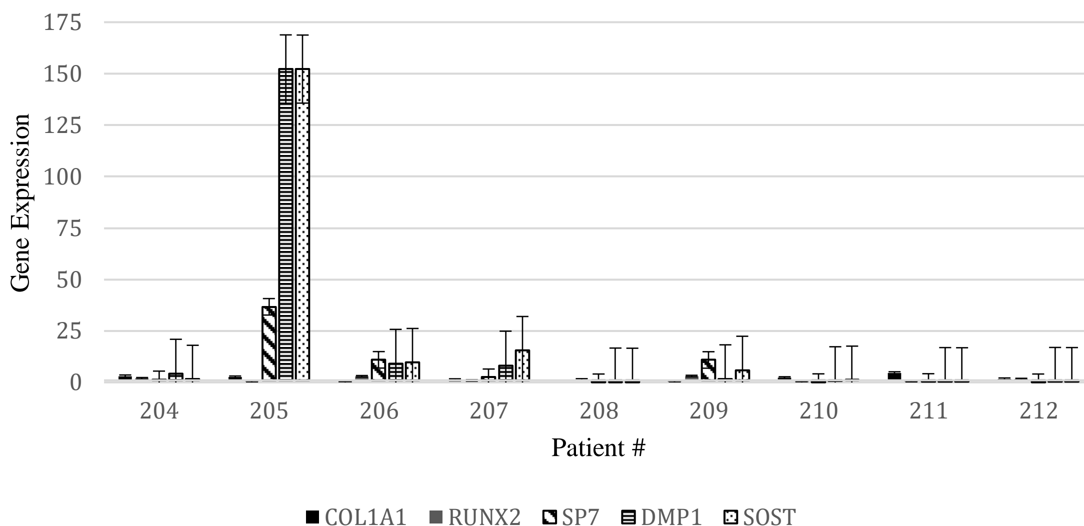
Table 4. Mean Log Transformed ΔCt Values for All Samples. Mean 2^(-ΔCt) values for all sample groups and genes of interest with standard deviations.

Genes	CM	CM-D	AFM	Bone	Cartilage
COL1A1	0.0028 ±0.002	0.0064 ±0.004	0.011 ±0.01	0.0020 ±0.02	0.0018 ±0.003
RUNX2	2.0E-4 ±9.9E-5	0.00021 ±0.0002	0.00049 ±0.001	5.7E-5 ±6.3E-5	0.0031 ±0.008
SP7	1.9E-7 ±1.8E-7	9.7E-7 ±1.7E-7	1.1E-5 ±1.9E-5	1.3E-5 ±2.6E-5	6.2E-6 ±1.1E-5
DMP1	2.6E-8 ±1.2E-8	3.2E-7 ±5.6E-7	2.2E-5 ±5.9E-5	2.4E-6 ±4.3E-6	1.2E-6 ±1.9E-6
SOST	2.8E-8 ±1.9E-8	3.6E-7 ±5.4E-7	8.0E-8 ±8.5E-8	2.0E-6 ±4.4E-6	1.0E-6 ±1.8E-6

ΔΔCt Comparisons

The ΔΔCt value is an important calculation because it shows how much more or less the gene of interest is expressed in the experimental group compared to the control group, which for the purpose of these calculations was CM. A value less than one for the sample group indicates that the control has a higher expression of the gene of interest,

and ΔΔCt value greater than one indicates the comparison group has the higher expression, and at what fold level. ΔΔCt calculations for our genes of interest show a general increase in gene fold expression of COL1A1, RUNX2, SP7, DMP1, and SOST in both CM-D (Figure 13A and 13B) and AFM (Figure 14A and 14B) media compared to the control media. For these calculations and graphs, patient 208 COL1A1 samples were not used because they were greater than two standard deviations from the mean, and was an extreme outlier.



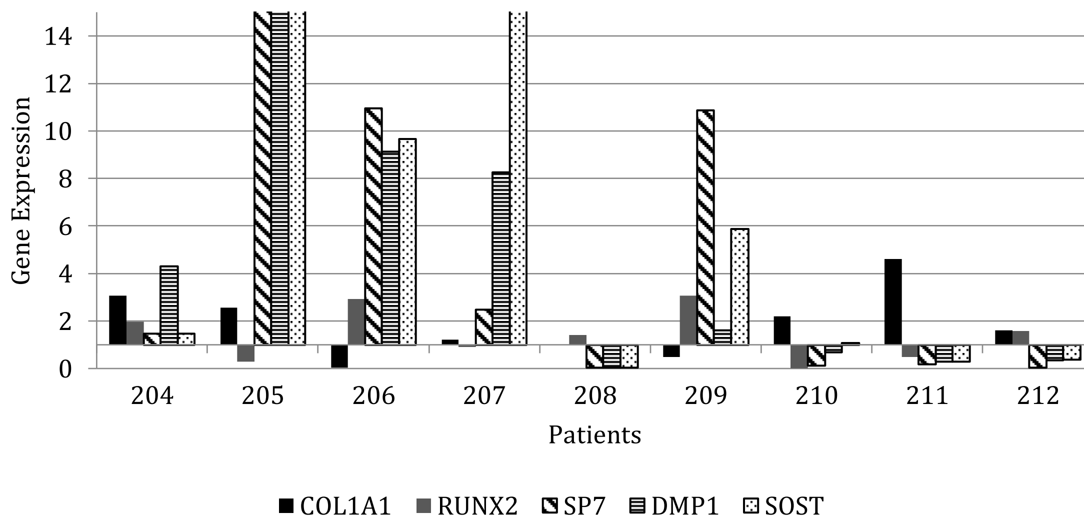
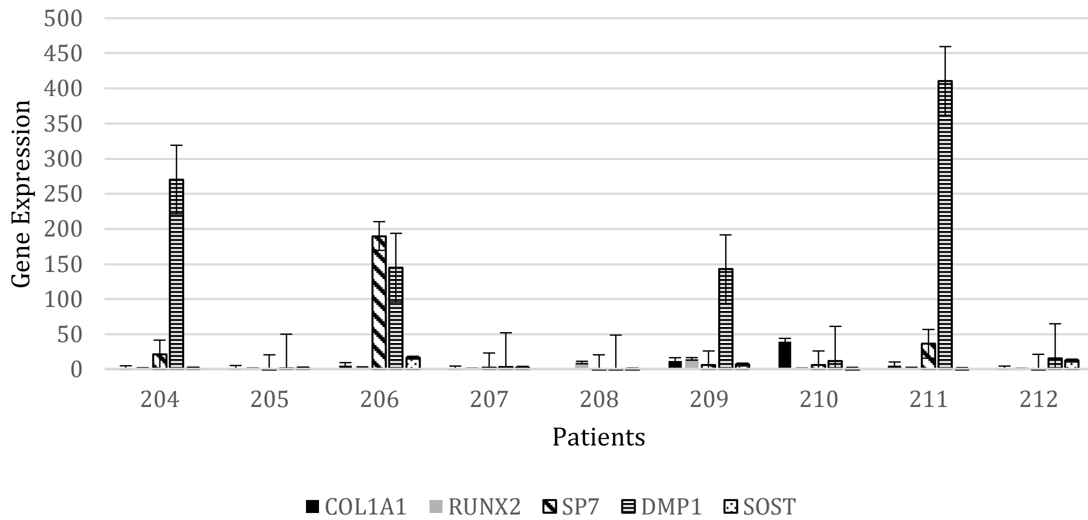


Figure 12. Gene Fold Expression Changes of CM-D Samples Compared to CM Samples. (A. All Samples B. Zoomed in View of Graph) Gene fold expression was calculated using the ΔΔCt method from expression levels obtained with qRT-PCR and shows the gene expression of CM-D compared to CM samples.



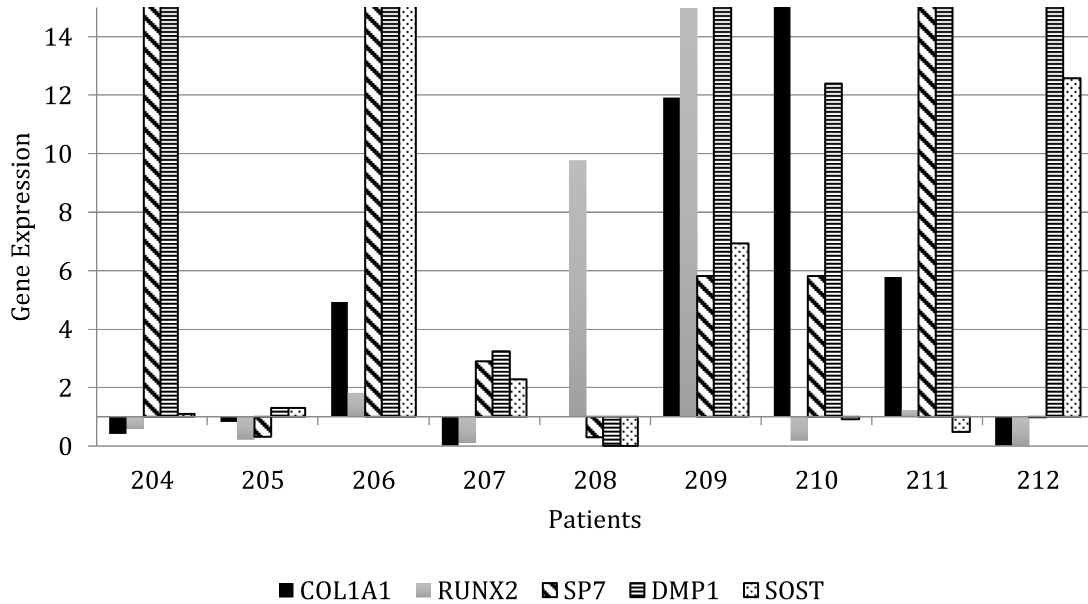


Figure 13. Gene Fold Expression Changes of AFM Samples Compared to CM Samples. (A. All Samples B. Zoomed in View of Graph) Gene fold expression was calculated using the ΔΔCt method from expression levels obtained with qRT-PCR and shows the gene expression of AFM compared to CM samples.

Table 5. Mean ΔΔCt Values for All Samples. Means and standard deviations of ΔΔCt values obtained from qRT-PCR. CM values are 1 and CM-D and AFM values are either greater or lesser than one to indicate whether they express greater or lesser amounts of the gene of interest, and at what level.

Genes	CM	CM-D	AFM
COL1A1	1	97.42±286	105.8±294
RUNX2	1	1.41±1.1	3.21±5.4
SP7	1	6.99±12.0	29.29±61.4
DMP1	1	19.66±49.8	111.27±146
SOST	1	20.72±49.6	4.70±6.1

Table 6. Statistical Summary. A summary of significant differences in gene expression for each gene of interest between the different sample groups. * = $p<0.05$, **=$p<0.01$, ns=not significant, $p>0.05$

Groups	COL1A1	RUNX2	SP7	DMP1	SOST
All Media	ns	ns	**	**	*
CM vs. CM-D	ns	ns	ns	ns	ns
CM vs. AFM	ns	ns	*	*	ns
CM vs. Bone	ns	*	**	*	*
Bone vs. Cartilage	ns	ns	ns	ns	ns

DISCUSSION

Several genes of interest were compared in this study. Each was chosen because of the role it plays in bone regulation and possible relation to osteoporosis pathology. The expression of these genes was used to assess the differentiation state of marrow mesenchymal stem cells grown in tissue culture under differing conditions. Each gene is expressed at different levels during the differentiation of MSCs into osteoblasts and then into osteocytes.

COL1A1

COL1A1 encodes collagen I, which is the major protein of human bones. Polymorphisms occurring in COL1A1 result in low bone mass, indicating the important role this gene plays in bone and cartilage tissues (S. F. A. Grant et al., 1996). We expected to see the highest expression of this gene compared to other genes of interest in both human samples and cultured cells, and across all media types. COL1A1 is often used as a positive control for osteoblastic differentiation (Hsu, Hsu, Hung, Shen, & Hsu, 2018). All cell culture samples tested in this study had at least some expression of COL1A1, so we can tell that at least some MSCs began the osteoblastic differentiation path, other genes tested will indicate how far down the path the cells are, and will be discussed further.

AFM had the lowest mean ΔCt value for COL1A1, and therefore the highest relative expression of this gene. The human tissue samples had the highest ΔCts, and the lowest relative gene expression. There were no significant differences between media

when it came to ΔCt measurements of COL1A1. This is likely because COL1A1 is a highly prevalent gene and is the first gene in the path of osteoblastogenesis so all samples are producing this at a relatively high rate. The ΔΔCt values showed that both CM-D and AFM samples had increased expression of COL1A1 compared to the CM, and both were one of the largest fold increases of any gene tested. This could show that both samples are producing more cells that are ready to enter the osteoblast stage.

RUNX2

RUNX2 is the next gene of interest in the pathway and is the initial transcriptional determinant of osteogenic differentiation from MSCs (Komori, 2009). This transcription factor is required for osteoblastogenesis to occur in both endochondral and intramembranous ossification. It is also necessary for proper function of mature osteoblasts as well as terminal hypertophic differentiation of chondrocytes during tissue mineralization. This makes this specific transcription factor an important measure because it can provide insight as to whether the bone tissue still has osteoblastic activity, and if the bone tissue is still actively turning over (Giner et al., 2013). Since RUNX2 is also crucial for chondrocyte hypertrophy, so we can expect to see some level of RUNX2 expression in the cartilage samples (Long & Ornitz, 2013).

There were no significant differences in levels of RUNX2 between any media conditions or human tissue samples. This is likely because RUNX2 is so prevalent in different types of cells and stages of development of those cells, as discussed above. All samples have a higher mean 2^(-ΔCt) value for COL1A1 than RUNX2, showing that

RUNX2 expression is relatively lower in all samples. This is further backed up by looking at log transformed ΔCt values for all samples, CM, CM-D, and AFM values are generally all clustered around the same values and therefore have similar relative expression of RUNX2 compared to 18S RNA.

ΔΔCt values showed an increase in gene fold expression for both CM-D and AFM compared to CM samples. This was the lowest fold increase of any gene of interest in this study. This could show that CM samples are achieving entry into the osteoblast lineage and are mainly in the osteoblast stage at 21 days, and are producing higher levels of RUNX2. These results point to CM samples being in an early stage of osteoblastogenesis with high RUNX2 expression, while other samples are further along in the lineage with lower RUNX2 expression.

SP7

SP7, also known as osterix (OSX), is a transcription factor that is required for osteoblast differentiation and bone formation. Mice with SP7 knockouts do not form bone, even if RUNX2 is present, which leads to the conclusion that SP7 is downstream from RUNX2 in the differentiation path (Long & Ornitz, 2013). SP7 is expressed in osteoblasts and in some chondrocytes, and expression in osteoblasts increases as differentiation occurs (Peng et al., 2013). SP7 is expressed during initial bone development, but is also expressed at significant levels throughout the life of the bone, indicating that the gene is important for bone homeostasis and maintenance. This would

also indicate that age should not have as strong as an effect on SP7 expression as it does on some of our other genes of interest (Zhou et al., 2010).

SP7 is the first gene in the differentiation pathway that showed a significant difference between different cell culture media samples. There was a significant difference between the CM and AFM samples. From this we can conclude that the media is making a difference in the level of differentiation that the MSCs are able to achieve at 21 days. CM had the lowest relative expression of this gene, even compared to human tissue samples, so at 21 days CM is in the RUNX2 osteoblastogenesis phase, and differentiates slower than AFM samples. AFM and CM-D both had an increase in fold expression over CM samples for SP7, however AFM had a much larger increase and strengthens the claims that cells in AFM are differentiating faster than cells in the other two types of media.

DMP1

Dentin Matrix Protein 1 (DMP1) is a protein that is required for mineralization of skeletal bone as well as dentin in teeth. It is highly expressed in osteocytes and is critical for osteocyte differentiation (Toyosawa et al., 2001). Mice lacking DMP1 have little or poor mineralization of bone and teeth. Humans with mutations of DMP1 will often present with hypophosphatemic rickets, a disorder of phosphate homeostasis. DMP1 is found in both early and mature osteocytes so we can expect to see expression of this protein in samples that are just starting to differentiate, as well as in samples that have reached the end of the osteocyte lineage (Inagaki et al., 2015).

There was a significant difference noted between DMP1 expression in CM and AFM. There was a 100-fold increase in AFM DMP1 expression compared to CM. AFM also has the highest relative expression of DMP1, indicating that some number of cells grown in AFM are differentiating from osteoblasts into osteocytes. Human bone samples also have a higher relative expression of DMP1 than CM samples, which suggests that CM samples are not as differentiated as human bone or AFM cells. CM-D had a large fold increase compared to CM, as well. These findings further indicate that the CM samples have not moved as far down the lineage as either the CM-D or AFM samples. CM samples could be more slowly differentiating, or something could have happened with the CM samples that has limited their ability to differentiate past the osteoblast stage.

SOST

SOST is responsible for the production of sclerostin, which is produced by osteocytes. Its primary role is to inhibit bone formation. If SOST is nonfunctional, it can result in undetectable sclerostin and produces a phenotype of sclerosteosis and hand abnormalities (Sebastian & Loots, 2017). SP7 and RUNX2 are known to activate the expression of SOST and the production of sclerostin (Pérez-Campo et al., 2016). SOST is the final gene in the path of differentiation that has been studied in this experiment. Samples with SOST expression are the most differentiated, and samples with no expression or little expression of SOST have likely not yet reached the osteocyte stage or were unable to differentiate that far in the lineage. It is a possibility that samples were

impaired in sclerostin production simply because of the age of the patients that cells were obtained from. Studies have shown that SOST production is significantly less in older individuals (Kwan et al., 2011).

Statistical analyses showed a difference between all media conditions for relative SOST expression, and a difference between the CM and bone samples. In fact, human bone had the overall highest relative expression of SOST in this study, which would be expected because many cells of adult bone are at the terminal stage of the osteocyte lineage. Some cells grown from the MSCs might have reached the point of sclerostin production, but it is unlikely that they could have gotten to a similar level as fully formed bone tissue.

The ΔΔCt values for both CM-D and AFM showed an increase in fold expression over CM. From this we can predict that CM-D and AFM cells are both further differentiated than CM cultured cells. This further backs up evidence from DMP1 results that CM samples had little or no osteocytes, and are likely made up mostly of MSCs and osteoblasts.

It is interesting to note that SOST has been found in some osteoblasts and chondrocytes in some studies (Delgado-Calle, Sato, & Bellido, 2017). This could explain the similar levels of SOST in human bone and cartilage samples. Since SOST is unpredictable in its expression, SP7 and DMP1 could give a bit more insight into the actual level of differentiation of the cells when it comes to comparison across different media.

Control Media without Dexamethasone

In order to properly grow MSCs into viable osteoblasts, stem cells should be cultured with dexamethasone (DEX), ascorbic acid, and beta-glycerophosphate (Q. Chen et al., 2016). DEX accelerates osteoblastic activity, although too high of a dose can induce apoptosis. A low level of DEX has been shown to increase cell viability (Zhang, Liu, & Liang, 2018). Our study did not find a significant difference in osteoblastic or osteocytic markers between cells grown in the control media and control media without DEX. Our CM-D samples did, however, show an increase in fold expression over CM for all genes of interest, which was unexpected. These unexpected results could be due to the small sample size used in this study. Additionally, the MSCs were harvested from the acetabular reamings of patients undergoing total hip replacements for various reasons. Any of the health conditions that the patients have, including osteoporosis or obesity, could have had an unknown effect on gene expression and affected any of the cells plated in any of the media. Other issues in plating and growing the control media cells so that they did not become as differentiated as CM-D or AFM could have occurred over the course of the study as well.

Bone and Cartilage

As an individual ages, bone marrow increases in adiposity because more MSCs are directed to the adipocyte lineage instead of the osteoblastic lineage. This could account for the decrease in osteocyte differentiation in human tissue samples in older patients, and possible gene expression abnormalities (H.-T. Chen et al., 2012). There

were no significant differences found between any of the bone and cartilage samples for any gene. This could be due to issues with methods, such as bone tissue not being completely separated from cartilage tissue, or issues with samples themselves. The age range of patients is skewed older, and this could affect the gene expression values that we expect to find. As one ages, transcriptional noise increases and can cause changes in gene transcription (López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013). This could also affect the expression of these genes involved in the osteoblastogenesis pathway. As mentioned above, disease processes of the bone and cartilage, such as osteoporosis, likely affected the expression of our genes of interest.

Conclusions and Future Directions

The results indicated that an ideal media to grow marrow derived stem cells into differentiated osteocytes is artificial media. The AFM had a higher mean $2^{(-\Delta Ct)}$ value for most genes of interest, and was significantly higher in expression for SP7 and DMP1, which are markers of osteocyte activity.

The patient population of this study had a mean age of 58.3 years. Previous studies have shown that MSCs obtained from bone marrow have a decreased potential to proliferate and differentiate as patients age (Yu et al., 2011). This should be taken into consideration for future studies and offers an interesting road for future analysis of these patients' gene expression. Further limitations of this study include the methods with which human bone and cartilage samples were prepped. In the future, it could be useful to be more specific in separating bone and cartilage tissue to ensure that there is no

crossover of this tissues or contamination by blood or fat tissues that are present in the original reaming sample. This could be achieved through use of a dissecting microscope and additional prep work, such as more washes before the sample is frozen. Another way to check for contamination of tissues would be to include more markers in the qRT-PCR analysis, including hematopoietic markers and markers specific for cartilage.

Future studies should continue to look at the expression levels of these genes and more to determine what role they play in the process of osteoporosis and should look into possible future directions for treatment and preventative health care measures in order to make osteoporosis a less prevalent and less life threatening condition.

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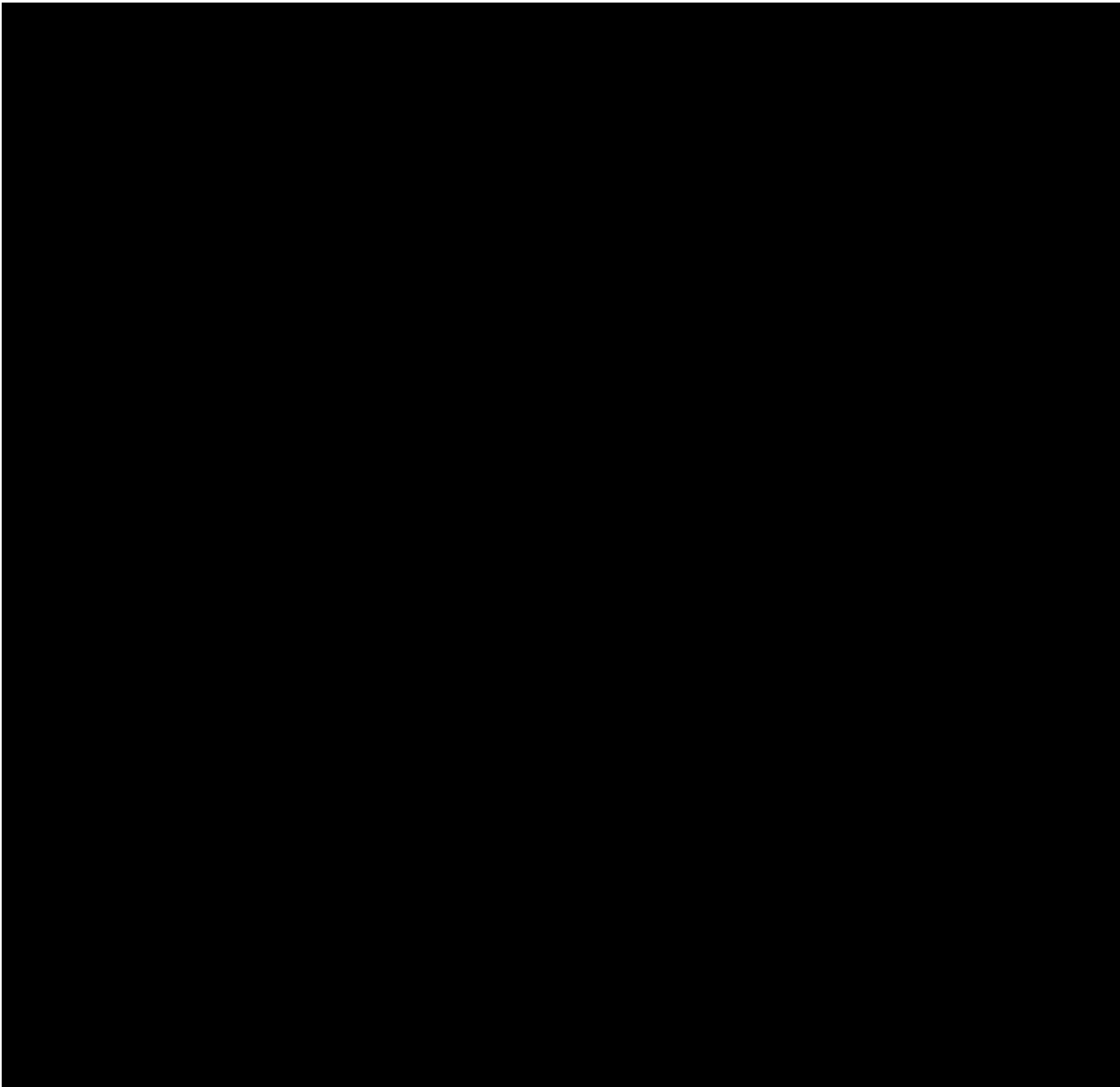

53