# Osteogenesis Effects Of Growth Factors On An In Vitro *Osteogenesis Imperfecta* Disease Model

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**Abstract**

Osteogenesis Imperfecta is a brittle bone disease in which there is an inadequate amount of Bone Collagen protein produced by cells. It has no cure and its treatments are highly ineffective. This project aimed to create a disease model using gene knockdown cells, seeded into a silk scaffold. We attempted to spur bone growth in the model by adding Bone Morphogenic Protein 2 (BMP-2), Transforming Growth Factor β (TGF- β) or both cytokines to the growth media fed to the scaffolds. The goal was to gain insight about healthy and diseased bone development by comparing results to that of normal mesenchymal stem cells (MSCs) in the same scaffolds. Well established qualitative tests and quantitative assays were used to assess the results. These included scanning electron microscopy (SEM), polymerase chain reaction (PCR) and the Sircol collagen content Assay.

Eventually it was shown that BMP-2 increased collagen production even in knockdown cells. BMP-2 also increased calcium mineralization. Knock down cells treated with both growth factors showed higher gene expression of COL1α1 than the untreated group.

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# CHAPTER 1: INTRODUCTORY MATERIAL

## 1.1 Clinical Significance

*Osteogenesis Imperfecta* (OI), or brittle bone disease, is a genetic disorder that can lead to extreme skeletal fractures, skeletal deformation, harmful sclera anomalies, poor muscle tone, hearing loss or respiratory issues, depending on the severity of the case. Current treatment is limited to physical therapy, bisphosphonate drugs and invasive surgery. There is no cure. Approximately one out of every 10,000 people is born with the disorder (Glorieux, 2008) and it pervades all racial and ethnic backgrounds (Huber 2007). Current treatments involve invasive rehabilitation techniques or focus on inhibiting bone resorption rather than promoting new healthy bone growth. Current research also suggests that gene therapy may be a viable answer. In order to provide a solution, it is important that we fully understand the underlying mechanism behind all forms of OI, including the results of both point mutations and non-sense mutations of the COL1A1 gene with regards to collagen formation and the effects that growth factors have during osteogenesis.

There exists some contradiction with regards to the effects of growth factors in differing situations. While studying animals or human tissue by biopsy is effective, it is costly and invasive. Instead, an accurate disease model with which to study growth factor effects would provide a superior alternative. This disease model is a significant contribution because it is expected to help illuminate confusion about Type I osteogenesis imperfecta, the most frequently occurring type.

Furthermore, the information ascertained from this experiment will also help to make important contributions to the field including insights into treatments relevant to other bone related disorders such as osteoporosis or osteoarthritis.

### 1.2 Specific Aims and Hypotheses

Specific Aim One is to establish an OI disease model that mimics forms of the disease in which inadequate amounts of collagen I are produced. Our working hypothesis is that seeding COL1A1 knock downs of human mesenchymal progenitor cells (MSCs) will accurately emulate Type I OI for in vitro studies. This is unique because it is a stem cell based technique which could potentially create a more accurate disease model than those that are not cell based. The use of silk is also important because it replaces past techniques of using collagen.

Specific Aim Two is to identify the effects that specific growth factors have on osteogenesis on an in vitro OI disease model. Our working hypothesis is that administered growth factors, specifically TGF-$\beta$ and BMP-2 will affect cell proliferation, differentiation and mineralization. The outcome of this aim will be an increased knowledge for the development of a drug to treat the disorder.

### 1.3 Long Term Goals

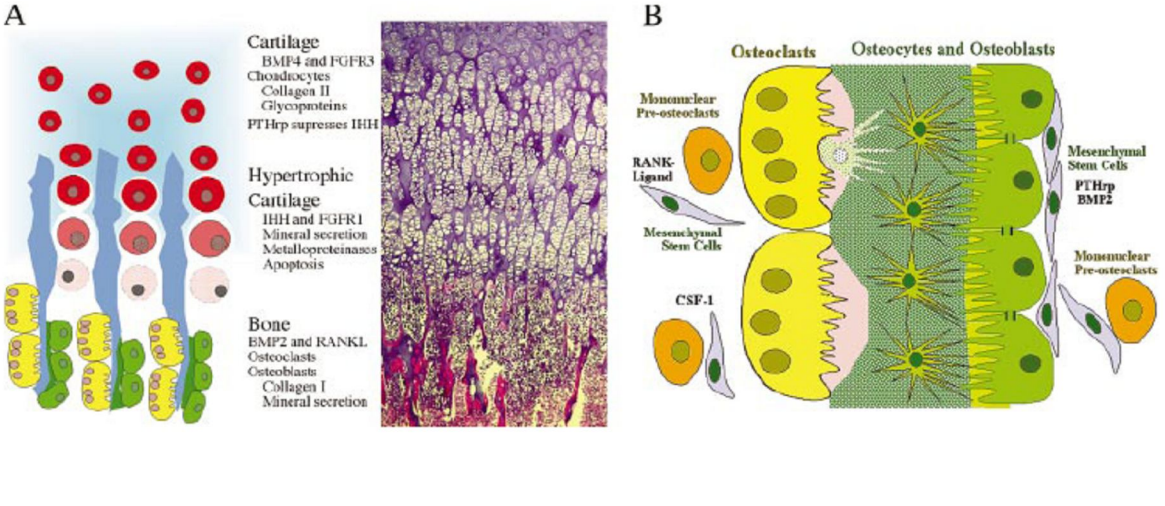
The long term goal of our studies is to alleviate symptoms of OI by promoting the production of active type I and II collagen based on administration of growth factors. Our objective is to stimulate osteogenesis in OI tissue. Our central hypothesis is that the administration of growth factors may promote bone formation even in OI tissue by stimulating osteoblast proliferation, differentiation and/or extracellular matrix

mineralization. The rationale of our hypothesis is based on growth factor studies done on healthy human osteoblasts (Bosetti 2007; Huang 2006). The objective of this application is to work with Transforming growth factor-β (TGF-β), and Bone morphogenetic protein 2 (BMP-2) in order to determine effects on osteogenesis on OI tissue.

## CHAPTER 2: BACKGROUND

### 2.1 Bone Physiology

Many mammalian tissues form initially during embryonic development, but continue to undergo modifications as the organism develops. One such tissue is human bone. It cycles through tissue as new cells and proteins are synthesized, mineralized and then resorbed. Initial osteogenesis (formation of the bone) does differ slightly from the tissue remodeling that the body undergoes throughout its lifespan. Embryonically, bone is derived from the mesenchyme (Blair et al, 2002). Mesenchymal stem cells are pleuripotent cells, which after proliferating enough to yield a significant cell mass, can differentiate in a number of different ways. One way these cells can differentiate is into chondrocites, or cartilage cells. Cartilage is closely related to bone. The location of bone




(A) Differentiation between cartilage and bone. A schematic view is shown adjacent to a 1 × 2 mm section from a human growth plate (haematoxylin and eosin; cartilage proteoglycans are blue and bone collagen is red). Once a cell differentiates into cartilage or bone, it cannot revert lineage. Ongoing differentiation in bone requires mesenchymal stem cells, which differentiate into several cell types. In the transition between cartilage and bone, feedback between Indian hedgehog and PTH-rp is critical, as are differences in FGFRs and BMP expression. Many other factors are required for normal differentiation, including vitamin D. The formation of hypertrophic chondrocytes in columns divides the developing bone into apoptotic regions, which become inter-trabecular spaces, and the mineralized columns, which are the scaffold for osteoblast bone deposition as the remaining mineralized cartilage is degraded. (B) Continuing differentiation of osteoclasts from marrow monocytes and osteoblasts from pluripotent mesenchymal stem cells. Osteoclasts differentiate under the influence of pro-apoptotic signals including RANK-ligand (RANKL) or TNFα, and CSF-1. Many accessory cytokines are important to the efficiency or regulation of the process, although intermediate stages of development are not well characterized. Development of osteoblasts is dependent on BMPs and PTH-rp. As in osteoclast development, other factors including vitamin D are necessary, and a host of cytokines act as co-regulators.

**Figure A.** Differentiation in Bone from Blair et al, 2002

growth is embryonically developed by chondrocites. These cells secrete an extra-cellular matrix, which, once mineralized, results in a prime site for osteoclastic attack. Osteoclasts are among the cell types that orchestrate the formation of bone (Figure A). The three main osteogenic cell types are osteoclasts, osteocytes and osteoblasts (Bone, 2009).

Osteoclasts are involved in a signaling pathway which regulates and executes degradation activities. Upon appropriate stimulation, a signal causes the release of acid phosphate, hydrochloric acid (HCl) and enzymes for the break down of mineralized calcium crystals in both the cartilage matrix the bone matrix. Osteoclasts differetiate from mononucleic cells found in blood. Thus vascularization is very important and is built in to the system by the creation of these hollow channels. Furthermore, the vascular channels can deliver nutrients and mesenchymal stem cells which can also differentiate into osteoblasts. For this reason, mesenchymal stem cells are also considered osteoprogenitor cells.

The osteoblasts are responsible for the production of bone extra-cellular matrix (ECM). Once they find their way to the osteoclast-formed cavities, they deposit ECM which includes mainly structural proteins such as type collagen I and then provide for the mineralization of this matrix by guiding the accumulation of calcium phosphate particles (Blair et al, 2002). Osteoblast cells can last on the order of days or weeks. They have a much slower throughput rate than osteoclasts, which only exist in the order of hours or days. Once completely surrounded and trapped by hardened ECM, however, osteoblasts become osteocytes. These cells maintain the bone tissue, lasting nearly 25

years (Bone, 2009). They have several lengthened sections which connect to others in order to create an information pathway within the matrix.

The most abundant protein in bone is Collagen I. This protein is a triple helix of polypeptide chains. These chains are coded for by the genes COL1α1 and COL1 α2. COL1α2 codes for one of the chains, while COL1α1 codes for the other two identical chains (Blair et al, 2002) with repeating sequence Gly-X-Y where X and Y are other amino acid residues. Often, X is proline and Y is hydroxyproline (Kofron and Laurencin, 2006). These collagens are rooted in proteoglycans (Li et al, 2006). Other bone proteins in the ECM include osteocalcin, alkaline phosphatase and bone sialoprotein (Blair et al, 2002; Safran et al, 2009). Osteocalcin is involved in calcium binding and alkaline phosphatase helps with bone mineralization (Blair et al, 2002). More specifically, alkaline phosphatase hydrolyzes phosphate ester, thereby spurring calcium phosphate precipitation. Its expression peaks at the cusp of cell proliferation and differentiation into osteoblasts (Bosetti et al, 2007). Bone sialoprotein, or integrin-binding bone sialoprotein (IBSP) is a major protein in bone that is also involved in calcium and hydroxyapetite binding. This protein also mediates cell attachment due to its Arginine-Glycine-Aspartic Acid (RGD) sequence (Safran et al, 2009). The compounds that crystallize during mineralization consist of calcium and phosphorous hydroxide (Blair et al, 2002).

There are a variety of molecules that act as co-factors in osteogenesis or as intermediates in causing differentiation or protein secretion. These come in the form of growth factors, cytokines, hormones, ligands or proproteins. For example, parathyroid

hormone, or PTH (Figure B) causes osteoclast formation in response to lower levels of Calcium. This signaling mechanism is in place in order to degrade the mineralized ECM and release entrapped calcium so that it becomes more available to the body, and more specifically the heart. One important ligand involved is receptor activator of nuclear factor κB (RANKL). This molecule regulates osteoclastic bone resorption (Kofron et al, 2006). Another family of signaling factors are Bone Morphogenic Proteins (BMPs). These growth factors have a variety of effects depending on cell or tissue type, but cause growth in osteoblasts. BMP-2 is highly expressed in bone and is thought to spur osteoblast differentiation (Blair et al, 2002; Phimphilai, 2006). Other chemicals

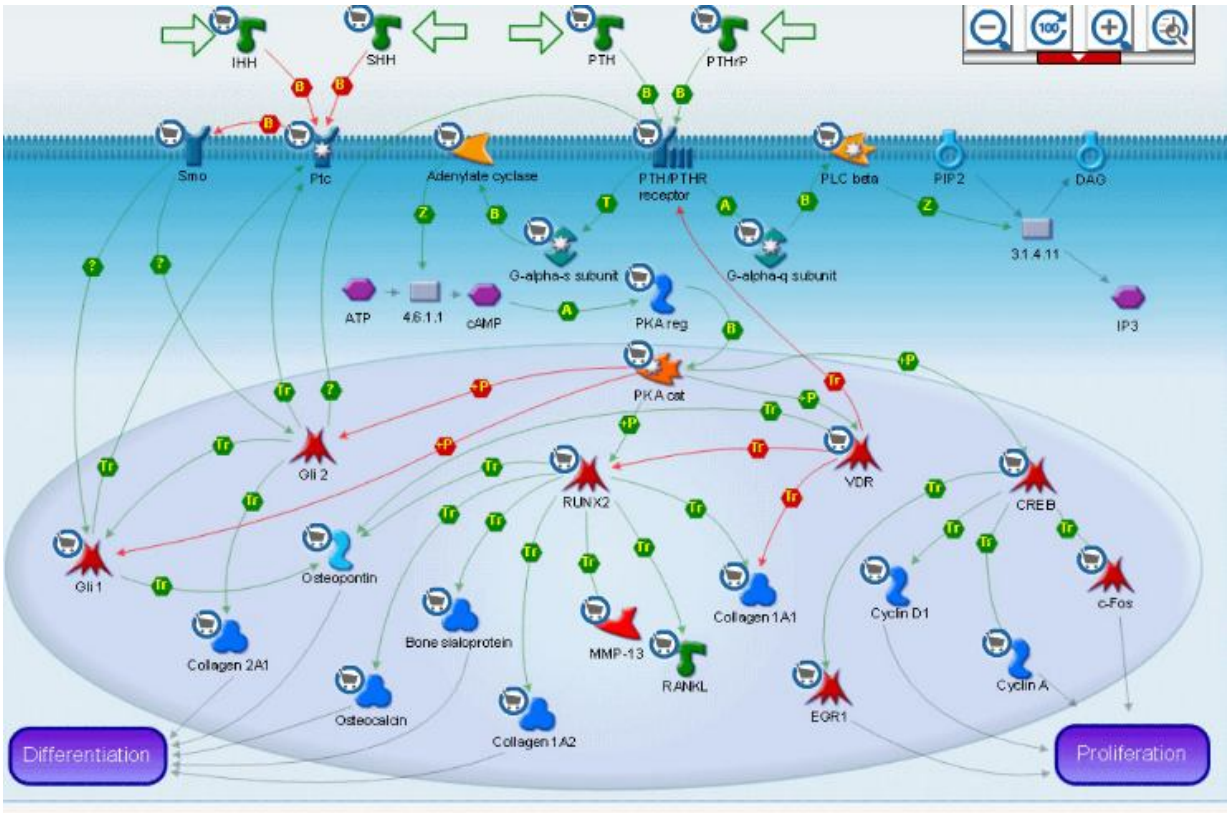



**Figure A.** Hedgehog and PTH signaling pathways participation in bone and cartilage development show the production of proteins and signaling molecules and their effects on differentiation and proliferation

involved in this differentiation include fibroblast growth factors (FGF) and Indian hedgegog (IH). Transforming growth factor beta (TGF-β) is another relevant molecule to osteoblast cells. It enhances proliferation and collagen and alkaline phosphatase formation. In healthy cells, transforming growth factor-β (TFG-β) is also known to spur cell division in the bone marrow). Once the significant mass of stem cells has accrued, the cells differentiate into osteoblasts. This differentiation is aided by the growth factor BMP-2, bone morphogenetic protein (Huang et al. 2007; Bosetti et al. 2007).

### 2.2 Osteogenesis Imperfecta

The complexity of the bone formation process leaves many opportunities for mistakes to be made. Osteogenesis Imperfecta (OI) is a disorder involving bone formation malfunctions. The multiplicity of error origins triggers varying forms of the disease with differing severities. This variation has led to a system that classifies each patient into a group between I and VII. Most of the types can be attributed to problems with type I collagen, which stem from mutations of the COL1A1 gene (Glorieux 2008). The most severe cases (type II) are fatal. Other cases are extremely debilitating and can lead to a life confined to a wheelchair. These cases mostly involve a mutation that replaces the glycine residue with another amino acid. This results in misfolded proteins with improper function and structure. Although severe, these cases are less prevalent (Figure C). More common are premature stop codons within COL1A1 that leads to “non-sense-mediated decay” and to a drastic decrease in collagen manufacture (Glorieux 2008).

Table I. Features of OI types I-IV

| *OI Type* | *Clinical Features* | *Prevalence* |
|---|---|---|
| I | Bone fragility and blue sclera.<br>First fracture when learning to stand or walk<br>Sensorineural deafness during late teens; > 90% by 30 years of age.<br>Typical normal stature, fully ambulatory with no bowing of the long bones. | 1: 15,000-20,000 |
| II | Lethal either in utero or during the perinatal period because of severe bone fragility and the subsequent development of pulmonary insufficiency, congestive heart failure, or pneumonia.<br>Newborns exhibit soft calvarial bones; a distinctive triangular face with beaked nose; blue sclera; short and deformed extremities with multiple fractures; and characteristic frog-like position.<br>Sixty percent and 80% of these patients die within the first day or month, respectively. | 1:20,000-60,000 |
| III | Progressive limb deformation, muscle weakness, marked limitation of mobility, and severe growth retardation.<br>Bone deformation at the base of the skull may lead to brain stem compression (basilar invagination) and possible catastrophic tetraplegia, respiratory compromise, and sudden death.<br>Enlarged head; characteristic triangular facies may develop, manifesting a broad and bossed forehead and small chin.<br>Early hearing loss.<br>High fracture rate; > 200 in a lifetime not uncommon.<br>Wheelchair bound at a very early age.<br>Shortened lifespan usually because of respiratory compromise. | 1:50,000-100,000 |
| IV | Phenotype that does not clearly fit Types I-III.<br>Progressive impairment of mobility.<br>Short stature, bowing of the long bones, and vertebral fractures.<br>Fractures occur throughout life with a relative period of quiescence between 20 and 40 years of age.<br>Minimal shortening of life expectancy. | Unknown |

*OI*, osteogenesis imperfecta.

**Figure B.** Classification of Osteogenesis Imperfecta Disease Types (Huber, 2006)

## 2.3 Disease Models

Tissue models are very useful in studying both healthy and unhealthy tissues because they circumvent the need for biopsy, which is invasive and costly. These models exist for all types of tissues and can often lead to new solutions in tissue regeneration. One effective healthy bone tissue model involves seeding mesenchymal stem cells (MSCs) onto appropriate scaffolds. MSCs are used because they have the ability to differentiate into a number of different specialized cells when provided with an appropriate fluid and structural environment. In the case of current bone tissue models, MSCs are used because they are found in vivo, in bone marrow, thus they exist in prenatal tissue but

also in adult tissue. They have also shown to be easily proliferated and compatible with eventual mineralization and ossification (Luyten et al, 2001). In one successful case, 3D silk fibroin sponge-like scaffolds with different pore sizes provide the structure, while a specific osteogenic media provides nutrients necessary for differentiation and growth (Kim et al 2007). Experimenters have also used silk in other scaffolds, such as in the form of electrospun fibers (Li et al, 2006). Silk is a biomaterial with a lot of promise, due to its non-toxicity, biodegradability and its mechanical properties (Li et al, 2006). Another reason silk is useful for *in vitro* applications, is that it is easy to differentiate cell derived proteins from those provided by the scaffold. Others have used collagen, hydroyapetite, or polylactic/polyglycolic acid scaffolds in the form of gels, sponges, or electrospun matrices (Luyten et al, 2001; Kanczler, 2008).

Another current relevant disease model mimics type II – VII tissues. In this model, Collagen-like peptides with mutated glycine residues were first synthesized using fmoc chloride (9-fluorenylmethyl chloroformate) as a protecting group and then assembly and mineralization were induced to create in vitro OI-similar tissues (Xu et al).

### 2.4 RNA Interference

One potential way to account for type I OI tissue, however, may be to use gene knockdown technology. Gene knockdowns use RNA interference technology to downregulate, or block, genes within cells of interest. Short interfering RNA (siRNA) is 21-23 nucleotides long and produced by the enzyme Dicer as an antiviral mechanism in

nature. SiRNA can also be artificially synthesized and integrated into cells by DNA-vector mediated transfection or by lipofection (Cheng, 2007). When siRNA was cloned into plasmids they were successfully used to downregulate the expression COL1A1 polymorphisms in human MSCs (Millington-Ward et al, 2004). If siRNA knockdowns are made of healthy MSCs and then seeded onto silk scaffolds, the result will serve as an effective way to study bone formation that mimics type I OI, effectively acting as a disease model where inadequate collagen is formed. However, methods using siRNA transfection are often short lasting and have unstable results (Khvorova, 2003). Furthermore, siRNA only attacks after the double stranded RNA is transcribed. It is not encorporated into the genome, but rather remains in the cytosol. For this reason, shRNA should be used. Its hairpin structure helps its stability. It can be delivered via a viral vector, which is effective in transfection and targeting. It does become integrated into the genome, either remaining as a plasmid or by random combination. This recombination can be tested for, using PCR (polymerase chain reaction). Transduction by shRNA techniques has been established in the literature in several mammalian cell types (Paddison et al, 2002 )

# CHAPTER 3: METHODS

## 3.1 Scaffold Preparation

3D silk scaffolds were prepared using previously established methods of silk manipulation (Kim et al 2005, Kim et al 2007, Kludge 2008). First, Bombyx mori cocoons were cut and emptied of the larvae. The cocoon pieces were boiled in an aqueous solution of 0.02 M $Na_2CO_3$ for 30 minutes with continued stirring to ensure adequate dispersal of the silk fibroin. The sericin supernatant was disposed of and the remaining fibroin was rinsed with distilled water for 20 minutes. This was done three consecutive times and then excess liquid was squeezed out, and the material was spread out by hand and allowed to dry in a fume hood for 12 hours. Afterwards, a 20% w/v solution of dried silk in 9.3 M LiBr solution was made and allowed to dissolve in an oven at 60˚C for 4 hours. Following this, the silk-LiBr solution was dialyzed in dialysis cassettes with milliQ water changes after 1, 4, 6, 12, 24, and 32 hours. The silk was collected by centrifuging for 20 minutes at 5-10˚C (9,000 rpm), 2 times. A 1 mL sample was then taken and weighed and allowed to dry completely for 24 hours. The dry weight was taken in order to measure the actual concentration of the silk. The silk was then diluted to obtain an 8% solution with distilled water.

After this solution was made, it was used to dissolve granular NaCl of particle size 500-700 μm to a concentration of 2 g/mL. In order to obtain this size granules, stocks of NaCl were sifted using respective sized sifts. This NaCl-silk solutions were added to Teflon cylindrical containers and allowed to sit at room temperature for 24 hours. After

adequate solidification, the NaCl particles were leached out by immersing in water for 2 days. The resulting scaffolds were cut using a biopsy punch with a 5mm diameter. The resulting cylinders were further cut to 3 mm thicknesses. The resulting scaffolds were autoclaved in order to sterilize them. Afterwards they were soaked in osteogenic media for 24 hours prior to cell seeding.

## 3.2 2D hMSC Cell Culture

### 3.2.1 Preparation of Proliferation Media

In order to make proliferation media, all materials were sterilized by pippetting into a 500 mL filter flask, pore size 0.2 μm. First, the Minimal Essential Medium (1X αMEM) was added to a final concentration of 88% v/v. 10% Fetal Bovine Serum (FBS), 1% Non-Essential Amino Acids Solution, and 1% Antibiotic-Antimycotic was also added to the flask. The flask was activated by attaching to a vacuum. The resulting solution was used for 2D cell expansion and remade when necessary. Each time the media was used with cells, it was first warmed in a water bath unless otherwise noted.

### 3.2.1 hMSC Isolation, Seeding and Proliferation

Human mesenchymal stem cells derived from bone marrow was extracted using previously established methods (Hoffman et al, 2007). Frozen stock of hMSCs with DMSO was thawed by hand and washed with at least 5 times the amount of media. The resulting solution was then centrifuged at 4°C, 1250 rpm for 10 minutes. After checking for a cell pellet, the DMSO and media were aspirated. The cells were then resuspended with media and distributed to T-185 tissue culture flasks. Flasks were then incubated in

37° C. Proliferation media was changed 2-3 times per week. At each media change, cells were inspected under a microscope and checked for confluency. Old media was aspirated and 25 mL of new media was added to each tissue culture flask, taking care not to blast the cells with the media. Upon reaching 85-90% confluency, cells were passaged. Trypsin was thawed as all media from the flasks was aspirated. Next, the cells were washed with 20mL PBS. After the wash, 5mL of warm Trypsin was added to each flask and incubated for 5 minutes in order to detach the cells from walls of the T-185 flasks. Just before the five minute marker, flasks were removed from the incubator and checked under the microscope for cell-detachment and round morphology. Cold media was added to the flasks in order to stop the reaction in a concentration of two times the amount of trypsin. The solution was mixed and transferred to a conical for centrifugation at 4 degrees, 1250 rpm, and 10 minutes. The resulting trypsin and media were aspirated from the cell pellet and then the cells were resuspended in 10 mL warm media.

At this point, an aliquot of the mixture was removed for cell counting and diluted 1:9 by volume with PBS. 0.1 % of the counting solution (~10µL) as then added to each side of a hemocytometer with a cover slip on top of the slide. The cells inspected under a microscope and counted clockwise within the 8 boxes. The resulting number was adjusted based on volumetric dilutions in order to acquire a total number of cells. This was done by first dividing by 8 to get an average per box, then multiplying by 10K cells per mL, then multiplying by 10(due to dilution) and lastly multiplying by 9 (due to total

mL). New flasks were seeded by adding cell-media solution in a concentration of 2 *$10^6$ cells per flask and a final volume of 25 mL of media. Normal hMSCs were continued to be grown for gene knockdown studies and until a total cell number of 5x$10^6$ normal hMSCs was reached.

## 3.3 Gene Knockdown

### 3.3.1 Puromycin Titration

In order to perform gene knockdown a puromycin titration was first carried out. The puromycin was thawed and sterilized with PBS using a syringe and a 0.2 μm filter. Before re-seeding all the cells back into tissue culture flasks during one of the hMSC passages, 2x$10^5$ cells were added to each of 10 wells in a set of 6-well plates. To each well, 2mL of proliferation media was added and the seeded plates were incubated for one 24 hours in order to allow cell attachment. After the first day, differing concentrations 0, 1 μg/mL, 2 μg/mL, 4 μg/mL and 8 μg/mL of puromycin were added to each pair of wells. The media was changed every two days, keeping track of which cells had died. The concentration which killed the cells eventually, but not all of them right away was recorded.

### 3.3.2 Hexadimethrine bromide Treatment

Before re-seeding all the cells back into tissue culture flasks during one of the hMSC passages, 2x$10^5$ cells were added to each of six wells with 2mL proliferation media and overnight incubation for the hexadimethrine bromide treatment. To each well, 1 mL of a 16 μg/mL hexadimethrine bromide in media solution was added (final concentration

ended as 8 µg/mL). A ten-fold serial dilution was prepared with the shRNA lentiviral particles to create a final lentiviral particle concentrations of $10^3$,$10^4$,$10^5$,$10^6$ and $2x10^6$ particles per well and a final volume of 2mL per well. In addition, one of the wells was kept as a nontransduced control group. The plates were then incubated for 24 hours. The media containing the lentiviral particles was then removed and replaced with fresh proliferation media. After another 24 hours, the media was again aspirated and replaced with proliferation media containing 2 µg/mL puromycin in order to kill the nontransduced cells. The puromycin media was changed every two days and inspected for living cells.

### 3.3.3 Lentiviral Transduction

Before re-seeding all the cells back into tissue culture flasks during one of the hMSC passages, $2x10^5$ cells were added to each of nine wells with 2mL proliferation media and overnight incubation for the lentiviral transduction. This was named day 1 of the lentiviral transduction. The lentiviral particles contained pLKO.1 vectors with shRNA to knock down the COL1A1 gene. On day 2, the media was aspirated and a final concentration of 8 µg/mL hexadimethrine bromide in media was added to five of the wells in order to enhance the transduction. After this addition, $2x10^6$ lentiviral particles were added to four of those five wells (Figure 1). To the last well with hexadimethrine bromide, the Green Fluorescent Protein (GFP) control was added in order to optically confirm the transduction of the cells using the GFP marker. The remaining four wells served as a control and were not transduced. After 24 hours of incubation, on day 3, the media containing the lentiviral particles was removed and replaced with proliferation

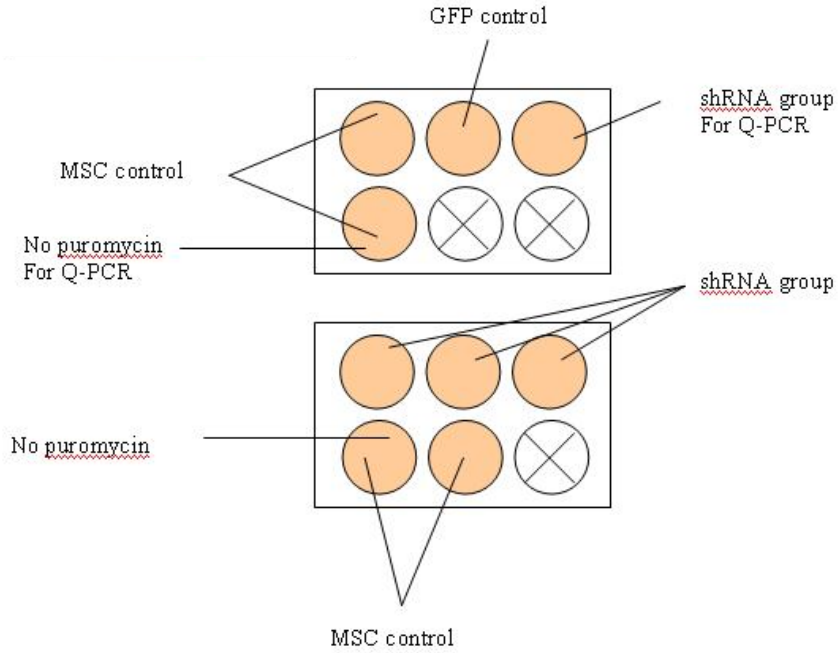



**Figure 1.** Lentiviral Transduction Preparation Schematic

media. On day 4, after overnight incubation, the media was aspirated and replaced with media containing 2 µg/mL puromycin. Half of the control cells (nontransduced) were treated with media containing puromycin and half without the puromycin. This media was changed again on day 6.

### 3.3.4 Knockdown confirmation

On day 7, the GFP well and the control wells were inspected using fluorescence and phase contrast microscopy. Upon reaching 90% confluence, the three wells of the shRNA group, were passaged into a T-185 flask and expanded. The last single well of the shRNA group and the control group without puromycin was used for Q-PCR.

In order to begin RNA extraction for Q-PCR, the single well of transduced cells and the control well of normal MSCs were treated with 1 mL Trizol. The cells were also washed with 1mL chloroform. The contents were mixed thoroughly and transferred to

eppendorf tubes. The samples were then centrifuged at 4°C at 13,000 rpm for 15 minutes. This yielded a two-tiered liquid. The upper layer was clear and was separate from the pink and contained the RNA. This supernatant was transferred to another eppendorf tube, where 350μL of 7% ethanol was added. After this step, the QIAGEN protocol for RNA purification was followed. This involved first transferring the RNA solution to an RNeasy column and then adding 700 μL of RW1 Buffer to each tube. This was followed by centrifugation at 12,500 rpm for 15 seconds. After discarding the eluent, 500 μL RPE Buffer was added to each tube and again centrifuged for 12,500 rpm for 15 seconds. This step was repeated but centrifuged for another 2 minutes. The pink section containing the filter was then transferred into a new eppendorf tube and treated with 50 μL RNase free water (directly onto the filter) and centrifuged at 12,500 for a minute. The eluents were then treated with the High Capacity cDNA RT kit. To each tube 21 μL DNAse free water, 10 μL 10x buffer, 25x dNTPs, 10 μL 10x random primers and 5 μL multiscribe was added. RT-PCR was then carried out in the thermocycler using the program DIHRT. This program took approximately 2 hours. When finished, the samples were stored in -20°C. In order to finish Real Time PCR, 17.5 μL DNAse free water, 2.5 μL AoD probe for COL1a1 and 25 μL 2x TaqMan reagent was added to each sample and loaded into the PCR plate and capped. The plate was then loaded into the PCR machine and the Mx Pro software calculated gene expression relative to GAPDH as a calibrator.

## 3.4 Treatment Group Preparation

### 3.4.1 Osteogenic Media Production

Osteogenic media was prepared by adding 90% alpha MEM, .1% dexamethazone, .1% 1000X Ascorbic Acid, 1.08g Glycerol-2-phosphate, 10% FBS, 1% antibiotic-antimycotic, and 1% Nonessential Amino Acids.

### 3.4.2 Scaffold Cell Seeding

Scaffolds, after sterilization and soaking in media were seeded with cells. Cells were first trypsinized from their culture flasks by adding 5mL of trypsin and incubating for 5 minutes. The reaction was stopped by adding double the amount of cold media. The solution was pipetted against the walls of the flask in order to assure detachment and then collected in a conical for centrifugation at 4° Celcius at 1250 rpm for 10 minutes. Afterwards, the media was aspirated from the cell pellet and the cells were resuspended in 20uL of cell suspension with about 5 x $10^5$ cells per scaffold. Each scaffold was gently handled and half of the cells were micropipetted onto the top and half onto the bottom of the scaffold and then placed within the well of a six well plate. The cells were allowed to attach and then osteogenic media was added, 5ml per scaffold.

### 3.4.3 Growth Factor Treatment

Two hours later, the treatment groups with cytokines began (Figure 2). Group one was the positive control of normal MSCs, and was treated with 100 ng/mL of BMP-2. Group two was a negative control, thus these knockdown MSCs were treated with media containing no growth factors. Group three contained knockdown MSCs as well and was treated with 5 ng/mL TGF-B1. Group four contained knockdown MSCs and 100ng/mL

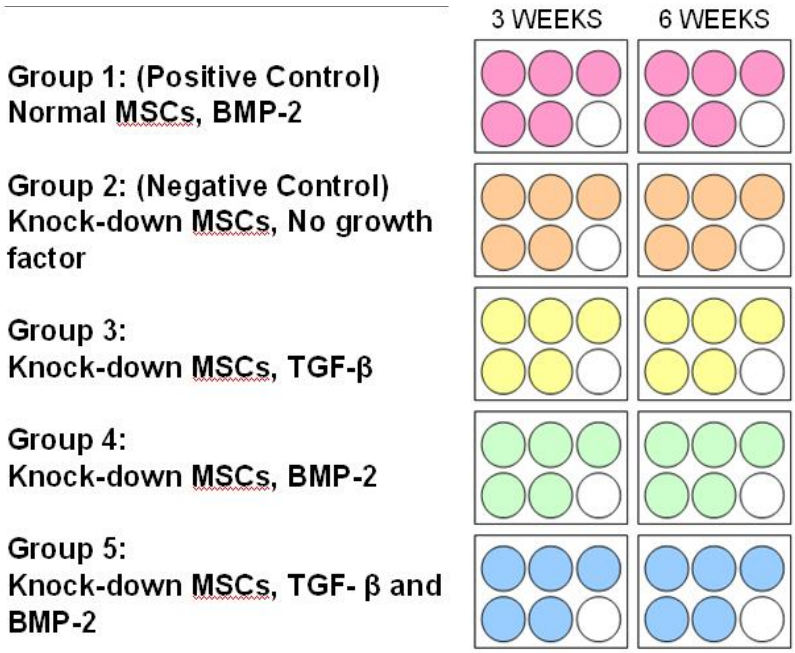



**Figure 2.** Treatment group Preparation Schematic

BMP-2. Group five contained knockdown MSCs and was treated with both 100ng/mL BMP-2 and 5ng/mL TGF-B1. Media was changed two times per week and each well was aspirated first and then treated with 5mL of fresh media.

### 3.5 Chemical Analysis

Approximately half the samples were treated for three weeks and half for six weeks and then subject to testing. At this each time point, scaffolds were cut, divided and placed into groups of labeled eppendorf tubes as shown. After drying the Calcium and Collagen Samples in an oven for 24 hours, their weights were measured and chemical analysis was performed as indicated (Figure 3). Fewer scaffolds were subjected to histology and SEM for week three samples due to fungal contamination. Mechanical analysis only done on week six samples (Figure 4)

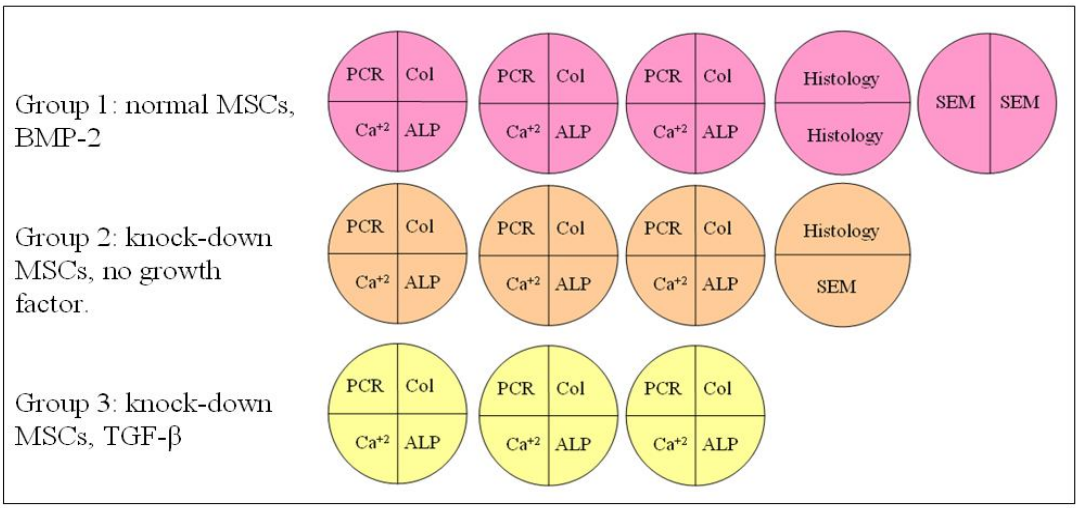



**Figure 3.** Week 3 Testing Schematic

### 3.5.1 Collagen Content Assay

In order to test the levels of collagen produced by the cells, the Sirius Red Assay was performed. First, 1mL of pepsin was added to each of the nine samples. Each sample was chopped with a microscissor and then centrifuged at 12000 RPM for 3 minutes. In the first row of a 96 well plate, bovine tracheal cartilage collagen of 1mg/mL was added to six wells and diluted with D2O to yield the following concentrations: 0, 0.2, 0.4, 0.6, 0.8, 1.0 mg/mL and a final volume of 20uL. In the succeeding rows, 100 uL of each sample was added (Figure) and the plate was allowed to dry for 24 hours at 36 degrees C. Once drying in the oven was completed, 100 uL of the Sirius red dye solution was added 1mg/mL and the samples were incubated for one hour. Next, stock fix solution was created which contained 71% w/w pitric acid, 23% w/w formaldehyde, and 4.7% glacial acetic acid. This stock was added to the samples and the standards, 100uL per well. After this addition, the wells were washed three times with 0.01 N HCl and allowed

to dry for 10 minutes. For resolving, 100 uL of 01 N HaOH was added to each well and shook gently. The absorbance was read at 550nm in an ELISA reader.

### 3.5.2 Calcium Assay

In order to test the levels of calcification, the Calcium Content Assay was used. First, 1mL of 5% trichloroacetic acid (TCA) Solution was added to each of the nine samples. Next, they were chopped using a microscissor and centrifuged at 12000 RPM for 3 minutes. In six wells of a 96-well plate, a 1:2 serial dilution was performed of the standard calcium solution starting with 1mg/mL and diluting with 5% TCA to a final volume of 10uL. The final concentrations of calcium were 1.0, 0.5, 0.25, 0.125, 0.0625, and 0.0 mg/mL. Below that row, 10 uL of each sample was added to separate wells. Next, 100 uL of calcium kit solution was added (50 uL of both A and B). After five minutes, the absorbance was read at 575nm in an ELISA reader.

### 3.5.3 ALP Activity Assay

In order to test the levels of alkaline phosphatase activity, the ALP Activity kit was used. First, 1mL of 0.2 % v/v Triton X-100 / 5mMMgCl2 solution was added to each of the samples. Next, samples were chopped with a microscissor and centrifuged at 12000 RPM for 3 minutes. 80 uL of 10 mM p-nitrophenol solution standard was pippetted into one well of a 96-well plate and serially diluted 1:2 with the Triton-X solution to create 6 wells with concentrations of 10, 5, 2.5 1.25, 0.625, and 0 mM. Below this row, each sample was added to separate wells, 80uL per well. Following this step, 20 uL of solution A and 100 uL of solution B from the kit were added to each well containing

sample or standard. The plate was incubated for one hour at 37 deg C. After incubation, absorbance was read at 405 nm in an ELISA reader.

### 3.5.4 PicoGreen dsDNA Quantification

In order to quantify dsDNA, this DNA content assay was used. First, 1mL of 0.2% v/v Triton X-100 / 5mMMgCl2 solution was added to each of the samples. Next, samples were chopped with a microscissor and centrifuged at 12000 RPM for 3 minutes. Next, the working solutions were created. Working B contained a 20 fold dilution of 20xTE Buffer (200mM Tris-HCl, 20mM EDTO, pH 7.5) with DNase-free H20. Working A contained a 200 fold dilution of picogreen dsDNA quantification reagent in DMSO with working B. This was covered with foil. Working C contained 30uL of 100 ug/mL in TE Lambda DNA standard in working B (ug/mL total, 1:2 dilution in Triton X-100). 25 uL

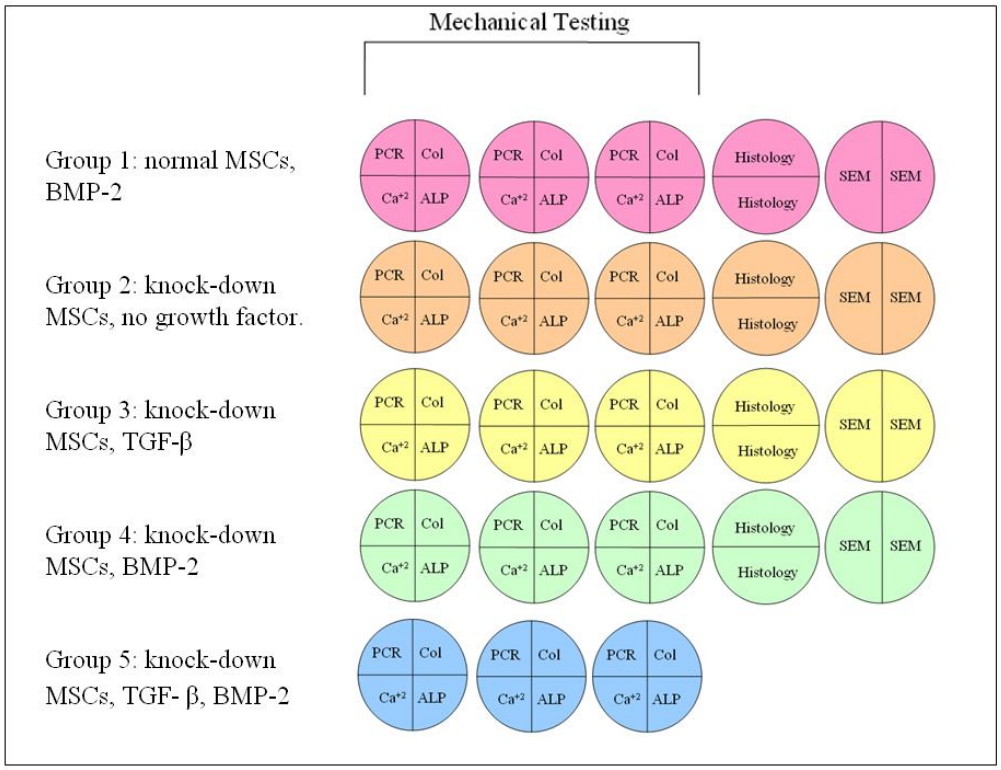



**Figure 4.** Week 6 Testing Schematic

of working C was added to each of six wells of a 96-well optical black plate. Below that, 25 uL of sample was added to each well. 75 uL of working B and 100 uL of working A was added to all the wells containing sample or standard. After five minutes of room temperature incubation, the excitation/ emission was read at 480/528 nm in an ELISA reader.

### 3.6 Mechanical Strength Testing

Before cutting week six samples for chemical analysis, mechanical strength was measured. Unconfined compression tests were performed on an Instron 3366 testing apparatus with a 10N maximum capacity load cell. Tests were conducted at room temperature on samples after having soaked in PBS for one hour. A displacement control mode was used, with a crosshead displacement rate of 5 $mm \cdot min^{-1}$. The compressive stress and strain were graphed based on the measured sample dimensions: cross-sectional area based on the sample punch size and sample height (measured automatically @ .02N tare load). The Yield Strength as well as the Compressive Modulus was determined after testing was complete. The Modulus was calculated based a least-squares' fitting typically between 5% and 10% strain (see exceptions below under the column header "Strain Range"), based on the new sample height measured automatically. The compressive yield strength was determined using an offset-yield approach. A line was drawn parallel to the modulus line, but offset by .5% of the sample gauge length. The corresponding stress value at which the offset line crossed the stress-strain curve was defined as the compressive yield strength of the scaffold.

### 3.7 Gene Expression

Week six samples were also subjected to PCR and chemical analysis using the same methods as previously stated. AoD primers that were used included GAPDH (Calibrator), COL1a1, OP, and BSP.

### 3.8 Histology

Samples for both three and six weeks that were cut for Histology or SEM were placed in labeled cassettes and fixed in 10% formalin together. The histology samples were eventually cut in horizontal cross sections at the laboratory at Tufts New England Medical Center and then subjected to Von Kossa Staining, Hematoxylin and Eosion (H and E) staining and Immunohistochemistry for Collagen I. Bright field images were taken of the stained slides using a Leica Fluorescence Microscope and the Leica Application Suite software.

### 3.9 Scanning Electron Microscopy

After fixing with formalin, samples were treated with ethanol in order to prepare them for Scanning Electron Microscopy. Samples were placed in 50%, 70% and 90% ethanol for 20 minutes each, followed by 100% ethanol for 30 minutes. Samples were cut in half, blotted and allowed to dry. After drying, sections were stuck on SEM imaging platforms so that both top surface and inside cross sections were visible (Figure 5). Samples were then taken to Harvard University for Scanning Electron Microscopy. First, images were coated with Platinum/Palladium using a Cressington Sputter Coater at 208 Hz and 90

sec/mA. These samples were then imaged using the Zeiss Gemini Scanning Electron Microscope and operated with the Smart SEM Program.

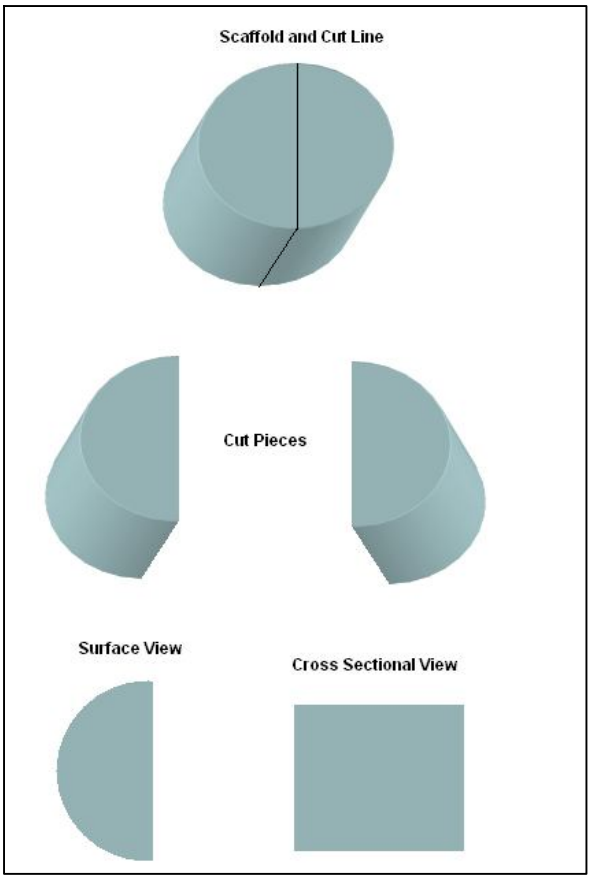



**Figure 5.** SEM Scaffold Preparation Schematic

### 3.10 Statistical Analysis

Independent experiments were conducted with a sample size of N=3 for each data point. Data was analyzed first using F-tests in order to determine whether pairs of data sets had equal variances. Depending on the result, statistical significance was ascertained using Student's t-tests. Each test was done based on comparisons to the negative control group.

## CHAPTER 4: RESULTS

### 4.1 Gene knockdown

The presence of successful gene knockdown cells was initially confirmed when the co-transduced Green Fluorescence Protein was visualized in the cells using fluorescence microscopy. Their general fibroblast morphology was also confirmed with phase contrast microscopy. The decreased mRNA transcript expression of the Col1a1 gene was further confirmed using real-time RT-PCR after 7 days in culture. Results were normalized to GAPDH and showed that knockdown cells expressed significantly less COL1a1 as compared to normal cells (Figure 6).

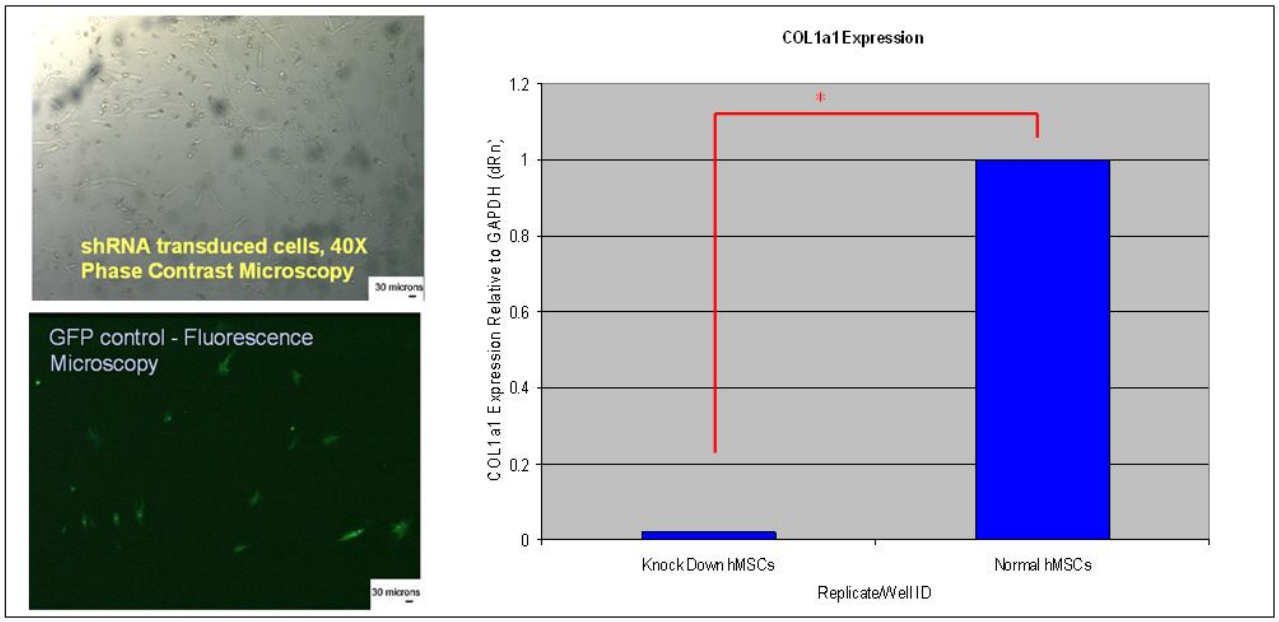



**Figure 6.** Quantitative and Qualitative Tests Confirm Gene Knockdown. Phase contrast and fluorescence microscopy visualizes transduction and fibroblast morphology. Transcript levels of COL1α1 were determined by PCR and normalized to GAPDH within the linear range of amplification. Data are shown as mean + standard deviation from N = 3, (*) represents statistically significant differences ($p < 0.05$)

### 4.2 Microscopy

Scanning electron microscopy showed varying results per treatment group. Images recorded of scaffolds from the positive control group of normal hMSCs showed proliferated cell-like spreading on the silk matrix in addition more string-like ECM synthesis. There were also balls of mineralized nodules (Figure 7). The negative control group from week three with knock down cells and no growth factors showed good cell surface and even some synthesized matrix but no mineralized nodules. The positive control group after six weeks also showed mineralized nodules on top of a cell-like spreading layer and stringier ECM-like layer. The negative control group ceased to show mineralization but did have plenty of cell proliferation. Densely synthesized ECM was discovered on the surface of some of the images captured of the week three TGF-β treated group. There was, however, relatively low cell density inside the scaffolds.

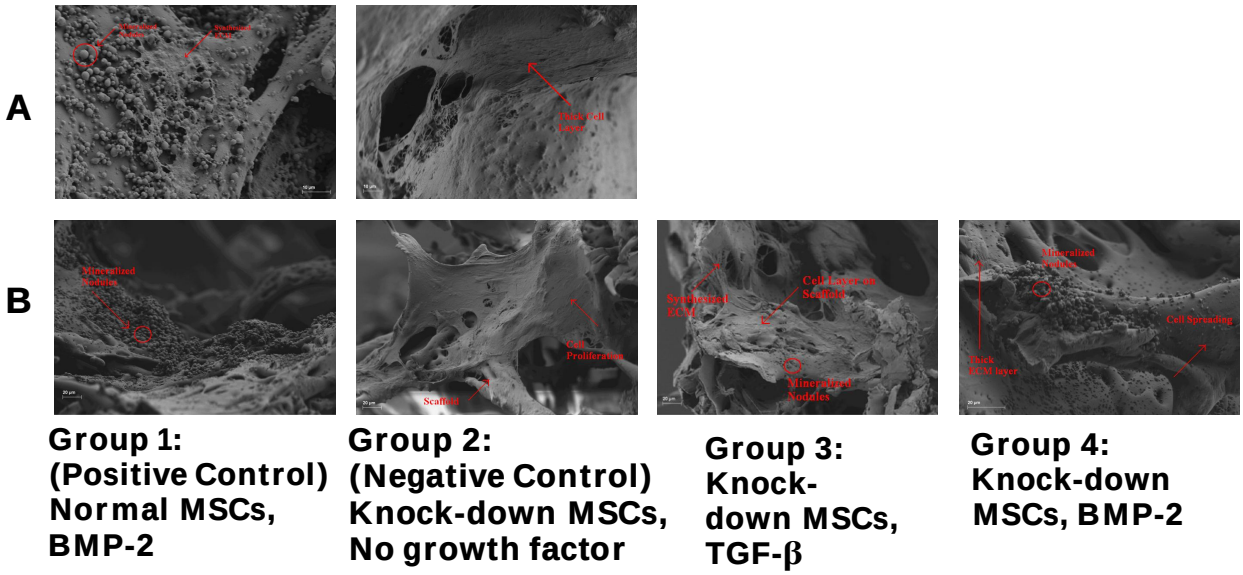



**Figure 7.** SEM images of silk scaffolds and different treatment or control groups after (A) three weeks and (B) six weeks. Mineralized nodules, cell proliferation and synthesized ECM is shown.

## 4.3 Histology

### 4.3.1 Von Kossa

For all samples tested, the mineralized calcium particles visualized as black spots.

### 4.3.2 H & E

In all samples tested, the silk stained a dark purple color, while the extracellular matrix stained bright pink. Fibroblast-like cells were visible, as well as the silk scaffolding and (Figure 8, Figure 9).

### 4.3.3 Imunohistochemistry (COL 1)

In all samples tested, the secreted collagen stained a dark brown color. Negative control groups showed less dark brown stain than other treatment groups.

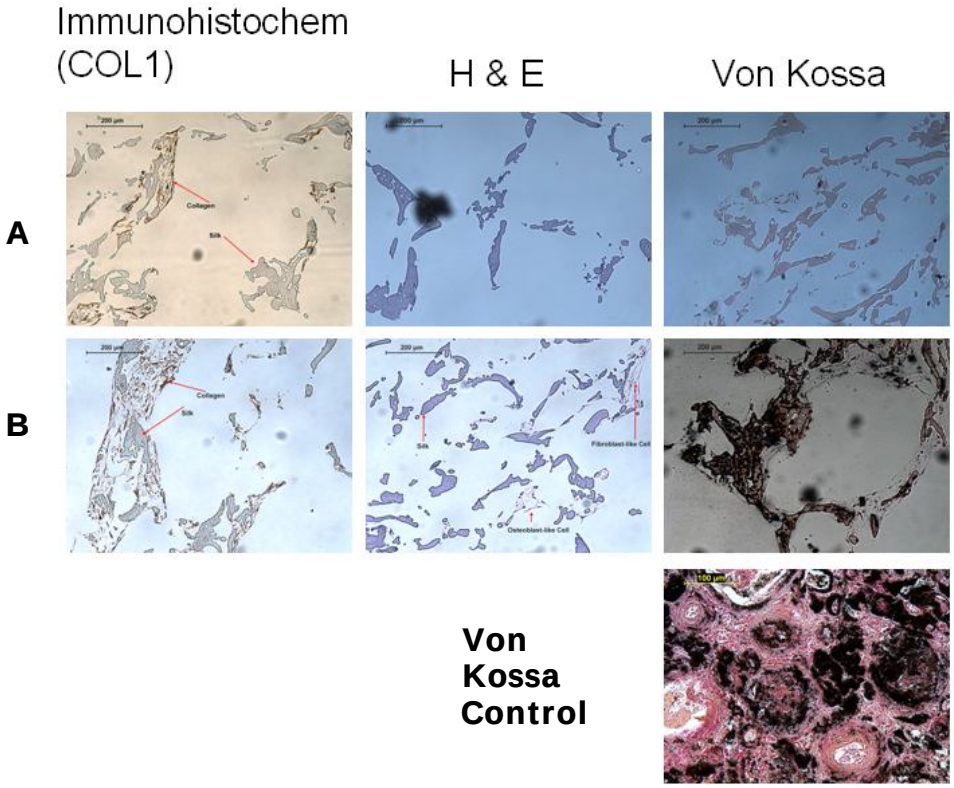



**Figure 8.** Histology stainging of Hematoxylin and eosin, Von Kossa and Immunohistochemistry for positive (A) and negative (B) control groups treated for three weeks. Visible structures include collagen, calcium and fibroblast-like cells.

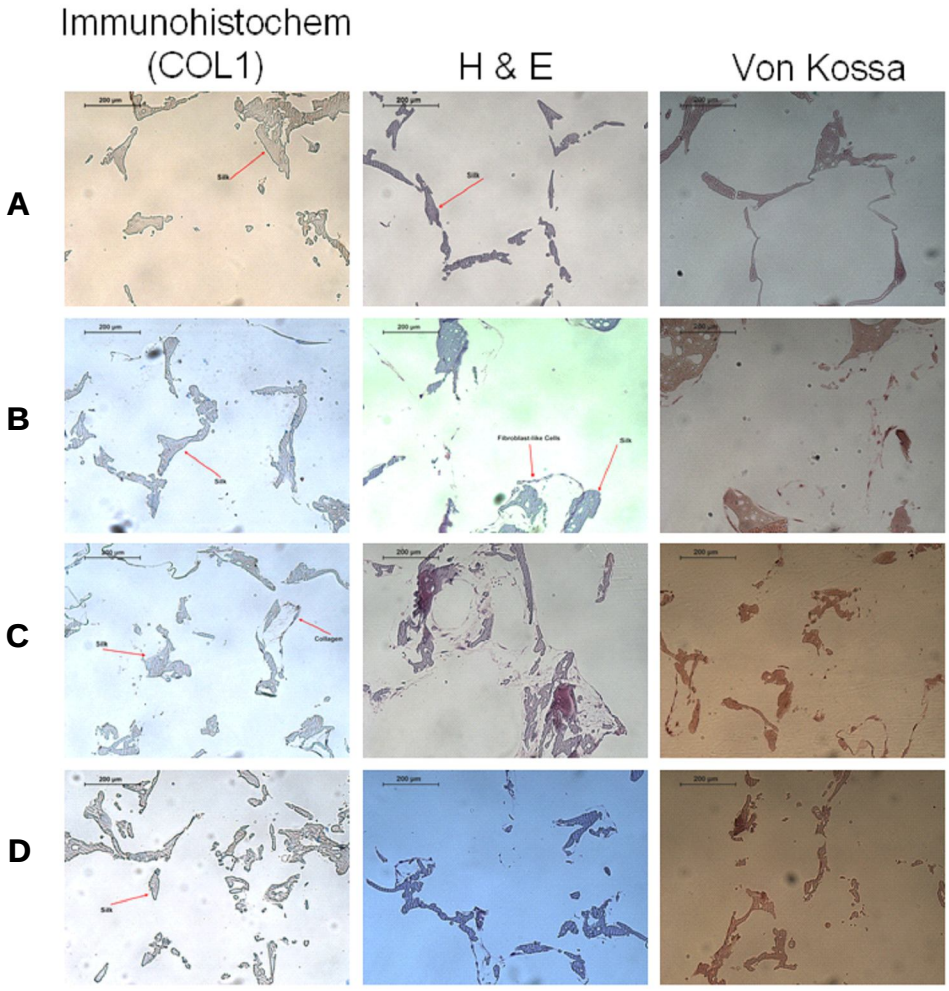



**Figure 9.** Histology stainging of Hematoxylin and eosin, Von Kossa and Immunohistochemistry for positive (A) and negative (B) control groups and(C) TGF-β and (D) BMP-2 treatment groups treated for six weeks. Visible structures include collagen, calcium and fibroblast-like cells.

## 4.4 Chemical Analysis

### 4.4.1 Collagen Content Assay

Scaffolds cultured for three weeks showed the most collagen expression by knockdown cells with TGF- β. The next highest collagen content was of the positive control, of normal cells with BMP-2. However, these results represent a non significant difference in collagen content as compared to the negative control. After six weeks of treatment,

the knockdown cell group with BMP-2 showed significantly higher collagen content than that of the negative control. The negative control group showed the lowest average collagen content. However, all other group differences of average collagen content as

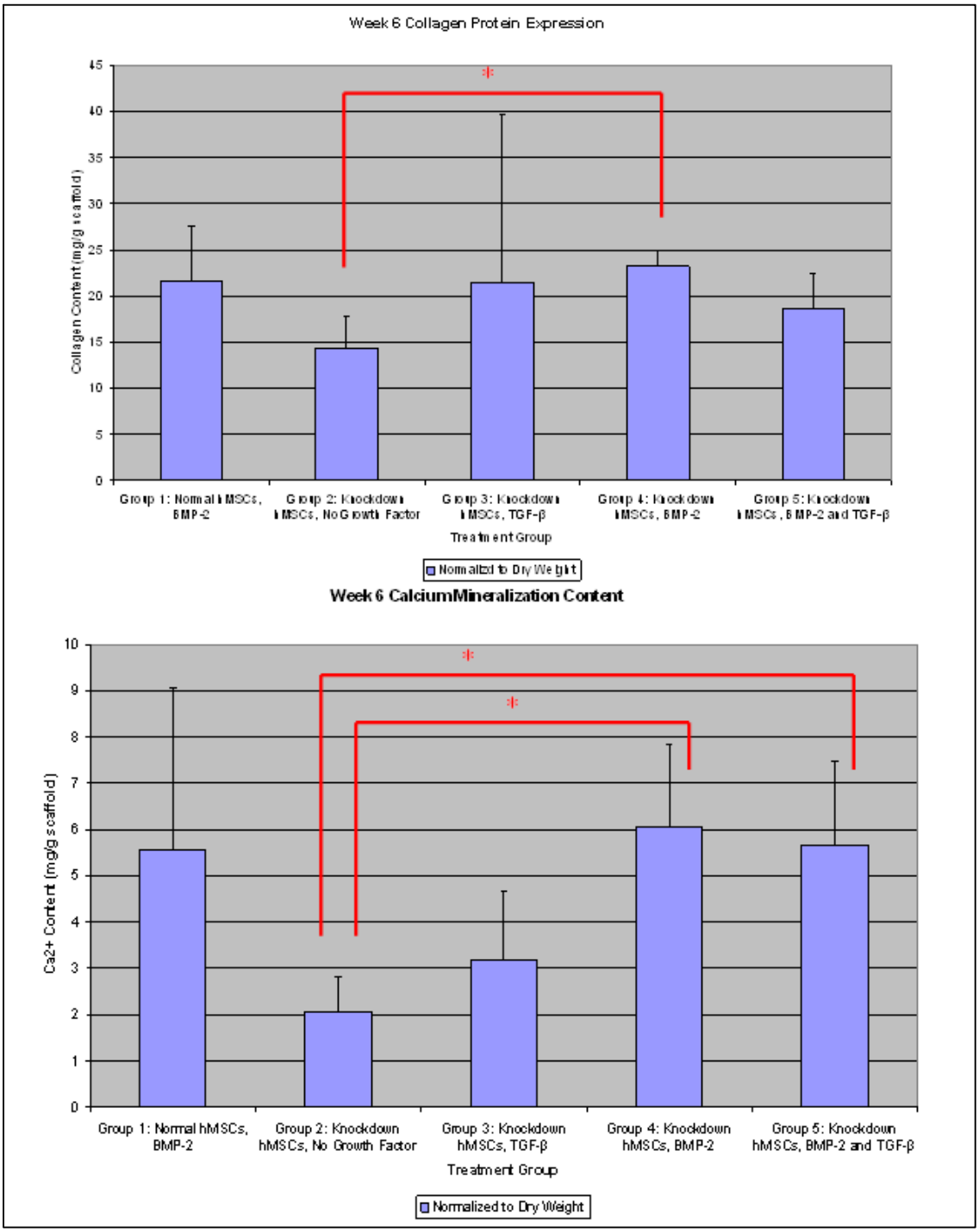



**Figure 10.** Chemical analysis by collagen content assay and calcium mineralization assay of growth factor treated scaffolds and control groups. Data are shown as mean + standard deviation from N =3 samples, (*) represents statistically significant differences ($p < 0.05$).

compared to the negative control were non-significant (Figure 11). There was also non-significant increase in positive control collagen from week three to week six.

### 4.4.2 Calcium Mineralization Assay

Scaffolds in culture for three weeks exhibited more calcium content by the TGF- β treatment group than by the positive or negative control. These results, however, were not significant. The amount of calcium increased non-significantly from three weeks to six weeks in the positive control. After six weeks, the negative control group showed the lowest amount of calcium produced, compared to all other groups of six week culture time. Furthermore, both the BMP-2 treated knockdown group and the dual growth factor group showed significantly more calcium content than the negative control. There was no significant difference between the negative control group and the TGF-β knockdown group (Figure 11).

### 4.4.3 Picogreen dsDNA Content Assay

After six weeks of culture, the picogreen Assay confirmed the presence of dsDNA in all groups. This was used to normalize the ALP assay data. There was significantly more DNA from the group with BMP-2 and knockdown cells as compared to the negative control (Figure 12).

### 4.4.4 Alkaline Phosphatase Activity

The positive control group showed ALP activity, while the other treatment groups expressed nearly no activity. The normal hMSC group showed significantly larger ALP activity than treatment group of knockdown cells with TGF-β (Figure 12).

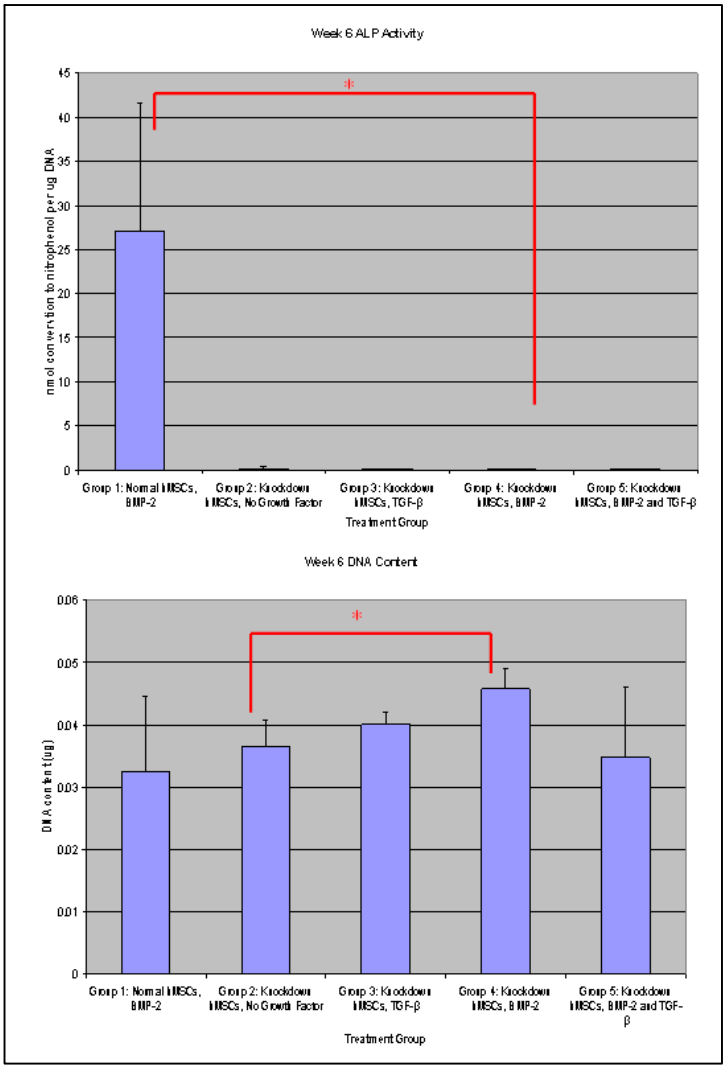



**Figure 11.** Chemical analysis by DNA content assay and ALPase activity assay of growth factor treated scaffolds and control groups. Data are shown as mean + standard deviation from N =3 samples, (*) represents statistically significant differences ($p < 0.05$).

## 4.5 Gene Expression

### 4.5.1 PCR-COL1a1

After three weeks of culture there was significantly more Collagen expression in the positive control group compared to the negative control group (Figure 13). After six weeks of culture, the positive control showed significantly more collagen expression than the negative control. Furthermore, the dual delivered growth factor group

displayed a significantly higher amount of collagen as compared to the negative control despite neither single growth factor groups exhibiting significant difference to the negative control (Figure 14).

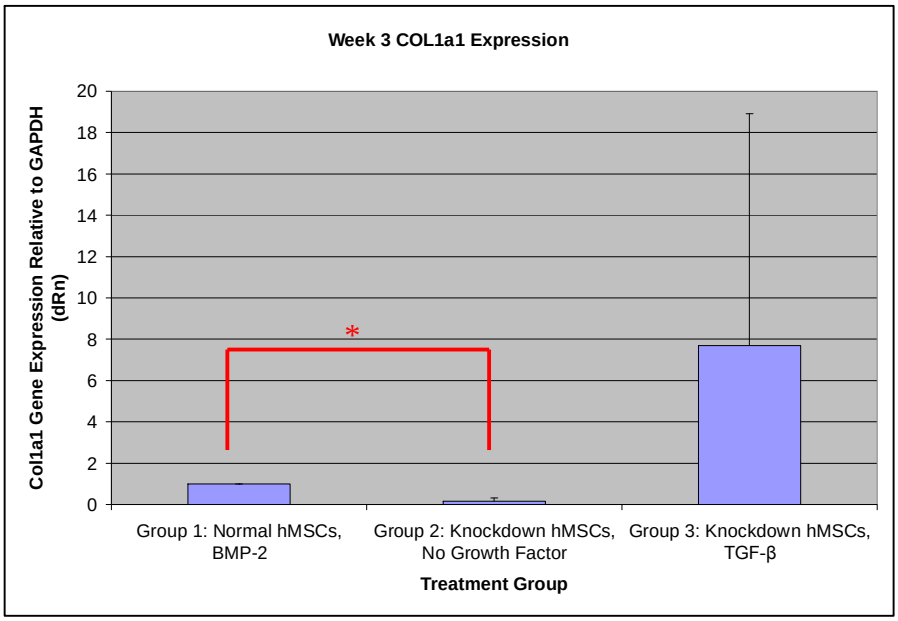



**Figure 12.** Transcript levels of COL1α1 determined by PCR and normalized to GAPDH within the linear range of amplification after three weeks of culture. Data are shown as mean + standard deviation from N = 3, (*) represents statistically significant differences ($p < 0.05$)

### 4.5.1 Osteopontin

After six weeks of culture, the negative control exhibited the least amount of OP expression as compared to both the positive control group and the dual growth factor group. These results, however, proved to be non-significant (Figure 14).

### 4.5.2 BSP

After six weeks of culture, the negative control exhibited the least amount of OP expression as compared to both the positive control group and the dual growth factor group. These results, however, proved to be non-significant (Figure 14).

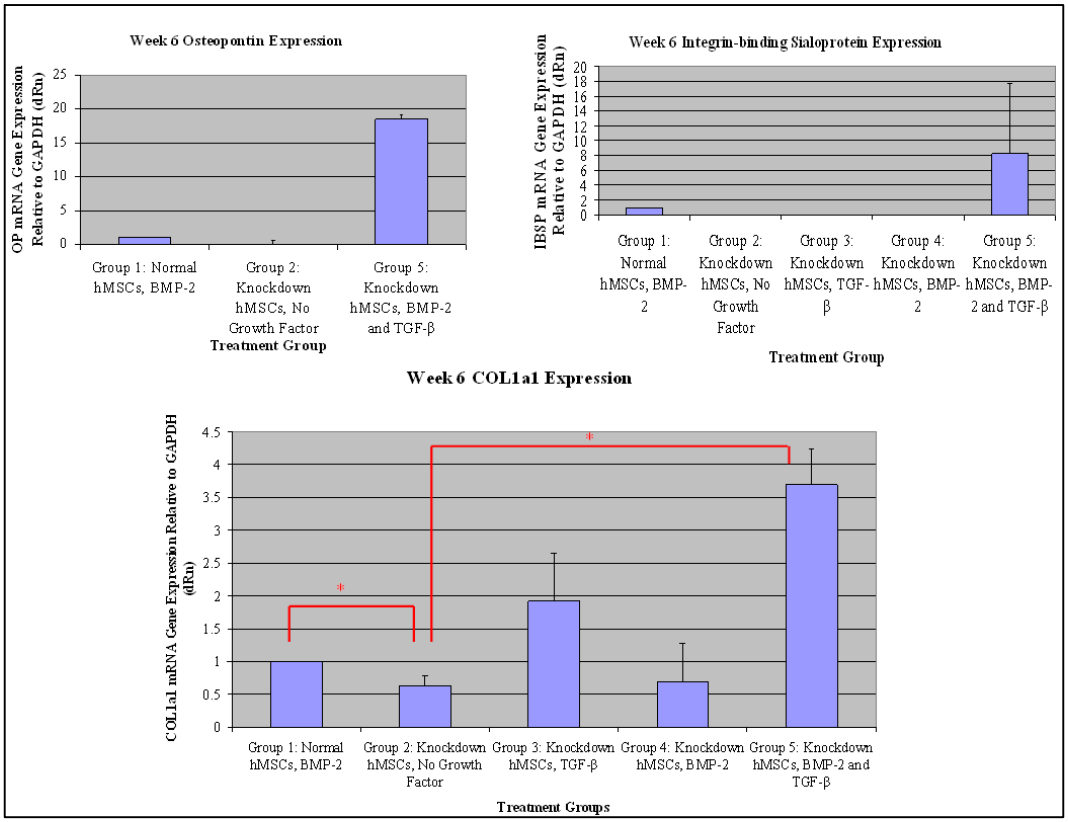
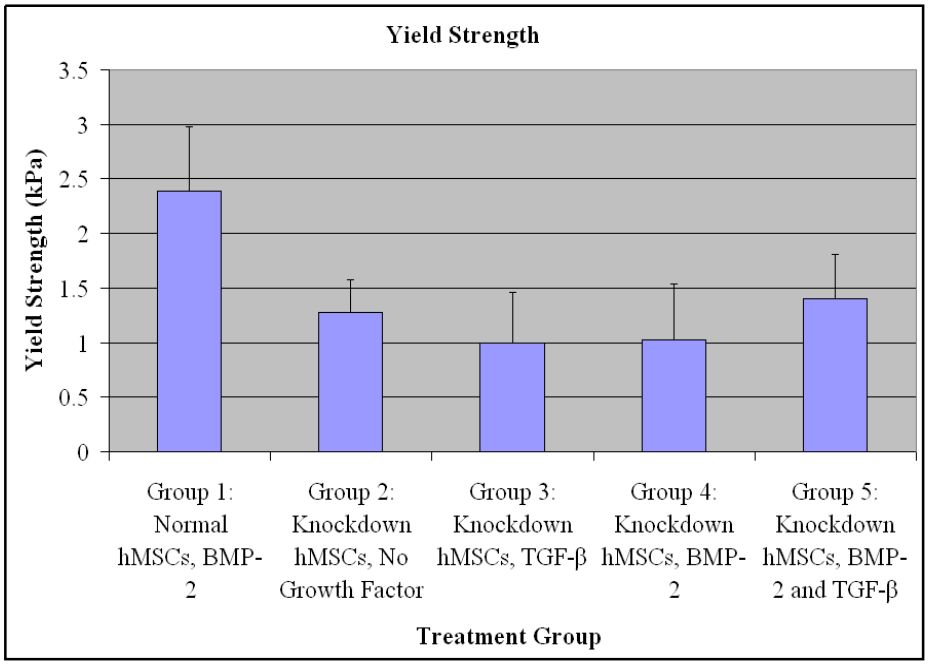



**Figure 13.** Transcript levels of osteogenic genes determined by PCR and normalized to GAPDH within the linear range of amplification after six weeks of culture. Data are shown as mean + standard deviation from N = 3, (*) represents statistically significant differences ($p < 0.05$).

## 4.6 Mechanical Analysis

Both yield strength and modulus were largest for the six week cultured scaffolds from the positive control group. The results showed, however, that there was a non-significant difference between the positive and negative control groups as well as between negative control group and the all the treatment groups (Figure 15).



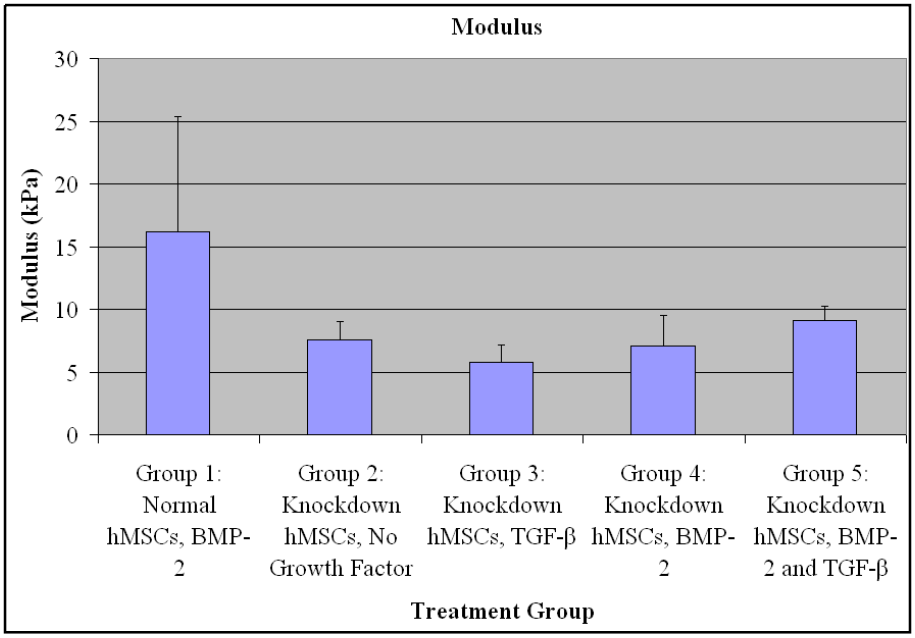




**Figure 14.** Compressive modulus and yield strength of treatment and control groups after six weeks of culture. Data are shown as mean + standard deviation from N = 3, (*) represents statistically

# CHAPTER 5: DISCUSSION

## 5.1 Scanning Electron Microscopy

Control groups after three and six weeks showed sections of silk scaffold with cells spread out in greater than a single cell layer with more fibrous looking material which is most likely synthesized ECM. This material most likely represents collagen fibers. The abundance of mineralized nodules confirms calcium deposition. They do not represent unwashed particles of salt from scaffold creation because these particles are more cuboidal and jagged in morphology as opposed to the spherical nodules found here. The highest calcium deposition was found in the group one constructs, the control group of normal hMSCs with BMP-2 added. This serves as a comparison for the other images, none which achieved the level of mineralization quite as high. This was expected , however, since this group represents normal healthy tissue, while the others represent both treated and untreated diseased tissue.

Although there were some sections found where mineralization and ECM production was present in group two samples (the negative control), the most representative image shows little or no mineralization and little or no ECM production.

Although difficult to determine significant differences in qualitative testing, it seems that the group four samples treated with BMP-2 had slightly greater mineralization than samples treated with TGF-β. This may be attributed to the fact that BMP-2 has stronger osteogenic effects. It also may be due to the fact that TGF- β has a wider range of effects in tissues, thus distributing the effects of the growth factor to cell signaling pathways that were not visible by SEM.

### 5.2 Histology

The cross sections stained and imaged by histology may not be representative of the best results. Because cross sections were sliced by the Tufts NEMC lab, it is likely that surface sections were not used, but rather only the middle sections. It was the surface sections, however, that maintained the highest cell densities and thus achieved ECM synthesis and mineralization confirmed in SEM. The slices in the middle were less likely to contain the cells, thus less likely to show stained collagen, calcium and ECM.

### 5.3 Chemical Analysis

Treatment groups with BMP-2 produced significantly more collagen and calcium than the negative control. These markers of osteogenesis indicate that the addition of BMP-2 was enough to overcome the osteogenesis imperfecta effects of the gene knockdown and elevate the cells to near normal production levels. The positive results of these chemical analyses also confirm the previous ideas that collagen and calcium was viewed in the SEM images. Furthermore, it confirms the better performance of BMP-2 treated samples with regards to osteogenesis, than the samples treated with only TGF-β in the SEM images. This may be due to the more direct signaling pathway that BMP-2 has in causing osteoblast differentiation than that of BMP-2 (Figure 15).

One reason the TGF-β group did not produce significant results is that the concentration of added growth factor may have been too low for quantitative results. Thus the significant difference in calcium between treatment group five and the negative control group can be attributed to the BMP-2 and not to the fact that growth factors were dual delivered.

The lack of significance between the positive and negative control groups does not detract from the validity of the other data. The growth factor addition (in the case of BMP-2) still helped improve calcium and collagen production. The positive control was merely for visual qualitative comparison. The groups are not suited to the same types of comparisons.

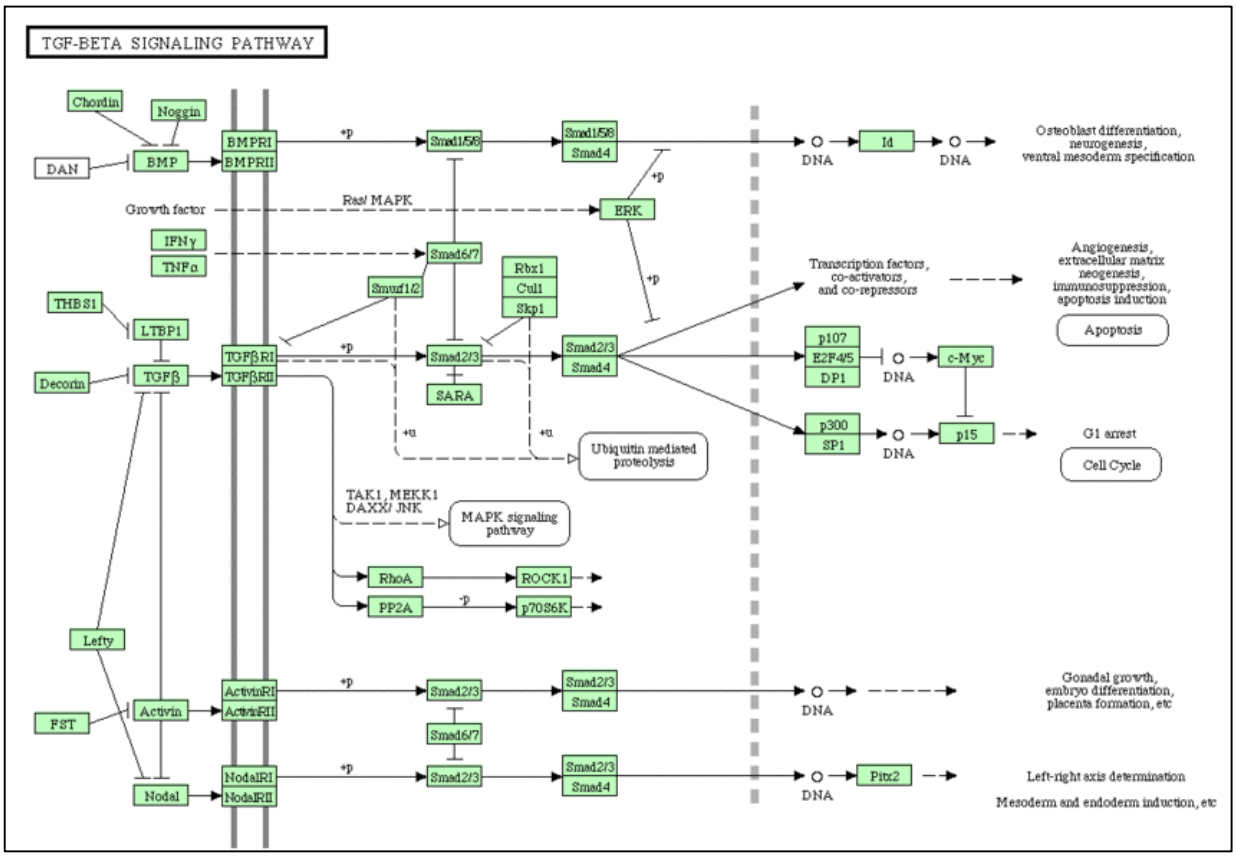



**Figure 15.** TGF-β Signaling Pathway Schematic.

DNA content was measured purely for normalizing the ALP assay. Thus significant differences in the data are not relevant to the study. None of the treatment groups, when compared to the negative control had any significant differences in ALP activity. The ALP activity was extrememly low. This is explained by the fact that after six weeks of culture, ALP is not expressed any more. The peak of ALP expression is at the end of

proliferation right before cell differentiation begins. For more meaningful ALP activity data, assays in future studies should be done at other, shorter time points.

### 5.4 Gene Expression

The three week and six week gene expression data again confirmed the knockdown of the COL1a1 gene in the comparison of the positive and negative control. After six weeks, the expression of COL1a1 in the dual delivered growth factor samples was significantly higher than the negative control although neither single growth factor achieved significant difference in transcript levels. Levels of actual collagen protein however, were significantly different in BMP-2 treated samples as compared to the negative control. This lack in congruity between actual protein and transcripts expressed is attributed to effects of time. The expression of COL1 is higher during the onset of osteoblast differentiation in normal tissue. Collagen produced at that time remains in the matrix and thus shows up in the protein chemical assay while the transcript levels eventually decrease as the osteoblasts mature and enter mineralization and apoptosis stages.

There was no significant difference in transcripts of BSP or OP. Since the gene knockdown targeted collagen production, it makes sense that the transcript expression of other proteins was not effected. These bone markers showed equivalent osteogenesis across the treatment groups.

### 5.5 Mechanical Strength

Because the same scaffolds were to be used in the mechanical strength test as the chemical analysis, the usually destructive compression tests were modified, thus allowing chemical analysis to remain quantitatively accurate.

Lack of statistical significance of the linear elastic regions examined in the results of mechanical testing is not something to be concerned with. Silk is a very stiff material thus contributions by cell and ECM material comparatively are so small as to go unnoticed. Specifically, the lack of significance between the negative control and treatment groups is not concerning, because there also was no statistical significance between the positive control and the negative control. Culture times need to be increased in order to mark significant increases in mechanical strength.

## CHAPTER 6: FUTURE WORK

First and foremost, the experiment should be repeated in future work in order to test the repeatability and viability of the study. The repetition should be focused on obtaining the high cell numbers and very careful cell seeding to ensure cell perfusion throughout the scaffolds and not just deposition on the scaffold surfaces. In this way we could obtain better histology images.

In further studies, higher concentrations of TGF-β should be added in order to better study its effects. Due to time and material constraints, other growth factors were not studied here but should be considered for future work. Specifically, the effects of IGF and VEGF show promise, especially when delivered in conjunction with the other cytokines as opposed to by itself. The growth factor delivery system may also be altered in subsequent experiments to microsphere application in other to improve the delivery kinetics.

In more advanced work, PCR should be used to test for the expression of the ALP and RUNX2 genes in addition to the genes already mentioned.

The study also my show more qualitative and quantitative results if scaled up, using larger scaffolds and higher cell numbers. In this case however, it might be wise to use a spinner flask or perfused media delivery system as opposed to static culture.

Other microscopy techniques including confocal microscopy should also be considered for use in the project perhaps to replace or supplement scanning electron microscopy.

Lastly, the same gene knockdown disease model studies can be investigated for use with related disorders such as osteoporosis and osteoarthritis.

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