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The Effect of Bmp-13 on the Chondroinduction of Mesenchymal Stem Cells

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THE EFFECT OF BMP-13 ON THE CHONDROINDUCTION OF MESENCHYMAL STEM CELLS

By

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Articular cartilage is a smooth, white connective tissue that covers and protects the ends of long bones to allow for a smooth, frictionless surface on which to glide for easy movement. Once the tissue is damaged, articular cartilage lacks a direct blood supply, which results in a limited ability to repair itself. This study explores the effect of the growth factor BMP-13 on the chondroinduction of primary human bone marrow-derived mesenchymal stem cells.

The results demonstrate the limited ability of BMP-13 to exert a strong chondroinductive effect on human bone marrow-derived MSCs. However, the results do indicate that BMP-13 has the ability to sustain chondroinduction to a certain extent for up to 18 days following initiation by 3 days of exposure to TGF-β3. Results are encouraging for future work that involves growth factor influence on MSCs in articular cartilage tissue engineering.

DEDICATION

This work is dedicated to my parents, Randy and Sissy, for all the love and support they have always given me. Thank you for everything.

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TABLE OF CONTENTS

Page

LIST OF TABLES

TABLE Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Prevalence

Joint and articular cartilage injuries account for over 6 million hospital visits in the United States each year [1]. Wear and tear on articular cartilage can lead to a loss in the tissue, further exposing the ends of long bones and leaving them without protection. Further tissue degradation leads to the most common type of arthritis, osteoarthritis. Osteoarthritis, or degenerative joint disease, affects an estimated 27 million adults in the United States [2].

Unlike other tissues, articular cartilage has a low regenerative capacity and once injured finds it much more difficult to self-repair. The composition and structure of articular cartilage give rise to the tissue's unique properties. Due to the complexity of articular cartilage composition, it has become challenging to restore full function to damaged or diseased tissue. Currently, there are many different methods employed for treating articular cartilage lesions; however, many shortcomings exist with these therapies.

Tissue engineering may provide alternative solutions for articular cartilage repair and regeneration by developing tissue substitutes that mimic the composition of articular cartilage. The objective of this research is to determine the precise nature and magnitude

of growth factors that regulate chondrogenic mesenchymal stem cell (MSC) differentiation. In order to make progress towards introducing a tissue-engineered technique, a basic understanding of articular cartilage structure, composition and function must first be established.

Articular Cartilage

Articular cartilage is a smooth, white connective tissue that covers and protects the ends of long bones to allow for a smooth, frictionless surface on which to glide for easy movement. Serving as shock absorber, articular cartilage assists in distributing the load of pressure and weight over the entire surface of a joint. When cartilage is damaged, the smooth gliding motion that bones were once able to achieve is disrupted, and movement becomes painful. If a joint experiences improper alignment, excessive weight, overuse, or injury, cartilage wears away. Once damaged, cartilage lacks a direct blood supply, resulting in a limited ability of the tissue to repair itself. If damaged cartilage is left untreated, disease can spread throughout the entirety of the tissue causing degradation, followed by pain, limited ability of joint movement, and possibly the contraction of osteoarthritis. Orthopedic researchers are continuing to search for new ways to treat damaged cartilage. Currently, there have been many advances in the surgical treatment of articular cartilage defects. However, newer techniques involving tissue-engineered cartilage hold some promise, but their effectiveness and long-term outcomes have not been currently established.

The knee is a hinge joint that is formed by two bones and held in place by several different ligaments. As seen in Figure 1.1, the ends of both bones are covered in articular

cartilage. The synovial membrane surrounds and protects the knee joint, and also functions to produce synovial fluid. Synovial fluid acts as a lubricant for our joints and is responsible for providing cartilage with its slippery surface. The small amount of fluid that the synovial membrane provides allows forces on joints to not be directly applied to bones. Cartilage receives nutrients and oxygen, and removes carbon dioxide and waste from cells within surrounding cartilage by diffusion through the synovial fluid. When the joint is loaded, fluid carrying waste and carbon dioxide is forced out of cartilage. Once relieved, the fluid is allowed to diffuse back into the tissue, carrying oxygen and nutrients with it.

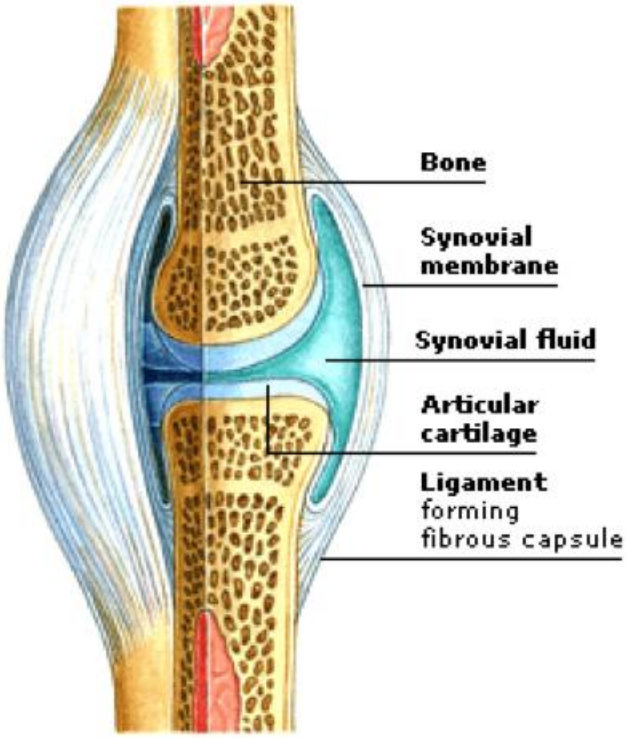

Figure 1.1 Illustration depicting the structure of the knee joint. From (factmonster.com)

Articular Cartilage Injury

Cartilage injuries and damage can occur from trauma, disease, or repetitive loading to the joint, and in turn, articular cartilage tissue will lose partial or complete function. Without a direct blood supply, cartilage is incapable of initiating the healing process. Articular cartilage trauma injuries can occur either as a chondral fracture or an osteochondral fracture. Chondral fractures occur when there is trauma to the joint surface usually in the form of a tear, but which does not affect the underlying subchondral bone. Osteochondral fractures occur when there is trauma to the joint surface that affects the underlying subchondral bone. If left untreated, these joint surface fractures can become progressively worse causing degradation of articular cartilage tissue and possibly the contraction of osteoarthritis [3]. Osteoarthritis, or degenerative joint disease, is a joint disease caused by cartilage degeneration and loss in a joint, and those who suffer from the disease experience pain and stiffness [4].

Composition

Articular cartilage is composed of cells known as chondrocytes and is surrounded by a complex extracellular matrix (ECM) (Figure 1.2). Chondrocytes account for only 1% of the volume of articular cartilage, and the ECM accounts for the remaining 99%. Water makes up as much as 80% of articular cartilage tissue weight. The remaining solid fraction of the tissue is composed of structural macromolecules. Collagen, proteoglycans, and noncollagenous proteins are the structural macromolecules present in articular cartilage ECM, and contribute 20% to 40% of the tissue weight.

Only one type of cell, the chondrocyte, is present in articular cartilage. Chondrocytes are formed by a chondroblast, which originates from a MSC. Chondrocytes are highly specialized cells responsible for producing and maintaining the ECM. These cells are sparsely distributed and embedded throughout the ECM. Chondrocytes play an essential role in maintaining the tissue by synthesizing all matrix components. As the cells secrete matrix material, chondrocytes become confined into small chambers called lacunae. The lacunae separate chondrocytes from one another, preventing interaction between cells in the ECM. Because articular cartilage is avascular, chondrocytes receive nutrients and oxygen through diffusion from the synovial fluid into the cartilage matrix.

The articular cartilage ECM consists of tissue fluid and the framework of structural macromolecules. The interaction between the tissue fluid and the structural macromolecules provides articular cartilage with its stiffness and resilience [5, 6]. Water contributes to the majority of the wet weight of articular cartilage tissue fluid. Articular cartilage tissue fluid also includes gases, small proteins, metabolites, and high concentrations of cations. Some water is capable of moving freely in and out of the tissue. However, the volume, concentration, and behavior of water at a particular time in the tissue depend primarily upon the interaction with the structural macromolecules [7].

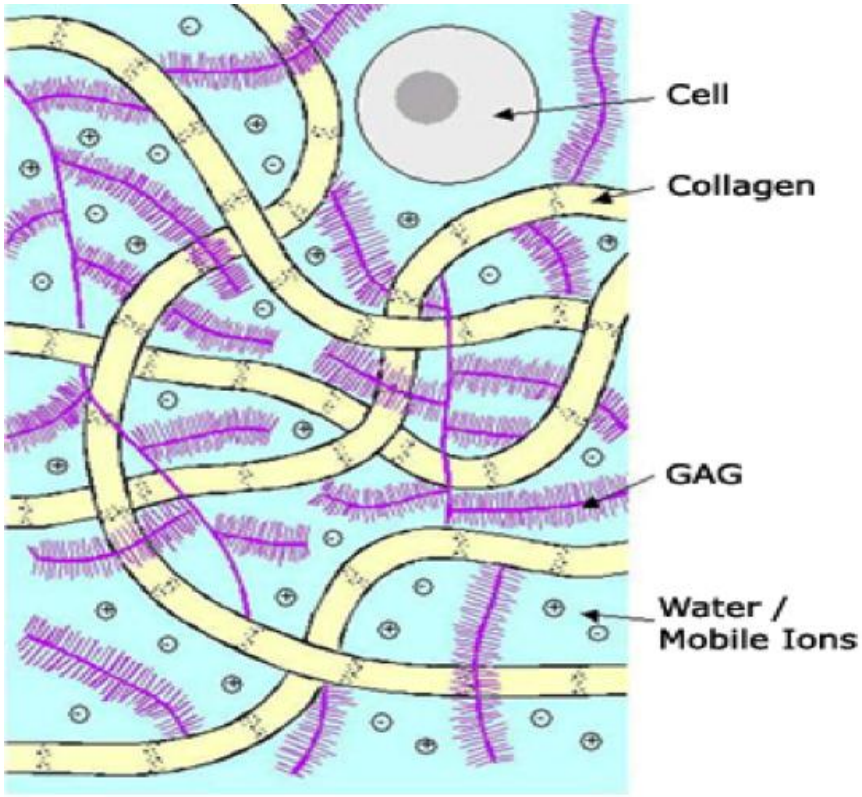

Figure 1.2 Illustration depicting the composition of articular cartilage. From (bidmc.org)

There are three classes of structural macromolecules present in articular cartilage, collagen, proteoglycans and non-collagenous proteins. These structural macromolecules exist throughout articular cartilage in varying concentrations and contributions to the tissue. Of the total 20% to 40% contributed wet weight, structural macromolecules account for the following, collagen (10%), proteoglycans (10%), and noncollagenous proteins (5% - 10%).

Collagen configuration can be described as a fibrous protein composed of three chains of amino acids that take that shape of triple helix. Making up approximately 60% of the dry weight of cartilage; collagen functions to create a framework housing the other components of cartilage. Type II collagen is the most abundant collagen found in

articular cartilage, and accounts for 90% - 95% of the collagen content present in the tissue. Type II collagen fibers cross-link to create the major support network for the ECM, giving articular cartilage its tensile strength. Collagen types III, VI, IX, X, XI, XII, and XIV are also present in articular cartilage tissue in smaller amounts.

Proteoglycans can be described as macromolecules composed of a central core protein with highly fixed negatively charged glycosaminoglycan (GAG) subunits attached to form a bottlebrush-like structure. The two main types of GAG found in articular cartilage are chondroitin sulfate and keratin sulfate. Proteoglycans can exist as individual monomers or also bind to a hyaluronic acid backbone by link proteins to form a macromolecule, the aggrecan. The aggregating proteoglycans function to help maintain the fluid within the ECM and the concentration of electrolytes in the tissue fluid [7]. Due to their highly negative charge, GAG molecules are extremely hydrophilic and can trap large amounts of water within the ECM, providing articular cartilage with its definitive elastic behavior. The large size of the aggrecan function to trap proteoglycans within the network of collagen, and in turn provides articular cartilage tissue with its stiffness. Figure 1.3 illustrates the structure of the proteoglycan macromolecule.

Non-collagenous proteins and glycoproteins contribute 15% to 20% of the dry weight of articular cartilage. For the purposes of this study, we will concentrate on only one of these non-collagenous proteins, cartilage oligomeric matrix protein (COMP). COMP is an acidic protein and is concentrated within the territorial matrix of the chondrocyte. COMP appears to be present only within cartilage, and has the capacity to bind to chondrocytes [8, 9]. Previous studies support the belief that COMP plays a role in cell growth and division and the self-destruction of cells, as well as in the regulation of

cell movement and attachment [10, 11]. However, the precise function of COMP remains unknown.

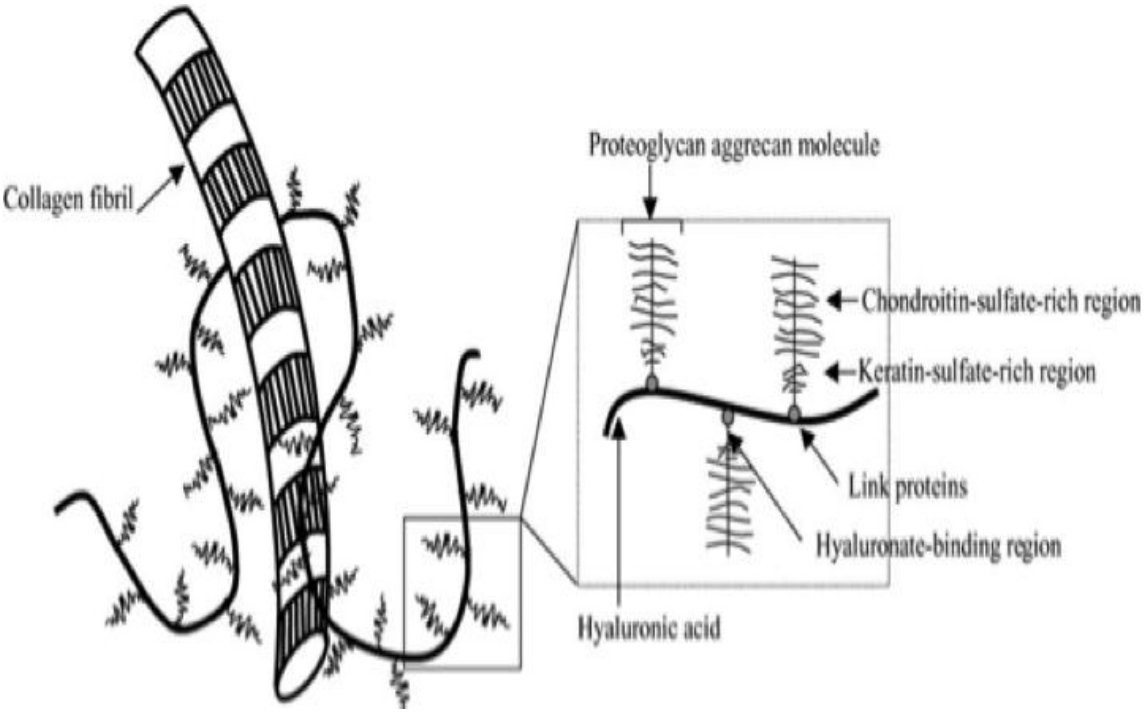

Figure 1.3 Illustration depicting the components of the ECM of articular cartilage. From (arthritis-research.com)

Tissue Engineering

The engineering of living tissue, tissue engineering, can be defined as the use of living cells, manipulated through their extracellular environment to develop biological substitutes for implantation into the body and/or to foster the remodeling of existing tissue [12]. The purpose of tissue engineering is to repair, replace, enhance, or maintain the function of a particular tissue or organ. Current surgical therapies in articular cartilage repair have yielded unsatisfactory and short-term results in the method of healing cartilage tissue. Tissue engineering is a fast-growing area of research that may serve as an

alternative approach for providing articular cartilage tissue repair or replacement without the negative shortcomings associated with current therapies. Advancements in science have brought tissue engineering to the forefront of the scientific research community. However, due to its complex composition and unique properties, cartilage is considered one of the most difficult tissues to recreate.

Cell Source

MSCs are unspecialized, undifferentiated cells that are characterized by their ability for long-term self-renewal and potential to produce different cell types in differentiation. Ernest A. McCulloch and James E. Till first identified bone marrow derived MSCs in the 1960s as being a clonal source of cells [13]. MSCs are capable of dividing and replicating themselves over long periods of time through a process called proliferation.

Additional work, in the 1970s and 1980s, conducted by Friedenstein, et al., expanded upon the potential of MSCs by demonstrating their capacity for multilineage differentiation [14, 15]. MSCs lack any tissue-specific structures that allow the cell to perform specialized functions. Through a process called differentiation MSCs can give rise to tissue specific cells. MSCs have proven to demonstrate the capability to differentiate into cells of multiple lineages, including chondrocyte, osteoblast, myoblast, adipocyte, and fibroblast [16-23]. Figure 1.4 illustrates the mechanism by which MSCs undergo proliferation and differentiation. These characteristics have made MSCs a popular cell source among current experimental approaches to articular cartilage tissue engineering. However, such approaches rely on efficient lineage-specific differentiation

of MSCs to a desired mature cell, which frequently involves addition of a transforming growth factor to the culture medium.

Chondrogenesis is the term used to describe the process by which a stem cell is differentiated into a mature chondrocyte. However, MSCs can only undergo chondrogenic differentiation when induced by various intrinsic and extrinsic factors. Growth factors represent a group of biologically active polypeptides produced by the body, which can stimulate cell proliferation and differentiation to a particular phenotype. In articular cartilage, growth factors control homeostasis and development [24]. Both MSCs and growth factors are promising tools in the ever-evolving field of cartilage tissue engineering. However, determining the growth factor, growth factor concentration, and growth factor combination to apply to MSCs in order to achieve a cartilage-like tissue has proved challenging.

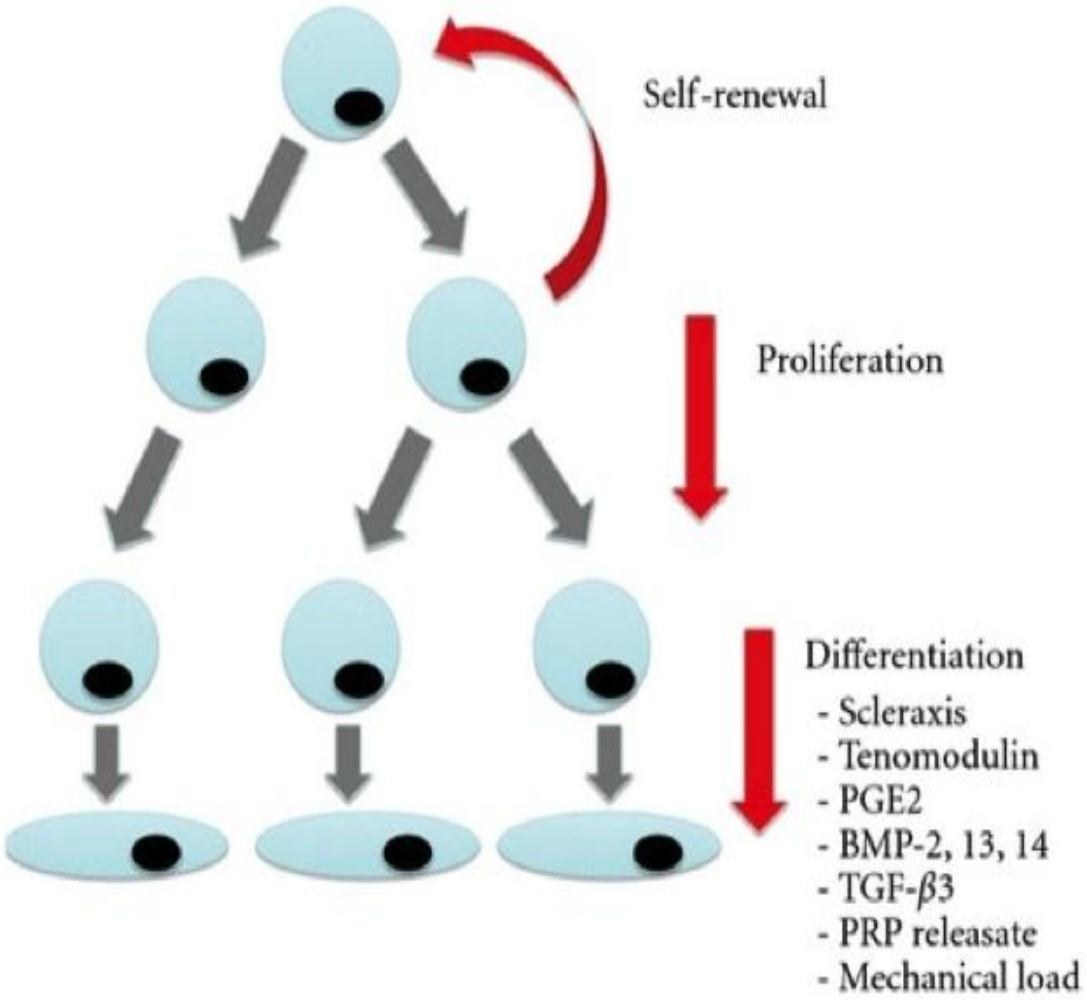

Figure 1.4 Illustration depicting the cellular proliferation and chondrogenic differentiation mechanism of MSCs. From [25].

Growth Factors

Numerous studies have reported efficient chondroinduction of MSCs using either transforming growth factor beta-1 (TGF-β1) [26-29] or TGF-β3 [30-36]. For example, adult stem cells from bone marrow cultured in defined medium containing 10 ng/ml TGF-β1 steadily increased mRNA expression of COMP, aggrecan, and type II collagen

over a 14-day period [34]. In a head-to-head comparison, TGF-β3 was found to stimulate expression of cartilage ECM components to a greater extent than TGF-β1 [30].

A potential drawback of the use of TGF-β3 for chondroinduction is that it may stimulate continued differentiation towards a hypertrophic phenotype as evidenced by increasing type X collagen expression [30]. By contrast, bone morphogenetic protein-13 (BMP-13) has been shown to support chondrogenesis in C3H10T1/2 cell cultures, but not terminal differentiation to hypertrophic chondrocytes [37]. A separate study using articular chondrocytes confirmed that BMP-13 does not promote hypertrophy [38]. The presence of type X collagen, only near the cells of the calcified cartilage zone of articular cartilage and the hypertrophic zone of growth plate, suggests that it has a role in cartilage mineralization.

BMP-13 was first identified and isolated as a component of bovine cartilage [39]. BMP-13, also known as cartilage-derived morphogenetic proteins-2 (CDMP-2), and the human homolog of mouse Gdf-6, belong to the TGF-β superfamily. Members of the superfamily are essential for the formation of cartilaginous tissue during early limb development [39, 40]. Previous reports (38, 41-44) have demonstrated the effects of BMP-13 in osteochondrogenesis using recombinant or adenovirus-mediated human BMP-13 (hBMP-13), and have also demonstrated that BMP-13 promoted chondrogenic differentiation in a variety of cells [41] and positively regulated growth and maintenance of articular cartilage [42]. However, two studies have reported that BMP-13 induced neotendon/ neoligament formation in vivo [45, 46]. The biological effects of BMP-13 remain controversial, and no studies have examined the effects of overexpression of hBMP-13 in cell culture models on primary human bone marrow MSCs.

The purpose of the current study was to determine the potential for BMP-13 to initiate and maintain chondroinduction of primary human bone marrow MSCs.

CHAPTER II

EXPERIMENTAL METHODS

Experimental Design

In order to determine the effect of growth factor BMP-13 on chondrogenesis of human bone marrow-derived MSCs, a pellet culture experiment was designed in order to compare the effects of growth factors, TGF-β3 and BMP-13. Four experimental groups were formed: a control group, a group supplemented with TGF-β3, a group supplemented with BMP-13 and a group supplemented with both TGF-β3 and BMP-13. The experiment was conducted over a 21-day period. The pattern of expression of key ECM components, as well as components known to stimulate a hypertrophic phenotype, were studied in order to develop a better understanding of the steps in the pathway leading from undifferentiated stem cell to mature chondrocyte.

Cell Source

Human multipotential marrow stromal cells (21 year-old female) frozen at passage 1 were provided by the Texas A&M Health Science Center College of Medicine Institute for Regenerative Medicine. These cells were extensively characterized and confirmed to meet the minimal criteria which define human MSCs as established by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular

Therapy [47]. These criteria include expression of the CD105, CD73 and CD90 surface molecules and display of multilineage differentiation in vitro.

Cells were thawed into expansion medium (Dulbecco's modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution) and plated at approximately 5×10^3 cell/cm^2 in two T-175 flasks. Cultures were maintained at 37°C in a humidified environment containing 95% air and 5% CO_2, and the cells were subcultured prior to reaching confluence. When passage 3 cells approached confluence, they were detached using trypsin, which was then neutralized by the addition of 10% FBS. RNA was isolated from five independent samples of these monolayer expanded cells to serve as a baseline for calculating subsequent changes in gene expression. These cells will be referred to as undifferentiated MSCs.

Pellet Culture

After centrifugation and aspiration of trypsin, cells were resuspended in differing media from which one control and three experimental groups were formed. Control group cells were resuspended in expansion medium. The three experimental groups were BMP-13, TGF-β3, and (TGF-β3→BMP-13). Cells in the BMP-13 group were resuspended in defined chondrogenic medium (DCM) containing 10 ng/ml BMP-13, and those in the remaining two experimental groups were resuspended in DCM plus 10 ng/ml TGF-β3. DCM consisted of high-glucose DMEM, 1% ITS+Premix (6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 µg/mL selenious acid, 1.25 mg/mL bovine serum albumin, and 5.35 µg/mL linoleic acid), 0.1 µM dexamethasone, 50 mg/mL ascorbate-2 phosphate, 1 mM sodium pyruvate, 40 µg/mL L-proline, 1% antibiotic-antimycotic solution (100

U/mL penicillin, 0.1 mg/mL streptomycin, and 0.25 µg/mL amphotericin B). Each cell suspension was evenly divided among 36 15-mL conical polypropylene centrifuge tubes so that each tube contained approximately 4×10^5 cells in 0.75 ml of medium. Tubes were centrifuged at 500g for 3 min and the resulting pellet cultures maintained in culture at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Figure 2.1 displays the gross morphology of a pellet culture in phosphate buffered saline (PBS) at the final day of the experiment.

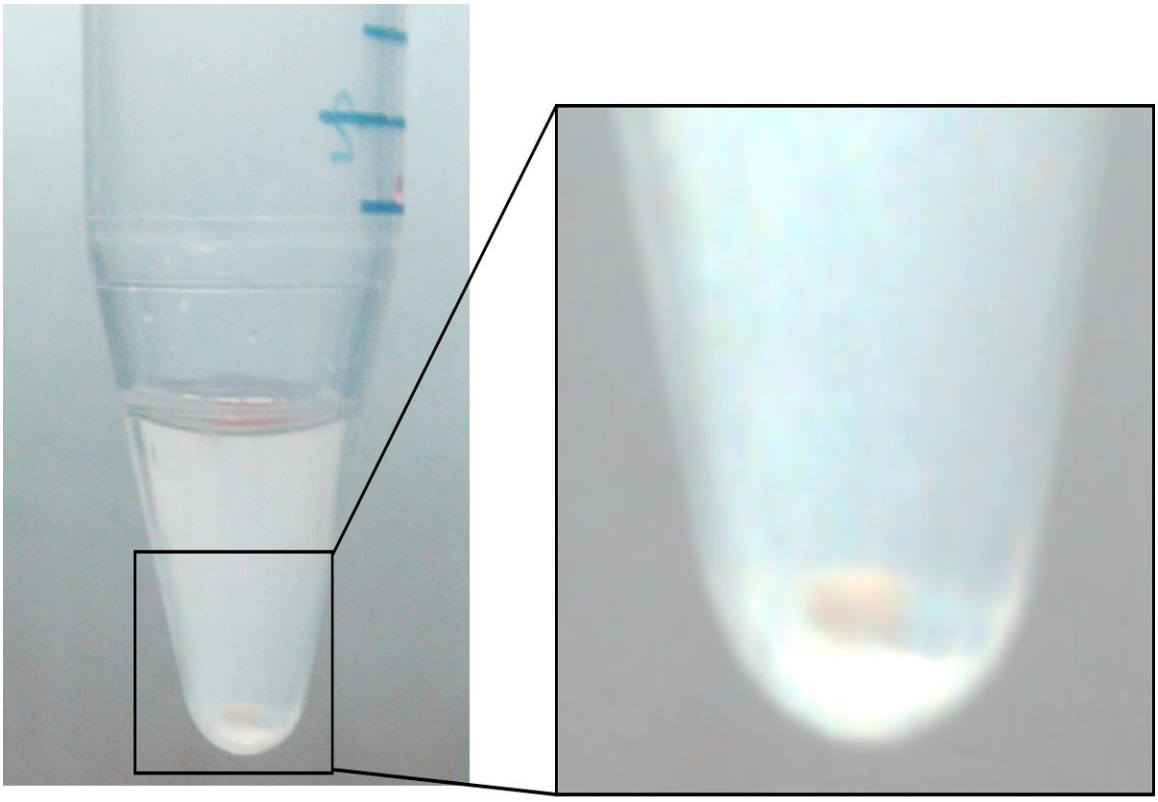

Figure 2.1 Gross morphology of pellet culture in PBS.

The medium was replaced every 3-4 days for 21 days. After 3 days of culture in DCM with TGF-β3, all media in the (TGF-β3→BMP-13) group was completely replaced

with DCM plus 10 ng/ml BMP-13, in which the cells were cultured for remainder of the experiment. Chondroinduction was evaluated based on gene expression, histology, and immunohistochemistry of pellets following 3, 10, and 21 days of culture. The medium content for each group over the course of the experiment is displayed in Table 2.1.

Table 2.1 Groups defined according to medium content at each experimental time point.

Group Name	Day 0 - Day 3	Day 3 - Day 21
Group I	Expansion Medium	Expansion Medium
Group II	DCM + TGF-β3	DCM + TGF-β3
Group III	DCM + BMP-13	DCM + BMP-13
Group IV	DCM + TGF-β3	DCM + BMP-13

Histological and Immunocytochemical Analysis

In order to evaluate the chondrogenic differentiation of the pellets, samples grown at three time points were extracted and immediately fixed in 10% buffered formalin. The pellets were then infiltrated and embedded in paraffin and sectioned to slides (University of Alabama-Birmingham's Center for Metabolic Bone Disease). Sections were stained with Hemotoxylin and Eosin (H&E) to evaluate the internal structure or Toluidine Blue to evaluate proteoglycan concentration.

Additional sections were immunostained for detection of collagen types II and X. Primary antibodies for immunohistochemistry were rabbit anti-human Collagen Type II

polyclonal antibody from MD Bioproducts (St. Paul, MN) and mouse anti-pig Collagen X monoclonal antibody from Abcam (Cambridge, MA). Pellet sections for immunohistochemistry were deparaffinized and rehydrated in xylene and graded ethanol, respectively. GAG removal and collagen exposure were performed by incubating slides in hyaluronidase (2 mg/ml in tris buffered saline) for 25 minutes at 95°C. Antigen retrieval was then performed by incubating slides in pronase (0.5mg/ml in PBS) for 10 minutes at 95°C. Subsequent incubations were all at room temperature, beginning with the primary antibody for one hour. Primary antibody diluted 1:10 in PBS with 1% bovine serum albumin was applied to sections and allowed to infiltrate for one hour at room temperature. Following the primary antibody, slides were stained following instructions from the SuperPicture 3rd Gen IHC Detection Kit (Invitrogen Corp., Frederick, MD). Multiple washes with PBS were performed between all incubations. Type II collagen and type X collagen positively stain dark orange to brown in color. High-resolution images were taken with the Leica DM 2500 microscope (Leica Microsystems Inc., Bannockburn, IL) in transmitted light mode under the same exposure for all slides.

Glycosaminoglycan and DNA Measurement

Three pellets from each group at every time point were digested in 1% papain and 50 mM sodium acetate at 60°C overnight. The total DNA content of the digestate was measured using the Hoechst method. Aliquots of the digestate were added to 0.1 µg/ml bisBenzimide solution in Fluorescent Assay Buffer, and fluorescence intensity was measured using a GloMax®-Multi Jr Single Tube Multimode Reader (Promega,

Corporation, Madison, WI). DNA content was obtained from a standard curve prepared with calf thymus DNA.

Total GAG content from the same papain digestate used in DNA analysis was quantified using the Blyscan Glycosaminoglycan Assay kit (Biocolor, Newtonabby, Northern Ireland), based on dimethyl methylene blue dye binding. The absorbance at 656 nm was measured with a BioTek μQuant Microplate Spectrophotometer (BioTek Instruments, Winooski, VT), and GAG concentration was calculated using a standard curve established from a chondroitin sulfate standard.

RNA Preparation and RT-PCR

Total RNA was prepared from undifferentiated MSCs (5 independent samples) and from pellets (3 per experimental group) cultured for 3, 10, and 21 days. The expression of selected genes was determined by TaqMan® qRT-PCR using pre-designed and validated probe/primer sets (Asuragen Inc., Austin, TX). Genes of interest were aggrecan, cartilage oligomeric matrix protein (COMP), and collagen types I, II, and X. Total RNA (200ng) was reverse transcribed in a total reaction volume of 20 μL. Assuming 100% cDNA synthesis efficiency, 10ng total RNA equivalent cDNA was added to the master mix for a final volume of 15 μL in each PCR well. All amplifications were performed in triplicate on a validated Applied Biosystems 7900HT real-time thermocycler. PCR was initialized by incubation at 95°C for 10 m, followed by 40 cycles of amplification: 95°C for 15 s, then 60°C for 1 m. Beta-2-microglobulin was used as a normalizer gene for ΔC_T calculations and the undifferentiated MSCs used as a calibrator for calculation of fold changes by the $\Delta\Delta C_T$ method.

Results of the RT-PCR data were represented as C_T values. C_T can be defined as the threshold cycle number for PCRs at which an amplified product was first detected [48]. The average C_T was calculated for the normalizer gene, beta-2-microglobulin, and treatment group samples, and ΔC_T was determined as (the mean of the triplicate C_T values for the normalizer gene) minus (the mean of the triplicate C_T values for treatment group samples). $\Delta\Delta C_T$ can be calculated by taking the difference between ΔC_T of treatment group sample and ΔC_T of undifferentiated MSCs from Day 0. Fold change can be described as a differential expression in the normalizer gene of a treatment group sample compared to the Day 0 undifferentiated MSCs counterpart and calculated by $2^{-\Delta\Delta CT}$ [48].

Follow-Up Experiments

Based on results of the primary experiment described above, two follow-up experiments were performed to further clarify the effectiveness of BMP-13 for sustaining chondroinduction of primary human MSCs through the use of RT-PCR analysis. The first follow-up experiment was designed to determine whether BMP-13 was responsible for any of the elevation in chondrocyte gene markers after 3 days of chondroinduction by TGF-β3. The initial gene expression experiment was repeated using cells from a 24 year-old male donor, but the only two experimental groups were (TGF-β3→BMP-13) and a new group, (TGF-β3→DCM). At the same time the TGF-β3-containing medium was replaced with the BMP-13-containing medium in the former group, DCM without any growth factor replaced the medium and growth factor in the latter group. The second follow-up experiment was designed to investigate the potential for BMP-13 to mitigate

the TGF-β3-induced upregulation of type X collagen RNA. The initial gene expression experiment was repeated using cells from a 22-year old male donor. Again, only two experimental groups were included, TGF-β3 and a new group, (TGF-β3→Combination), in which 10 ng/ml BMP-13 was added to the TGF-β3 containing medium after Day 3.

Statistical Analysis

All data were analyzed by using SPSS for Windows software, version 12.0 (SPSS, Chicago, IL). Numerical results are expressed as the mean ± standard deviation. A one-way analysis of variance and LSD posthoc test (α=0.05) were performed to test for the interaction between treatment groups over time.

CHAPTER III

RESULTS

Histological and Immunocytochemical Evaluation

H&E staining were done in order to analyze the cellular structure of the samples. The stained sections for each group display a loss in cellular content over time. At each progressive time point for all treatment groups following Day 3, cellular debris collected in the center of the sphere of each of the pellet constructs. However, the perimeter of each spherical section indicates live cellular content from the appearance of cell nuclei and fibrous-like material. Figure 3.1 displays sections stained for H&E for each group at each time point.

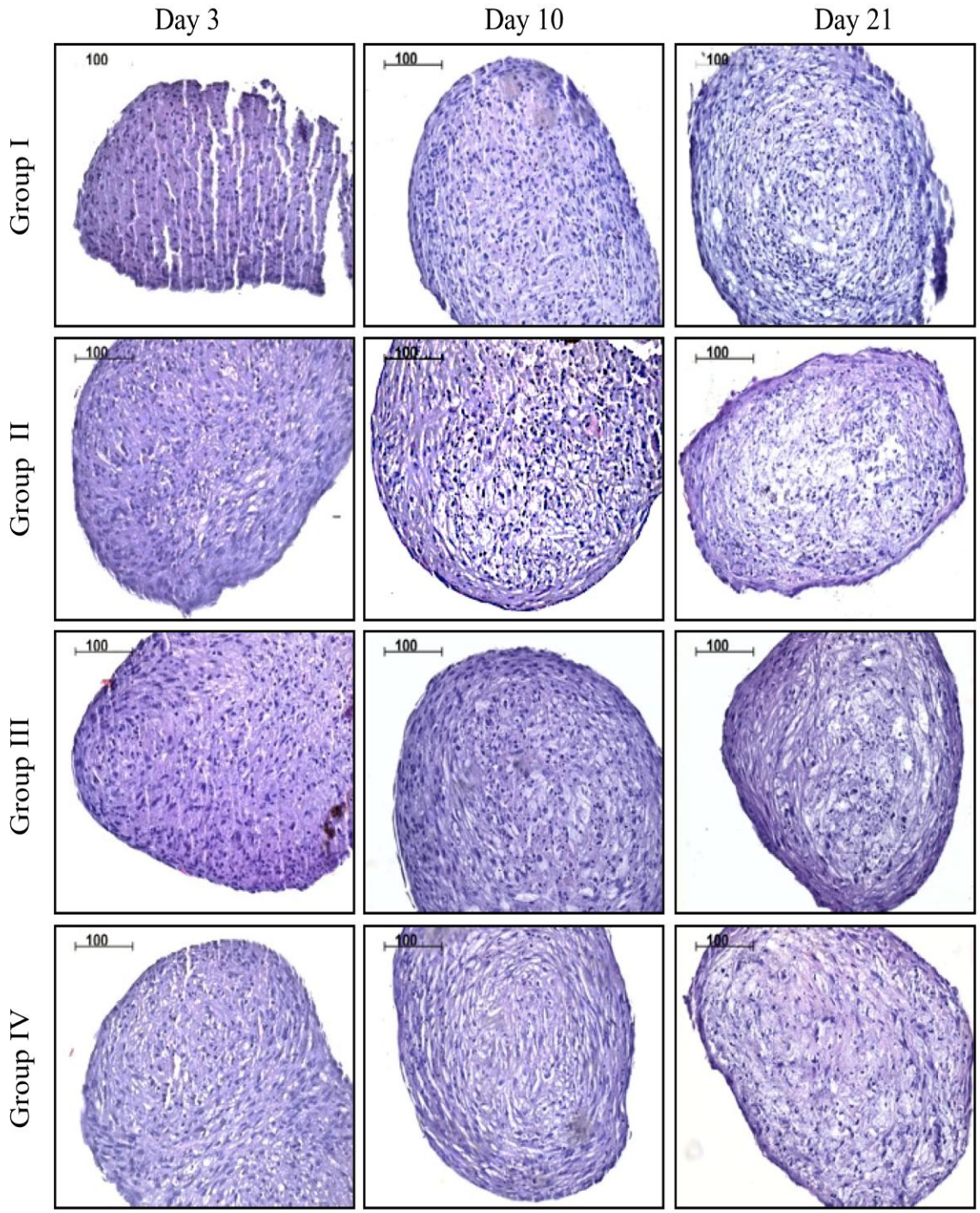

Figure 3.1 20X Histological sections of Groups stained with H&E.

Histological evaluation shows an intense staining with toluidine blue in all treatment groups across time, indicating an ECM rich in proteoglycans. Following the initial time point, a higher intensity of toluidine blue can be seen in the groups at both Day 10 and Day 21 compared to that of Day 3. Groups do not seem to show a distinct staining compared to one another. However, groups do show a higher staining intensity from Day 3 to Day 10. Figure 3.2 displays sections stained for toluidine blue for each group at each time point.

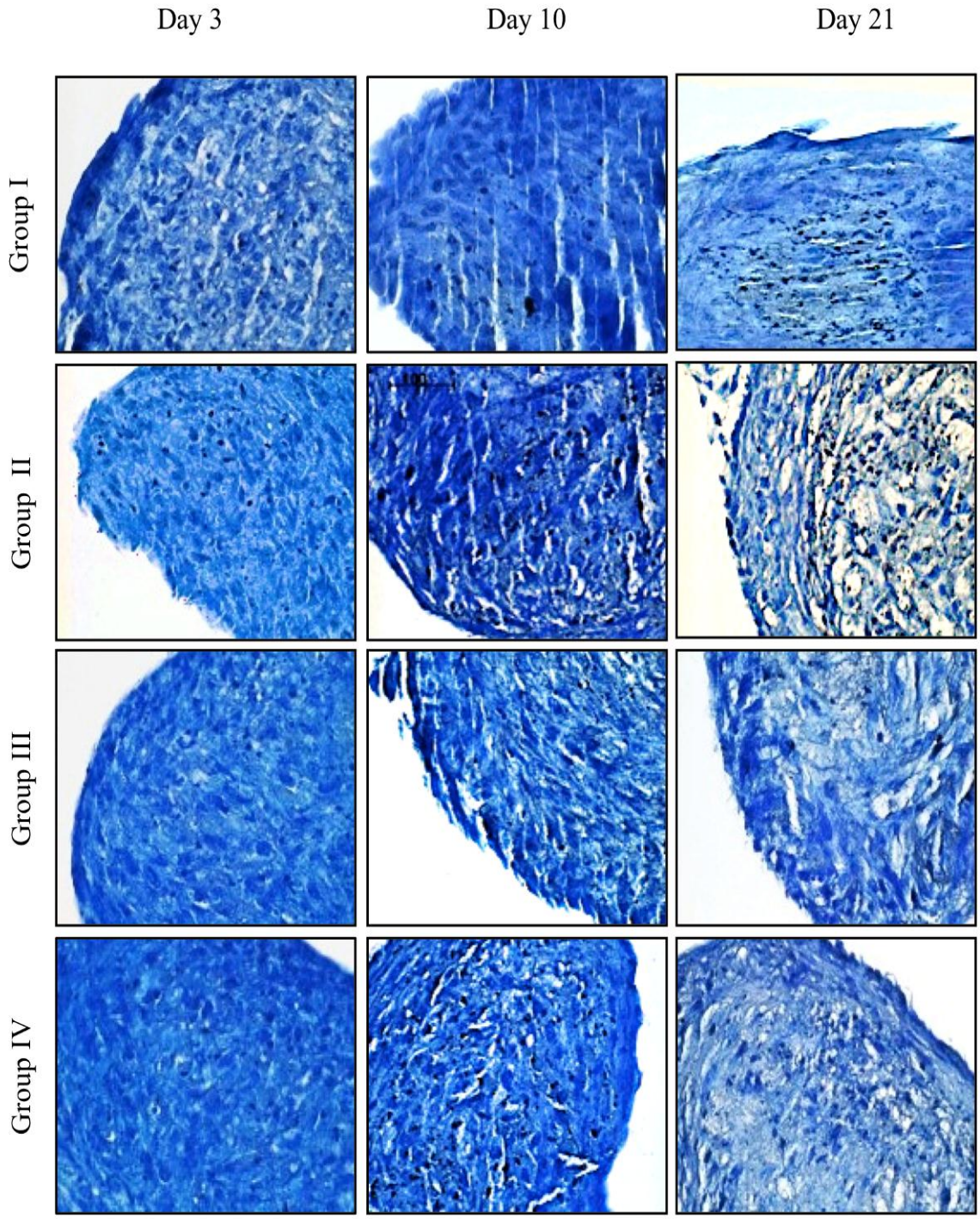

Figure 3.2 40X Histological sections of Groups stained with Toluidine Blue.

Immunocytochemical analysis of type II collagen shows a distinct staining in Group II and Group IV at different time points, indicating the presence of type II collagen. Group I at Day 3 stains relatively light for type II collagen, and no change is exhibited over the remaining time points. Group II type II collagen staining at Day 3 displays a positive staining for type II collagen, and such fibers can be seen. Group II displays no change in intensity of type II collagen staining over time. Group III at Day 3 stains very light in color, indicating little or no existence of type II collagen. Group III remains light in staining over the course of the experimental time points. Group IV at Day 3 displays a positive staining for type II collagen, and such fibers can be seen. At Day 10, there is a dramatic decrease in type II collagen staining intensity for Group IV. From Day 10 to Day 21, there is no relative change in type II collagen staining intensity for Group IV. Figure 3.3 displays sections immunostained for type II collagen for all groups at each time point.

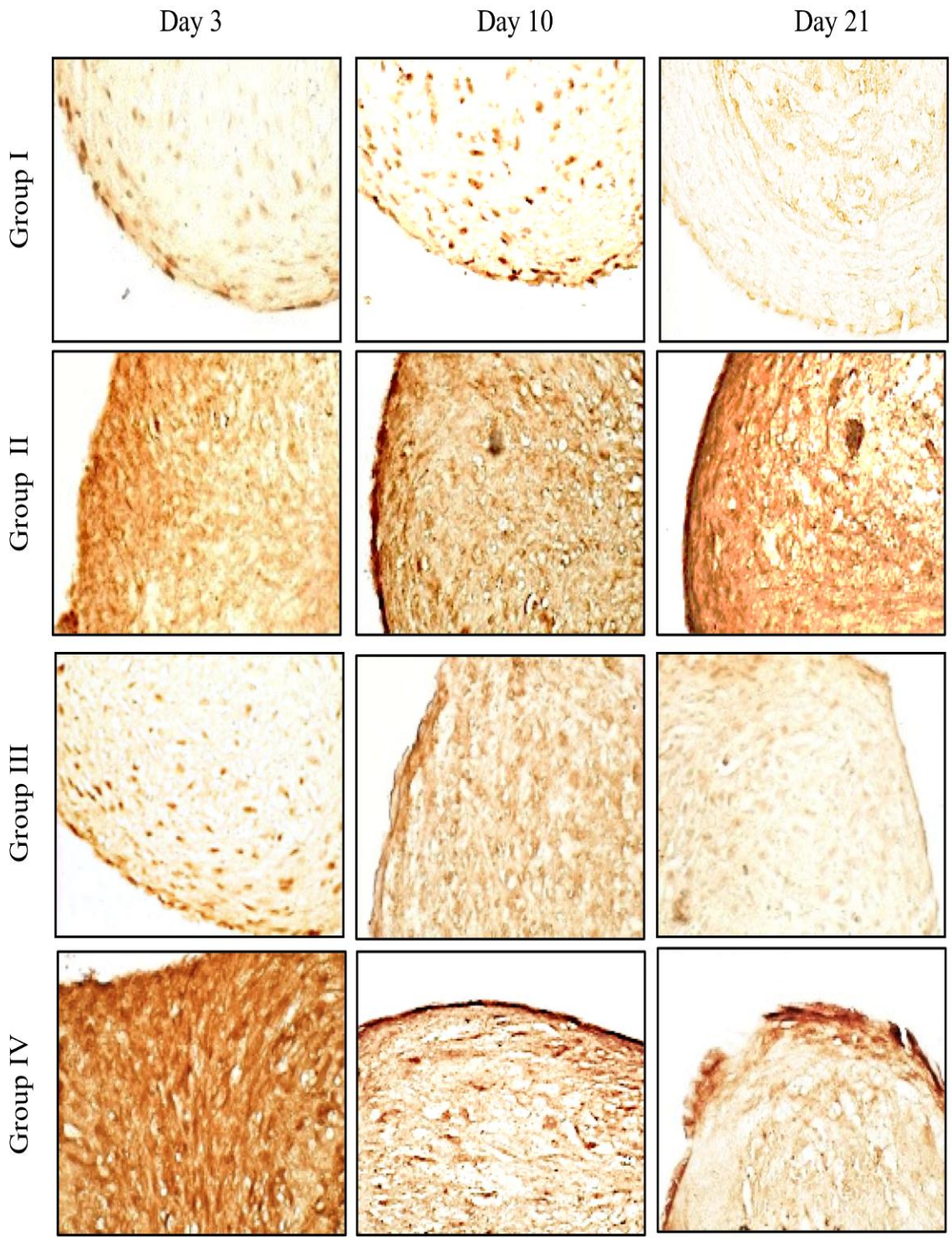

Figure 3.3 40X Histological sections of Groups immunostained for type II collagen.

Immunocytochemical analysis of type X collagen shows a distinct staining in Group II and Group IV at different time points, indicating the presence of type X collagen. Group I at Day 3 shows a light stain indicating no existence of type X collagen. Over time, the staining intensity for Group I remain unchanged with no type X collagen present over the course of the experiment. Group II displays an intense type X collagen stain at Day 3, indicating the presence of type X collagen. At Day 10, Group II type X collagen stain appears more intense. From Day 10 and Day 21, the existence of type X collagen is even more prevalent in the dark staining color and fibers. Group III at Day 3 shows a light stain indicating the little or no presence of type X collagen. Group III type X collagen staining intensity remains unchanged with no presence of type X collagen over time. Group IV displays an intense type X collagen stain at Day 3, indicating the presence of the fiber. However, at Day 10, there is a dramatic decrease in type X staining intensity, indicating a major loss in the fiber expression between time points. At Day 21, type X collagen staining remains light in color similar to that of the previous time point for Group IV. Figure 3.4 displays sections immunostained for type X collagen for each group at each time point.

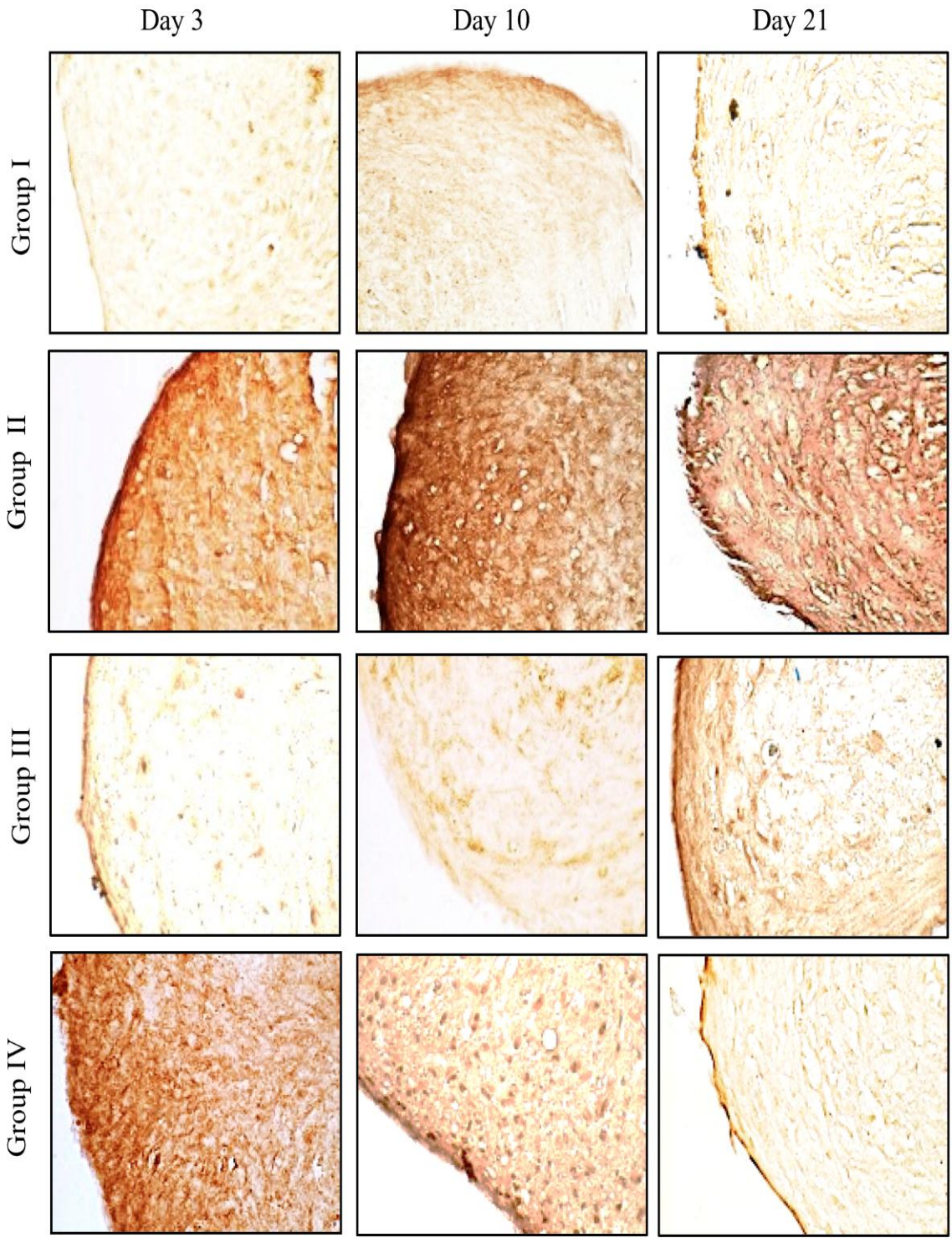

Figure 3.4 40X Histological sections of Groups immunostained for type X collagen.

Biochemical Analysis

Total GAG content (normalized to DNA) for the treatment groups at each time point is displayed in Figure 3.5. GAG content for Group I at Day 3 averages a (2.35±0.93) mean across six pellets. At Day 10, Group I undergoes a significant increase in mean GAG content at (4.21±1.15) across pellets. Between Day 10 and Day 21, Group I exhibits no significant change in GAG content averaging a mean of (2.96±1.10) across pellets. GAG content for Group II at Day 3 averages a (1.31±0.24) mean across six pellets. At Day 10, Group II undergoes a significant increase in mean GAG content at (3.98±1.21) across pellets. Between Day 10 and Day 21, Group II exhibits no significant change in mean GAG content averaging a mean of (3.42±0.93) across pellets. GAG content for Group III at Day 3 averages a (1.85±0.62) mean across six pellets. At Day 10, Group III undergoes a significant increase in mean GAG content at (4.36±1.17) across pellets. Between Day 10 and Day 21, Group III exhibits no significant change in mean GAG content averaging a mean of (2.58±0.64) across pellets. GAG content for Group IV at Day 3 averages a (1.42±0.22) mean across five pellets. At Day 10, Group IV undergoes a significant increase in mean GAG content at (3.93±0.92) across pellets. Between Day 10 and Day 21, Group IV exhibits no significant change in mean GAG content averaging a mean of (3.49±1.01) across pellets.

At Day 3, Group I demonstrated a significantly higher mean GAG content compared to that of Group II and Group IV. Group III was not considered significantly different from any of the other groups. GAG content for all groups did not significantly differ at Day 10. However, GAG content on Day 10 was significantly higher than on Day

3 by 135% (4.12±1.06 vs. 1.75±0.69 µg/µg, n = 6 per group). From Day 10 to Day 21, groups again exhibited no significant difference when compared to one another.

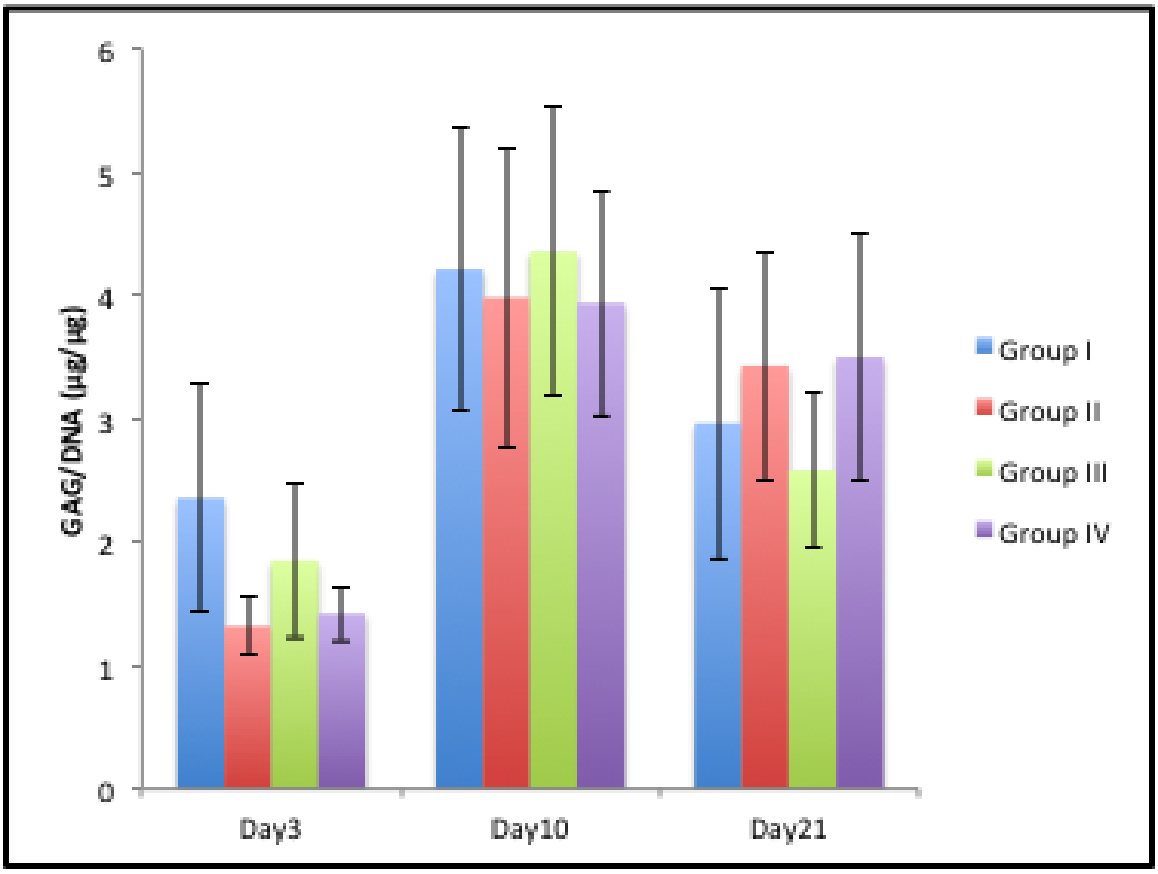

Figure 3.5 Total GAG content averaged across pellets for each Group over time.

DNA content for Group I at Day 3 averages a (737.22±38.95) mean across six pellets. At Day 10, Group I undergoes a significant decrease in mean DNA content at (453.37±32.35) across pellets. Between Day 10 and Day 21, Group I exhibits no significant change in DNA averaging a mean of (431.32±39.32) across pellets. DNA content for Group II at Day 3 averages a (739.85±36.90) mean across six pellets. At Day 10, Group II undergoes a significant decrease in mean DNA content at (354.42±58.89)

across pellets. Between Day 10 and Day 21, Group II exhibits no significant change in DNA averaging a mean of (305.06±17.33) across pellets. DNA content for Group III at Day 3 averages a (785.27±100.25) mean across six pellets. At Day 10, Group III undergoes a significant decrease in mean DNA content at (438.17±19.01) across pellets. Between Day 10 and Day 21, Group III exhibits no significant change in DNA averaging a mean of (375.44±21.28) across pellets. DNA content for Group IV at Day 3 averages a (808.01±29.99) mean across five pellets. At Day 10, Group IV undergoes a significant decrease in mean DNA content at (341.50±53.31) across pellets. Between Day 10 and Day 21, Group IV exhibits no significant change in DNA averaging a mean of (307.97±29.88) across pellets.

At Day 3, treatment groups exhibited no significant difference between one another. DNA content for Group I and Group III displayed significantly higher mean values than that of Group II and Group IV at Day 10. From Day 10 to Day 21, Group I and Group III again expressed a higher mean DNA content than that of groups Group II and Group IV. However, Group I exhibited a significantly higher mean DNA content than that of all groups. On Day 10 and Day 21, pellets from all groups contained about 50% of the DNA at which they had contained on Day 3. Total DNA content for the groups at each time point is displayed in Figure 3.6.

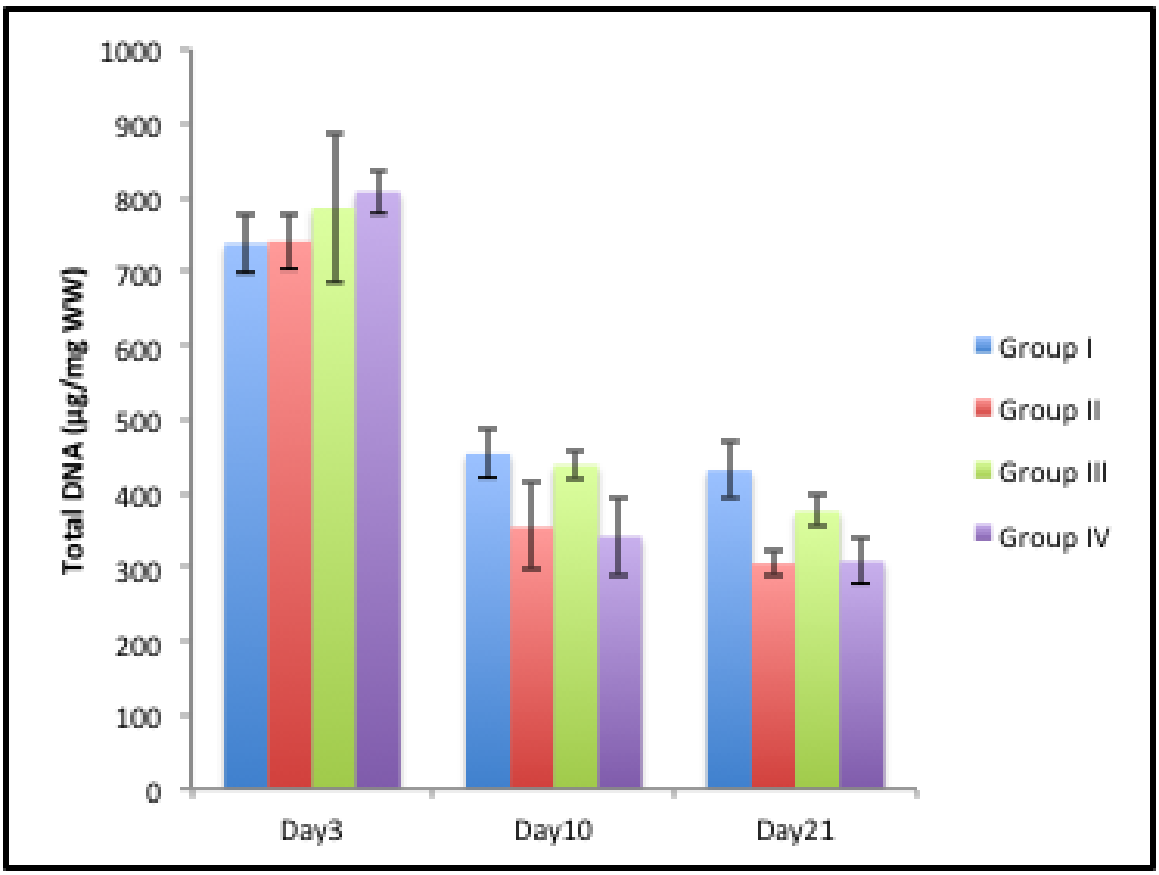

Figure 3.6 Total DNA content averaged across pellets for each Group over time.

Following statistical analysis for each gene's mean fold change, the significant comparison between treatment groups over time could be determined. The data is presented as the fold change in target gene expressions for all treatment groups and is relative to five independent samples of undifferentiated MSCs from Day 0.

Aggrecan expression for Group I at Day 3 averages a (-11.99±1.24) mean fold change across pellets. At Day 10, aggrecan expression for Group I experiences a significant increase in mean fold change at (-7.61±2.43). Between Day 10 and Day 21, aggrecan expression increases but does not experience a significant change at a (-6.36±0.78) mean fold change for Group I. Aggrecan expression for Group II at Day 3

averages a (-3.40±1.83) mean fold change across pellets. At Day 10, aggrecan expression for Group II experiences an increase in mean fold change at (-1.34±0.42). Between Day 10 and Day 21, aggrecan expression significantly increases at a (2.86±0.72) mean fold change for Group II. Aggrecan expression for Group III at Day 3 averages a (-4.12±1.59) mean fold change across pellets. At Day 10, aggrecan expression for Group III experiences a decrease in mean fold change at (-11.07±6.10). Between Day 10 and Day 21, aggrecan expression significantly increases at a (1.14±0.09) mean fold change for Group III. Aggrecan expression for Group IV at Day 3 averages a (-3.12±1.35) mean fold change across pellets. At Day 10, aggrecan expression for Group IV experiences a significant decrease in mean fold change at (-7.05±2.06). Between Day 10 and Day 21, aggrecan expression significantly increases at a (-2.78±1.06) mean fold change for Group IV. Group I and Group II aggrecan expression significantly differ between Day 3 and Day 21.

At Day 3, aggrecan expression is significantly lower for Group I compared to that of the other treatment groups. Group II and Group III exhibit the only significant difference in mean fold change relative to aggrecan expression at Day 10, with Group II having a significantly higher mean fold change value. At Day 21, Group I aggrecan expression experiences a significantly lower mean fold change compared to that of the other treatment groups. Also, Group II has a significantly higher mean fold change than all groups at Day 21. All treatment groups at Day 21 experience a significant difference from all other treatment groups with respect to aggrecan expression of mean fold change. Total aggrecan mean fold change for the groups at each time point is displayed in Figure 3.7.

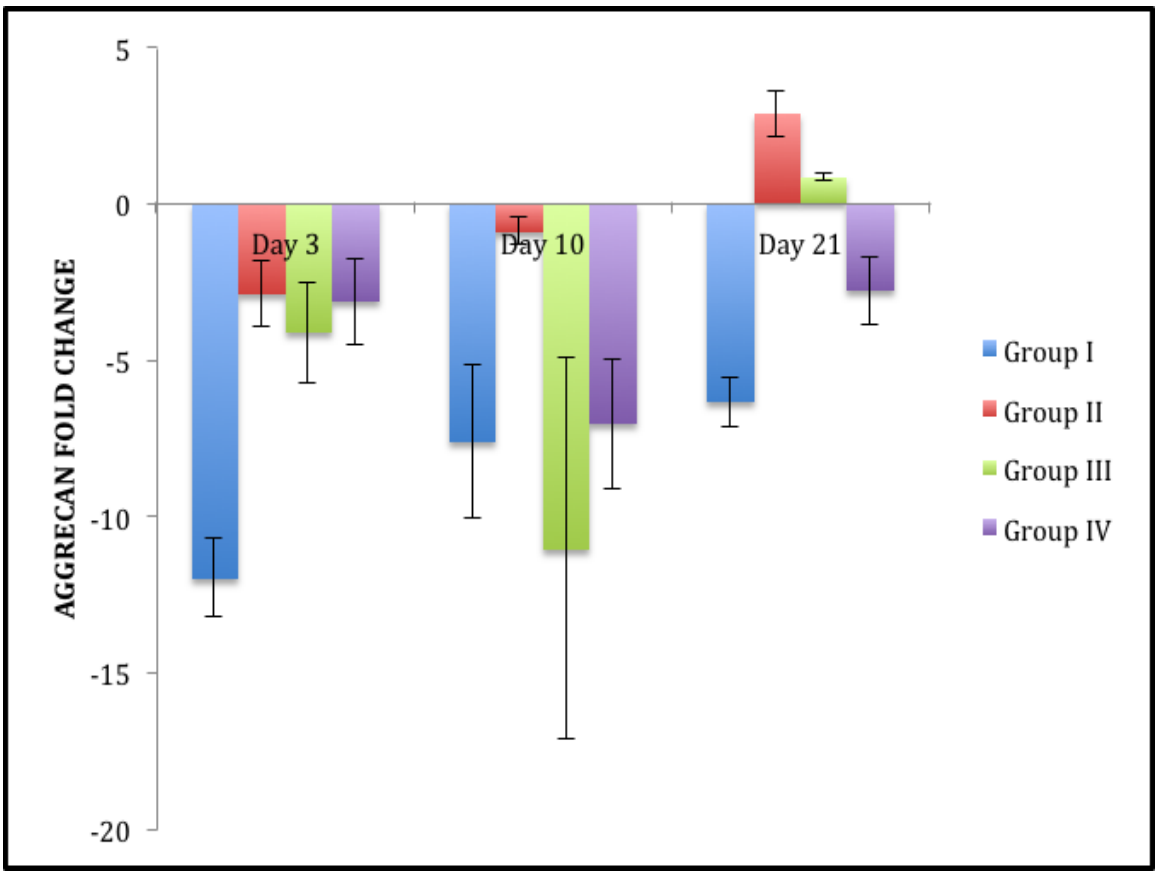

Figure 3.7 Total aggrecan mean fold change in pellets over time for initial experiment.

COMP expression for Group I at Day 3 averages a (1.14±0.21) mean fold change across pellets. At Day 10, COMP expression for Group I experiences a decrease in mean fold change at (-2.03±0.07). Between Day 10 and Day 21, COMP expression experiences a significant increase at a (4.43±3.83) mean fold change for Group I. COMP expression for Group II at Day 3 averages a (76.57±22.54) mean fold change across pellets. At Day 10, COMP expression for Group II experiences a significant increase in mean fold change at (728.70±172.75). Between Day 10 and Day 21, COMP remains relatively unchanged at a (675.89±231.69) mean fold change for Group II. COMP expression for

Group III at Day 3 averages a (-0.19±1.49) mean fold change across pellets. At Day 10, COMP expression for Group III experiences a decrease in mean fold change at (-1.33 ±0.36). Between Day 10 and Day 21, COMP expression significantly increases at a mean fold change (7.11 ±0.87) for Group III. COMP expression for Group IV at Day 3 averages a (117.54±15.24) mean fold change across pellets. At Day 10, COMP expression for Group IV experiences a significant decrease in mean fold change at (48.28±15.28). Between Day 10 and Day 21, COMP expression decreases at a (22.62±13.37) mean fold change for Group IV. Only Group I COMP expression did not significantly differ between Day 3 and Day 21.

At Day 3, COMP expression is significantly lower for Group I and Group II compared to Group II and Group IV mean fold change. COMP expression for Group II expresses a significantly higher mean fold change compared to the other groups at Day 10. Group II COMP expression, again at Day 21, experiences a significantly higher mean fold change compared to the remaining groups. Total COMP mean fold change for the groups at each time point is displayed in Figure 3.8.

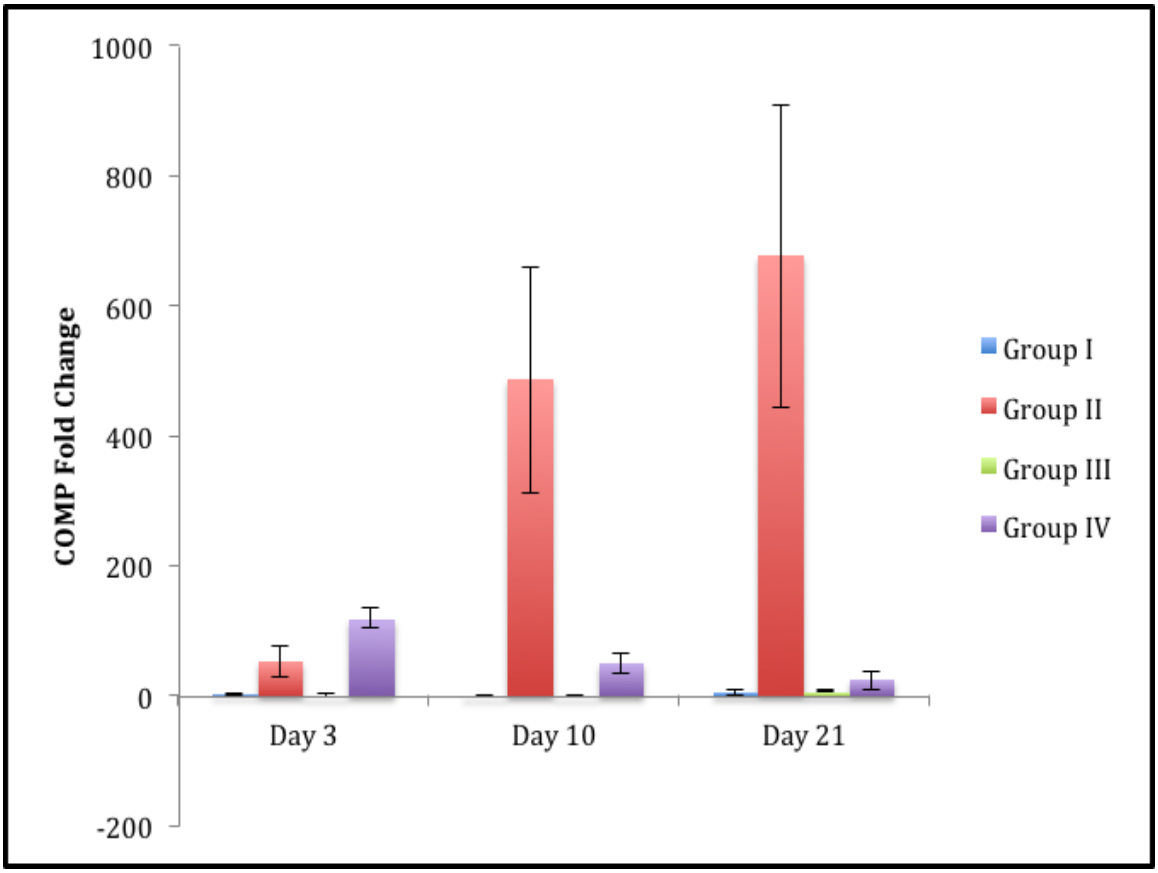

Figure 3.8 Total COMP mean fold change in pellets over time for initial experiment.

Type I collagen expression for Group I at Day 3 averages a (-1.49±0.28) mean fold change across pellets. At Day 10, type I collagen expression for Group I experiences an increase in mean fold change at (-0.34±1.22). Between Day 10 and Day 21, type I collagen expression experiences a significant increase at a (1.88±0.30) mean fold change for Group I. Type I collagen expression for Group II at Day 3 averages a (2.84±0.28) mean fold change across pellets. At Day 10, type I collagen expression for Group II experiences a significant increase in mean fold change at (6.17±1.45). Between Day 10 and Day 21, type I collagen increases at a (8.27±1.24) mean fold change for Group II. Type I collagen expression for Group III at Day 3 averages a (1.31±0.40) mean fold

change across pellets. At Day 10, type I collagen expression for Group III experiences an increase in mean fold change at (1.71±0.17). Between Day 10 and Day 21, type I collagen expression significantly increases at a mean fold change (5.12±0.30) for Group III. Type I collagen expression for Group IV at Day 3 averages a (2.56±0.45) mean fold change across pellets. At Day 10, type I collagen expression for Group IV experiences an increase in mean fold change at (3.57±0.24). Between Day 10 and Day 21, type I collagen expression significantly increases at a (4.65±0.78) mean fold change for Group IV. All groups type I collagen expression differ significantly between Day 3 and Day 21.

At Day 3, type I collagen expression is significantly lower for Group I compared to the remaining groups. Group II and Group IV also exhibit a significantly higher mean fold change than Group III in type I collagen expression at Day 3. Again, Group I type I collagen expression exhibits a significantly lower mean fold change, and Group II exhibits a significantly higher mean fold change compared to remaining groups at Day 10. Group IV also expresses a significantly higher type I collagen expression than Group III. Group II, Group III and Group IV type I collagen expression, at Day 21, experiences a significantly higher mean fold change compared to that of the remaining group, Group I. However, Group II has a significantly higher mean fold change type I collagen expression than all groups at Day 21. Total type I collagen mean fold change for the groups at each time point is displayed in Figure 3.9.

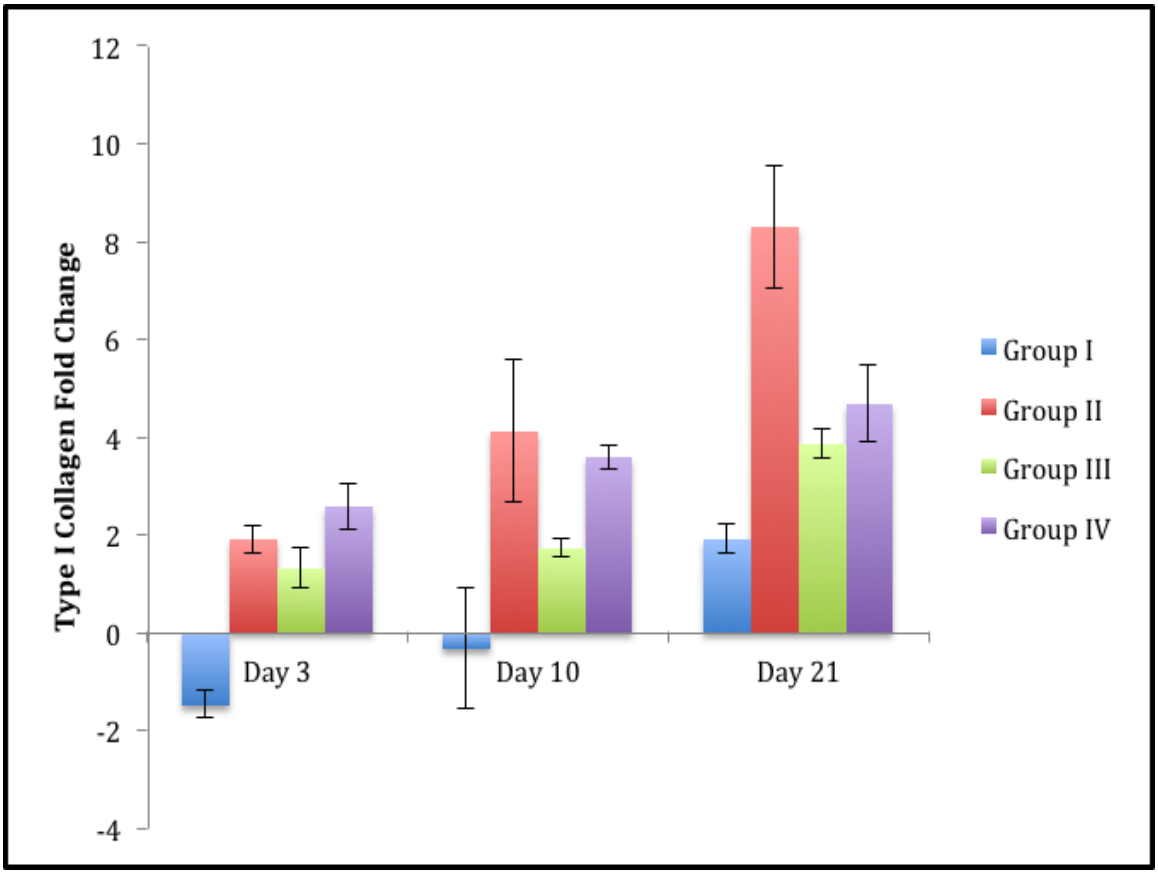

Figure 3.9 Total type I collagen mean fold change in pellets over time for initial experiment.

At Day 10, type II collagen expression for Group I experiences a mean fold change at (2.15±0.55). Between Day 10 and Day 21, type II collagen expression experiences an increase at a (2.72±1.36) mean fold change for Group I. Type II collagen expression for Group II at Day 3 averages a (11.87±8.72) mean fold change across pellets. At Day 10, type II collagen expression for Group II experiences an insignificant decrease in mean fold change at (9.33±7.97). Between Day 10 and Day 21, type II collagen remains relatively unchanged at (8.95±4.03) mean fold change for Group II. The type II collagen expression for Group III at Day 3 averages a (0.37±1.42) mean fold change across all pellets. At Day 10, type II collagen expression for Group III

experiences an increase in mean fold change at (2.24±0.99). Between Day 10 and Day 21, type II collagen expression decreases at a mean fold change (0.46±1.76) for Group III. The type II collagen expression for Group IV at Day 3 averages a (14.67±4.57) mean fold change across all pellets. At Day 10, type II collagen expression for Group IV experiences a significant decrease in mean fold change at (4.83±1.73). Between Day 10 and Day 21, type II collagen expression decreases at a (2.76±0.604) mean fold change for Group IV. Group IV type II collagen expression differs significantly between Day 3 and Day 21. Also, all treatment groups exhibit no significant change in type II collagen content from Day 10 to Day 21.

At Day 3, type II collagen expression is significantly lower for Group III compared to the remaining groups present. Type II collagen expression does not exhibit a significantly different mean fold change among all groups at Day 10. Group II type II collagen expression experiences a significantly higher mean fold change compared to that of the remaining groups at Day 21. Total type II collagen mean fold change for the groups at each time point is displayed in Figure 3.10.

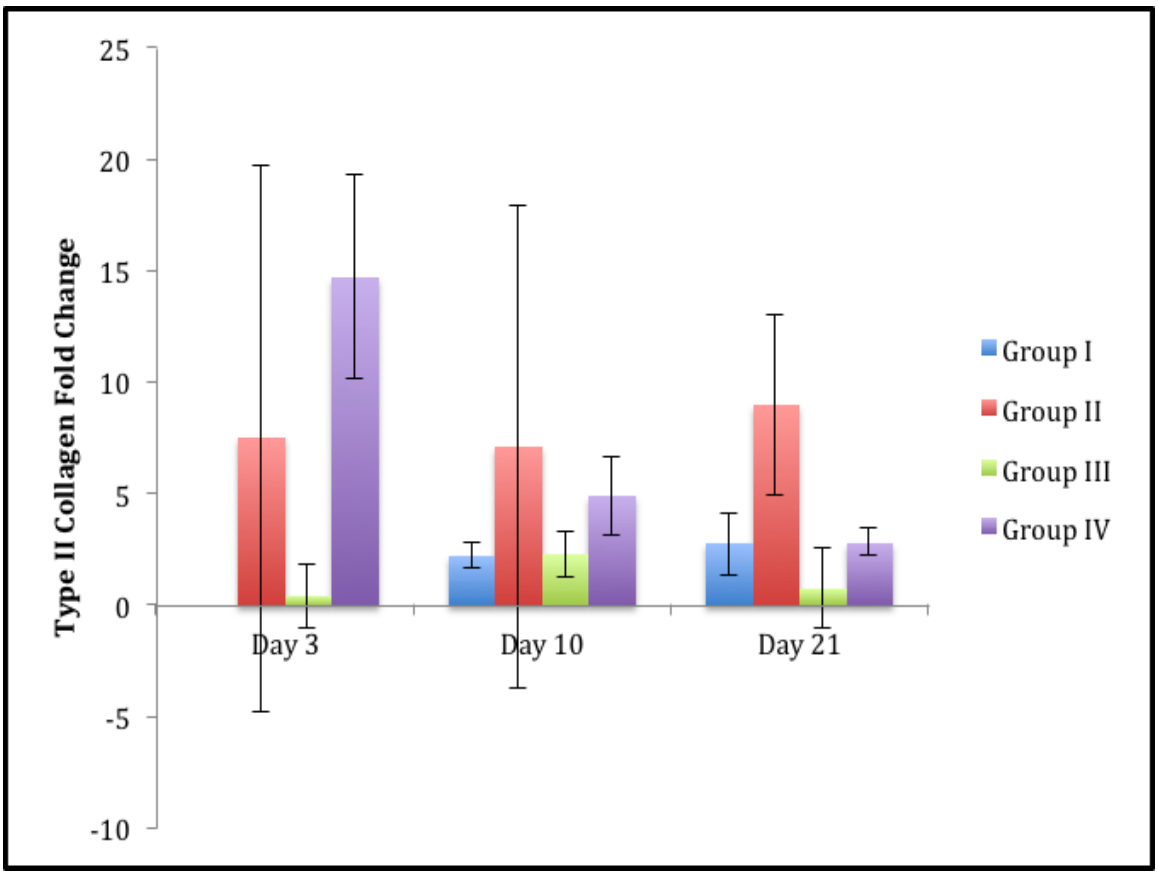

Figure 3.10 Total type II collagen mean fold change in pellets over time for initial experiment.

Type X collagen expression for Group I at Day 3 averages a (-3.53±0.79) mean fold change across pellets. At Day 10, type X collagen expression for Group I experiences a significant decrease in mean fold change at (-12.20±2.45). Between Day 10 and Day 21, type X collagen expression experiences a significant increase at a (0.56±3.93) mean fold change for Group I. Type X collagen expression for Group II at Day 3 averages a (449.57±70.27) mean fold change across pellets. At Day 10, type X collagen expression for Group II experiences an increase in mean fold change at (547.52±17.84). Between Day 10 and Day 21, type X collagen significantly increases at a (875.31±177.72) mean fold change for Group II. Type X collagen expression for Group

III at Day 3 averages a (3.41 ±1.59) mean fold change across pellets. At Day 10, type X collagen expression for Group III remains relatively unchanged in mean fold change at (5.77±3.15). Between Day 10 and Day 21, type X collagen expression significantly increases at a mean fold change (44.45±12.71) for Group III. Type X collagen expression for Group IV at Day 3 averages a (361.23±78.19) mean fold change across pellets. At Day 10, type X collagen expression for Group IV experiences a significant decrease in mean fold change at (20.86±3.56). Between Day 10 and Day 21, type X collagen expression remains relatively unchanged at a (44.63±27.65) mean fold change for Group IV. Group II, Group III, and Group IV type X collagen expression differ significantly between Day 3 and Day 21.

At Day 3, type X collagen expression is significantly higher for Group II and Group IV compared to that of Group I and Group III. Type X collagen expression for Group II expresses a significantly higher mean fold change compared to the other groups at Day 10. Again, Group II type X collagen expression experiences a significantly higher mean fold change compared to that of the remaining groups at Day 21. Total type X collagen mean fold change for the groups at each time point is displayed in Figure 3.11.

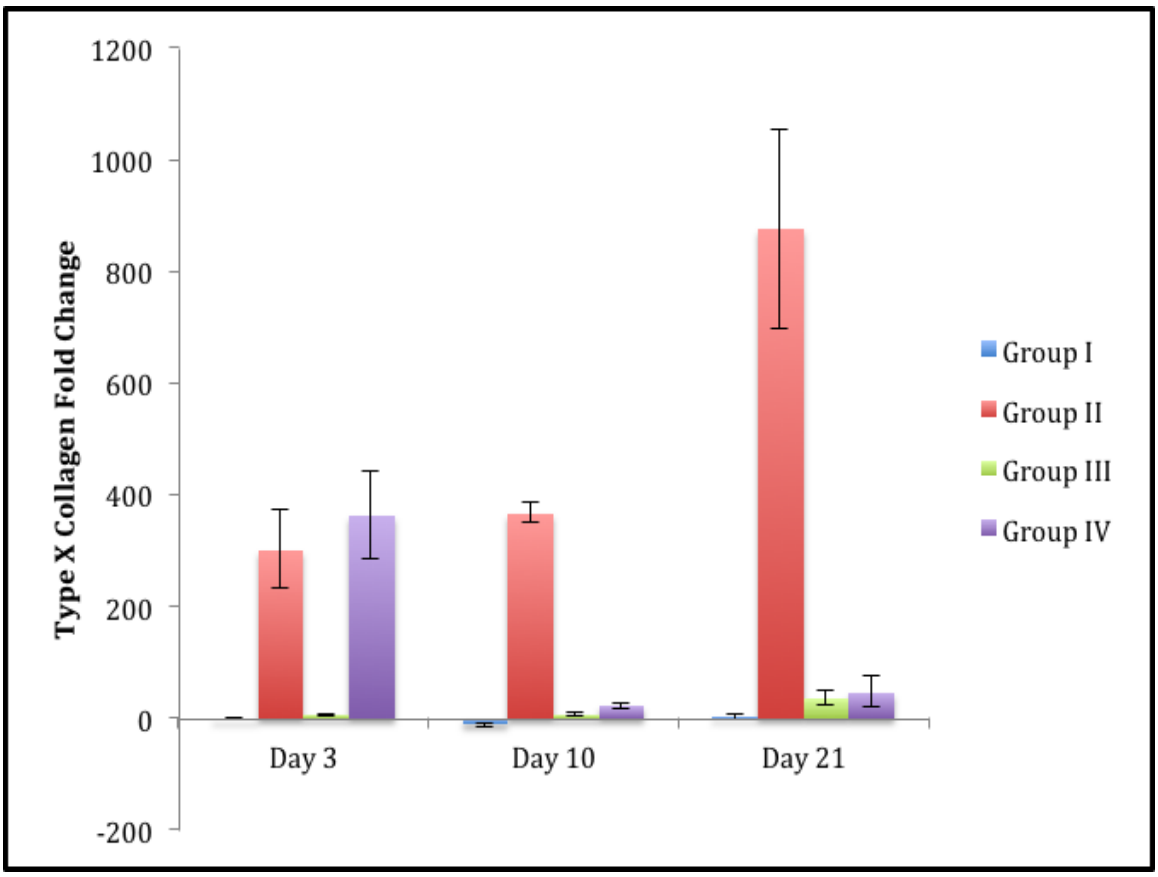

Figure 3.11 Total type X collagen mean fold change in pellets over time for initial experiment.

Follow-Up Experiments

RT-PCR Analysis

The first follow-up experiment was designed to determine whether BMP-13 was responsible for any of the elevation in chondrocyte gene markers after 3 days of chondroinduction by TGF-β3, with only two experimental groups present, (TGF-β3→BMP-13) and (TGF-β3→DCM). Total RNA was prepared from undifferentiated MSCs and also from pellets cultured at days 3 and 10 using the Qiagen RNeasy mini kit. RT-PCR analysis followed the same experimental outline as discussed previously. Group (TGF-β3→BMP-13) exhibits a mean (-14.80±1.74) fold change at Day 3 aggrecan

expression. At Day 10, (TGF-β3→BMP-13) undergoes a significant decrease in aggrecan expression at a mean fold change of (-114.14±17.26). Group (TGF-β3→DCM) exhibits a mean (-14.56±4.07) fold change at Day 3 aggrecan expression. At Day 10, (TGF-β3→DCM) undergoes a significant decrease in aggrecan expression at a mean fold change of (-105.95±0.00). At Day 3 and Day 10, Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) do not exhibit a significant difference in aggrecan expression. Total aggrecan mean fold change for the groups at each time point for the first follow-up experiment is displayed in Figure 3.12.

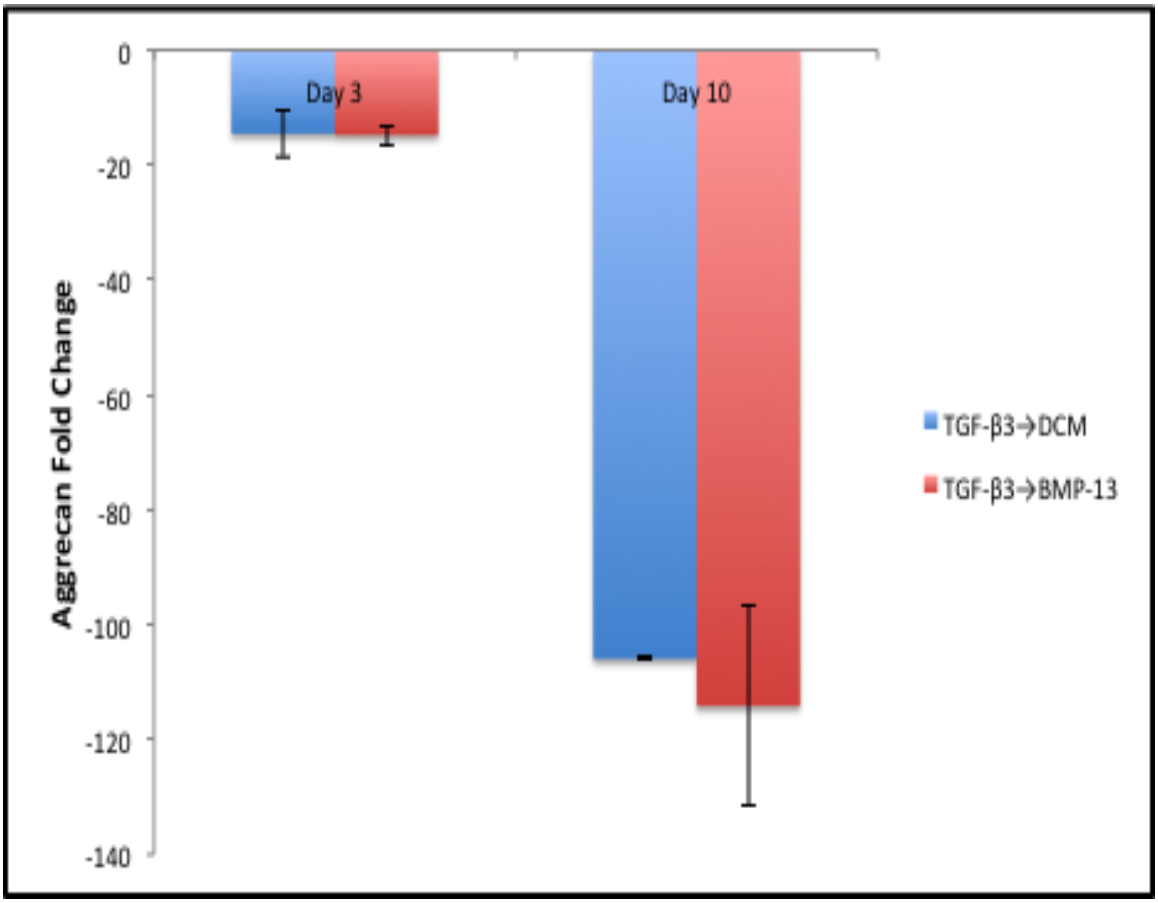

Figure 3.12 Total aggrecan mean fold change in pellets over time for first follow-up experiment.

Group (TGF-β3→BMP-13) exhibits a mean (-4.44±0.37) fold change at Day 3 COMP expression. At Day 10, (TGF-β3→BMP-13) undergoes a significant decrease in COMP expression at a mean fold change of (-43.28±3.62). Group (TGF-β3→DCM) exhibits a mean (-4.81±0.71) fold change at Day 3 COMP expression. At Day 10, (TGF-β3→DCM) undergoes a significant decrease in COMP expression at a mean fold change of (-37.55±9.55). At Day 3 and Day 10, Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) do not exhibit a significant difference in COMP expression. Total COMP mean fold change for the groups at each time point for the first follow-up experiment is displayed in Figure 3.13.

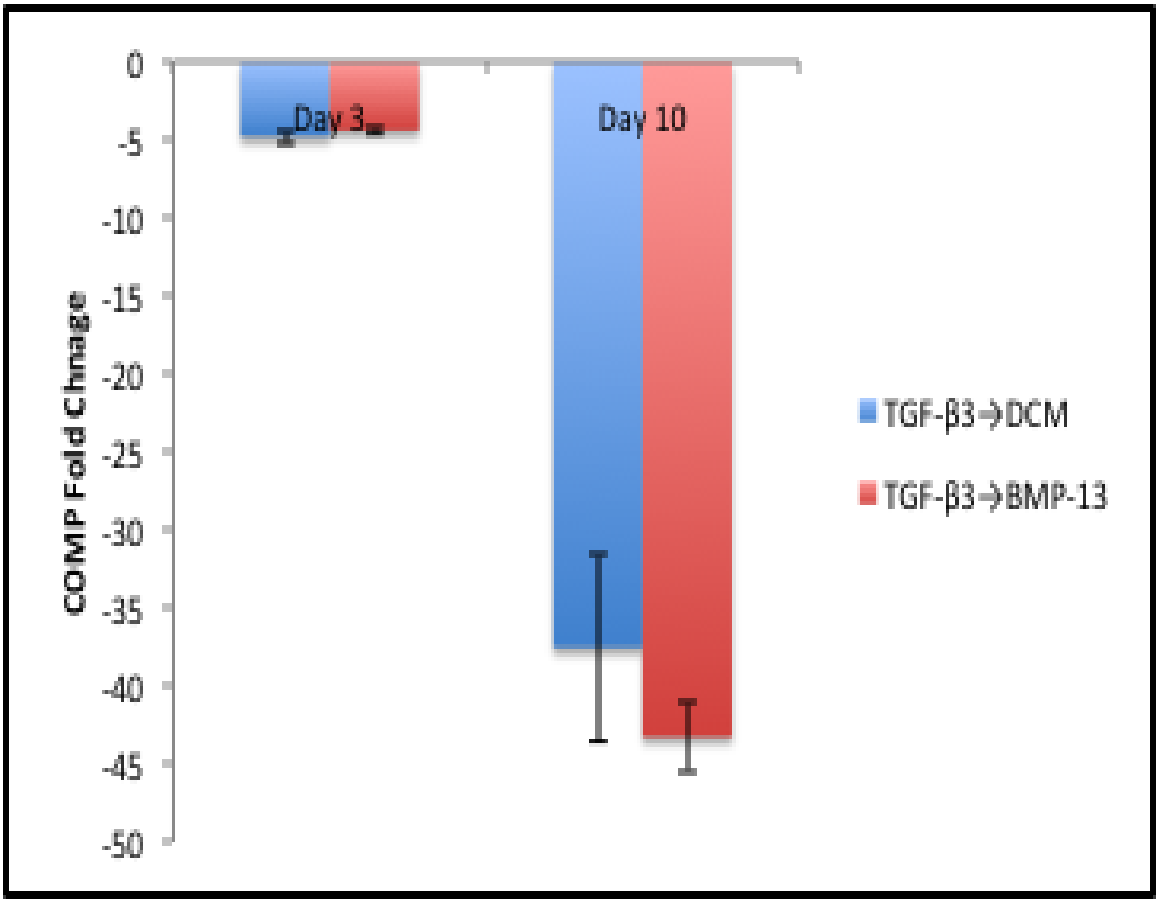

Figure 3.13 Total COMP mean fold change in pellets over time for first follow-up experiment.

Group (TGF-β3→BMP-13) exhibits a mean (0.24±1.51) fold change at Day 3 type II collagen expression. At Day 10, (TGF-β3→BMP-13) remains relatively unchanged in type II collagen expression at a mean fold change of (1.11±2.21). Group (TGF-β3→DCM) exhibits a mean (-2.29±0.82) fold change at Day 3 type II collagen expression. At Day 10, (TGF-β3→DCM) remains relatively unchanged in type II collagen expression at a mean fold change of (-0.40±1.31). At Day 3 and Day 10, Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) do not exhibit a significant difference

in type II collagen expression. Total type II collagen mean fold change for the groups at each time point for the first follow-up experiment is displayed in Figure 3.14.

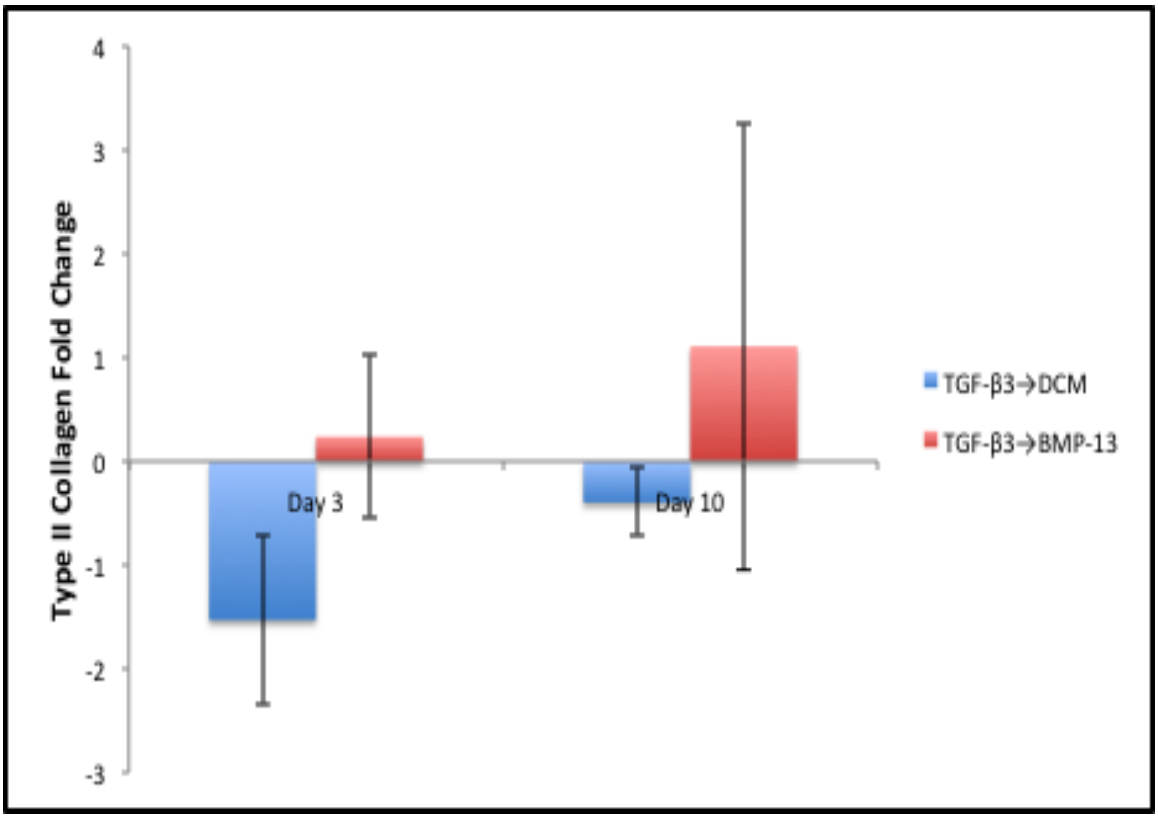

Figure 3.14 Total type II collagen mean fold change in pellets over time for first follow-up experiment.

Group (TGF-β3→BMP-13) exhibits a mean (377.85±76.63) fold change at Day 3 type X collagen expression. At Day 10, (TGF-β3→BMP-13) undergoes a significant decrease in type X collagen expression at a mean fold change of (4.21±0.52). Group (TGF-β3→DCM) exhibits a mean (441.715±19.58) fold change at Day 3 type X collagen expression. At Day 10, (TGF-β3→DCM) undergoes a significant decrease in type X collagen expression at a mean fold change of (3.74±0.38). At Day 3 and Day 10, Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) do not exhibit a significant difference

in type X collagen expression. Total type X collagen mean fold change for the groups at each time point for the first follow-up experiment is displayed in Figure 3.15.

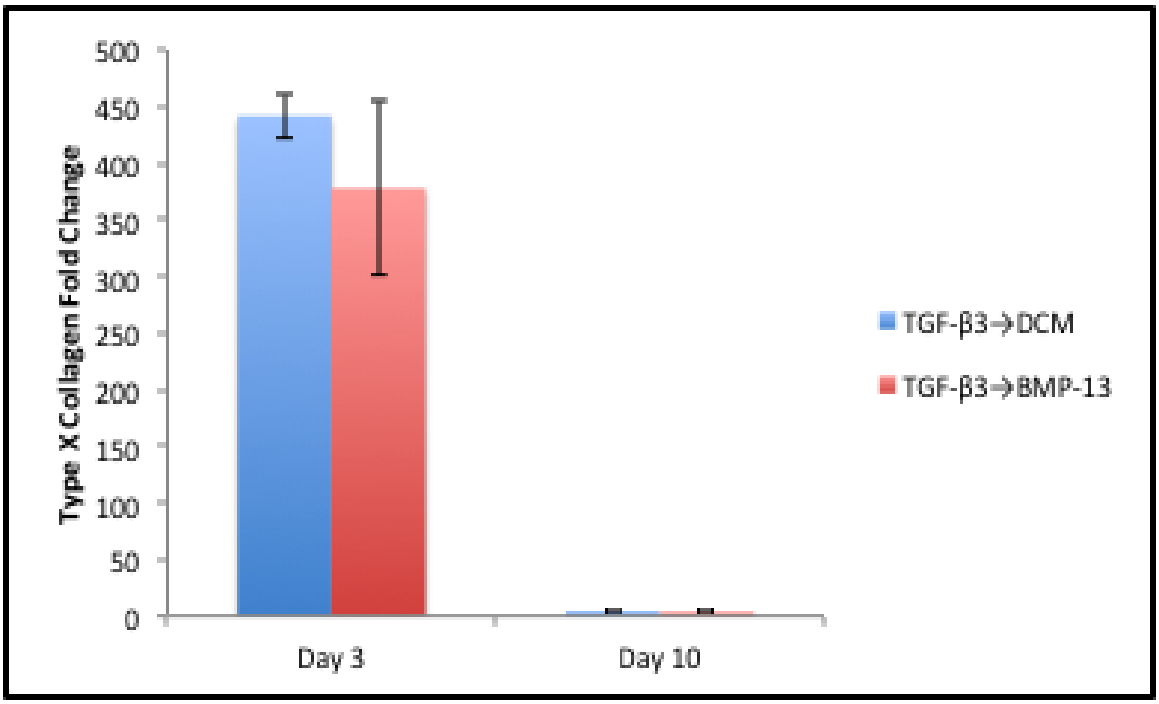

Figure 3.15 Total type X collagen mean fold change in pellets over time for first follow-up experiment.

The second follow-up experiment was designed to investigate the potential for BMP-13 to mitigate the TGF-β3-induced upregulation of type X collagen expression. The experimental groups were TGF-β3 and a new group, (TGF-β3→Combination), in which 10 ng/ml BMP-13 was added to the TGF-β3 containing medium after Day 3. Total RNA was prepared from undifferentiated MSCs and also from pellets cultured at day 10 using the Qiagen RNeasy mini kit. RT-PCR analysis followed the same experimental outline as discussed previously.

Group TGF-β3 exhibits a mean (18.76±3.38) fold change at Day 10 type X collagen expression. Group (TGF-β3→Combination) exhibits a mean (21.66±0.00) fold change at Day 10 type X collagen expression. By Day 10, Group TGF-β3 and Group (TGF-β3→Combination) do not exhibit a significant difference in type X collagen expression. Total type X collagen mean fold change for the groups at each time point for the second follow-up experiment is displayed in Figure 3.16.

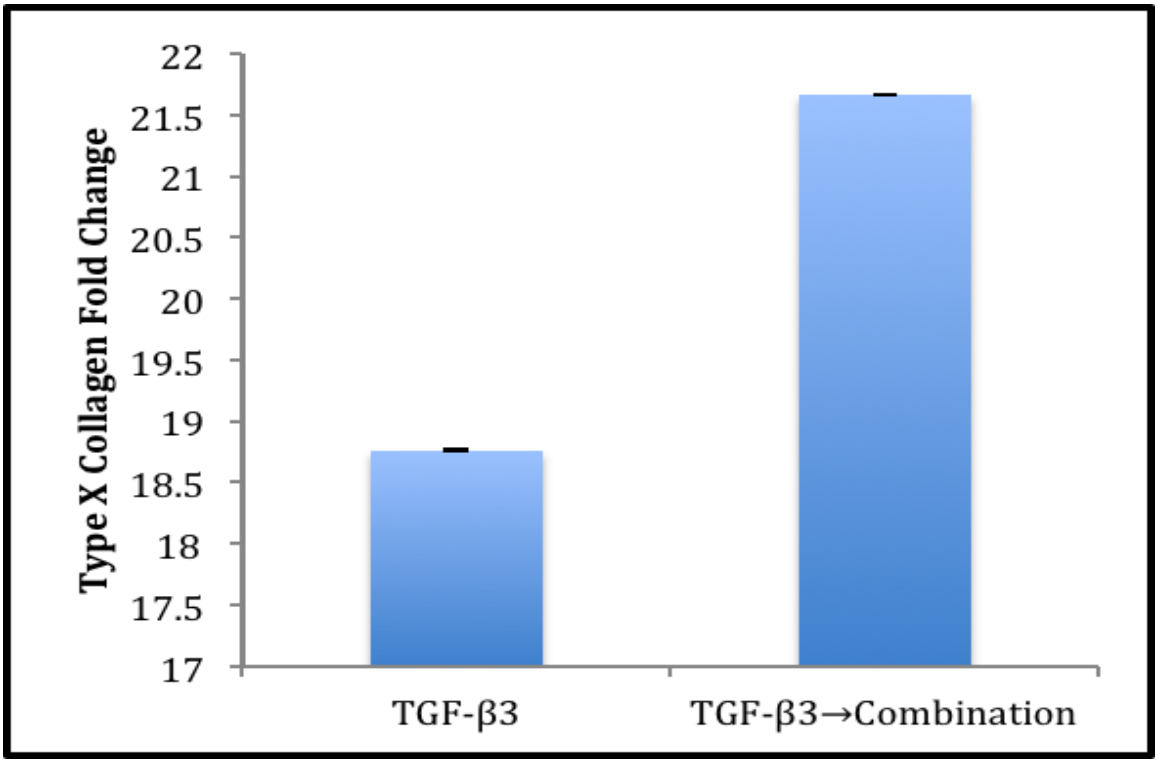

Figure 3.16 Total type X collagen mean fold change in pellets over time for second follow-up experiment.

CHAPTER IV

DISCUSSION

In this study, the concentration and time interval of growth factors, TGF- β3 and BMP-13, are manipulated in order to determine their effect on chondrogenic differentiation of bone marrow-derived MSCs. The hypothesis, that human bone marrow-derived MSCs can be driven to differentiate toward a chondrogenic lineage after the application of BMP-13, was tested by the analyses presented. The results indicate that the application of BMP-13 alone does not exert a strong chondroinductive effect on MSCs. However, the data does suggest that BMP-13 has the ability to sustain chondroinduction to a certain extent for up to 18 days following initiation by 3 days of exposure to TGF-β3.

The total GAG content was found to be significantly higher for Group I compared to that of Group II and Group IV at the first experimental time point. Group III was not considered significantly different from any of the other treatment groups at Day 3. Following the initial time point, GAG content for all treatment groups was significantly higher than on Day 3 by 135%, and all groups did not significantly differ with regards to GAG content. The significant increase in GAG content suggests that all Groups promote differentiation of MSCs from Day 3 to Day 10. From Day 10 to Day 21, GAG content was not significantly different for all treatment groups between time points, and treatment groups again exhibited no significant difference in GAG content when compared to one

another. Previous studies have reported an initial increase, and the leveling off of GAG content in cartilage tissue engineering [49]. This leveling of GAG content could be associated with a higher turnover per cell accompanying the decrease in DNA content presented across groups [30]. Previous studies, comparing seeding cell density, have reported that a higher initial cell-density has been proven to produce more proteoglycan in the presence of TGF- β3 [50, 51].

The total DNA content was found to be similar among the treatment groups at the first experimental time point. Following the initial time point, there is a significant decrease in DNA content in all treatment groups, suggesting cellular death. Group I and Group III had significantly higher DNA content at Day 10 and Day 21 compared to that of Group II and Group IV. Group I also had a significantly higher DNA content than Group III at Day 21. All treatment groups containing growth factors, Group II, Group III, and Group IV, had a significantly lower DNA content compared to pellets cultured in expansion medium, Group I. Groups do not demonstrate a significant change in DNA content from Day 10 to Day 21, indicating the maintenance of cell density. The results suggest that MSCs were lost at the initiation of chondroinduction. The sections stained with H&E also indicate the loss of cells, which is indicated by the collection of cellular debris in the center of all pellets over time. The loss of MSCs in pellet culture with additional growth factors has been reported by previous studies [52, 53]. These studies also reported loss of DNA content in pellets induced by TGF-β and BMP growth factors, suggesting little cell proliferation or apoptosis throughout the course of differentiation.

Aggrecan fold change was found to be significantly higher for Group II, Group III, and Group IV compared to that of Group I at the first experimental time point.

Aggrecan expression for all treatment groups express a negative fold change value, indicating little presence or absence of the aggrecan protein at Day 3. Following the initial time point, Group II expresses a significantly higher mean fold change compared to that of Group III at Day 10. After the removal of growth factor TGF-β3 and the addition of growth factor BMP-13, Group IV experiences a significant decrease in mean fold change in aggrecan gene expression from Day 3 to Day 10. At Day 10, all treatment groups again express a negative aggrecan mean fold change. Treatment groups at Day 21 all exhibit a significant difference from one another. Group II expresses a significantly higher mean fold change and Group I a significantly lower mean fold change. Group II and Group III both exhibit positive mean fold change aggrecan expression by Day 21. Aggrecan message was not detected in the MSCs of Group I by RT-PCR, but appeared after treatment with TGF-β3 and BMP-13 after 21 days. The results suggest that growth factor BMP-13, similar to that of TGF-β3, originally do not encourage the expression of aggrecan, but slowly seem to support the presence of aggrecan with a positive mean fold change by Day 21. The initial absence of aggrecan expression for groups may be an effect of pelleting at the initiation of the experiment. Previous studies have also reported that growth factor BMP-13 induced upregulation of the mature chondrocyte marker, aggrecan [37].

COMP mean fold change was found to be significantly higher for Group II and Group IV compared to that of the remaining groups at the initial experimental time point. Following Day 3, Group II continues to express a significantly higher mean fold change compared to the remaining treatment groups at both Day 10 and Day 21. Group II is the only treatment group that is able to initiate, sustain and increase COMP gene expression

throughout the experiment. Group IV initiates the production of COMP at Day 3, but following the removal of TGF-β3 and addition of BMP-13 COMP mean fold change significantly decreases. COMP message was not detected in the MSCs of Group I by RT-PCR and appeared after treatment with TGF-β3. The results and previous studies indicate the ability of TGF- β3 to initiate COMP expression, and the limited ability BMP-13 to initiate or sustain the expression of the protein [30].

Type I collagen expression for Group II and Group IV exhibit a significantly higher mean fold change compared to the remaining groups at the initial experimental time point. Following Day 3, Group II continues to exhibit the highest significant mean fold change among treatment groups at Day 10 and Day 21. Group II, Group III, and Group IV have significantly higher mean fold change type I collagen expression compared to that of the MSCs of Group I at Day 21. Following the removal of TGF-β3 and the addition of BMP-13 to DCM in Group IV at Day 3, type I collagen expression remains relatively unchanged. The results suggest that BMP-13 may be capable of sustaining the type I collagen expression initiated by TGF-β3. Type I collagen is detected under the influence of both growth factors.

In terms of chondrogenic differentiation, type I collagen is not desirable. Growth factor BMP-13 demonstrates a significantly lower expression of type I collagen, compared to that of the growth factor TGF- β3. Barry et al. also report the type I collagen uniform expression throughout differentiation in the presence of TGF- β3. Collagen type I gene was expressed in the TGF- β3 and BMP-13 treated pellets. These results suggest that the MSCs may not have completely differentiated into chondrocyte-like cells after a 21-day treatment of growth factors, and has also reported seen in previous studies [50].

Nochi et al. also reported no suppression of type I collagen by the growth factor BMP-13, although the osteogenic phenotype associated with type I collagen had been diminished through the use of another member of the bone morphogenic protein family, BMP-2.

Type I collagen message showed a gradual increase in the untreated MSCs of Group I, and was readily detected after 21 days in pellet culture. The presence of type I collagen in the control group, Group I, at Day 21 suggests the osteogenic potential of human bone marrow-derived MSCs, which has been suggested in previous studies in regards to the C3H10T1/2 cell line [37].

Type II collagen expression is significantly higher for Group II and Group IV compared to that of Group III at the initial experimental time point. Following Day 3, all treatment groups express no significant difference in mean fold change for type II collagen expression. However, Group IV undergoes a significant decrease in mean fold change in type II collagen expression, following the removal of growth factor TGF-β3 and addition of growth factor BMP-13 to the DCM. At Day 21, Group II shows a significantly higher type II collagen expression compared to that of the remaining treatment group, suggesting that TGF- β3 may have the ability to increase type II collagen expression more than that of BMP-13. Type II collagen expression remains relatively unchanged for Group I, Group II and Group III over the course of the experiment. Type II collagen message was uniformly detected in the MSCs of Group I and throughout differentiation in pellet culture. Previous studies indicate a gradual increase over time in type II collagen under the influence of TGF- β3 [30], and an upregulation in type II collagen of MSCs stimulated by BMP-13 [37, 54]. However, this

could be a reflection of the differentiation state of cells under study, rather than the signals being transduced by the BMP-13 growth factor.

Type X collagen expression is significantly higher for Group II and Group IV compared to that of Group I and Group III at the initial experimental time point. At Day 10, Type X collagen expression for Group II expresses a significantly higher mean fold change compared to the remaining treatment groups. Group IV experiences a significant decrease in type X collagen expression from Day 3 to Day 10. Group II type X collagen expression experiences a significantly higher mean fold change compared to that of the remaining treatment groups at Day 21. As expected, Group II, under the influence of TGF-β3, experiences a significantly higher mean fold change compared to that of all treatment groups, and undergoes significant increases in type X collagen over time. Previous reports also have stated that in the presence of TGF-β3, MSCs display an up-regulation of type X collagen over time [30, 55]. However, Group IV undergoes a significant decrease in type X collagen expression following the removal of growth factor TGF- β3 and addition of growth factor BMP-13 to DCM. The results suggest that BMP-13 does not promote the expression of type X collagen.

Following initial experiments, the question still remained whether BMP-13 was responsible for suppressing the expression of certain genes, or if it was the removal of TGF-β3 that lead to the decrease in gene expression. Group IV undergoes a significant decrease in gene expression for aggrecan, COMP, type II collagen, and type X collagen at Day 10, following the removal of TGF-β3 and the addition of BMP-13. Follow-up experiments were conducted in order to further determine the influence of BMP-13 on MSCs.

The first follow-up experiment was designed to determine whether BMP-13 was responsible for any elevation in chondrocyte gene markers after 3 days of chondroinduction by TGF-β3. The results suggest the limited ability of BMP-13 to induce chondroinduction of MSCs. The aggrecan expression for Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) undergoes a significant decrease following the removal of TGF- β3 at Day 3. Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) exhibit no significant difference in aggrecan expression from one another at Day 10. The results suggest that the removal of TGF-β3 is responsible for a significant decrease in mean fold change of aggrecan expression. BMP-13 is not responsible for the suppression of the aggrecan gene, but may experience an initial decrease in mean fold change like that seen in the initial experiment, to be followed by a significant increase in aggrecan mean fold change.

The COMP expression for Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) undergoes a significant decrease following the removal of TGF- β3 at Day 3. Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) exhibit no significant difference in COMP expression from one another at Day 10. The results suggest that the removal of TGF-β3 is responsible for a significant decrease in COMP expression. BMP-13 is not responsible for the suppression of the COMP gene.

Type II collagen expression for Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) remains relatively unchanged following the removal of TGF- β3 at Day 3. Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) exhibit no significant difference in type II collagen expression from one another at Day 10. The results suggest that the initial presence of TGF- β3 is responsible for the expression of type II collagen.

BMP-13 does not seem to play a vital role in the up-regulation or sustainability in the expression of type II collagen.

Type X collagen expression for Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) undergoes a significant decrease following the removal of TGF- β3 at Day 3. Group (TGF-β3→BMP-13) and Group (TGF-β3→DCM) exhibit no significant difference in type X collagen expression from one another at Day 10. The results suggest that the removal of TGF-β3 is responsible for a significant decrease in type X collagen expression. BMP-13 is not responsible for the suppression of the type X collagen gene.

The second follow-up experiment was designed to better understand the affect of BMP-13 on the alleviation of type X collagen in TGF- β3 induced MSCs. The results suggest the limited ability of BMP-13 to repress type X collagen following initiation with TGF-β3. Type X collagen expression for Group (TGF-β3) and Group (TGF-β3→Combination) exhibit no significant difference relative to type X collagen expression at Day 10. From the initial experiment, we recognize that TGF-β3 is responsible for a significant increase in type X collagen expression. BMP-13, when added in combination with TGF-β3, exhibits no capabilities of suppressing type X collagen expression.

In summary, this study has demonstrated that chondrogenesis of human bone marrow-derived MSCs can be sustained with the treatment of BMP-13 as attested by the results of gene expression analyses and histological and immunohistochemical assessments. BMP-13 appears to have some limited capacity for chondroinduction, much less than that of pellet cultures stimulated by TGF-β3. However, the chondroinduction stimulated by BMP-13 is not accompanied by an increase in type I collagen and type X collagen expression as demonstrated with TGF-β3.

In this study, we proved that BMP-13 is capable of stimulating proliferation and the aggregation of undifferentiated MSCs. BMP-13 is able to sustain type II collagen expression following initiation with TGF-β3, but alone does not have the capability to express the protein considered to be the basis of articular cartilage. BMP-13 does not exert a strong influence on the expression of chondrogenic specific markers, aggrecan, COMP, and type II collagen, but BMP-13 does not act to suppress the expression of these genetic markers. Initial experiments prematurely suggested that BMP-13 might be responsible for the suppression the expression of aggrecan, COMP, type II collagen and type X collagen. However, follow-up experiments reported that the removal of TGF-β3 was responsible for the significant decrease in gene expression. In order to improve this method of tissue engineering, future studies should be conducted to establish the model growth factor and MSC conditions required to simulate the properties consistent with articular cartilage.

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