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# The Chondrogenic Potential of Hematopoietic Stem Cells

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by

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ABSTRACT

Cartilage is a complex tissue that has a very low regenerative capacity, which significantly hinders its ability to repair itself. Stem cell therapy for the purpose of cartilage regeneration has gathered much attention. Bone marrow consists of two types of stem cell populations, the mesenchymal stromal cell (MSC) and the hematopoietic stem cell (HSC). While MSCs have been demonstrated to have the capacity of differentiating into osteoblasts, chondrocytes, and adipoctyes, recent studies are beginning to delve into the possibility of hematopoietic stem cells also having this differentiation capability. Our laboratory has demonstrated an HSC origin for cells such as osteoblasts, cancer-associated fibroblasts, and immature adipocytes, revealing the ability of HSCs to give rise to cell types not typically associated with this lineage. Of particular relevance to this study are our *in vivo* studies using a clonal cell transplantation model that demonstrated that HSCs can give rise to hypertrophic chondrogenic cells during non-stabilized fracture repair. This work has led to the hypothesis that chondrocytes differentiate through an HSC lineage. To address this hypothesis, *in vitro* studies first elucidated culture conditions for HSC-derived chondroprogenitors. Using the defined cell types of articular cartilage chondrocytes and the chondrogenic cell line ATDC5 morphology, and *in vitro* chondrogenic potential was examined. The ability of TGF-β1, TGF-β3, BMP-2, and BMP-7 to promote chondrogenesis in the ATDC5 cell line was determined. The potency of these factors on chondrogenic differentiation was demonstrated with Alcian Blue staining and gene expression profiling by qRT-PCR analysis. Culture conditions and endpoints as defined

using this cell line then served as a basis for culture of HSC enriched cells. Novel HSC origins for chondroprogenitors were examined through chondrogenic induction of two HSC-derived cell sources: non-adherent bone marrow fraction (the fraction enriched for HSCs) and a monocytic precursor lineage. Chondrogenic differentiation and maturation was confirmed by comparing morphology and Alcian Blue staining to those derived from articular cartilage chondrocytes and ATDC5 cells and by the presence of immunofluorescent staining for cartilage specific markers, Collagen II and Aggrecan. Together, these data demonstrate the potential of the HSC to give rise to chondrocytes via the monocyte lineage. Future studies directed at determining the mechanisms regulating the HSC contribution to chondrogenic lineages and the associated process of differentiation/maturation has the potential to enhance stem cell therapies for cartilage repair.

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LIST OF ABBREVIATIONS

HSC – Hematopoietic Stem Cell

MSC – Mesenchymal Stromal Cell

TGF-β – Transforming Growth Factor Beta

BMP – Bone Morphogenetic Protein

AA – Ascorbic Acid

Dexa – Dexamethasone

L-glut – L-glutamine

Col II – Collagen2α1

Acan – Aggrecan

Sox9 – Sex-determining region Y – Box9

DMEM – Dulbecco's Modified Eagle Medium

αMEM – Alpha Minimal Essential Medium

$CoCl_2$ – Cobalt Chloride

VEGF – Vascular Endothelial Growth Factor

FBS – Fetal Bovine Serum

PS – Penicillin/Streptomycin

GAGs – Glycosaminoglycans

PBS-Az – Phosphate Buffed Saline with Sodium Azide

BSA – Bovine Serum Albumin

ITS – Inuslin, Transferrin, Selenium

M-CSF – Macrophage Colony Stimulating Factor

IL-3 – Interleukin-3

OA – Osteoarthritis

ECM – Extracellular Matrix

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## BACKGROUND

### *Introduction to cartilage*

Cartilage is a specialized connective tissue that is categorized into three different types: fibrocartilage, elastic, and hyaline. Fibrocartilage can be found in anatomical locations such as intervertebral discs, and its matrix is mainly composed of type I collagen; however it also contains collagen II. Elastic cartilage can be found in the external ear, and its matrix is composed mainly of elastin fibers. Hyaline cartilage is most commonly found on the articulating surfaces of long bones, but can also be found in places such as the ribs and trachea[15,29,41]. Articular cartilage is made up of hyaline cartilage. The components of articular hyaline cartilage are very specific for its function in providing a smooth, lubricated surface for articulation of joints[41]. Articular cartilage is a rare tissue in that it is naturally avascular, alymphatic, and is not innervated[10]. These qualities present unique conditions within the tissue. In order to receive nutrients, cartilage cells, termed chondrocytes, must rely heavily on the diffusion of nutrients from the synovial fluid. This avascularity, which contributes to a lack of ability for self-repair, also creates a hypoxic microenvironment for the cells within the tissue[18].

Chondrocytes are the only cellular component of cartilage tissue and represent only 1-2% of the total cartilaginous matrix volume. Despite this low cell density, chondrocytes synthesize, secrete, and maintain an abundant extracellular matrix (ECM) throughout the process of chondrocyte development and their mature

state. The ECM of articular cartilage is mainly composed of collagen II for tensile strength and glycosaminoglycans (GAGs) for compressive strength. Collagen II represents the majority of the collagens in the matrix while a lesser volume is attributed to other minor collagens, such as collagen IX, XI, III, XII, VI, I, and proteoglycans, such as Aggrecan, which are made up of GAGs. The remaining volume consists of water[10,28,41].

*Chondrocyte differentiation*

The different stages in chondrogenesis include the condensation of stem cells (i.e. mesenchymal stem cells), proliferation and differentiation of chondroprogenitors, differentiation and maturation of the chondroblasts into chondrocytes, and finally terminal differentiation of chondrocytes. Terminal differentiation refers to the process of chondrocytes becoming hypertrophic and beginning to express more Collagen X rather than Collagen II. These hypertrophic chondrocytes will eventually be involved in the development of bone through the process of endochondral ossification, which is the process by which bone develops through a cartilage intermediate. Chondrocytes not in the hypertrophic zone, those in the upper zones of articular cartilage, do not synthesize Collagen X unless damaged or in a disease state.

Chondrocyte differentiation is a highly regulated process involving multiple growth factors and differential signaling among a variety of genes. Transforming growth factor beta (TGF-β) is one of the major influencing factors in

chondrogenic differentiation. While TGF- β1, TGF- β2, and TGF- β3 are all biologically involved in inducing chondrogenesis, TGF- β1 is the main regulator. For research purposes, TGF- β1 and TGF-β3 are most commonly used for inducing chondrogenesis *in vitro*. TGF- β1 is a major initiator for the condensation of stem cells and is a driving factor for their differentiation as well[10]. TGF-β1 stimulates the proliferation and ECM production of chondrocytes and can also work to inhibit endochondral ossification through the Smad 2/3 signaling pathway[24]. TGF-β1 stabilizes the Sox9 transcription complex through Smad2/3[42]. Once condensation is established, the cells begin to differentiate and the transcription factor SOX9 initiates the expression of chondrogenic markers such as type II collagen and aggrecan[12]. Sox9 not only acts to promote chondrogenesis, but also serves to inhibit genes associated with endochondral ossification such as VEGF (Vascular Endothelial Growth Factor), OSX (Osterix), and RUNX2[10]. TGF- β1 can also prevent terminal differentiation by increasing PTHrP. This hormone inhibits Col X, VEGF, osteocalcin (OCN), and matrix metalloproteinase-13 (MMP-13), all markers of terminal differentiation and endochondral ossification. Other factors, such as bone-morphogenetic protein 2 (BMP-2) and bone-morphogenetic protein 7 (BMP-7), have also been established to play a role in the condensation and differentiation phases of chondrogenesis. BMP-2 can be involved in inducing SOX9 expression, the transcriptional regulator of chondrogenesis, and the formation of a richer ECM. Sustained BMP-2 activity at higher concentrations can promote hypertrophy of chondrocytes by causing expression of type X collagen. This eventually leads to calcified

chondrocytes developing into mineralized bone, the natural progression of endochondral ossification. BMP-2 also works through the Smad1/5/8 pathway to interact with the bone associated transcription factor Runx2 to increase hypertrophy and promote osteogenesis[10]. Smad2/3 and Smad1/5/8 work with Smad4 once activated to translocate to the nucleus[43]. In contrast to BMP-2, BMP-7 has been shown to have a more chondro-protective effect. Caron et. al demonstrated BMP-7 treated progenitor cells had higher expression of Col2a1 and lower expression of Col10a1 in comparison to BMP-2 treated cells[8]. Furthermore, expression of hypertrophic markers Runx2, ALP, and MMP13 were also lower in BMP-7 treated cells in comparison to BMP-2. This data would indicate BMP-7 has the potential to promote chondrogenesis while also suppress hypertrophy, perhaps making it a better growth factor for chondrogenic differentiation than BMP-2[8].

*Articular cartilage response to injury*

The importance of the function of articular cartilage to provide a smooth articulating surface can be greatly appreciated and better understood when the results of injuries are examined. Cartilage can be damaged either by one excessive load on the joint or by repetitive overloading of that joint. These injuries are classified into three categories based on the depth of the injury: microdamage, chondral fracture, or ostesochondral fracture.

Microdamage may not be visible on the surface of the cartilage, however, it still results in death of chondrocytes in the area of injury and disruption of the

surrounding matrix. Over time this will lead to degradation of the matrix resulting in a rougher surface compared to the normal smooth surface. If the initial injury is caused by repetitive overloading, the damage often goes unnoticed due to the lack of innervation in cartilage tissue.

A chondral fracture stays within the thickness of the articular cartilage and does not penetrate the subchondral bone, thus does not involve the vascular system. Because chondrocytes are not migratory and are mostly trapped in their respective lacuna, they will not migrate into the site of the defect. Although an initial proliferative response may occur by adjacent chondrocytes, this proliferation is brief and does not make up for the loss of chondrocytes or matrix. Therefore, with time the surrounding regions will wear away exposing the surface of the subchondral bone below. This exposure then allows bone surfaces to grind against each other upon movement of the joint, which will cause development of symptoms such as pain and locking of the joint.

Osteochondral fracture is different in that it penetrates the subchondral bone allowing the release of growth factors and stem cells and progenitor cells from the bone marrow below. A hematoma can form within the defect that will eventually develop into a fibrin clot. This repair tissue will contain chondrocytes after six to eight weeks that will begin to synthesize a new matrix. However, the resulting matrix is of a fibrocartilage nature rather than hyaline and contains more type I collagen instead of type II collagen. The mechanical stability of the fibrocartilage that is formed will fail over time and cause eventual degradation of the tissue and exposure of bone.

All three of these types of injuries lead to degradation of articular cartilage and increase the likelihood of joint diseases such as osteoarthritis (OA), a disease in which the cartilage surface is worn away[45]. OA is the most common joint disease in the world affecting 60% of men and 70% of women over the age of 65 and is becoming increasingly prevalent due to the aging population and rising level of obesity[37,46,27]. Therapeutic interventions have been utilized attempting to prevent OA from occurring by treating initial chondral defects with drilling to stimulate the bone marrow release, osteochondral allograft, or autologous chondrocyte implantation (ACI); however, these therapies are highly limited and provide minimal improvement. Eventually OA may occur and progress to the point where a joint replacement is the only repair method available. Stem cell therapies are now being investigated for use due to a higher proliferative capacity as compared to chondrocytes in culture and the potential to eliminate the consequence of donor site morbidity as seen with ACI[44]. The plasticity of stem cells would also potentially increase the likelihood for regeneration of the tissue versus repair. Regeneration of the tissue would restore the natural structure and function of the original compared to the fast process of repair, which attempts to resolve an injury, yet results in a tissue that is not identical to the original and is not integrated with the existing tissue[3]. Regenerating the natural cartilage tissue would help prevent the incidence of OA and eliminate the need for joint replacement.

*Origin of Articular Chondrocytes*

Cartilage makes the framework for the developing skeleton before its transition into bone, a process known as endochondral ossification. The development of this cartilage framework occurs in stages beginning with the formation of a series of specific zones culminating from the condensation of progenitor cells that are in close proximity to each other and the aggregation into an interzone that forms a future joint. These interzone cells are suggested to give rise to the articular layer of long bone anlagen while more transient chondrocytes are derived from the mesenchymal condensations that form the bulk of the anlagen. Thickening of the transient interzone contributes to the lengthening of the long bone anlagen, and differentiation of these interzone cells into chondrocytes outlining the soon-to-be bone structure. However, the outer population of these chondrocytes that are to form the articular surfaces differ from the chondrocytes that reside in the layers below. The articular surface chondrocytes maintain a specific phenotype. In contrast, the more transient chondrocytes of deeper layers have a more dynamic phenotype over time displaying the ability to more readily undergo proliferation, maturation, hypertrophy, and apoptosis in order to eventually be replaced by osteoblasts. This duality of the chondrocyte perhaps implies a distinct embryonic origin of the articular chondrocyte as compared to that of the more transient chondrocyte[32]. Given this implication, it is possible that articular chondrocytes differentiate separately from transient chondrocytes owing to potential differences in the stem cell from which they arise. In other words, articular chondrocytes may differentiate from stem cells not commonly thought to create cartilage. If a

transient chondrocyte's major purpose is to eventually turn over and allow bone development while an articular chondrocyte remains a stable chondrocyte for its entire lifespan, it is possible the two cell types have varying developmental lineages.

*MSC Origin of Chondrocytes*

While it is known that chondrogenesis begins with the condensation of stem cells, the identity of these stem cells is less clear. Mesenchymal stromal cells (MSCs) are known to give rise to multiple mesenchymal lineages including myocytes, adipocytes, chondrocytes, osteoblasts, and tenocytes[27]. Of particular interest are the studies that have demonstrated that MSCs from the bone marrow have the potential to give rise to chondrocytes *in vitro.* Pittenger et al demonstrated that adult mesenchymal cells lacking hematopoietic markers could be isolated from bone marrow and differentiated under the influence of TGFβ-3 into chondrocytic cells as shown by morphological changes and staining with C4F6 monoclonal antibody to type II collagen[33]. Numerous studies thereafter conducted by various research groups have tested a variety of enhancing factors on these isolated MSCs such as dexamethasone[31], fibroblast growth factor (FGF-2)[14], and hyaluronic acid (HA)[6] in addition to TGFβ. Although the resulting data is convincing, evidence for a true lineage of the chondrocyte is still lacking. One issue is the lack of specific definition of MSCs. An *in vivo* study conducted by Wakitani, et. al. showed autologous human bone marrow mesenchymal cells embedded in a collagen gel and transplanted into cartilage defects in

osteoarthritic knee joints had higher arthroscopic and histological grading scores than that of the cell-free group 28-95 weeks after transplantation[47]. However, the only way the study defines their mesenchymal stem cells is by the adherence to plastic. No cell surface markers were identified, no other cell separation techniques were applied, and no tri-lineage differentiation capabilities were demonstrated. Interestingly, the authors do acknowledge it is unknown whether the transplanted cells differentiated or solely produce factors that promote the differentiation of other existing cells. The *in vitro* study mentioned above by Handorf et. al. that tested the effects of priming MSCs with FGF-2 for chondrogenic differentiation also only used the adherence to plastic as their determining factor for identification of their MSCs[14]. In contrast, Pittenger et. al. defined the MSCs by adherence, positive for common mesenchymal cell surface proteins, negative for common hematopoietic surface markers, and tri-lineage differentiation[33]. However, a definitive set of universal mesenchymal surface markers has yet to be determined and implemented into current research.

*HSC Origin of Chondrocytes*

Although chondrocytes have been established to originate from a mesenchymal lineage, recent studies have indicated that alternative precursors and varying lineages may also be a source of this cell type. Of particular relevance for the proposed study are publications suggesting that the hematopoietic stem cell (HSC) can contribute to chondrogenic tissues. Like the MSC, the HSC resides in the bone marrow and serves as the progenitor cell for all blood lineages. In

addition to blood lineages, the HSC has been shown to give rise to mesenchymal cells in tissues including fibroblasts[20], adipocytes[39], and osteoblasts/osteocytes[26], cell once thought to be derived solely from the MSC. In addition, the HSC is thought to be the precursor of the fibrocyte, a blood borne cell that expresses both hematopoietic and fibroblastic markers[7]. Fibrocytes are known to express hematopoietic stem cell antigens and monocyte lineage markers in addition to fibroblast product markers and serve as bone-marrow derived mesenchymal progenitors[5]. Taken together, these studies suggest that the HSC may also give rise to chondrocytes. In support of this, a study conducted by Choi et al. presented data that show the differentiation of human circulating fibrocytes into chondrocytes[9]. These fibrocytes were isolated from peripheral blood mononuclear cells and cultured in a chondrogenic media containing TGFβ-3. Since the hematopoietic stem cell marker CD34 surface antigen and CD45 were expressed on these cells, this could suggest an HSC origin of these cells despite the expression of common MSC markers as well. The implication would be the progression of HSC -> fibrocyte -> chondrocyte, with the fibrocyte being a potential intermediate in this proposed lineage. Another study involving articular cartilage regeneration demonstrated improved results when autologous peripheral blood stem cells (PBSCs) were injected into the defect in combination with hyaluronic acid (HA) compared to HA injection alone[38]. While this study did not specifically define the stem cells used, it raises the question of which stem cells in the peripheral blood were able to aid the regeneration of the articular hyaline cartilage seen in the results. Furthermore, a study conducted by Pufe et.

al. demonstrated that cells of monocytic origin derived from peripheral blood were able to be differentiated into collagen II-producing chondrocytes when cultured with specific growth factors[35]. In addition to this, a study conducted by Kuwana et. al. also demonstrated monocytic cells to have the ability for chondrogenic differentiation[19]. In this study, a cell population collected from peripheral blood mononuclear cells with the phenotype $CD14^+$ $CD45^+CD34^+$ type I $collagen^+$ were cultured in serum-free medium with TGF-β1 for three weeks. After this time period, the newly termed monocyte-derived mesenchymal progenitor (MOMP) showed positive collagen II staining as well as positive CD45 and Sox-9 immunohistochemical staining[19]. Thus, these studies support a monocytic origin for chondrocytes or in the least a peripheral blood stem cell source for chondrocytes[35,19].

*Clinical Impact*

Identifying a novel stem cell source to be used as cartilage defect therapies could potentially reduce the incidence of OA or minimize the need for surgical intervention. Furthermore, due to stem cell plasticity, stem cells may have the ability to regenerate the tissue rather than simply repair it. A study conducted by Wakitani et. al demonstrated that osteochondral defects treated with bone marrow stem cell transplantation showed articular cartilage growth arthroscopically and histologically 42 weeks postoperatively; however, clinical outcomes between control groups and transplant groups were similar[47]. Hematopoietic stem cells having the ability to differentiate into chondrocytes

would provide a new source of stem cells that not only is easily attainable (i.e. from mobilized peripheral blood), but also more readily identifiable. Due to definitive cell surface markers, hematopoietic stem cells can be isolated in a more straight-forward manner, whereas mesenchymal stem cells do not have a universally agreed upon phenotype. Cells with a more specific phenotype would help ensure that the right cells are being used to heal the defect rather than cells that may cause potential issues.

# CHAPTER 2: RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

## *Rationale*

Osteoarthritis and other related orthopaedic conditions stem from the degeneration of articular cartilage whether from direct trauma or natural biological processes, resulting in pain and disability. Due to the low regenerative capacity of articular cartilage, treatment options are limited. Current clinical procedures such as autologous chondrocyte implantation (ACI), drilling, and osteochondral allograft have inadequate success because generally fibrocartilage, rather than hyaline cartilage, results and donor site morbidity may occur[44]. Hyaline cartilage on articular surfaces is specifically designed to withstand the compressive forces of everyday activities; fibrocartilage is less mechanically suited and has a greater risk of breaking down and leading to earlier degeneration. Therefore, the ultimate therapeutic goal is to regenerate the normal hyaline cartilage structure and restore full function. In recent years, the use of stem cells in therapeutics has greatly increased and regeneration of articular cartilage through the use of stem therapies has become an attractive goal. To utilize adult stem cells for potential stem cell therapy in cartilage defects, it is first necessary to explore the origins of chondrocytes. Better defining a chondrogenic lineage will aid in the application of differentiating these cells for therapeutic purposes. Bone marrow consists of two types of stem cell populations, the mesenchymal stromal cell (MSC) and the hematopoietic stem cell (HSC). While MSCs have been demonstrated to have the capacity of

differentiating into osteoblasts, chondrocytes, and adipocytes, recent studies are beginning to delve into the possibility of HSCs also having this differential capability. Our laboratory has previously demonstrated an HSC origin for cells such as osteoblasts[26], cancer-associated fibroblasts[25], and immature adipocytes[39], revealing the ability of HSCs to give rise to cell types not typically associated with this lineage. Of particular relevance to this study are our *in vivo* studies using our clonal cell transplantation model which demonstrated that HSCs can give rise to hypertrophic chondrogenic cells during non-stabilized fracture repair as well as give rise to chondrocytes in a normal knee joint[26]. Based on these studies, we sought to examine the ability of HSC/HSC lineage cells to give rise to chondrocytes *in vitro*.

***Hypothesis***

**We hypothesized that hematopoietic stem cells can differentiate into chondrocytes.**

***Specific Aims***

**This hypothesis was tested through the following specific aims:**

**Specific Aim 1:** ***Establish in vitro culture methods for differentiation of cells to chondrogenic lineage through defined cell types.*** Articular cartilage cells were first harvested and cultured for observational purposes of growth patterns and served as a positive primary cell end-point model for the differentiation studies. Methods of chondrocyte differentiation were then tested using specific inducing factors including ITS, TGF-βs, and BMPs on the chondrogenic cell line ATDC5. End-point profiles of these cultures were established through staining for specific molecular markers by immunofluorescence and immunohistochemistry and by the specialized chemical staining of Alcian Blue. Confirmation of the staining was carried out by qRT-PCR.

**Specific Aim 2:** ***Demonstrate the differentiation of chondrocytes from an HSC lineage in vitro.*** Sources of primary cells (non-adherent HSC-enriched bone marrow and monocyte progenitors) were investigated along with isolation methods and culture conditions in order to determine the best culture conditions to yield HSC-derived chondroprogenitor cells. The end-point molecular marker profiles and gene expression profiles of HSC-derived chondrocytes were compared to those of the chondrogenic cell line ATDC5 (from Aim 1) in order to profile differentiation and maturation from an HSC source to the chondrogenic lineage.

# CHAPTER 3: ESTABLISHMENT OF *IN VITRO* CULTURE METHODS FOR CHONDROGENIC DIFFERENTIATION

**Specific Aim 1: *Establish in vitro culture methods for differentiation of cells to chondrogenic lineage through defined cell types.***

## 3.1 Introduction

Primary articular chondrocytes are often harvested in research to study growth patterns of these cells and learn more about ways to use them in clinical practice for cartilage repair. Because these cells are already differentiated and committed to a specific cell type, they often serve as a good end-point model for differentiation studies. Articular chondrocytes are currently used clinically in autologous chondrocyte transplantation. In addition to native articular chondrocytes, the ATDC5 cell line is used frequently in research to study cartilage development and endochondral ossification due to its growth process that is analogous to chondrocyte differentiation. The cell population in a model cell line is more homogenous and consistent while being less influenced by passage number as compared to isolated primary cells, and thus provides a better platform for standardization of culture conditions, characterization, and endpoint analyses. These features also allow molecular mechanisms to be studied in an easier manner. In this aim, we first used primary cells in order to establish how these cells may grow *in vitro,* determine cell morphology and examine the presence of cartilaginous nodule formation. These primary

chondrocytes were cultured in an undefined serum containing (FBS) media because these cells were already in a differentiated state. ATDC5 cells were then used to better define culture conditions. Identification of which growth factors and supplements that promoted chondrogenesis and the concentration at which these factors most influenced cell differentiation was then determined. Findings from this aim were then applied to primary HSC-enriched/derived cells in Aim 2.

### 3.2 Materials and Methods

*Isolation and Culture of Articular Cartilage*

Articular cartilage was isolated for digest or explant culture by removal of the femoral and tibial condyles of the 5-9 week old C57Bl/6 or enhanced green fluorescent protein (EGFP) male or female mice. The cartilage pieces were incubated at 37 degrees C and underwent collagenase digestion by collagenase D (Roche) at a concentration of 3mg/mL for at least 30 min to an hour. The solution was collected and pipetted successively through decreasing pipette sizes in order to disperse cell aggregates. The solution was then filtered through a sterile 40 μm cell strainer and collected for centrifugation. Cells were resuspended and plated in Dulbecco's Modified Eagle Medium (Gibco), 10% Fetal Bovine Serum (Atlanta Biologicals), 1% Penicillin/Streptomycin (Atlanta Biologicals or Quality Biologicals), supplemented with 2mM L-glutamine (Sigma), and plated on 8-well fibronectin coated plates. For explant cultures, the femoral condyle was collected from the digest dish after incubation and plated on an 8-well fibronectin coated plate in DMEM/10% FBS/1%PS, supplemented with 2mM

L-glut. An explant culture was used as a means of harvesting residual articular chondrocytes that were not collected by the digestion alone. For explant culture, the femoral condyle was placed in a well of an 8-well fibronectin plate. Media was changed every 2-3 days for all cultures and the cells were cultured for 2-3 weeks. The method of harvesting and culturing the articular cartilage was adapted from the protocol of Gosset et. al.[13].

*Culture of ATDC5 cells*

ATDC5 (Sigma) cells were plated at a starting concentration of 4 X $10^4$ cells/well for a 12-multiwell plate. The cells were cultured in a basic media containing 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Gibco) supplemented with 5% fetal bovine serum (Atlanta Biologicals) and 2mM L-glutamine (Sigma) at 37 degrees C under 5% $CO_2$. The culture medium was changed every 2-3 days.

*Differentiation and Supplementation of ATDC5 cells*

ATDC5 cells were cultured in 12-well plates for 21 days in a base media with 5% serum. The supplement ITS (insulin, transferrin, selenium) (Sigma) was added at a concentration of 1% in order to stimulate initial differentiation of the cell line. The growth factors TGF-β1, TGF-β3, BMP-2, and BMP-7 (R & D Systems) were tested separately to promote chondrogenesis in conjunction with the ITS supplement. TGF-β1 and TGF-β3 were added at a concentration of 10ng/mL. For the experiment to select optimal BMP-2 and BMP-7 concentrations

for chondrogenesis of ATDC5 cells, the following concentrations of BMP-2, 0, 10, 50, 100, and 500 ng/mL, and the following concentrations of BMP-7, 0, 5, 10, 50, and 100 ng/mL were tested. Quantitative analysis carried out for the Alcian Blue staining (as described below) in order to better identify the appropriate concentration of growth factors for subsequent experiments. The supplements L-Ascorbic Acid (AA) (Wako) and dexamethasone (dexa) (Sigma) were tested separately in order to determine potential contribution to ATDC5 chondrogenesis. AA was added at a concentration of 0.1mM while dexamethasone was added at $10^{-7}$ M. Media was changed every 2-3 days. For all experiments, growth factors and supplements were added fresh for each media change.

*Alcian Blue Staining*

To evaluate cartilage-specific proteoglycan synthesis, sulfated glycosaminoglycans (GAGs) were visualized by staining with Alcian Blue. After three weeks of culture (21 days), cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, and then washed 3-5 times with distilled water. The cells were then stained with Alcian Blue (1% in 3% acetic acid, pH 2.5, 8XG solution - Sigma) for 30 minutes and rinsed with distilled water until the water ran clear (about 5 rinses). Staining was assessed visually using a Nikon Eclipse Ti scope and quantitatively using a Synergy HT microplate reader (Biotek). Absorbance for Alcian Blue staining was read at a wavelength of 630 nm.

*Immunofluorescent Staining*

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for ten minutes at room temperature. Cells were washed with PBS containing 0.05% sodium azide 3 x 5 minutes. For aggrecan staining, cells were incubated with 5 x $10^{-5}$ U/µl Chondroitinase ABC for three hours at 37 degrees Celsius post-fixation then washed 3 x 5 minutes with PBS-Az prior to permeabilization. Chondroitinase treatment allows for better exposure for the antibody to bind to the matrix component that may otherwise be masked by other proteins. Cells were then permeabilized with Triton X-100 (0.02% in PBS) for ten minutes. Cells were washed with PBS-Az 3 x 5 minutes and then blocked in 5% donkey serum in 3% BSA-PBS for thirty minutes at room temperature. Block was removed, and the primary antibody was added (Rabbit-anti mouse Collagen II polyclonal antibody at 1:50 or Rabbit- anti-Aggrecan polyclonal antibody at 1:100– Millipore) for 45 minutes at room temperature. Cells were washed 3 x 5 minutes with PBS-Az and then blocked for thirty minutes in 5% donkey serum in 3% BSA-PBS. The secondary antibody, Donkey anti-Rabbit Cy3 (Jackson), was added at a 1:100 dilution for thirty minutes at room temperature. Cells were washed 3 x 5 minutes with PBS-Az. Hoechst (Invitrogen) was added at a 1:25000 dilution in PBS for eight minutes. Cells were washed 3 x 5 minutes with PBS-Az, and they were then mounted and cover-slipped. Immunofluorescence was visually assessed using a Nikon 90i microscope with digital camera.

*Isolation of RNA and qRT-PCR Analysis*

Cell samples were harvested at day 21 of culture for RNA extraction. Total RNA was extracted using the RNeasy Plus Mini Kit and QIAshredder. RNA concentration was measured using the Qubit 2.0 fluorometer. 1 µg of total RNA was reverse transcribed in a 20 µl reaction using the Bio-Rad iScript cDNA synthesis kit. qRT-PCR was performed in a 10 µl reaction with 2.5 µl of 1:20 diluted reverse-transcribed cDNA, the Bio-Rad SsoFast Probes Supermix, and univeral probe library (UPL) system in a Roche LightCycler 96. The cycling conditions were performed as follows: preincubation, 95° C for 10 seconds; 45 cycles of denaturation at 95° for 5 seconds; annealing at 60° C for 30 seconds with a single data acquisition taken at the end of each extension; and cooling at 40°C for 30 seconds. Triplicate reactions were run for each cDNA sample. The relative expression of each gene was quantified on the basis of Ct value measured against an internal standard curve for each specific primer set using the LightCycler 96-supplied software. The concentration of each sample was then normalized to Hprt[48]. qRT-PCR was carried out at endpoints for expression of Collagen II, Aggrecan, and Sox9 using the LightCycler96. Collagen II, Aggrecan, and Sex-determining region Y - Box9 (SOX9) are all specific cartilage markers. Collagen II and Aggrecan are the main components of the extracellular matrix of cartilage while Sox9 is the main regulatory transcription factor of chondrocytes (Table 3.1).

Table 3.1: Primers and probes for each gene used for qRT-PCR analysis.

| Gene | Forward Primer | Reverse Primer | UPL Probe |
|---|---|---|---|
| Collagen 2α1 | 5'-ccaggatgcccgaaaatta-3' | 5'-gaggtcctctgggtcctatgat-3' | 80 |
| Sox9 | 5'-gtacccgcatctgcacaac-3' | 5'-ctcctccacgaagggtctct-3' | 66 |
| Hprt | 5'-tcctcctcagaccgctttt-3' | 5'-cctggttcatcatcgctaatc-3' | 95 |

*Statistical analysis*

Data is presented as mean ± standard deviation of the value. To compare one variable condition between groups, the two-tailed Student's *t*-test was used with the statistical significance value of $P < 0.05$.

## 3.3 Results

*Articular chondrocytes form cartilaginous nodules* in vitro *and show positive Alcian Blue staining*

Articular chondrocytes were harvested from 5-9 week old mice, plated and cultured as described in the methods, and observed for morphology and growth patterns associated with harvested articular chondrocytes *in vitro* as previously described in the literature[22]. Within the first week, the cells began to show fibroblastic like morphology, very elongated and stretching across the plate. After two to three weeks in culture, both the digest wells as well as the explant well showed the formation of multiple cartilaginous nodules. Positive Alcian Blue staining indicated glycosaminoglycan deposition (Fig 3.1). The morphology and

staining pattern of these primary cells was then compared to the ATDC5 cell line.

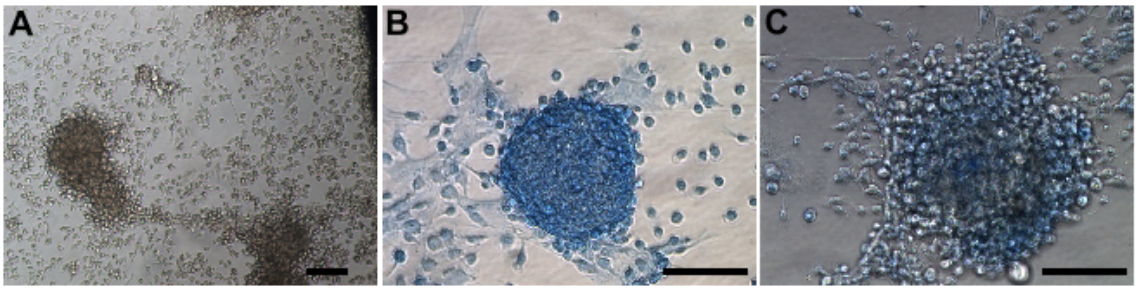


Fig. 3.1: Harvested articular cartilage cultured in DMEM/10% FBS/1% PS, supplemented with 2mM L-glutamine, for 2-3 weeks. (A) Unstained articular cartilage nodules after two weeks in culture. (B) Explant culture showing formation of cartilaginous nodule and positive Alcian Blue staining. (C) Digest well demonstrating formation of cartilaginous nodules and positive Alcian Blue staining. Bar = 100 µm.

*ITS supplement is necessary for inducing chondrogenesis of ATDC5 cells*

To determine specific factors necessary for chondrogenesis, the ATDC5 cell line was tested under defined conditions. Insulin is a supplement widely used to induce chondrogenesis in the ATDC5 cell line. The induction of chondrogenesis by ITS (insulin, transferrin, selenium) supplementation was assessed visually by Alcian Blue staining and quantitatively by the Synergy HT Microplate reader. Both the staining and the plate reader analysis indicated that the addition of ITS to the ATDC5 culture media was necessary to induce chondrogenesis of the cell line (Fig 3.2).

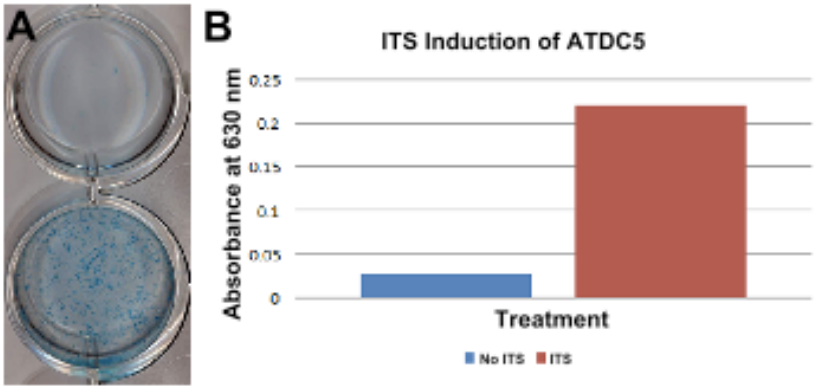


Fig. 3.2: ATDC5 cells cultured for three weeks in DMEM:Ham's F12/ 5% FBS, supplemented with 2mM L-glutamine, with or without 1% ITS. (A) ATDC5 cells cultured without ITS (top) or with ITS (bottom) and stained with Alcian Blue. (B) Absorbance of each well at 630 nm.

*TGF-β1 and TGF-β3 promote chondrogenesis of ATDC5 cells*

TGF-β1 and TGF-β3 are both known regulators of chondrogenesis and widely used to induce *in vitro* chondrogenesis. There is discrepancy as to which of these two isoforms more readily promotes chondrogenesis. For this reason, the chondrogenic potential of both isoforms was investigated[10,24,3,2]. ATDC5 cells were cultured for three weeks (21 days) in a basal media of DMEM/Ham's F12 (1:1) / 5%FBS and supplemented with 1% ITS and 2mM L-glutamine. TGF-β1 and TGF-β3 were separately added at a concentration of 10 ng/mL to the ATDC5 cell culture media containing ITS with each media change in order to compare their ability to promote chondrogenesis over the ITS only control. Alcian Blue staining revealed that the two isoforms of TGF-β were both able to promote chondrogenesis of the ATDC5 cells as compared to the ITS only control (Fig 3.3). Addition of either TGF-β to the culture media resulted in a change in the

morphology of the cells from a distinct round shape to more fibroblastic in nature within the first week of culture. Positive Alcian Blue staining in the ITS only controls showed punctate staining while TGF-β treated wells showed more diffuse staining, suggesting more widespread GAG deposition throughout the culture wells potentially extracellular versus deposition only in nodules. This would indicate that TGF-β is either upregulating the production of GAGs in existing cells or potentially inducing more cells within the culture to differentiate and produce GAGs. While there was a significant difference between both isoforms compared to the ITS only control, there was no significant difference between the isoforms when compared to each other indicating that both are capable of enhancing chondrogenesis. However, it must be noted that TGF-β1 did show a higher averaged absorbance over TGF-β3 at 0.276 and 0.223 respectively.

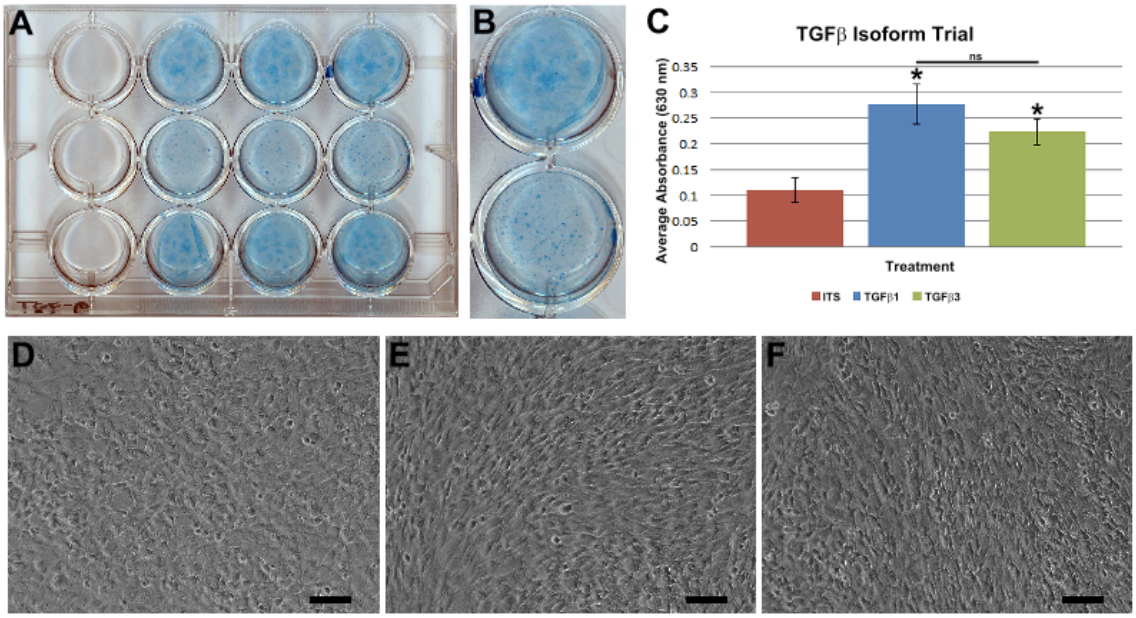


Fig. 3.3: ATDC5 cells cultured for three weeks with TGF-β1 + ITS, ITS alone, or TGF-β3 + ITS. (A) Alcian Blue staining of ATDC5 cells. Three wells of each culture condition are represented: TGF-β1 + ITS (top row), ITS only (middle row), TGF-β3 + ITS (bottom row). (B) Enlarged image of a well cultured with TGF-β1 + ITS (top) and a well with ITS only (bottom). (C) Average absorbance at 630 nm for each culture condition. Stars (*) represent a significant difference as compared to ITS treatment alone where $p < 0.05$. N.S. indicates a non-significant value. (D) Morphology of ITS only well after one week in culture. (E) Morphology of TGF-β1 + ITS well after one week. (F) Morphology of TGF-β3 + ITS well after one week. Bar = 100 μm.

*TGF-β1 and TGF-β3 increase expression of collagen II and Sox9*

As collagen II is the major component of the extracellular matrix of hyaline cartilage, the mRNA expression levels were determined in ATDC5 cells treated with and without the addition of TGF-β1 or TGF-β3. Expression levels were normalized to the reference gene Hprt. Both isoforms demonstrated a significantly higher expression of collagen II as compared to the ITS only control. Interestingly, TGF-β3 had a significantly higher expression level compared to TGF-β1. As Sox9 is the main regulating transcription factor of chondrogenesis, the mRNA expression levels were determined in ATDC5 cells. Sox9 expression

levels showed a similar trend to that of Collagen II expression. Both TGF-β1 and TGF-β3 treated cells showed a significantly higher expression compared to the ITS only treated cells. Additionally, TGF-β3 treated cells had a significantly higher expression level compared to TGF-β1 (Fig 3.4).

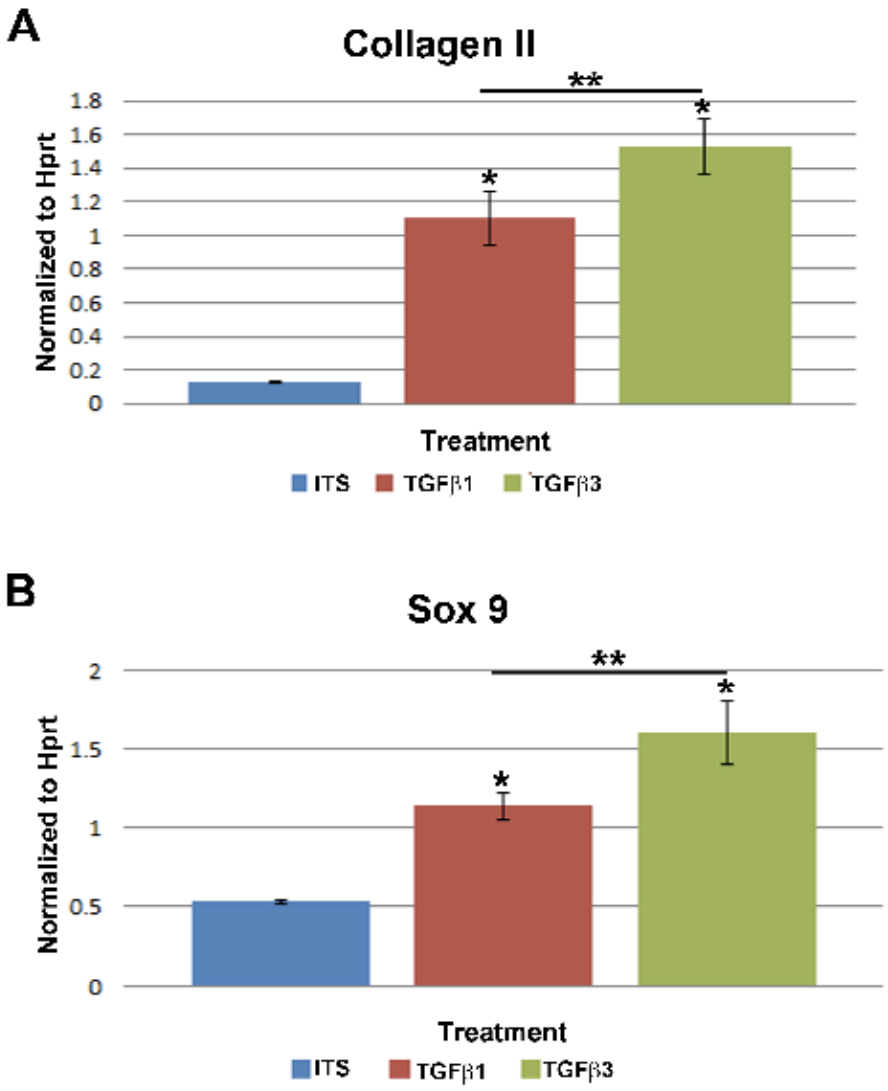


Fig. 3.4: qRT-PCR analysis of ATDC5 cells after three weeks in culture with ITS, TGF-β1 + ITS, or TGF-β3 + ITS. (A) Expression of Collagen II normalized to Hprt. (B) Expression of Sox9 normalized to Hrpt. Single star (*) represents a significant difference as compared to ITS treatment alone, $p < 0.05$. Double star (**) indicated significant difference between TGF-β1 and TGF-β3.

*BMP-2 promotes chondrogenesis of ATDC5 cells at low concentrations*

BMP-2 has been shown to play a role in chondrogenesis by regulating the expression of Sox9 and is thought to promote cell proliferation and chondrocyte matrix synthesis[8]. Because it is known that BMPs have concentration dependent effects, it was necessary to first conduct a titration using BMP-2. As determined by Alcian Blue staining, the optimum concentration for promoting chondrogenesis of ATDC5 cells with BMP-2 and ITS was 10 ng/mL as it showed the highest average absorbance of all the concentrations and the highest significant difference when compared to the ITS only control (Fig 3.5). The Alcian Blue staining from wells containing 50 ng/ml and 100 ng/ml BMP-2 were also significantly higher than the ITS only control. The highest concentration of 500 ng/mL appeared to not to contribute to chondrogenic differentiation based on the lack of positive Alcian Blue staining, which was comparable to that of the ITS only control. The average absorbance of BMP-2 at a concentration of 500 ng/ml was not significantly different from the ITS only control. Several studies have suggested an additive effect of TGF-βs and BMPs in induction of chondrogenesis. Specifically, studies have examined the combinatorial effects of TGF-β3 and BMP-2[40]. Based on this study, we examined the efficacy of these combinations to promote chondrogenesis in our ATDC5 cells. A combination of 10ng/ml BMP-2 with 10 ng/ml TGF-β3 and ITS was also investigated in order to determine whether or not combining these two growth factors enhanced chondrogenesis compared to BMP-2 alone. Alcian Blue staining and plate reader

analysis determined that combining BMP-2 and TGF-β3 did not enhance chondrogenesis compared to BMP-2 alone as assessed by the level of absorbance (Fig 3.6).

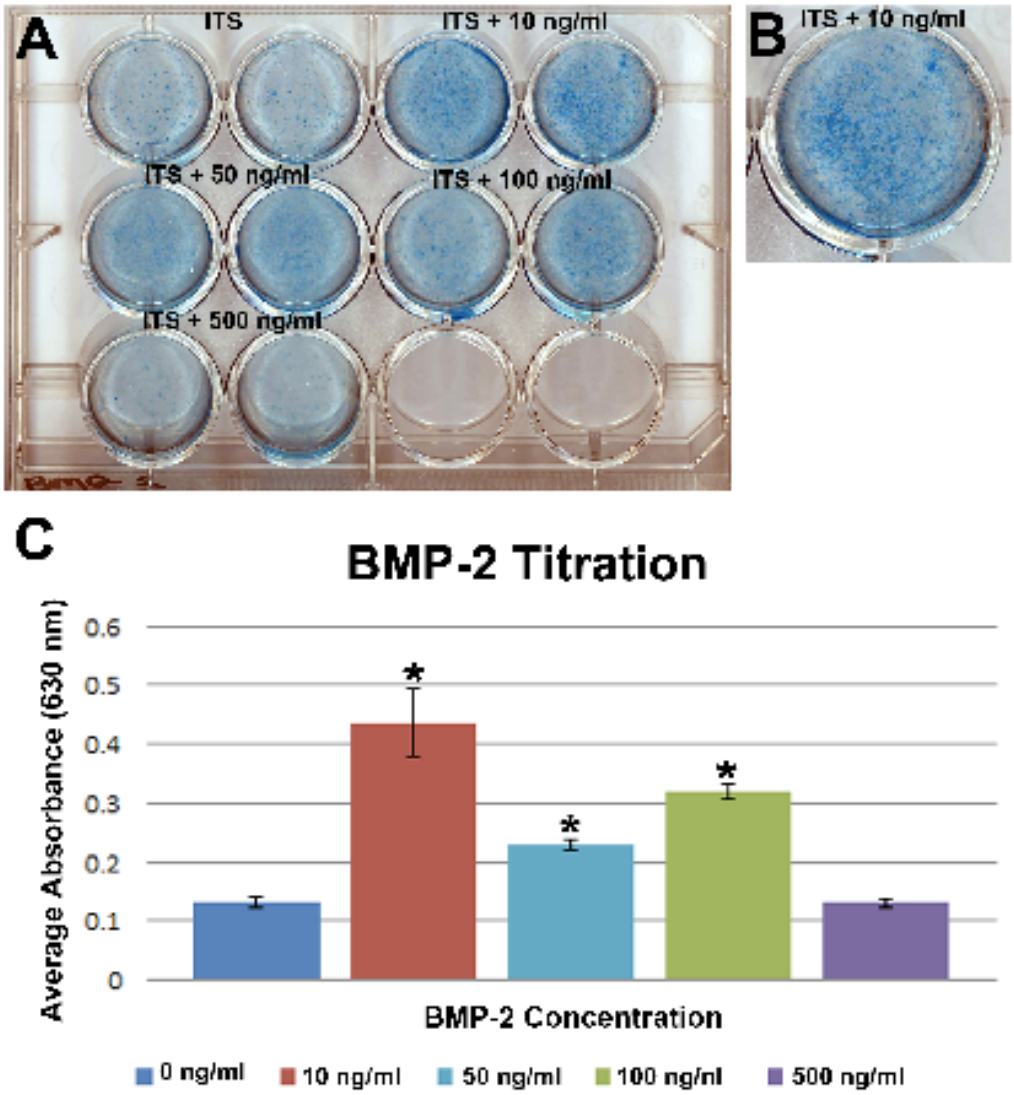


Fig. 3.5: ATDC5 cells cultured for three weeks in varying concentrations of BMP-2. (A) Alcian Blue staining of ATDC5 cells. Each concentration of BMP-2 was added to two wells of the 12-well pate. (B) Enlarged image of well cultured with 10 ng/mL BMP-2. (C) Average absorbance at 630 nm for each concentration of BMP-2. Stars (*) indicate significant difference compared to ITS alone, $p < 0.05$.

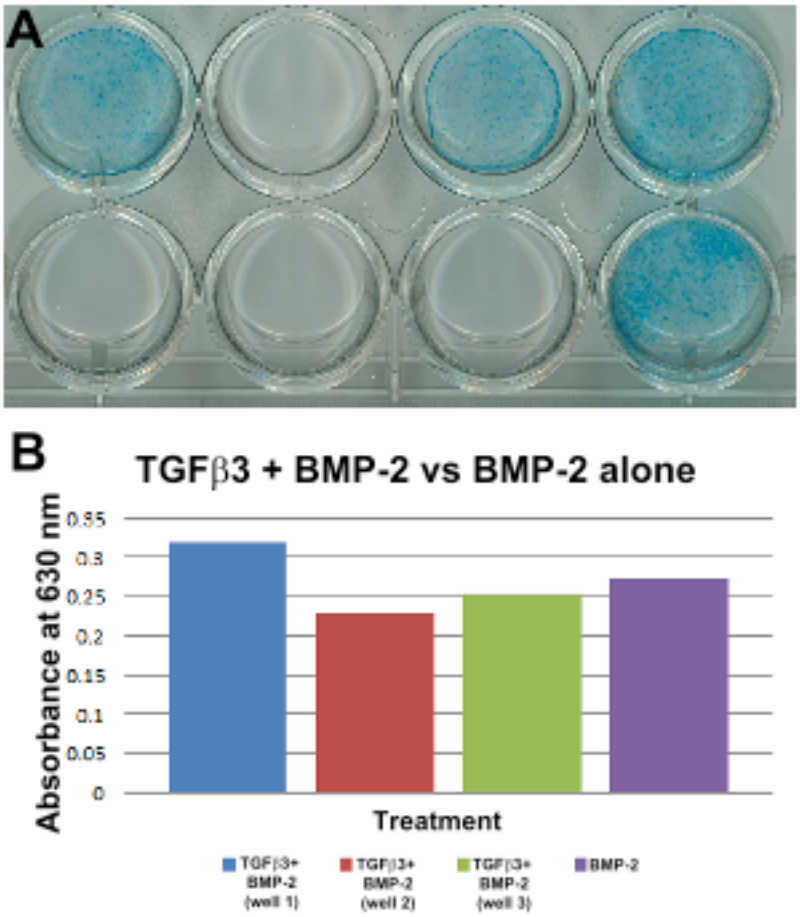


Fig. 3.6: ATDC5 cells cultured for three weeks with TGF-β3 + BMP-2 + ITS or BMP-2 + ITS. (A) Alcian Blue staining of each well. TGF-β3 + BMP-2 + ITS (top row), BMP-2 + ITS (bottom row). (B) Absorbance at 630 nm for each individual well.

*BMP-7 minimally promotes chondrogenesis of ATDC5 cells*

The effect of BMP-7 on ATDC5 cells was investigated because it is thought to enhance the synthesis of chondrogenic extracellular matrices. Furthermore, it is clinically approved for use in bone tissue regenerative purposes along with BMP-2[8]. A study by Caron *et. al.* demonstrated the potential of BMP-7 to increase Collagen II expression and suppress Collagen X expression, a marker of chondrocyte hypertrophy, thereby suggesting a chondroprotective effect. A titration was also conducted for BMP-7 in order to determine the appropriate concentration for experiments. Quantitative analysis of the Alcian Blue staining performed on the BMP-7 and ITS cultures determined that only the concentration of 50 ng/ml of BMP-7 showed a significant difference over the ITS only control (Fig 3.7). As stated before, an additive effect of TGF-βs and BMPs have been suggested in other studies. Specifically, studies have examined the combinatorial effects of TGF-β1 and BMP-7[16]. Based on this study, we examined the efficacy of this combination to promote chondrogenesis in the ATDC5 cells. BMP-7 was tested in conjunction with TGF-β1 and ITS in order to determine the effect of combining growth factors. The combination of these factors was found to enhance chondrogenesis of ATDC5 cells when compared to the ITS only control. The average absorbance of the combination was significantly higher than that of ITS alone (Fig 3.8).

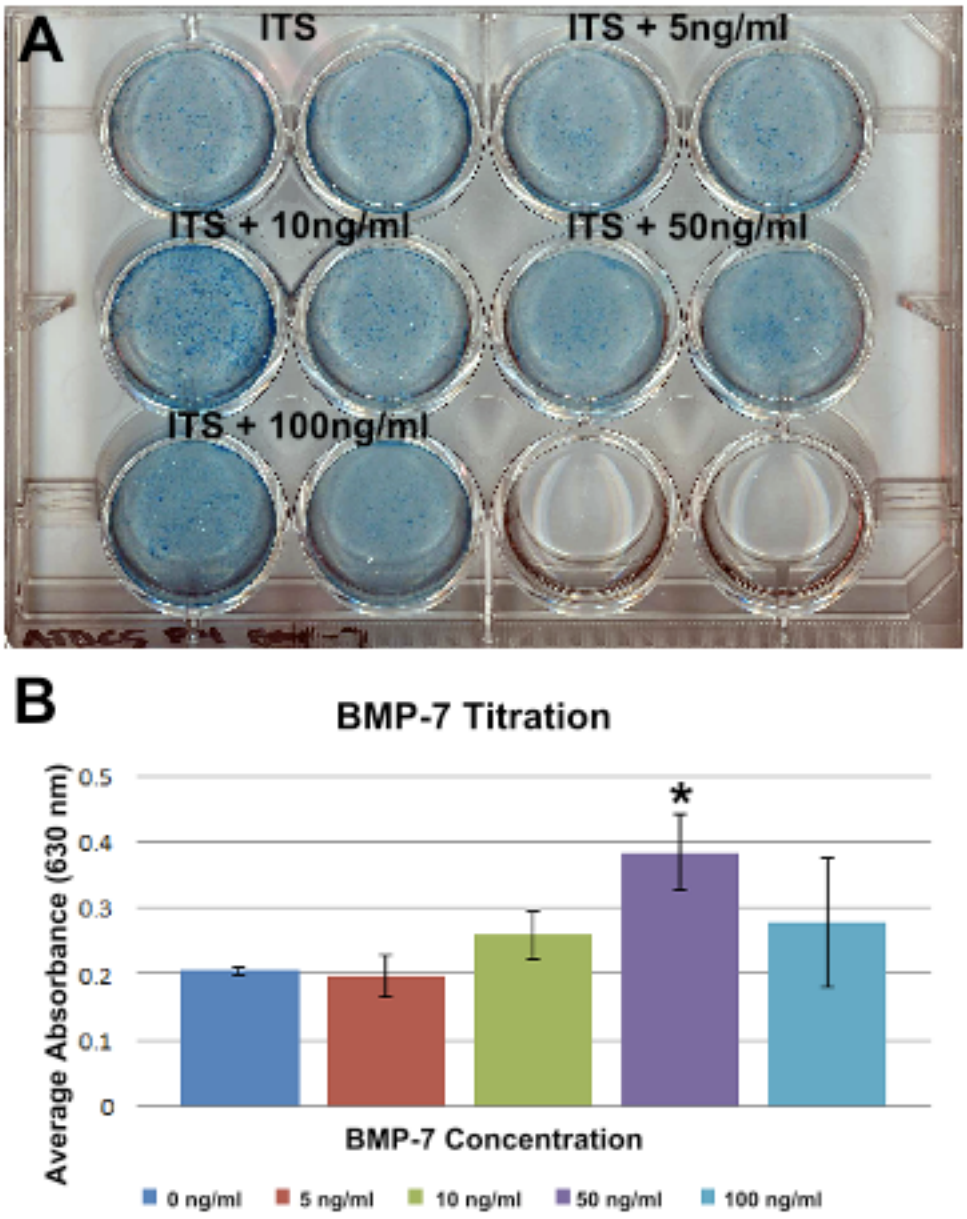


Fig. 3.7: ATDC5 cells cultured for three weeks with BMP-7 at varying concentrations. (A) Alcian Blue staining of ATDC5 cells. Each concentration of BMP-7 was added to two wells of the 12-well pate. (B) Average absorbance at 630 nm for each concentration of BMP-7. Stars (*) indicate significant difference compared to ITS alone, $p < 0.05$.

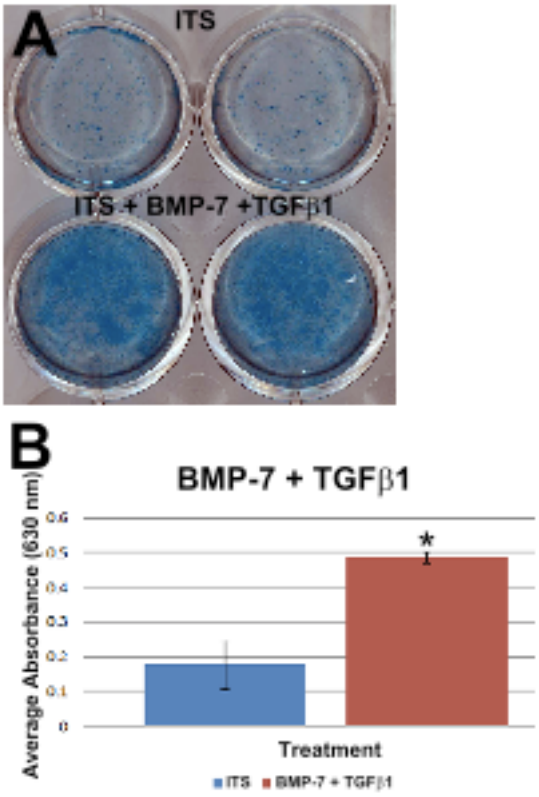


Fig. 3.8: ATDC5 cells cultured for three weeks with TGF-β1 + BMP-7 + ITS or ITS alone. (A) Alcian Blue staining of each well. ITS alone (top) and TGF-β1 + BMP-7 + ITS (bottom). (B) Average absorbance at 630 nm for each treatment.

*Ascorbic acid assists in chondrogenesis of ATDC5 cells while dexamethasone does not*

Supplements, ascorbic acid and dexamethasone, were separately added to the ATDC5 culture media containing ITS in order to assess their contributions to ATDC5 chondrogenesis. Ascorbic acid is a vitamin that is involved in the hydroxylation of proline residues in collagen and therefore would assist in the development of extracellular matrices. Furthermore, ascorbate has been shown to enhance chondrogenesis of ATDC5 cells by Altaf *et. al.*[1]. Dexamethasone is a steroid often used in cell cultures. It may increase proliferation of cells, however,

has been shown to potentially have inhibitory effects on ATDC5 cells[49]. Alcian Blue staining indicated that the addition of ascorbic acid may promote chondrogenesis. The addition of dexamethasone, however, does not as it showed little to no positive Alican Blue staining at the macroscopic level when compared to the wells containing ascorbic acid (Fig 3.9). These ATDC5 that were treated with dexamethasone or ascorbic acid were both used to optimize the antibody staining for immunofluorescence (Figs 3.10 and 3.11). The resulting optimal concentrations for Collagen II (1:50) and Aggrecan (1:100) are represented.

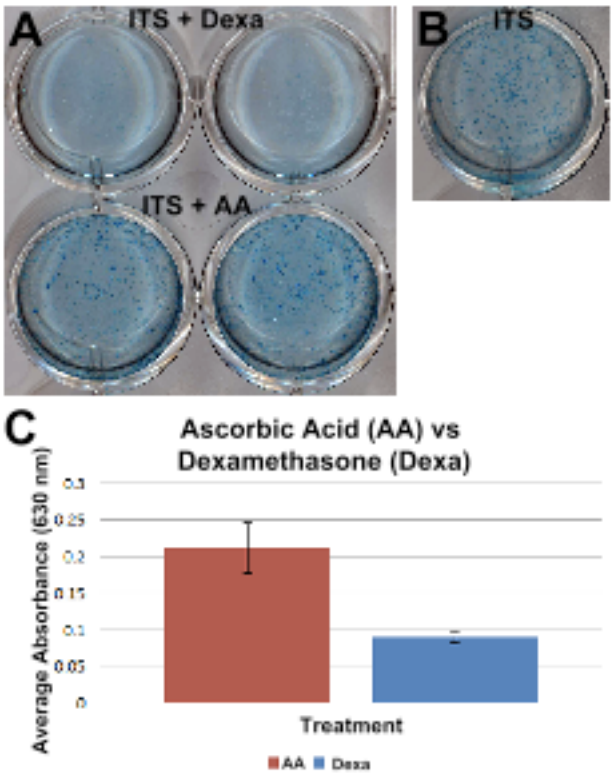


Fig. 3.9: ATDC5 cells cultured for three weeks with Ascorbic Acid+ ITS or Dexamethasone + ITS. (A) Alcian Blue staining of each well. Dexa + ITS (top) and AA + ITS (bottom). (B) Alcian Blue staining of ITS only well for comparison. (C) Average absorbance at 630 nm for AA + ITS or Dexa + ITS.

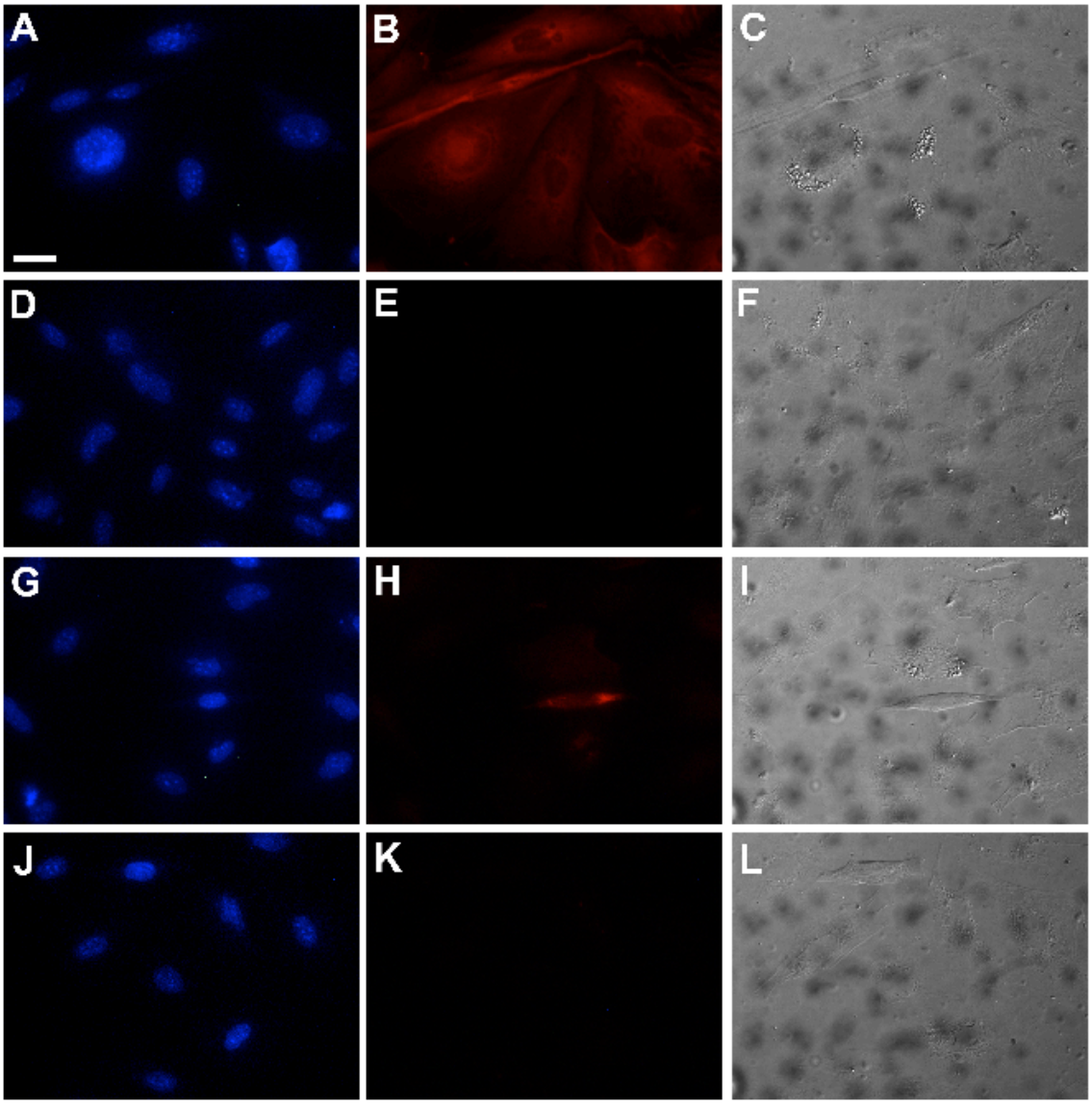


Fig. 3.10: ATDC5 cells cultured for three weeks with Dexamethasone + ITS. (A, D, G, J) Hoechst nuclear staining. (B) Collagen II staining. (C, F, I, L) DIC images. (E) Secondary only control for Collagen II staining. (H) Aggrecan staining. (K) Secondary only control for Aggrecan staining. Bar = 50 μm.

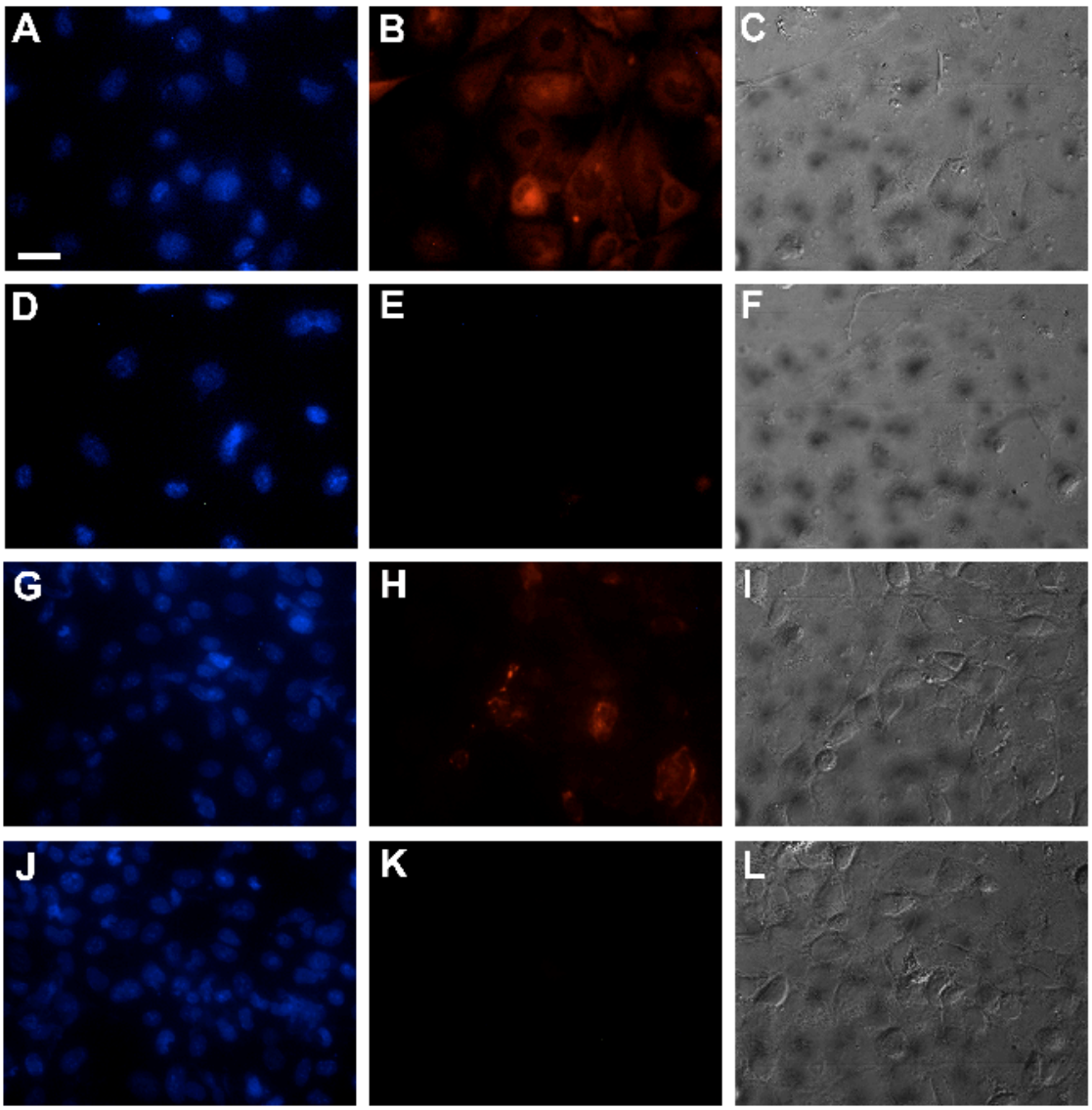


Fig. 3.11: ATDC5 cells cultured for three weeks with Ascorbic Acid + ITS. (A, D, G, J) Hoechst nuclear staining. (B) Collagen II staining. (C, F, I, L) DIC images. (E) Secondary only control for Collagen II staining. (H) Aggrecan staining. (K) Secondary only control for Aggrecan staining. Bar = 50 μm.

*Effects of chemically induced hypoxia on chondrogenesis of ATDC5 cells*

Cartilage is naturally an avascular tissue meaning it has a hypoxic microenvironment compared to that of other tissues in the body. Therefore, creating a hypoxic environment *in vitro* would more or less mimic a more *in vivo* environment. Cobalt Chloride ($CoCl_2$) is a chemical inducer of hypoxia by which it sequesters oxygen from the media. $CoCl_2$ was added fresh with each media change every 2-3 days at a concentration of 75 μM. The concentrations of 75 μM and 100 μM were tested previously, and 100 μM was found to cause cell death within the cultures. As assessed by Alcian Blue staining, Cobalt Chloride ($CoCl_2$) did not seem to promote chondrogenesis after 48 hrs or 3 weeks of treatment (Fig 3.12).

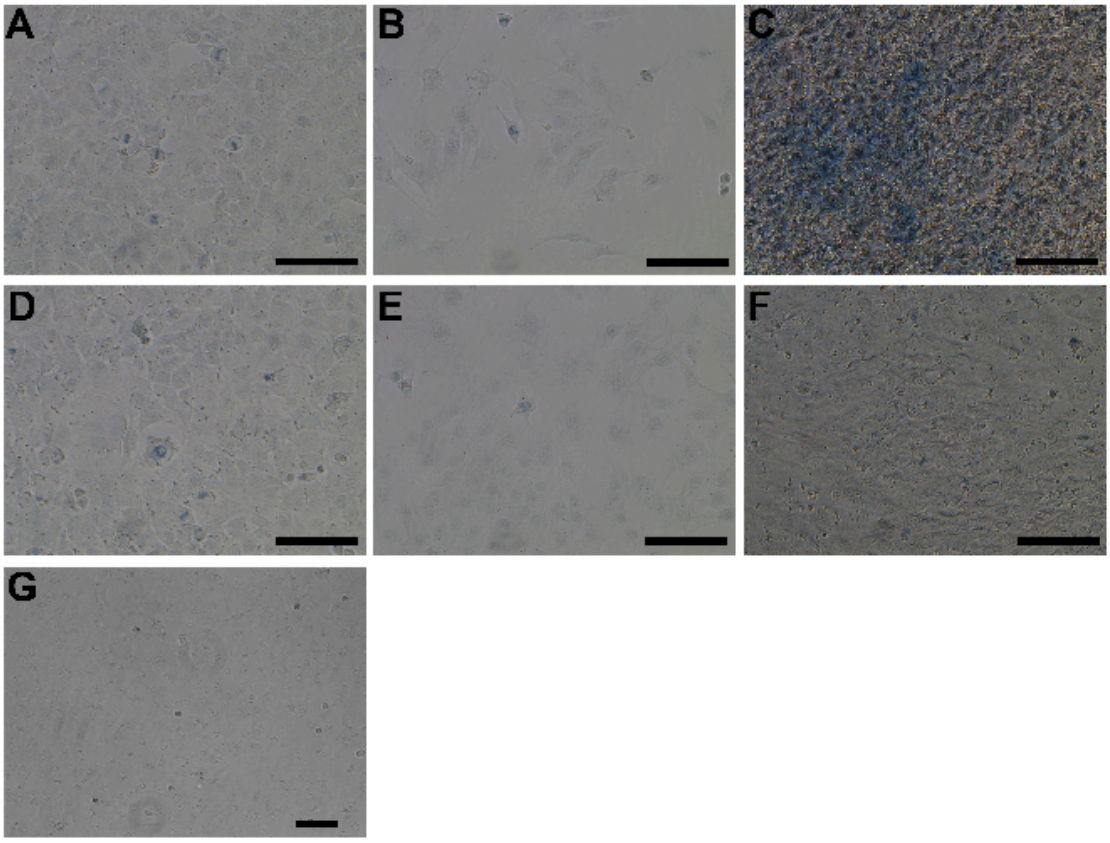


Fig. 3.12: Alcian Blue staining of ATDC5 cells cultured for with $CoCl_2$ with or without ITS at 75 µM or 100 µM. (A) 75 µM $CoCl_2$ + ITS for 48 hrs. (B) 75 µM + No ITS for 48 hrs. (C) 75 µM $CoCl_2$ + ITS for ~3 weeks. (D) 100 µM + ITS for 48 hrs. (E) 100 µM $CoCl_2$ + No ITS for 48 hrs. (F) 75 µM + No ITS for ~3 weeks. (G) 100 µM $CoCl_2$ demonstrating cell death. Bars = 100 µm.

### 3.4 Discussion

Both defined cell types, harvested articular cartilage and the ATDC5 chondrogenic cell line, proved to be suitable models for *in vitro* chondrogenesis to serve as a reference for our primary cell cultures. Articular cartilage cultures demonstrated the ability to form cartilaginous nodules within the three week culture period. At the end of the three week culture period, positive Alcian Blue staining indicated the presence of proteoglycans. All nodules within the articular cartilage cultures stained positively, however, the explant culture stained visibly darker than the digest cultures. This difference could be due to a variety of factors such as higher cell density in the explant cultures or more growth factors being produced from the chondrocytes growing directly off of the explant. Ideally in future studies harvested and cultured articular cartilage would be used for molecular analysis as well as comparison of further staining using immunofluorescence. Therefore, in future temporal differentiation/maturation studies, the mRNA expression and protein expression of articular cartilage cells in addition to the ATDC5 cell line would be compared to differentiated primary cells of Aim 2.

The use of the ATDC5 cell line allowed for identification of specific factors necessary for induction of chondrogenesis, as articular cartilage was cultured in FBS, an undefined serum. The supplement ITS was found to be necessary for inducing chondrogenesis of the ATDC5 cell line. For this reason, it was added to all subsequent experiments and used as the baseline control for chondrogenic differentiation of this cell type. The addition of growth factors to the ITS

containing media demonstrated further promotion of the chondrogenic lineage. Specifically, the addition of TGF-β1 and TGF-β3 were both found to promote chondrogenesis in the cell line significantly over the ITS only control. However, a significant difference was not found between the two isoforms based on Alcian Blue staining, indicating that both have the potential to drive chondrogenesis of the progenitor primary cells tested in Aim 2. A major change in the morphology of these cells under the influence of TGF-β indicates their responsiveness to this growth factor regardless of isoform. Interestingly, the mRNA level expression of Collagen II was significantly different between the two isoforms with TGF-β3 being higher. Given this information both isoforms are tested in Aim 2 and analyzed by immunofluorescent staining for protein expression.

The effect of the addition of BMPs to the culture media was found to be dose dependent. BMP-2 had the highest effect at a concentration of 10ng/mL, whereas BMP-7 had the highest effect at 50 ng/mL. The combination of BMP-2 with TGF-β3 was not found to be significantly differently than BMP-2 alone. For this reason, the combination of these growth factors was not tested in Aim 2. The combination of BMP-7 with TGF-β1 was found to be significantly higher than ITS alone, but was not compared to BMP-7 alone and therefore was also not tested in Aim 2.

The supplements ascorbic acid and dexamethasone were tested separately on the ATDC5 cell line in order to determine their contribution to the promotion of chondrogenesis as both are common supplements to *in vitro* culture media. Both were tested with the addition of ITS, because these supplements

alone would not induce chondrogenesis. Ascorbic acid appeared to promote chondrogenesis, however, its addition did not produce a response comparable to that of growth factors, which was not expected. However, because ascorbic acid is simply a vitamin involved in the synthesis of collagen, it would make sense that its contribution to chondrogenesis would not be overly striking, but may be necessary for proper production of ECM molecules. It is important to note that dexamethasone did not enhance chondrogenesis and may inhibit expression of ECM components to a small extent based on analysis of Alcian Blue staining for GAGs and immunofluorescent staining for Collagen II and Aggrecan. For this reason, dexamethasone was excluded in certain culture conditions in Aim 2.

The effect of chemically induced hypoxia with $CoCl_2$ was found to be insignificant at both time points investigated, 48 hrs and ~3 weeks. It is possible the $CoCl_2$ was not creating an appropriate hypoxic microenvironment for the cells. The efficiency of the chemical actually creating a hypoxic mircroenvironment was not tested, however, it had some effect within the culture due to the presence of cell death with the 100μM concentration. Because no significant changes were noted in either time point tested, this culture condition was not pursued further for the purposes of this study. In addition to this, other potential effects of the chemical on the cells themselves is not known, and therefore would introduce more variables when attempting to differentiate primary cells in Aim 2. However, future studies may need to address the idea of hypoxia by other means in order to mimic the *in vivo* avascular nature of cartilage tissue as this is an important component to recognize for tissue engineering.

**Significance:**

It is important to determine specifically what growth factors and supplements best promote differentiation of cells *in vitro* towards a chondrogenic lineage. Using the chondrogenic cell line ATDC5 was a more efficient, standardized, and quicker method for identifying individual factors that would promote chondrogenesis rather than testing each on primary cells of Aim 2. Having a more defined culture media better controls the variables within the culture providing a better baseline when attempting to differentiate primary cells for a specific purpose, which in this case is cartilage regeneration.

CHAPTER 4: CHONDROGENIC DIFFERENTIATION FROM AN HSC LINEAGE

**Specific Aim 2:** ***Demonstrate the differentiation of chondrocytes from an HSC lineage*** **in vitro.**

## 4.1 Introduction

Methods to isolate HSC-derived chondrocytes were established in order to demonstrate the potential of HSCs to give rise to chondrocytic cells. Identifying a novel stem cell source for chondrocytes would provide a new resource for cartilage regeneration stem cell therapies. The two sources of cells that were tested for their ability to give rise to chondroblasts/cytes were non-aderent bone marrow cells and monocytic precursor derived cells. Given that there are two types of stem cells that reside in the bone marrow, it is important to make a distinction between the two. The non-adherent fraction of bone marrow cells, meaning those that do not adhere to plastic culture dishes, is the fraction in which is enriched for HSCs and HPCs[34,33]. The adherent bone marrow fraction is thought to be enriched for MSCs and has been shown *in vitro* to give rise to chondrocytes[33]. In the present study, we first examined the ability of the HSC-enriched, non-adherent fraction of bone marrow to give rise to chondrocytes *in vitro*. Adherent bone marrow was used as a positive control. To further investigate the chondrogenic potential of the HSC lineage, using a more defined population of HSC derived cells, we examined monocytic precursor cells in chondrogenic culture conditions. Growth factors and additional supplements to

the basic culture media were identified from Aim 1 that demonstrated a pro-chondrogenic effect on the chondrogenic cell line ATDC5 and were tested in this aim using primary Non-Adherent bone marrow cells and monocytic derived cultures. Chondrogenesis was examined based on morphology, Alcian Blue staining, immunofluorescence (IF), and molecular profiles.

## 4.2 Materials and Methods

*Isolation and culture of Non-Adherent and Adherent bone marrow cells*

Bone marrow was isolated from the femurs and tibias of 5-9 week old C57BL/6 or EGFP male or female mice. The ends of the femurs and tibias were cut to create an opening on both ends. Bone marrow was then flushed out of the bone using an insulin syringe. Mononuclear cells were collected from the bone marrow by Lympholyte-M (Cedar Lane Tech) density centrifugation. Mononuclear cells were plated and cultured for 5 days at 37 degrees C in Dulbecco's Modified Eagle Medium (Gibco), 10% Fetal Bovine Serum (Atlanta Biologicals), 1% Penicillin/Streptomycin (Atlanta Biologicals) or DMEM/15% FBS/1%PS in order to separate adherent cells from non-adherent cells. After 5 days, the non-adherent fraction, enriched for HSCs, was collected and replated at 1 X $10^6$ cells/well on an 8-well fibronectin plate. The adherent fraction, enriched for MSCs, was trypsinized and cells were replated at 1 X $10^6$ cells/well on an 8-well fibronectin plate. Cells were cultured for ~6 weeks, and media was changed every 2-3 days. Various chondrogenic medias were tested including: StemXVivo Chondrogenic Media with supplement (R & D Systems), MSC-Gro Serum Free

Chondrogenic Media (VitroBiopharma), StemPro Chondrogenic Media (Gibco), DMEM/1% ITS/ 1% PS with 2mM L-glut, 0.1mM ascorbic acid, $10^{-7}$ dexamethasone, and 10 ng/mL TGF-β1, and DMEM/10% FBS/ 1% PS with 2mM L-glutamine.

*Isolation and culture of monocytic precursor cells*

Bone marrow was harvested as described above. Mononuclear cells were collected from the bone marrow by lympholyte-M centrifugation, plated on T75 tissue culture flasks and incubated for 24 hrs in αMEM (Gibco),15% FBS (Atlanta Biologicals), 1% PenStrep (Atlanta Biologicals) supplemented with 15 ng/mL IL-3 (BioAbChem) and 1 ng/mL M-CSF (BioAbChem). M-CSF (macrophage colony-stimulating factor) and IL-3 (Interleukin-3) were added because they influence HSC differentiation toward macrophages and myeloid progenitor cells, respectively. After 24 hrs, the non-adherent cells were collected, centrifuged at 2.3 rpm for 10 minutes, and underwent Pronase digestion with a 0.02% Pronase solution for 15 min. at 37 degrees C. The cell suspension was then placed on 10 mL horse serum (Atlanta Biologicals) on ice for 15 min. The suspension was then removed, layered onto another 10 mL of horse serum, centrifuged at 1.2 rcf for 10 minutes, and re-plated on a T75 in the αMEM media above. After 2 days, the non-adherent cells were again collected and the above Pronase treatment was repeated. Cells were then plated in one of the chondrogenic media listed below at a density of 2 X $10^6$ or 2.5 X $10^6$ cells/well of an 8-well fibronectin plate. Cells were cultured for ~6 weeks, and media was changed every 2-3 days. Various

chondrogenic medias were tested including: StemXVivo Chondrogenic Media with supplement (R & D Systems), MSC-Gro Serum Free Chondrogenic Media (VitroBiopharma), StemPro Chondrogenic Media (Gibco), DMEM/1% ITS/ 1% PS with 2mM L-glut, 0.1mM ascorbic acid, with or without $10^{-7}$ dexamethasone, and 10 ng/mL TGF-β1 or 10 ng/mL TGF-β3, and DMEM/10% FBS/ 1% PS with 2mM L-glutamine with or without 10 ng/mL TGF-β1.

*Alcian Blue Staining*

To evaluate cartilage-specific proteoglycan synthesis, sulfated GAGs were visualized by staining with Alcian Blue. After ~six weeks of culture, cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature, and then washed 3-5 times with distilled water. The cells were then stained with Alcian Blue (1% in 3% acetic acid, pH 2.5, 8XG solution) for 30 minutes and rinsed with distilled water until the water ran clear (about 5 rinses). Alcian Blue staining was assessed visually using a Nikon Eclipse Ti microscope.

*Immunohistochemical staining*

Cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for ten minutes at room temperature. Cells were washed with PBS containing 0.05% sodium azide 3 x 5 minutes. For Aggrecan staining, cells were incubated with 5 x $10^{-5}$ U/µl Chondroitinase ABC for three hours at 37 degrees Celsius post-fixation then washed 3 x 5 minutes with PBS-Az prior to permeabilization. Cells were then permeabilized with Triton X-100 (0.02% in

PBS) for ten minutes. Cells were washed with PBS-Az 3 x 5 minutes and then blocked in 5% donkey serum in 3% BSA-PBS for thirty minutes at room temperature. Block was removed, and the primary antibody was added (Rabbit-anti mouse Collagen II polyclonal antibody at 1:50 or Rabbit- anti-Aggrecan polyclonal antibody at 1:100– Millipore) for 45 minutes at room temperature. Cells were washed 3 x 5 minutes with PBS-Az and then blocked for thirty minutes in 5% donkey serum in 3% BSA-PBS. The secondary antibody, Donkey anti-Rabbit Cy3 (Jackson), was added at a 1:100 dilution for thirty minutes at room temperature. Cells were washed 3 x 5 minutes with PBS-Az. Hoechst (Invitrogen) was added at a 1:25000 dilution in PBS for eight minutes. Cells were washed 3 x 5 minutes with PBS-Az, and they were then mounted and cover-slipped. Immunofluorescence was visually assessed using a Nikon 90i microscope.

### 4.3 Results

*Non-adherent cells stain positive for Alcian Blue under chondrogenic conditions*

In order to determine the presence of glycosaminoglycan deposition as a marker for chondrogenic differentiation, adherent and non-adherent bone marrow cells were stained with Alcian Blue after six weeks in culture. Various culture methods as well as culture conditions were tested. Methods for culturing adherent and non-adherent cells included 96-well plate, pellet culture, hanging drop culture, micromass culture, and monolayer culture (Table 4.1). The

monolayer culture method was chosen because of the ability to observe changes in real time in the cell culture throughout the culture period. Positive and negative Alcian Blue staining was observed in both the adherent and non-adherent cell cultures indicating a mixed population (Fig. 4.1). Positive staining indicated the non-adherent cells had begun to differentiate into potential chondroblasts by demonstrating the production of GAGs, despite this presence being intracellular as compared to the extracellular staining seen in an *in vivo* environment demonstrated by the stained bone section (Fig. 4.7). While positive and negative staining was found in all of medias tested, the media conditions that had the healthiest looking cells were chosen to be investigated further (Table 4.2). These conditions were: DMEM/1% ITS/ 1% PS supplemented with 2mM L-glutamine with or without TGF-β1 or TGF-β3 and DMEM/10% FBS/ 1% PS supplemented with 2mM L-glutamine with or without TGF-β1. Successful culture conditions were based on morphology, positive Alcian Blue staining, and the formation of cartilaginous nodules. Because the non-adherent bone marrow culture provided evidence that a culture enriched for HSCs could give rise to potential chondroblasts, the monocytic precursor was investigated further as a more purified HSC-derived population for the remainder of experiments.

Table 4.1: Methods tested for culturing adherent and non-adherent cells under chondrogenic conditions. M (morphology): + indicates morphology of chondrocytes detected,? indicates unknown morphology, - indicates absence of chondrocyte morhphology; AB (Alcian Blue): + positive staining detected, ? indicates not tested, - indicates absence of AB staining.

| | **96- well plate** | **Pellet Culture** | **Micromass** | **Hanging Drop** | **Monolayer** |
|---|---|---|---|---|---|
| **Adherent** | M+, AB- | M?, AB? | Not tested | Dead | M+, AB+ |
| **Non-Adherent** | M+, AB+ | M?<br>AB+ (weak) | M+, AB+ | M+, AB+ | M+, AB+ |

Table 4.2: Initial media conditions tested for culturing adherent, non-adherent, and monocytic precursor cells. M (morphology): + indicates morphology of chondrocytes detected, ? indicates unknown morphology, - indicates absence of chondrocyte morphology; AB (Alcian Blue): + positive staining detected, ? indicates not tested, - indicates absence of AB staining, NF (nodule formation: + indicates nodules detected, ? indicates not determined, - indicates absence of nodule formation.

| | **StemXVivo** | **MSC-Gro** | **StemPro** | **Serum Media** | **Serum-free media with TGF-β1** |
|---|---|---|---|---|---|
| **Adherent** | M+/-, AB+ | M+/- | M+, AB+<br>*dying | M+, AB+,<br>NF+ | M+/-, AB+ (weak) |
| **Non-Adherent** | M+,AB+<br>*dying | M+ | Dead | M+, AB+,<br><br>NF+ | M+, AB+<br>*dying |
| **Monocytic precursor** | M+, AB+<br>*dying | M+, AB+<br>*dying | Dead | M+, AB+,<br>NF+ | M+, AB+ |

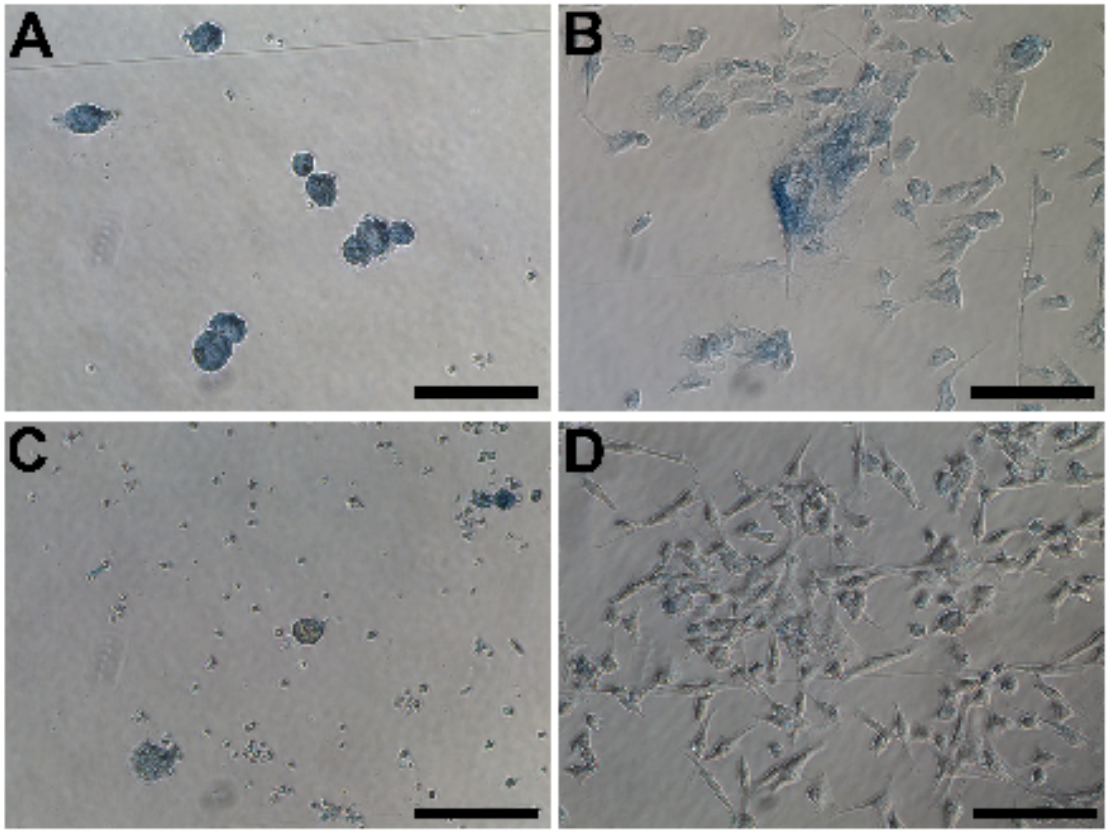


Fig 4.1: Non-Adherent and Adherent cells cultured in DMEM/ 10% FBS/1% PS and supplemented with 10ng/ml of TGF-β1 and 2mM L-glutamine. Cells were fixed and stained for Alcian Blue after six to nine weeks of culture. (A) Positively stained non-adherent cells at nine weeks of culture. (B) Positively stained adherent cells at six weeks of culture. (C) Negatively stained non-adherent cells at nine weeks of culture. (D) Negatively stained adherent cells. Bar = 100 μm.

*Monocytic precursor cells stain positive for Alcian Blue*

As above, monocytic precursor cells were stained with Alcian Blue to demonstrate glycosaminoglycan deposition after six weeks of culture in either DMEM/1% ITS/ 1% PS supplemented with 2mM L-glutamine with or without TGF-β1 or TGF-β3 and DMEM/10% FBS/ 1% PS supplemented with 2mM L-glutamine with or without TGF-β1. Positive staining indicated the presence of glycosaminoglycans and potential chondroblasts (Fig 4.2). In order to confirm

that the positive Alcian Blue stain was indeed the result of differentiation to the chondrogenic lineage and not due to a non-specific uptake of dye by monocytes, monocytic precursor cells were collected after the second Pronase treatment, plated in αMEM/1% FBS at a concentration of 1 X $10^6$ cells/well of an 8-well fibronectin plate for 2 hrs, and fixed and stained with Alcian Blue. Negative staining of these cells validated that the positive staining in cells resulting from culture in chondrogenic differentiation media were due to differentiation toward a chondrogenic lineage (Fig 4.3).

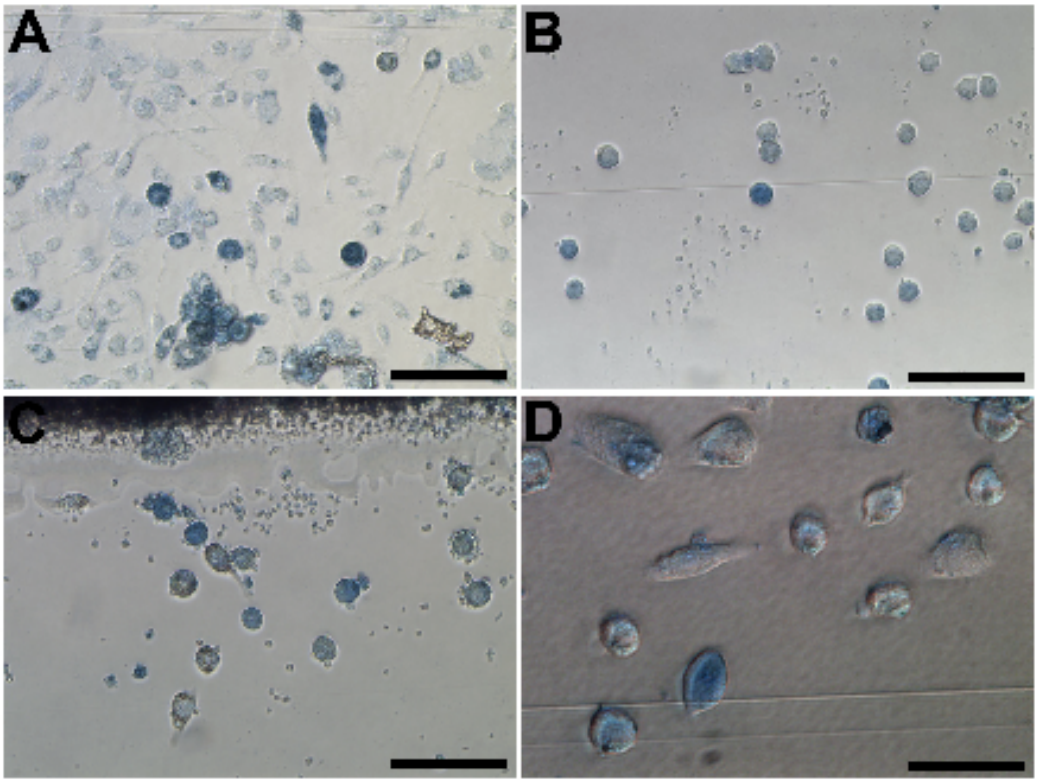


Fig 4.2: Monocytic precursor cells cultured in chondrogenic media for six weeks and stained with Alcian Blue. (A) Cells cultured in serum containing media: DMEM/10% FBS/ 1% PS supplemented with 2mM L-glutamine. (B-D) Cells cultured in serum free media: DMEM/1% ITS/1% PS supplemented with 2mM L-glutamine, 0.1 mM AA, $10^{-7}$M dexa, and 10 ng/ml TGF-β1. Bar in (A-C) = 100 μm. Bar in (D) = 50 μm.

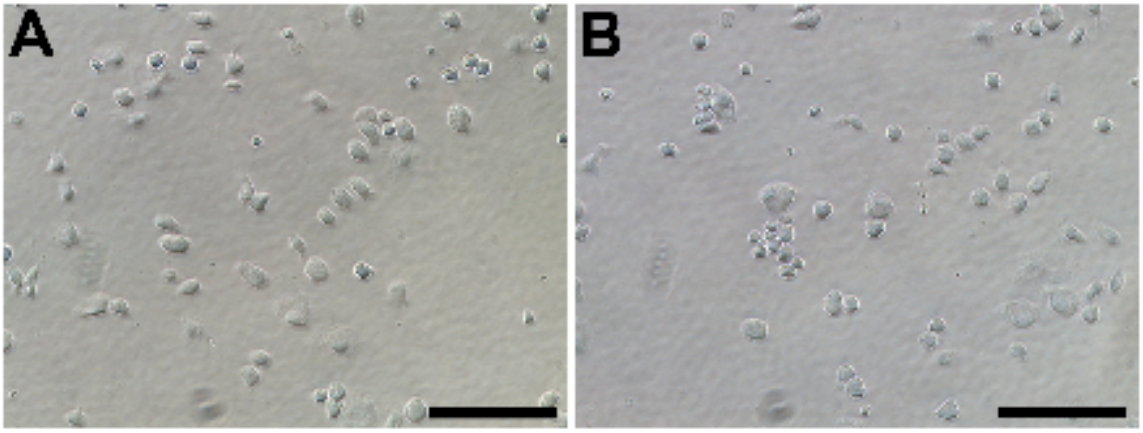


Fig 4.3: Monocytic precursor cells plated before chondrogenic induction for 2 hours in αMEM/1%FBS. Cells were fixed and stained with Alcian Blue. (A-B) Representative images of negative cells. Bar = 100 μm.

*Monocytic precursor derived cells form cartilaginous nodules in serum containing media*

Monocytic precursor derived cells under DMEM/10% FBS/ 1% PS supplemented with 2mM L-glutamine with or without TGF-β1 culture conditions demonstrated cell matrix spreading by two weeks in culture and nodule formation by the end of the six week culture period (Fig 4.4). The pattern in which these cells changed morphology over time and formed cartilaginous nodules were highly similar to those observed in the articular cartilage cultures (Fig 1, Chapter 3). Although nodule formation was not seen in every well containing cells, there were multiple nodules within the well that did form nodules. In addition to that, all the wells under these conditions produced cells that stained positive for Alcian

Blue, despite the lack of nodule formation. However, negatively stained cells were also observed as it is expected that there would be a mixed population.

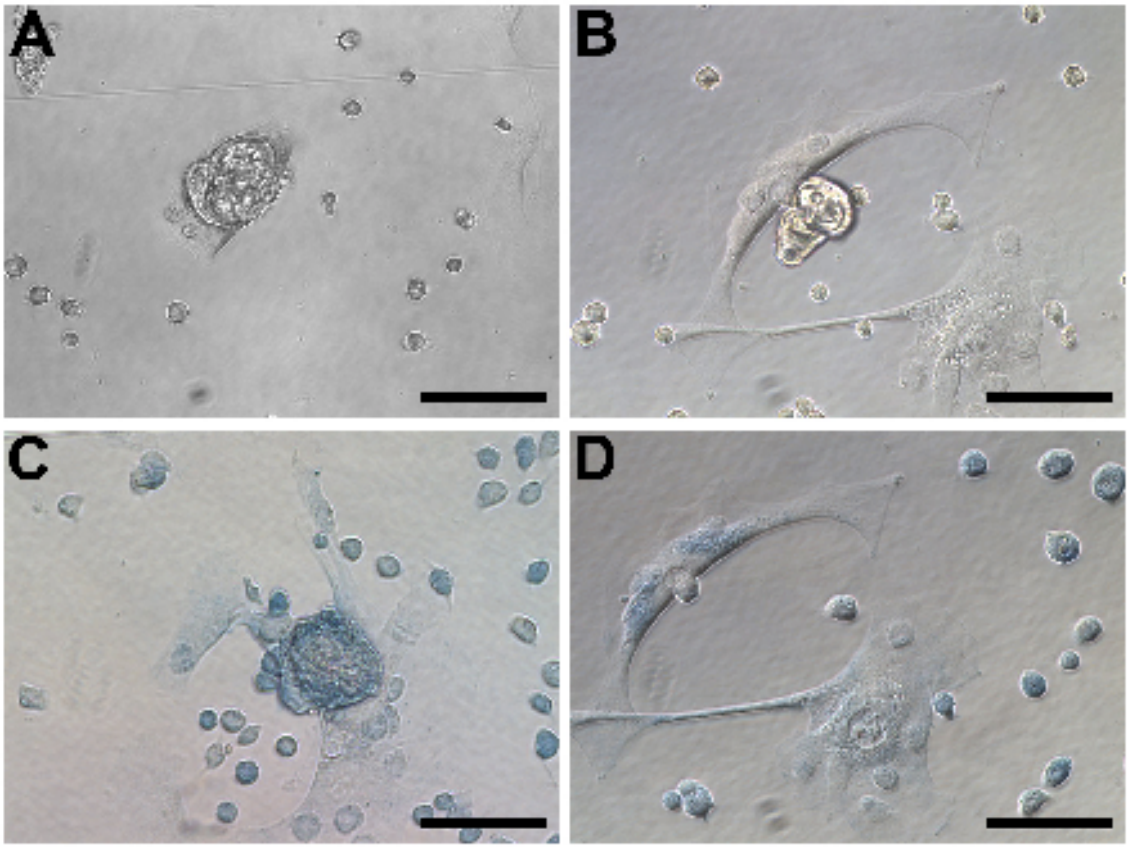


Fig 4.4: Monocytic precursor cells cultured for six weeks in serum containing chondrogenic media: DMEM/10% FBS/1% PS, supplemented with 2 mM L-glutamine and 10 ng/mL TGF-β1. (A) Representative nodule at week four of culture – mono filter. (B) Nodule and matrix before staining – color filter. (C) Stained cartilaginous nodule. (D) Stained matrix. Bar = 100 μm.

*Monocytic precursor derived cells express collagen II and aggrecan and lack expression of F4/80 after culture in chondrogenic conditions*

Monocytic precursor derived cells under serum free conditions containing DMEM/1% ITS/ 1%PS and supplemented with or without TGF-β1 or TGF-β3 and 2mM L-glutamine, 0.1 mM ascorbic acid, and in some cultures dexamethasone, demonstrated expression of Collagen II and Aggrecan, both specific cartilage markers, yet lacked the expression of F4/80 as assessed by immunofluorescence (Figs 4.5, 4.6). Collagen II is the major component of cartilage extra cellular matrix. Aggrecan is also a component of cartilage ECM. The presence of both of these proteins within these monocytic precursor derived cells further indicates that these cells have differentiated down a chondrogenic lineage. Because monocytic precursors have a known HSC origin, the expression of these proteins by these cells confirms that HSCs are able to differentiate into chondrogenic cells. F4/80 is a monocyte and macrophage marker indicating that these cells have lost their monocytic marker over the differentiation period and are no longer monocytic cells. Furthermore, monocytic precursor cells were stained for Collagen II, Aggrecan, and F4/80 before chondrogenic induction and lack expression of collagen II and aggrecan while demonstrating positive F4/80 expression. This indicates that the monocytic precursor cells express a monocytic marker prior to chondrogenic induction and do not express cartilage specific markers before induction.

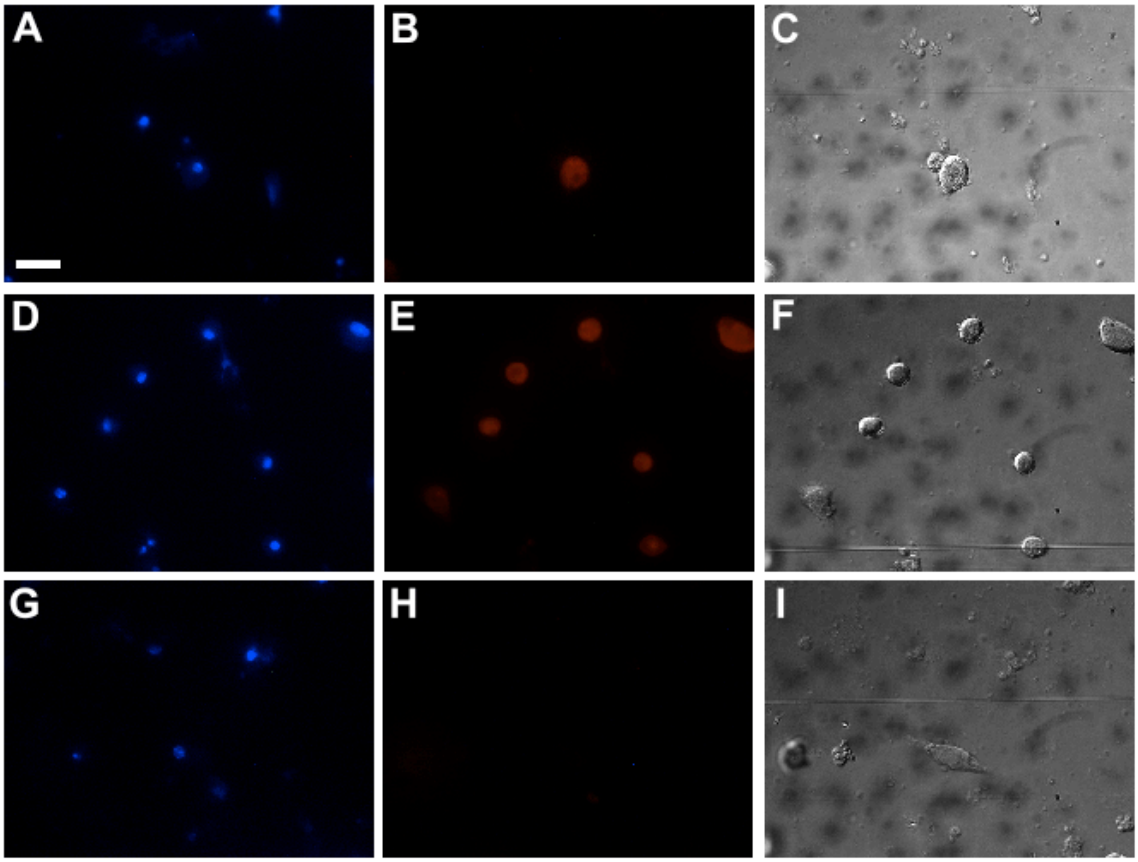


Fig 4.5: Immunofluorescent staining of monocytic precursor cells cultured for six weeks in serum free media: DMEM/1% ITS/1% PS supplemented with 2 mM L-glutamine, 0.1 mM AA, $10^{-7}$M dexa, and 10 ng/mL TGF-β3. (A, D, G) Hoechst nuclear stain. (B) Aggrecan staining (C, F, I) DIC images. (E) Collagen II staining. (H) Secondary only staining
Bar = 50 μm for all panels.

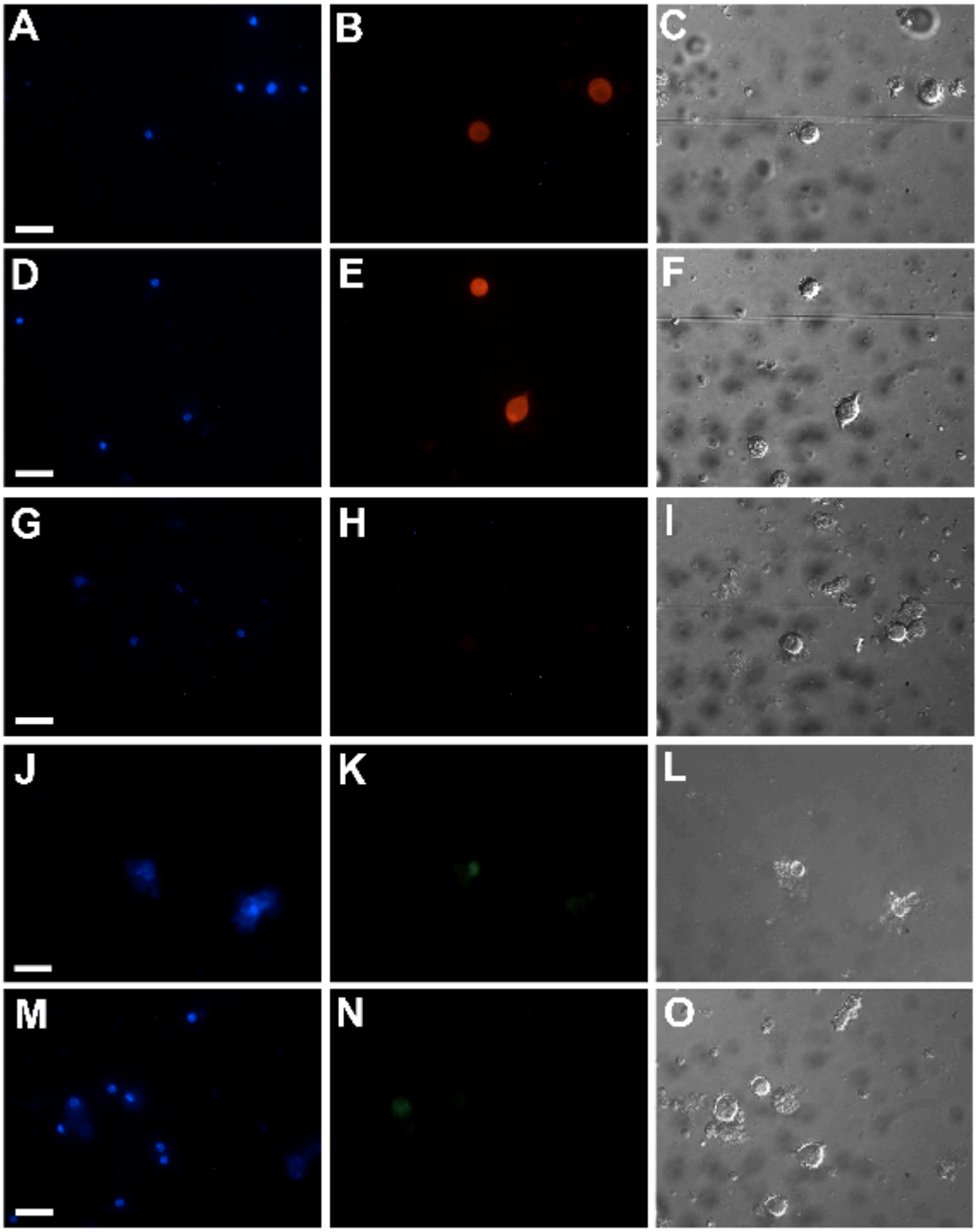


Fig 4.6: Immunofluorescent staining of monocytic precursor cells cultured for six weeks in serum free media: DMEM/1% ITS/1% PS supplemented with 2 mM L-glutamine, 0.1 mM AA, and 10 ng/mL TGF-β1. (A, D, G, J, M) Hoechst nuclear stain. (B) Aggrecan staining. (C, F, I, L, O) DIC images. (E) Collagen II staining. (H) Secondary only control for Aggrecan and Collagen II staining. (K) F4/80 staining. (N) Secondary only control for F4/80 staining. Bar = 50 μm.

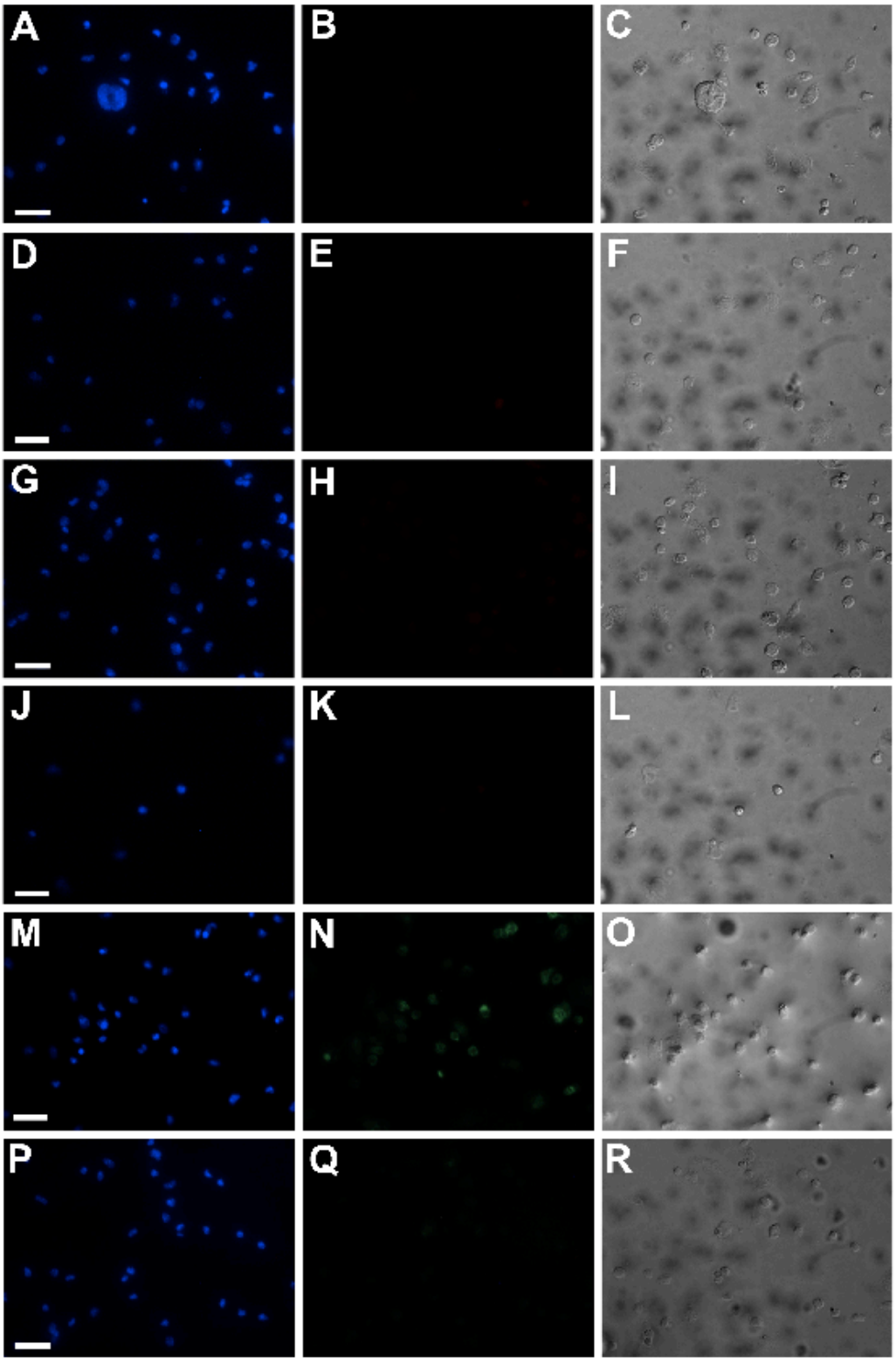


Fig 4.7: Monocytic precursor cells prior to chondrogenic induction. (A, D, G, J, M, P) Hoechst nuclear stain. (B) Aggrecan staining. (C, F, I, L, O, R) DIC images. (E) Secondary only control for Aggrecan staining. (H) Collagen II staining. (K) Secondary only control for Collagen II staining. (N) F4/80 staining. (Q) Secondary only control for F4/80 staining. Bar = 50 μm.

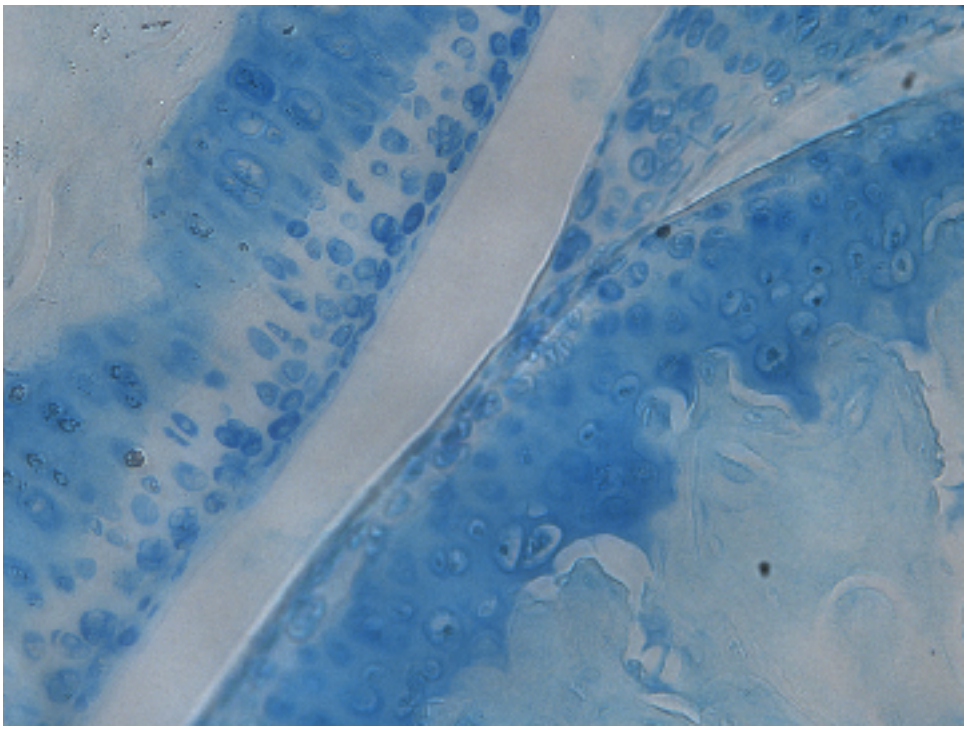

Fig 4.8: Knee joint of a bone section stained with Alcian Blue – 200x.

## 4.4 Discussion

Both primary cell types investigated, the non-adherent bone marrow fraction cells and the monocytic precursor derived cells, were found to have chondrogenic potential, thus, these studies identify a new source of cells with this ability.

The chondrogenic ability of the non-adherent bone marrow fraction, enriched for HSCs, was compared to that of the adherent fraction, enriched for MSCs, and both stained positive for Alcian Blue indicating the presence of GAGs and their chondrogenic potential. The non-adherent cells were cultured under

serum and serum free conditions. Although these cells stained positive for Alcian Blue under both conditions, it was only in media containing serum that these cells formed cartilaginous nodules. This perhaps is due to the presence of many different growth factors present within the serum compared to the single growth factor added to the serum free media. The complex combination of growth factors may be needed in order to provide the correct signals for cells to form these cartilaginous nodules. It may also be a factor of cell density within the culture or frequency of the chondrogenic progenitor cell within this enriched population, because not all the cultures cultured in serum media formed these nodules. In future studies, it would be interesting to identify other growth factors that may be involved in this process and/or the frequency of progenitor cells since the formation of cartilaginous nodules *in vitro* is a characteristic of harvested articular chondrocytes in culture as identified in Aim 1. Despite the inconsistency of nodule formation, both serum and serum free conditions were tested in monocytic precursor experiments to detect an effect of a single growth factor on chondrogenic differentiation and better control the variables within the culture so that these results could be replicated consistently.

Since the non-adherent, HSC-enriched population demonstrated the chondrogenic ability, it was necessary to further confirm these findings using a better defined HSC-derived population, the monocytic lineage. Other groups have demonstrated a chondrogenic precursor in the monocytic lineage[35] and our previous studies have shown an HSC origin for adipocytes[39,23] through a monocytic precursor lineage, we chose to examine these monocytic precursor

derived cells under chondrogenic conditions. Under serum containing conditions, the monocytic precursor derived cells formed cartilaginous nodules similar to that of the harvested articular cartilage cultures. This *in vitro* growth pattern could indicate similar lineage commitment during differentiation. This, in combination with the presence of positive Alcian Blue staining, demonstrated the potential for chondrogenic commitment. Validation for chondrogenic differentiation of these cells was the identification of cartilage specific protein expression of Collagen II and Aggrecan by immunofluorescence. Monocytes are not known to naturally express either of these proteins, and because both of these cartilage markers are expressed in our cell type after induction this provides significant evidence that these cells are of a cartilage nature and were derived from an HSC lineage.

An important point to address, however, is the staining pattern of the cells. *In vivo*, as in the cartilage of the knee, Alcian Blue as well as Collagen II and Aggrecan are all staining for extracellular matrix components, with a pattern of expression around or outside of the cell *in vivo* (Fig 4.7). However, the staining pattern for these cells in culture is intracellular. This pattern is in agreement with that seen in cultured cells in other studies[11]. This could be accounted for by a variety of reasons. The most likely is that these cells are grown in monolayer, which is not a realistic environment for cartilage cells. A three dimensional environment may provide signals for these cells to secrete their matrix molecules rather than simply synthesizing them (i.e. retaining them intracellularly) [21,30]. It is also possible that the cells are not at a high enough density or have enough cellular contact to secrete these proteins[36]. Finally, it could be that all the proper

factors are not present in these culture conditions in order for signaling of these cells to secrete properly. However, together, data in this chapter demonstrate that HSC-derived chondrogenic cells can be generated *in vitro*.

**Significance**:

These results indicate the potential of HSC-derived cells to differentiate into chondrogenic cells *in vitro*, providing evidence for a greater plasticity of HSCs than previously thought. The indication of this would be that HSC-derived cells have the potential to also be investigated for use in cartilage regeneration therapies.

# CHAPTER FIVE: GENERAL DISCUSSION

Osteoarthritis (OA) affects millions of middle-aged and elderly people and has become one of the most prevalent joint diseases as it can develop as a result of obesity, joint instability, or trauma. Its costs have significantly increased over the recent years and have increased the economic burden of society to the point where they "account for up to 1% - 2.5% of the gross national product of countries with aging populations such as the USA, Canada, the UK, France, and Australia"[46]. Since there are limited options for therapy, surgical intervention is often the treatment option. However, surgery brings risk for complications and potential side effects both of which can also affect society's economic burden[17]. More importantly, patient quality of life can be greatly diminished with the pain, immobility, and joint stiffness that can occur even with minimal damage to cartilage because of its lack of reparative properties. Even small lesions can contribute to the risk of osteoarthritis over time[12]. Trauma to cartilage can hold serious repercussions for the site of injury. For example, apoptosis of chondrocytes can occur during chondral injury often at a significant percentage of 34% as shown by D'Lima et. al. (referenced by Ulrich-Vinther et. al.)[45]. The edges of the chondral fracture will not fuse due to lack of migration of proliferating chondrocytes. In deeper osteochondral lesions release of stem cells from the bone marrow may secrete growth factors for differentiation and fill in the fracture; however, the resulting repair is of fibrocartilaginous matrix and can lead to further degeneration of the joint tissue due to lack of stability and structure[45].

Drilling, autologous chondrocyte implantation (ACI), and osteochondral allograft are common treatments currently being used in the clinic, however, the cartilage structure is not regenerated to its previous hyaline state preventing normal motion and leading to earlier degeneration[44].

Stem cells not only have differentiation and expansion potential but also are readily accessible. All of these features make them appealing for the potential use of cartilage regeneration[44]. Bone marrow, the source of HSCs, is already routinely harvested for a variety of medical treatments and so is a familiar procedure for clinical practice. Using it as a source for stem cells with the capability of chondrogenic differentiation has incredible potential. In this study we have identified a novel lineage of chondrocytes and therefore a novel source of stem cells with the ability to be cultured and expanded *in vitro.* This study has provided a basis by which to examine new cells for the use of cartilage regeneration. From Aim 1, knowledge was attained regarding the characteristics of chondrocytes *in vitro* by using harvested articular chondrocytes and identification of predominant growth factors involved in chondrogenic differentiation as assessed by the cell line ATDC5. Most importantly it was demonstrated that both isoforms of TGF-β enhanced chondrogenesis. This finding was then applied to Aim 2 to provide insight into newly identified chondrogenic capabilities of HSC-derived cells and the role that TGF-β played in differentiating the non-adherent HSC enriched population and monocytic precursor derived cells.

We demonstrated the ability of the monocytic precursor-derived cells to produce collagen II and aggrecan and differentiatie into cells with chondrogenic morphology, including nodule formation. Given the relative ease with which hematopoietic progenitor cells, including monocytic lineage cells, can be harvested from humans, it is likely that these cells would be of great use in developing a cell-based intervention therapy to enhance regeneration of damaged articular cartilage.

**Future Directions:**

Given these results, there are many future directions from which this research would benefit greatly:

First it would be important to conduct temporal profiling of the differentiation and maturation process towards the chondrogenic lineage from monocytic precursor cells. Not only would this establish the mRNA and protein expression levels of these cells for chondrogenic markers each week of culture, but it would also aid in pinpointing when these cells are differentiated and identifying opportunities for *ex vivo* manipulations/enhancement.

In addition to temporal profiling, the necessity of specific factors on gene expression and commitment of the lineage would be important to identify. The necessity of specific factors could be determined by adding and subtracting them to/ from the culture media during time points that are crucial for differentiation as identified by temporal profiling. This would provide a better understanding for the mechanism of differentiation from the HSC lineage to the chondrogenic lineage

and may also identify novel factors that regulate differentiation from this unique origin.

As stated in a previous chapter, further investigation into the effect of hypoxia on *in vitro* differentiation of these monocytic precursor derived cells would be important for creating a more realistic microenvironment comparable to that *in vivo*. $CoCl_2$ was tested in this study, but future studies could test creating a hypoxic environment using equipment such as a hypoxia chamber.

Conducting an indirect co-culture experiment with monocytic precursor cells and harvested articular chondrocytes (or conditioned media from the articular chondrocytes) would determine whether the articular chondrocytes could enhance the chondrogenic differentiation of the monocytic precursor cells or speed up the differentiation process in any way, suggesting a role for the microenvironment in promoting HSC-derived cell differentiation to the chondrogenic lineage[4].

Along these lines, it would be most advantageous to begin exploring ways of creating a more three dimensional environment not only for these cells to grow but also for the purposes of transplantation. For cartilage tissue engineering to be successful, the *in vitro* work must eventually be able to be translated into the clinic. Testing suitable scaffolds such as collagen sponges or encapsulation of cells in materials such as matrigel or fibrin glue in order to be implanted into cartilage defects is necessary for this research to have a major clinical impact.

Lastly, assessing mechanical loading on the differentiated cells, perhaps in or on a suitable scaffold, would give a better indication of the integrity of the

construct and how it would withstand the functional stresses of articular cartilage *in vivo*.

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