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SCHOOL OF MEDICINE

Thesis

CHONDROGENESIS OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN ALGINATE HYDROGELS UNDER VARIOUS CULTURE CONDITIONS – A PILOT STUDY

by

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Submitted in partial fulfillment of the

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2013

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CHONDROGENESIS OF BONE MARROW-DERIVED MESENCHYMAL STEM CELLS IN ALGINATE HYDROGEL UNDER VARIOUS CULTURE CONDITIONS – A PILOT STUDY

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ABSTRACT

Osteoarthritis (OA) is a major concern among healthcare providers as its increasing prevalence has not shaped sufficiently adequate therapies to slow or reverse the onset of OA. Further, traumatic injury to joints in the young population can lead to early arthritic changes characteristic of the elderly population. The spontaneous regeneration of injured articular cartilage (AC) is virtually non-existent and current treatments focus on pain management and reestablishing patient mobility. No current treatments restore the AC surface to its pre-injured state, biomechanically or biochemically. Cartilage tissue engineering has expanded the potential toolbox of treatment options. Bone marrow derived mesenchymal stem cells (BMSCs) and chondrocytes are the most common cell sources for AC tissue engineering. Yet, few studies have investigated the use of a co-culture model, combing both cell sources into one hydrogel. In this study, swine BMSCs and chondrocytes were co-cultured (1:1

ratio) in a 2% sodium alginate hydrogel and implanted subcutaneously into the backs of old nude mice for 6 weeks. For comparison, two other groups were designed to study the effectiveness of this co-culture model: BMSCs only and chondrocytes only. A previous study with BMSCs grown in chondrocyte conditioned media was also evaluated with the co-culture groups. All groups (n=4) spent 2 weeks *in vitro* under the influence of chondrogenic differentiation media prior to implantation. Histological examination showed extracellular matrix formation in all gels. The BMSCs only group showed the continuous formation of matrix, while the co-culture group showed isolated pericellular matrix. The conditioned BMSCs showed the most continuous matrix formation. Not surprisingly, glycosaminoglycan (GAG) and hydroxyproline assays reported the chondrocyte gel group as the most biochemically active. The co-culture group produced the lowest amounts of GAG and hydroxyproline. This study shows that BMSCs and chondrocytes co-cultured in a 1:1 ratio does not provide a significant advantage in cartilage matrix production in comparison with BMSCs and chondrocytes cultured separately. Further studies on the proper ratio and in vitro conditions will be the next step in defining a model for co-culture of BMSCs and chondrocytes.

TABLE OF CONTENTS

Title	i
Reader's Approval Page	ii
Acknowledgements	iii
Abstract	iv
Table of Contents	vi
List of Tables	viii
List of Figures	ix
List of Abbreviations	x
Glossary	xi
Introduction	1
Articular Cartilage	1
Injury & Osteoarthritis	7
Surgical Treatment	10
Tissue Engineering	11
Alginate	13
Specific Aims/Objectives	15
Methods	17
Overview	17
Cell Isolation and Culture	17
Devitalized Ring Creation	19

Alginate Gel Creation 20

Implantation and Harvest 21

Histological Evaluation 21

Biochemical Evaluation 22

Statistical Evaluation 23

Results 24

Macroscopic Evaluation 24

Histological Evaluation 25

Biochemical Evaluation 29

Discussion 31

References 37

Vita 43

LIST OF TABLES

Table	Title	Page
1	Type of biomaterials used in cartilage tissue engineering	13
2	Overview of Alginate Gels	16

LIST OF FIGURES

Figure	Title	Page
1	Articular Cartilage Structure	3
2	Swine articular cartilage stained in H&E and safranin-O	3
3	Hematoxylin and Eosin stain of hyaline cartilage	5
4	Proteoglycan aggregate and aggrecan molecule	7
5	Chemical structure of alginate	14
6	Macroscopic appearance of alginate hydrogel after 6 weeks *in vivo*	24
7	Macroscopic appearance of alginate gels with chondrocyte conditioned BMSCs (5 weeks *in vivo*)	25
8	sGAG content in alginate hydrogels and native cartilage	27
9	Hydroxyproline content in alginate hydrogels and native cartilage at 6 weeks *in vivo*	27
10	Histological presentation of BMSCs in alginate gel (6 weeks *in vivo)*	28
11	Histological presentation of swine chondrocytes in alginate gel (6 weeks *in vivo*).	28
12	Histological presentation of swine chondrocytes and BMSCs co-cultured in alginate gel (6 weeks *in vivo*)	29
13	Histological presentation of BMSCs cultured in chondrocyte conditioned media (5 weeks *in vivo*)	30

ABBREVIATIONS

AC	articular chondrocyte
ACI	autologous chondrocyte implantation
BMSCs	bone-marrow derived mesenchymal stem cells
$CaCl_2$	Calcium chloride
$CaCO_3$	Calcium carbonate
CM	chondrocyte media
DC	devitalized cartilage
EC	engineered cartilage
ECM	extracellular matrix
FBS	fetal bovine serum
G	guluronic acid
GAG	glycosaminoglycans
H&E	hematoxylin & eosin
IGF	Insulin-like growth factor
M	mannuronic acid
MF	microfracture
OA	osteoarthritis
OATS	Osteoarticular transfer system
P0	Passage 0
PBS	phosphate-buffered saline
sGAG	sulfated glycosaminoglycan

TGFβ3	Transforming growth factor beta 3

INTRODUCTION

Articular Cartilage

Cartilage is a highly specialized tissue found throughout the body serving many functions. There are three classification types - elastic, fibro-, and hyaline cartilage. Hyaline cartilage is a subtype of cartilage that has many implications in the field of orthopedics. It is found on the ends of long bones such as the femur, tibia, and humerus; lining the articular surfaces of all diarthrodial joints. It provides mechanical compressive support as our joints experience weight-bearing and load-bearing forces during activity and movement (Mow et al., 1989).

Articular cartilage (AC) is composed of four distinct zones: superficial, middle, deep and calcified (Figures 1 & 2). Each zone is characterized by the density and shape of chondrocytes as well as the orientation of type II collagen fibers. The superficial, or tangential, zone comprises approximately 10-20% of the AC thickness and is responsible for providing a smooth surface for ease of joint movement. The collagen fibers in the superficial zone are tightly arranged in a parallel manner to the surface (Pearle et al., 2005). The chondrocytes in this layer produce little proteoglycans compared to chondrocytes in the other zones of cartilage. Instead, they produce proteins for surface lubrication (Wong et al., 1996). Chondrocytes in this layer tend to be flatter or more elongated than those in the other three layers. The layer of cartilage deep to the superficial layer is the middle zone. Here, the collagen fibrils are less organized, thicker and arranged in a non-parallel fashion with respect to the surface. The middle zone normally

makes up about 40% to 60% of the AC layer. The next 30% is called the deep zone and chondrocytes here produce large amounts of proteoglycans. This layer also has the least amount of water, giving it the highest compressive modulus. Chondrocytes in this zone are easily identifiable due to their “stacked” arrangement aligned perpendicular to the subchondral bone. Collagen fibers in this zone are larger in diameter and arrange themselves perpendicularly to the articular surface (Pearle et al., 2005). The deepest zone, called the calcified zone, is situated directly on top of the subchondral bone. The calcification results from accumulating apatitic salts which surround the chondrocytes. The deep and calcified zones are separated by a tide mark. The spatial arrangement of chondrocytes and collagen fibers largely determines the compressive modulus of each zone, increasing as you approach subchondral bone from the superficial zone (Pearle et al., 2005).

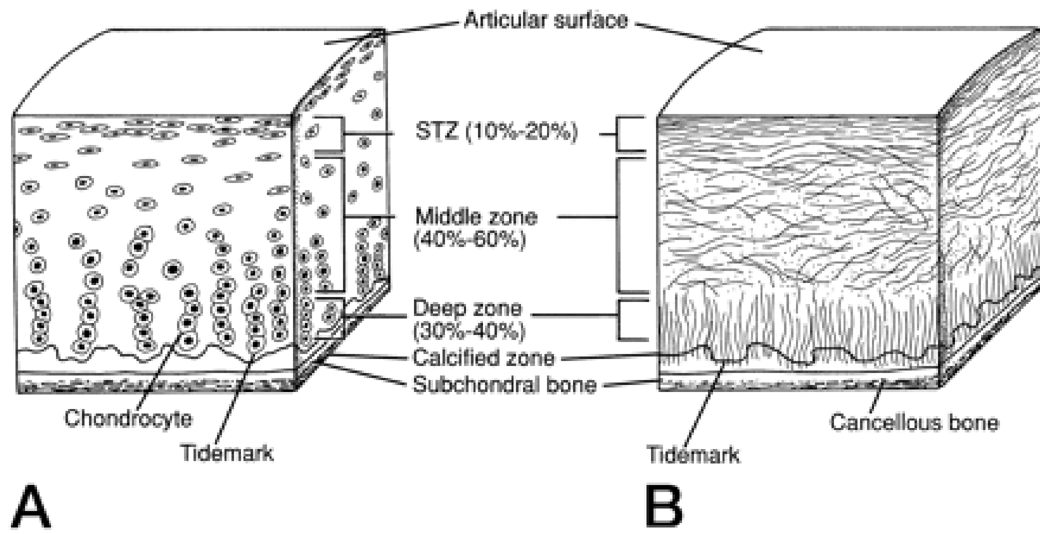

Figure 1: Articular Cartilage Structure. A, cellular organization in zones of articular cartilage; B, collagen fiber orientation (Sophia Fox, Bedi, & Rodeo, 2009)

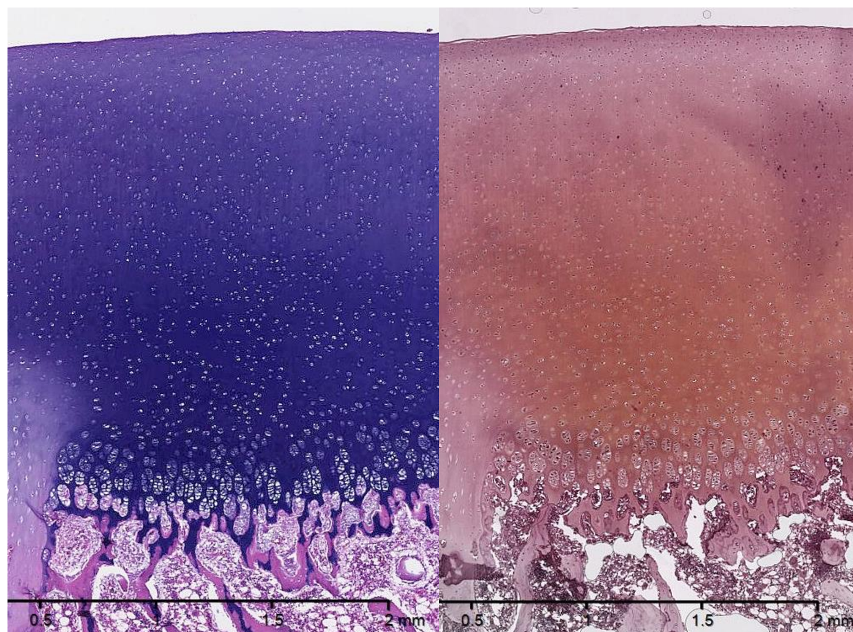

Figure 2: Swine articular cartilage stained in H&E (left) and Safranin-O (right). Comparison with the diagram shown in Figure 1 indicates the organization of the chondrocytes from the superficial zone to the deep zone. Tick marks are measured in millimeters.

The resident cell in articular cartilage is the chondrocyte and it is a highly specialized cell that plays a unique and important role in the development, maintenance, and repair of the extracellular matrix (ECM) (Alford & Cole, 2005a). Each chondrocyte is responsible for turnover and maintenance of the ECM which directly surrounds the cell. Consequently, chondrocytes are quite active, yet receive no blood supply, lymph drainage or nervous input. The water content of AC allows for the exchange of nutrients, gasses and waste products through diffusion. The normal mechanics of weight-bearing or load-bearing stresses on the AC present pressure differentials within the ECM, assisting gas, nutrient, and waste exchange (Athanasiou et al., 2001). In mature cartilage, chondrocytes have encapsulated themselves within the ECM in structures called lacunae (Figure 3). This is why chondrocytes don't appear to move after full development of the AC layer. Chondrocytes whose lacunae develop closely together form isogenous groups (Figure 3) (Kheir & Shaw, 2009).

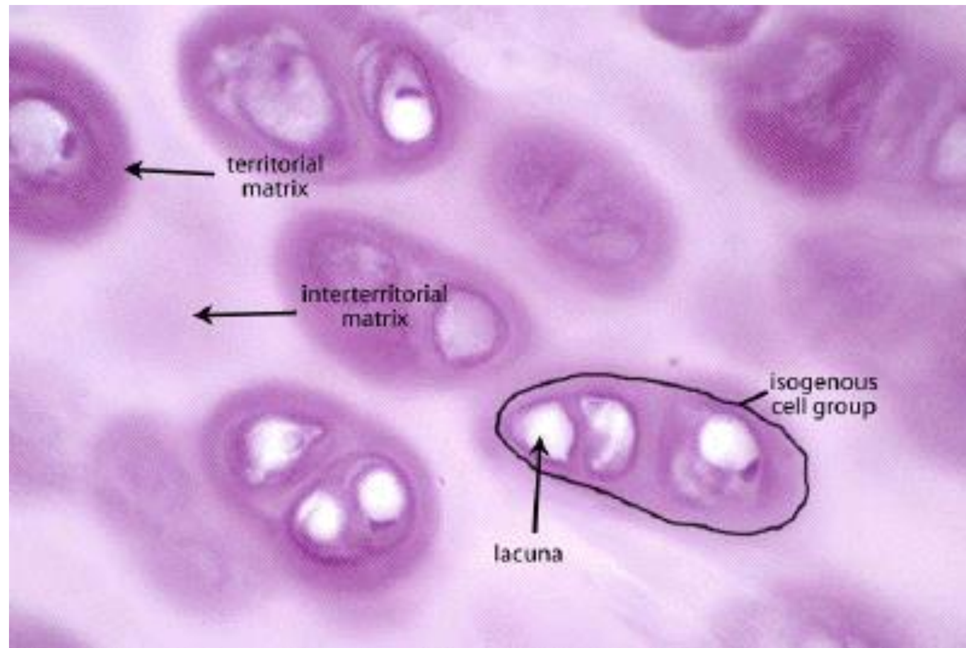

Figure 3: Hematoxylin and eosin stain of hyaline cartilage. The matrix regions are addressed above. Pericellular matrix is identified as the dark line directly adjacent to the outter edge of each lacuna. (Source: http://www.dartmouth.edu/~anatomy/Histo/lab_2/bone/DMS065/popup.html)

In AC, type II collagen provides the majority of the shear and tensile properties unique to hyaline cartilage, representing 90%-95% of the collagen (Buckwalter & Mankin, 1998). While other types of collagens are present, they merely help stabilize the type II collagen network (Buckwalter et al., 2005). The triple helix structure of type II collagen is the most influential physical characteristic on the shear and tensile properties of AC (Fox et al., 2009). These positively charged type II collagen fibrils are intertwined with the negatively charged proteoglycans. On a standard hematoxylin & eosin (H&E) stain (Figure 3), varying levels of proteoglycan distribution within the ECM are evident through

the gradient of hematoxylin staining around the lacunae (Fox et al., 2009). The dark-staining thin layer surrounding the cell is the pericellular matrix and consists of mostly proteoglycans. It has been hypothesized that this layer is important for the initiation of signal transduction during load bearing events (Eggli et al., 1985). Surrounding the pericellular matrix is the territorial matrix, which has an extensive fine collagen fibril network. This region is much thicker than the pericellular matrix (Guilak & Mow, 2000). It is also believed that this network is essential for mechanical protection of the chondrocytes during load-bearing instances (Buckwalter et al., 2005). The interterritorial matrix makes up the largest part of the matrix regions. Here, the collagen fibrils are larger and arranged in random bundles similar to that described by the zones (Figure 1B).

Proteoglycans are the second most abundant macromolecule in the ECM. They consist of a protein core with 1 or more glycosaminoglycans (GAGs) attached (Buckwalter & Mankin, 1998). Glycosaminoglycan chains are composed of many monosaccharides that exhibit charge repulsion creating a "distended state" (Newman, 1998). GAG side chains carry large amounts of negative charge, creating a hydrophilic environment for the high water content (60%-80%) that helps AC resist compressive loads (Alford & Cole, 2005a). Chondroitin sulfate, keratin sulfate, hyaluronic acid, and dermatin sulfate are the most common GAGs found in AC. Each one's concentration varies with increasing age, traumatic injury, and disease (Buckwalter et al., 2005).

Aggrecan is the major proteoglycan in AC consisting of one hundred chondroitin sulfate and 50 keratan sulfate GAG chains bound to a protein core. Many aggregan molecules attached to a hyaluronic acid molecule make up a proteoglycan aggregate (Figure 4) (Buckwalter et al., 2005).

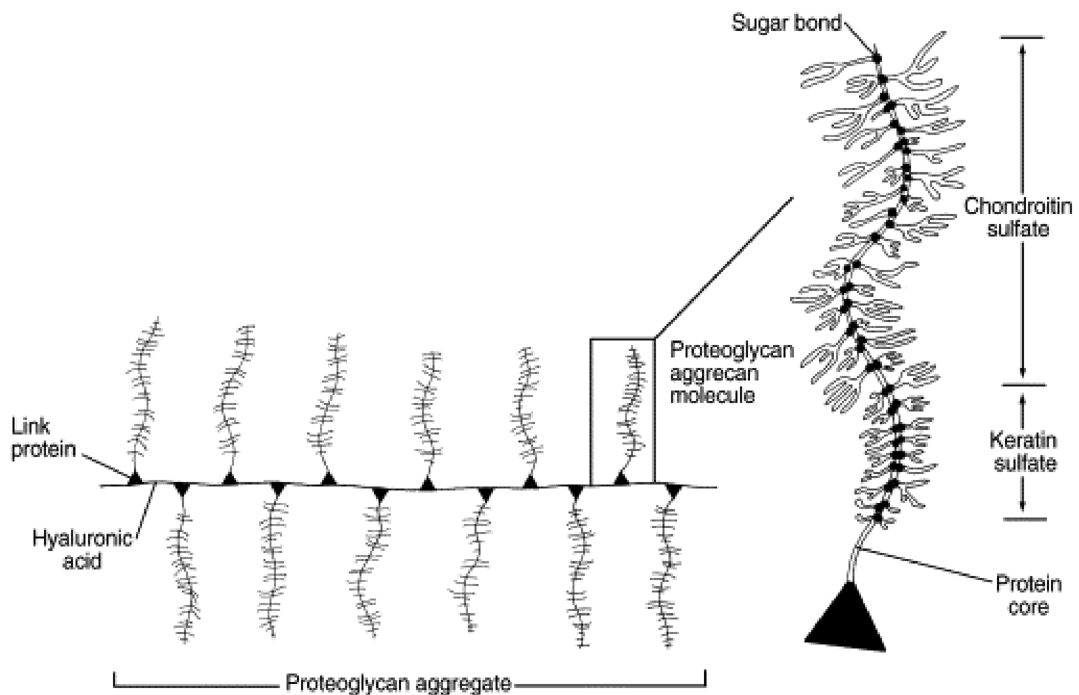

Figure 4: Proteoglycan aggregate and aggrecan molecule (Pearle et al., 2005). Aggrecan is composed primarily of chondroitin sulfate and keratin sulfate attached to a protein core. Chondroitin sulfate and keratin sulfate carry large amounts of negative charges and repel one-another. This gives the proteoglycan aggregate a large volume with which to attract water.

Injury & Osteoarthritis

Degradation and loss of the AC layer is commonly known as osteoarthritis (OA). Generally, OA is a degenerative joint disease whereby loss of hyaline

cartilage can result in severe pain and limited mobility (Alford & Cole, 2005a). The progression of this disease results in both mechanical and biochemical changes, affecting the overall health and utility of the joint. Macroscopically, the earliest evidence of OA may be "fraying" of the AC surface.

Two of the most common ways in which the integrity of hyaline cartilage is lost is through traumatic injury and normal aging. Traumatic injury to the articular surface can often lead to chondral lesions that can further degenerate (Messner & Maletius, 1996). Partial lesions do not heal and full-thickness lesions of AC can lead to fibrocartilage invasion. Fibrocartilage formation is a response that fills the void left by a lesion yet lacks the resiliency of normal AC, both mechanically and biochemically (Brittberg et al., 1999). Depending on the extent of the lesion, injury may also lead to decreased normal shear stresses in the knee, which is the main source for pressure gradients necessary for appropriate nutrient and waste exchange (Athanasiou et al., 2001).

The articular lesions created by this aging process only heal partially, if at all (Hunziker, 2002). Biochemically, the progression in OA is directly proportional to the loss of proteoglycan content and composition. A larger percentage of nonaggregated proteoglycans exist via proteolytic degradation. The decrease in proteoglycan aggregates is accompanied by a decrease in the GAG chains resulting in a larger influx of water. At the same time, the collagen network becomes less organized, possibly aiding in the increased water concentration among the spread out aggrecan molecules. Cumulatively, these changes

decrease the overall stiffness of the hyaline cartilage matrix through an increase in water permeability (Akizuki et al., 1987; Buckwalter et al., 2005). The high water content and decreased stiffness result in a softening of the articular layer known clinically as chondromalacia (Bentley, 1970). Collagen changes in AC during progression of OA result mostly in decreased organization, unlike proteoglycan changes where we see decreased content and formation of aggregates.

At the cellular level, chondrocytes proliferate around the area of increased osmolarity. With increased proliferation, there is an increase in matrix production. Interestingly, there is a simultaneous release of metalloproteases which degrade newly synthesized and existing matrix components (Gillogly et al., 1998). Overall, increased matrix production outweighs the activity of the metalloproteases and can lead to stabilization of the articular surface, delaying the spread of OA (Buckwalter et al., 2005).

With advancing age, there is an increasing prevalence of osteoarthritis. The pathological progression results in the eventual loss of the articular cartilage layer, exposing the underlying bone surface. In the knee, the loss of articular cartilage creates bone-on-bone contact, often resulting in chronic pain and limited mobility. OA is a growing concern among chronic diseases, currently affecting an estimated 50 million adults in the United States. Projections estimate an increase towards 67 million by 2030 (Hootman & Helmick, 2006).

Surgical Treatment

In the United States as of 2009, it is estimated that OA accounts for over $185 billion combined out-of-pocket and insurer expenditures (Kotlarz et al., 2009). OA can be attributed to other primary healthcare concerns such as diabetes and obesity. Further, OA ranks fourth in hospitalization causes (Murphy & Helmick, 2012). Cartilage replacement surgeries account for more than 1 million surgical procedures each year (Langer & Vacanti, 1993).

Among these surgical procedures, microfracture (MF) is a popular surgical technique currently used to treat cartilage damage. MF surgery aims to grow new cartilage by creating a blood supply to the damaged area, which is achieved by punching holes in the underlying bone to induce bleeding and thus expose bone marrow cells to the articular surface ("Articular Cartilage Restoration - OrthoInfo - AAOS," n.d.). As previously mentioned, the new tissue produced is mostly fibrocartilagenous and inferior to the previous tissue. MF provides temporary relief at best by delaying the onset or progression of arthritic changes. Strict patient compliance is needed post-operatively in order to achieve the best outcomes (Alford & Cole, 2005b).

Another technique employed is autologous chondrocyte implantation (ACI) in which cartilage from a non-weight bearing portion of the distal femur is removed and digested to yield chondrocytes. The chondrocytes are cultured in the lab and then injected back into the original defect. Often, periosteum taken from the proximal tibia is used to cover the area where chondrocytes were

injected. Donor site morbidity and low chondrocyte acquisition are common problems encountered with ACI. Further, this course of treatment requires two major invasive surgeries only a few weeks apart (Alford & Cole, 2005b).

Osteoarticular transfer system (OATS) is another widely used surgical treatment for chondral lesions. In this autograft technique, damaged cartilage is removed with a biopsy punch of variable diameters. Another biopsy punch is used to obtain a similarly sized osteochondral plug from a non-weight bearing portion of the distal femur and transplanted into the site of the original injury. The idea that healthy cartilage now replaces the same previously damaged area is the main proponent of this technique (Hangody et al., 2008). Yet, frequently the plugs transplanted do not fit the exact contour of the injured area of the condyle of the distal femur, resulting in graft failure and pain due to improper surface contact with the meniscus and tibial plateau (Garretson et al., 2004). Similarly, if a cartilage defect covers a significant portion of an articular surface, a mosaic OATS procedure can be employed. Yet it is difficult to construct the contour of the articular surface with multiple osteochondral plugs (Cain & Clancy, 2001; Pearce et al., 2001).

Tissue Engineering

Langer and Vacanti identified tissue engineering as

> "… an interdisciplinary field that applies the principles of engineering and the life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function." (Langer & Vacanti, 1993).

Tissue engineering presents a field where the current therapy shortfalls and costs can be overcome.

Tissue engineering aims to create new tissue, termed neotissue, using cells suspended with a scaffold material. Probably the biggest challenge in the field of tissue engineering is constructing a scaffold that can be durable and resilient yet be broken down over time and eventually be replaced by the ECM made from cells suspended within the scaffold. Further, the perfect scaffold must be able to provide adequate cell nutrition or diffusion of nutrients in order to maintain high cell viability over the course of neocartilage formation. Thus, a good scaffold must be able to act as an intermediate ECM while the cells adapt to their new physiological environment (Rowley et al., 1999).

Tissue engineering of cartilage most commonly utilizes chondrocytes and mesenchymal stem cells as cell sources. Chondrocytes represent an obvious choice for cells since they already express the necessary matrix components of AC (Chung & Burdick, 2008). Chondrocyte acquisition can be adversely affected in instances of elderly patients, limited donor sites or loss of chondrocyte phenotype during *in vitro* culture, requiring another source of cells (Tuan et al., 2003). In support, mesenchymal stem cells are a popular choice for their intrinsic

renewability and capacity to differentiate towards the chondrogenic lineage (Cancedda et al., 2003).

Scaffolds have the ability to provide expression of desirable phenotypes within a certain cell lineage in addition to supporting cell attachment and migrations (Table 1) (Danišovič et al., 2012).

Table 1. Type of biomaterials used in cartilage tissue engineering (Table adapted from Danišovič et al., 2012).

Natural Polymers	Synthetic Polymers
Aragose	Poly (α-hydroxy esters)
Alginate	Poly (ethylene glycol/oxide)
Cellulose	Poly (NiPAAm)
Collagen	Poly (propylene fumarate)
Chitosan	Poly (urethane)
Chondroitin Sulfate	Poly (vinyl alcohol)
Fibrin Glue	
Gelatin	
Hyaluronic Acid	
Silk Fibroin	

Alginate

Brown algae are the main source for alginate used in tissue engineering and was first discovered and characterized by Stanford in 1883 (Mautner, 1954). Alginate gels have long been used in the food (Steinbüchel & Rhee, 2005) and paper (Augst et al., 2006) industries as emulsifiers and shear thinning agents

respectively. Further, alginate has become popular in the pharmaceutical industry as a drug delivery mechanism (Ciofani et al., 2002).

The structural unit that comprises the alginate polysaccharide are two guluronic acid (G) and two mannuronic acid (M) residues connected via (1,4)-linkages (Figure 4) (Augst et al., 2006). -The amount and sequence of the G and M residues is variable and depends on the source of the alginate (Martinsen et al., 1989). Divalent cations, commonly Ca^{2+}, are used to ionically cross-link adjacent polymer chains by interacting with the carboxylic acid groups of G-blocks (Augst et al., 2006).

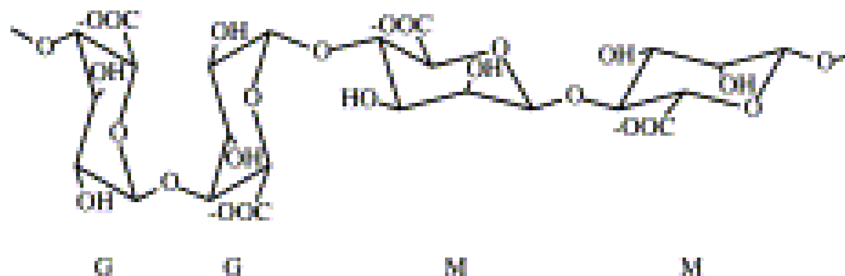

Figure 5: Chemical structure of alginate. Shows two guluronic acid (G) monomers and 2 mannuronic acid (M) monomers in a chain (Drury et al., 2004)

The stiffness, swelling and degradation rates of alginate are variable, producing a customizable hydrogel with tissue specific applications (Augst et al., 2006). Many of these properties are controlled by gelation rate, which is determined by the cross-linker used (Kuo & Ma, 2001). Calcium chloride ($CaCl_2$) and calcium carbonate ($CaCO_3$) solutions have been a popular source for calcium ions required in the ionic cross-linking of alginate (Kuo & Ma, 2001).

They have differing water solubilities and therefore different diffusion rates through the alginate solution, ultimately affecting the gelation rate. Slower cross-linking results in enhanced mechanical properties (Augst et al., 2006). Alternatively, alginate can be covalently cross-linked by carbodiimides, resulting in slower degradation times (Rowley et al., 1999). In tissue engineering, alginate hydrogels represent a favorable scaffold due to their biocompatibility and non-immunogenicity (Gu et al., 2004; Silva et al., 2006).

Specific Aims/Objectives

From previous studies, it is apparent that a successful AC engineered replacement is one that will be able to withstand the normal mechanical loads experienced by the knee throughout daily activities. Current research has yet to generate AC that has the compressive properties of adult tissue. Furthermore, there seems to be a connection between the type of scaffold that is used to deliver the cells and their ultimate resiliency *in vivo*. Limited research has questioned the efficacy of delivering chondrocytes and bone-marrow derived mesenchymal stem cells (BMSCs) in one gel. It is plausible that chondrocytes cultured with BMSCs in alginate can produce a neocartilage comparable to chondrocytes or BMSCs alone. It is hypothesized that chondrocytes will aid in directing BMSCs toward their chondrogenic potential while simulatenously producing ECM products. Combining BMSCs with chondrocytes minimizes the strict requirements of ACI therapies where chondrocytes are the only cell source.

The goal of this study was to assess the utility of alginate as a model hydrogel for AC tissue engineering in conjunction with use and viability of BMSCs co-cultured with chondrocytes as a hybrid cell source. Histological and biochemical evaluation of the chondrogenic capacity of BMSCs and subsequent collagen II matrix production within a sodium alginate hydrogel will be discussed. The results of this study will be compared to a previous alginate experiment in which BMSCs were cultured in chondrocyte conditioned media.

Table 2: Overview of Alginate Gels

Study	***Group***		***Timepoint***	***Size***
Co-Culture Study	BMSCs	Experimental	6 weeks *in vivo*	4
	Chondrocytes	(+) control		
	BMSCs/Chondrocytes (1:1 ratio)	Experimental		
Conditioned Media Study	BMSCs (conditioned media prior to gel seeding)	Experimental	5 weeks *in vivo*	

MATERIALS AND METHODS

Overview

All procedures were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. Sterility was maintained throughout all stages of the experiment. Briefly, swine articular chondrocytes and BMSCs were isolated and cultured. BMSCs and chondrocytes were suspended within a 2% sodium alginate hydrogel constructs and cultured *in vitro* under chondrogenic differentiation media for two weeks. After two weeks *in vitro*, constructs were implanted subcutaneously in nude mice for 6 weeks.

Cell Isolation and Culture

Knees were harvested from two 4-month-old Yorkshire swine (40 kg). The femoral and patellar articular cartilage layer was removed using a no. 10 surgical blade (Bard-Parker, Dublin, Ohio). The cartilage was chopped into 1 mm^3 chips and digested for 18 hours in 0.1% collagenase type 2 (Worthington Biochemical, Freehold, NJ), F-12 HAM (Gibco, Grand Island, NY) and 1% antibiotic antimycotic solution (Sigma, St. Louis, MO). The suspension was passed through a sterile 100 μm filter (Becton Dickinson, Franklin Lakes, NJ) to remove any undigested tissue. Cells were then centrifuged at 250 *g*'s for 10 minutes. The supernatant was aspirated and the cell pellet was resuspended in chondrocyte media (CM) consisting of F-12 HAM, 1% antibiotic antimycotic solution, 10% Fetal Bovine Serum (FBS) (Gibco), 50 μg/ml L-ascorbate (Sigma)

and 1% non-essential amino acids (Gibco). The cells were centrifuged and washed twice more. Cell number and viability were determined using the hemocytometer and trypan blue method. Chondrocytes were finally plated at a density of 10,000 cells/cm^2 in 150 cm^2 tissue culture treated flasks (BD Falcon, Bedford, MA) with 25 mL of CM and incubated at 37˚C and 5% CO_2 under humidified conditions. This culture period was identified as passage 0 (P0), since cells had not yet been split. Media was changed twice weekly.

Additional Yorkshire swine knees were harvested for bone marrow processing. The soft tissue and muscle layers were carefully removed to expose the distal femur and proximal tibia. A sterile saw was used to cut 1 cm off of the distal femur and proximal tibia. Bone curretts were used to scrape the interior of the trabecular bone on both sides of the cut. Bone fragments were collected in 0.9% saline and chopped with sterile scissors until no large fragments existed. The mixture was then passed through 3 layers of sterile gauze and 100 µm filters to remove any remaining large bone fragments. The remaining solution was then plated in 150 cm^2 tissue culture flasks in 10 mL aliquots. BMSC media (10% FBS, high glucose DMEM, 1% antibiotic antimycotic solution, and 50 µg/ml L-ascorbate) (15 mL) was added to each flask (P0). Flasks were placed in a 37°C humidified incubator with 5% CO_2. After one week, flasks were washed with 10 mL of phosphate-buffered saline (PBS) twice and refilled with 25 mL of BMSC media. Media was changed twice weekly. BMSCs were trypsined and replated

at 10,000 cells/cm^2 (P1). Cells were grown and trypsined until the end of the third passage (P3).

In a separate previous experiment, BMSCs were isolated as described above and cultured until P3. During the P3 phase, the cells were cultured with conditioned media from chondrocytes. P0 chondrocytes were harvested and plated into 150 cm^2 flasks at a cell density of 10,000 cells/cm^2. Chondrocytes were cultured with BMSC media instead of CM. Media was changed twice weekly and old media was stored for use on P3 BMSCs. Conditioned media was fed to BMSCs during the entire P3 culture period, which lasted 7 days.

Devitalized Ring Creation

Devitalized cartilage rings were used to mold the alginate gel. Knees were harvested from 4-month-old Yorkshire swine. Subcutaneous tissue and muscle were carefully removed to expose the femoral condyles and patellar groove. Osteochondral plugs were collected from the distal femur using an 8.5 mm surgical biopsy punch (Acufex, Smith & Nephew, Memphis, TN). Cartilage discs were created by using a custom designed cutting block, removing the bone layer from the original osteochondral plugs. A disposable biopsy punch (Miltex, Integra, Plainsboro, NJ) was used to create a ring with a 6 mm inner diameter. Cartilage rings were placed in PBS (Sigma) with 1% antibiotics and exposed to a minimum of five freeze-thaw cycles in order to kill the chondrocytes. Rings were

stored at -20˚C until gels were made. Final dimensions of the devitalized ring were 8.5 mm outer diameter, 6 mm inner diameter and 2.25 mm thick.

Alginate Gel Creation

Sodium alginate powder, Pronova UP LVG (NovaMatrix, Sandvika, Norway), was dissolved in 0.9% saline to create a 2% (weight) alginate solution. The solution was vortexed and placed in a 37°C incubator for 30 minutes. The alginate gel was then filtered using a 20 µm sterile syringe filter (Corning Incorporation, Corning, NY). Three alginate gel groups were organized for the experiment (n=4): BMSCs only, BMSCs and chondrocytes in a 1:1 ratio, and chondrocytes only. Chondrocytes in culture flasks were removed with 0.05% Trypsin-EDTA (Gibco). Collected cells were washed twice with PBS and centrifuged at 250 g's for 10 minutes. BMSCs were collected from the culture flasks in a similar fashion. The supernatant was discarded and the cell pellets resuspended in the 2% alginate gel at a concentration of 40 million cells per mL for each of the three experimental groups. Devitalized cartilage rings were thawed to room temperature and 10 µL of 10% calcium chloride solution (w/v) was pipetted into each ring construct. Then 100 µL of the alginate-gel solution was pipetted into each devitalized ring and another 10 µL 10% calcium chloride was pipetted on top of the alginate gel. Gels were allowed to set for 15 minutes at 37˚C and then placed into individual wells of a 24-well plate. Each well was filled with 1 mL StemPro Chondrogenic Differentiation media (Gibco) and

cultured at 37˚C and 5% CO_2 for two weeks under humidified conditions. Differentiation media was changed twice weekly until implantation.

The alginate gel creation for the chondrocyte conditioned BMSCs (n=4) was the same as just described except devitalized ring models were not used. Instead, alginate gel molds were formed using 1 mL polypropylene microcentrifuge tube (Fisher Science). These gels experienced the same in vitro culture conditions.

Implantation and Harvest

Under sterile surgical technique, gels were implanted subcutaneously into the backs of 6-week old nude mice (Massachusetts General Hospital, Boston, MA). Alginate co-culture study gels were harvested at 6 weeks and initially evaluated macroscopically. Alginate gels seeded with chondrocyte conditioned BMSCs were harvested at 5 weeks and also initially evaluated macroscopically. Conditioned BMSCs were not left *in vivo* for 6 weeks due to time constraints. All constructs from each group (n=4) were used in histological and biochemical evaluation.

Histological Evaluation

One fourth of each construct was used for histological evaluation by hematoxylin and eosin (H&E), toluidine blue, and safranin-O staining. After harvest, one-fourth of each construct was fixed in 10% phosphate buffered

formalin (Fisher Scientific) for 18 hours. Formalin was replaced with PBS prior to embedding and staining. Samples were paraffin embedded and stained with H&E for overall evaluation of neocartilage of the samples. Samples were stained with toluidine blue and safranin-O in order to evaluate the GAG content of the engineered tissue. Toluidine blue and safranin-O are charged dyes and therefore bind to negatively charged GAGs, revealing the relative synthesis of this ECM component.

Biochemical Evaluation

One-fourth of each construct was used to evaluate the sulfated glycosaminoglycan content using Blyscan sGAG Assay kit (Biocolor, Carrickfergus, UK). Briefly, samples were digested in papain extraction reagent (150 µg/mL papain; 200 mM sodium phosphate buffer, pH 6.4; 10mM sodium-EDTA; and 5mM cysteine-HCl) for 18 hours overnight at 65˚C. Additionally, hydroxyproline content was measured on one-fourth of each construct using a hydroxyproline colorimetric assay kit (Biovision, San Francisco, CA). Samples were homogenized using a motorized homogenizer, Tissue Ruptor (Qiagen, Hilden, Germany), and exposed to acid hydrolysis (concentrated HCl) at 120˚C for 3 hours. Swine AC served as the positive control and was included in the assay for sGAG and hydroxyproline. Partial constructs were weighed (wet weight) prior to each test.

Statistical Analysis

A Student *t*-test was performed among the biochemical data collected in this study. An alpha value of 0.05 was used for statistical significance and all values are reported as the mean ± standard deviation.

RESULTS

Macroscopic Evaluation

The alginate gels within the devitalized ring model were harvested at the end of 6 weeks in nude mice and presented in Figure 6 below. The gels appeared to maintain their shape and volume within the devitalized cartilage ring. Evidence of a possible neocartilage formation is seen in all samples, to varying degrees, within the chondrocyte-BMSC and BMSC groups. Inflammation and scar tissue was not present at the time of sample harvest.

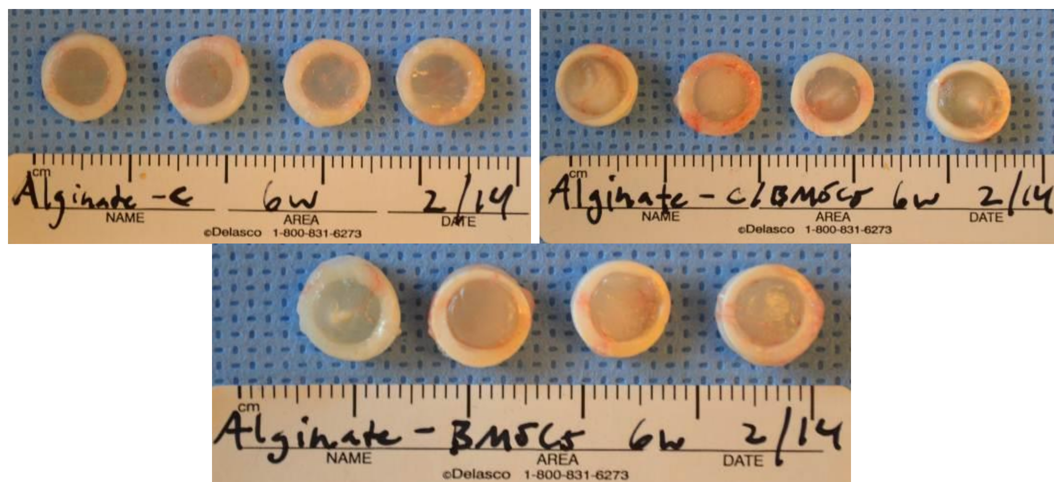

Figure 6: **Macroscopic appearance of alginate hydrogel after 6 weeks *in vivo*.** Gross appearance of chondrocytes (top left); chondrocytes and BMSCs co-cultured (top right); and BMSCs (bottom) in alginate gel.

The alginate gels with chondrocyte conditioned BMSCs were harvested after 5 weeks and indicated in Figure 7 below. Possible neocartilage formations is evidenced by the white opaque accumulations, but were not continuous throughout the gel. Upon touch, there seemed to be some calcifications present.

Likewise, there were no signs of inflammation or scar tissue buildup in the area of sample harvest.

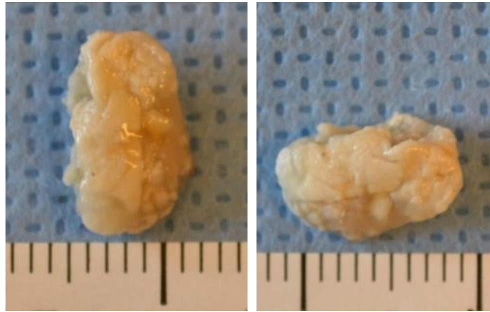

Figure 7: Macroscopic appearance of alginate gels with chondrocyte conditioned BMSCs (5 weeks in vivo). Neocartilage formation is readily apparent upon gross evaluation although not continuous throughout the gel. Tick marks indicate 1 mm.

Histological Evaluation

Alginate gels stained with H&E showed formation of ECM as evidenced by basophilic (purple) staining (Figures 10-13). Further, presence of sGAG was noted with the staining of samples with toluidine blue and safranin-O. It should be noted that safranin-O samples were understained, diminishing the red color in order to show sGAG accumulation. Safranin-O staining in the chondrocyte conditioned BMSCs showed the deepest staining at 5 weeks (Figure 11).

Figures 8 and 9 clearly demonstrate integration of the neotissue with the devitalized cartilage ring. The neocartilage formed by the BMSC gel was continuous throughout the partial sample used for histological evaluation, unlike the other two groups in the co-culture study (Figure 8).

Similar results were seen in conditioned media BMSC gels (Figure 11). Alternatively, in the chondrocyte gels, ECM accumulation resulted in cell "islands" of neocartilage, best evidenced in the toluidine blue stain (Figure 9, middle). The continuous nature of neocartilage in the H&E and safranin-O sections (Figure 9) were only seen in the pictured sections. While the experimental group, chondrocytes and BMSCs, did not show integration with the cartilage ring or neocartilage formation similar to other groups, evidence of cellular lacunae can be seen microscopically (Figure 10). Further, ECM production is indicated within each lacuna.

Except for the understained safranin-O sections, native cartilage staining was less intense due to the devitalization process by way of repeated freeze-thaw cycles. Cellular morphology appears normal for chondrocyte phenotype in all samples except for the conditioned BMSCs (Figure 11). Despite the most intense staining for ECM products, the cells in these gels retain a slight spindle-like morphology more closely associated with fresh BMSCs and not fully differentiated chondrocytes.

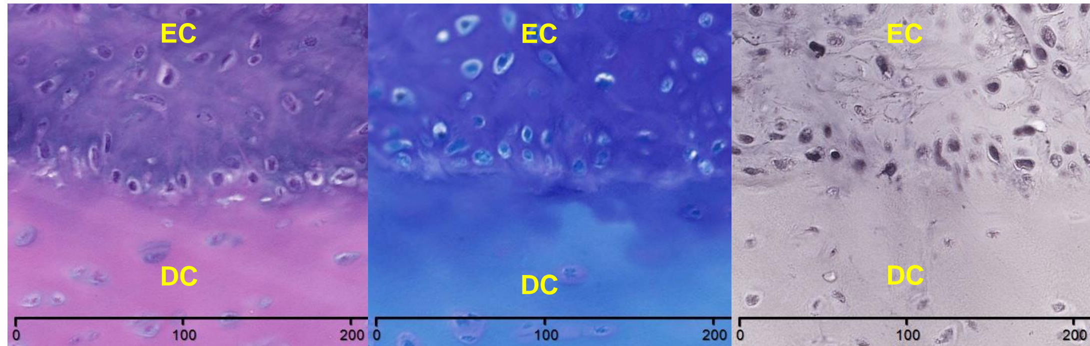

Figure 8: **Histological presentation of BMSCs in alginate gel (6 weeks *in vivo*).** Cross sections of alginate gels showing integration of devitalized cartilage ring (DC) with engineered cartilage (EC) in H&E (left), toluidine blue (middle), safranin-O (right). Staining for safranin-O was less than ideal, but integration with the DC ring is evident in all three stains. Scale bar is in μm.

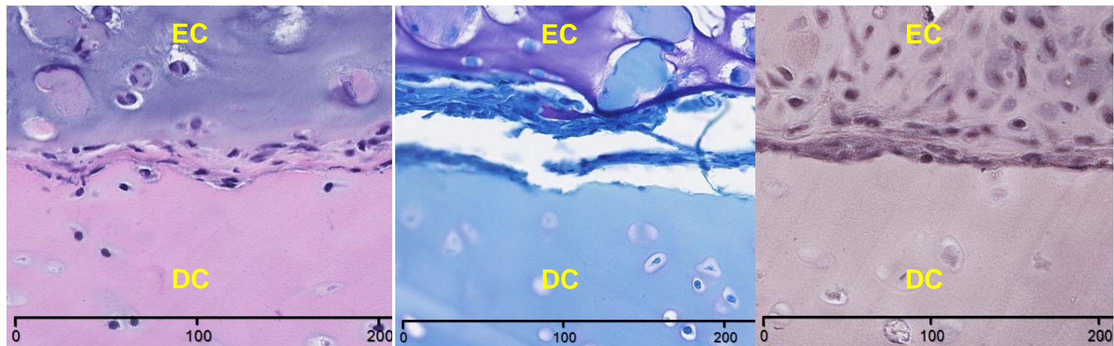

Figure 9: Histological presentation of chondrocytes in alginate gel (6 weeks *in vivo*). Cross sections of alginate gel showing integration of engineered cartilage (EC) with devitalized cartilage ring (DC) stained with H&E (left), toluidine blue (middle), and safranin-O (right). Most of the ECM produced by the chondrocytes resulted in a gel in microscopic appearance most similar to the toluidine blue stained section. The continuous EC cartilage pictured in the H&E and safranin-O sections were not evident anywhere else. Scale bar is in μm.

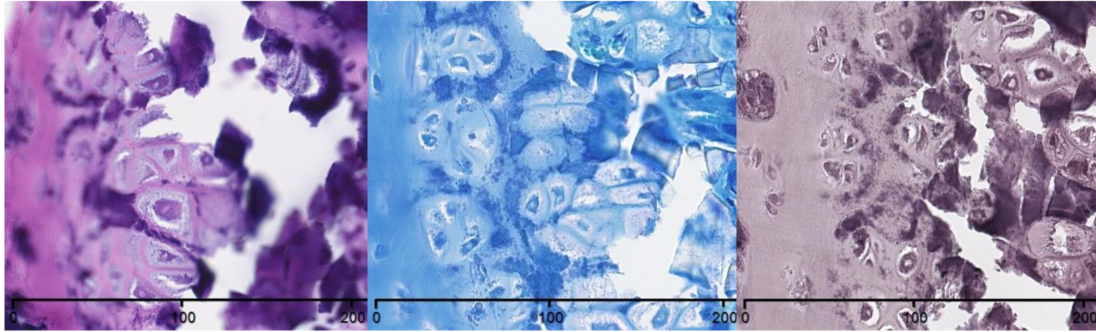

Figure 10: Histological presentation of swine chondrocytes and BMSCs co-cultured in alginate gel (6 weeks *in vivo*). Cross sections of alginate gel showing gel only stained with H&E (left), toluidine blue (middle), and safranin-O (right). Strict lacunae formation is evident although no continuous neocartilage formation was present. Pericellular ECM production is evident through the purple (left), blue (middle), and faint red (right) staining around each of the cells. These gels had to be sectioned several times before any gel could be surveyed. Scale bar is in μm.

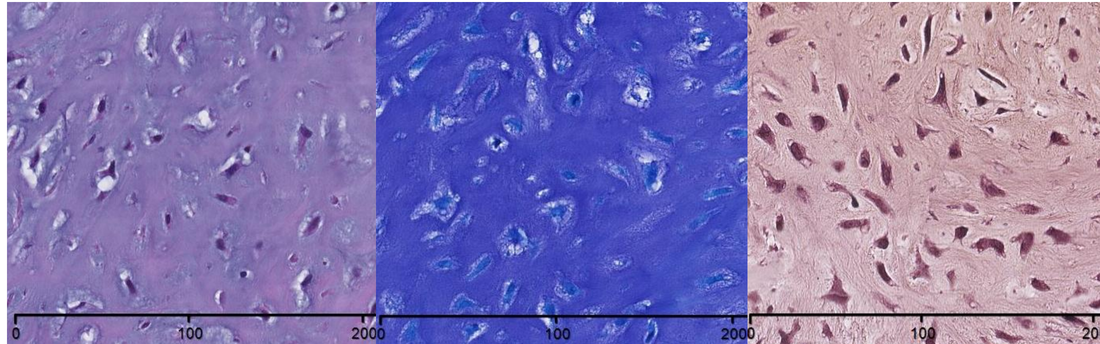

Figure 11: Histological presentation of BMSCs cultured in chondrocyte conditioned media (5 weeks *in vivo*). Cross sections of alginate gels stained with H&E (Left), toluidine blue (middle), and safranin-O (right). Abundant ECM production resulted in continuous neocartilage formation. It should be noted that these gels produced the best ECM in one less week than the co-culture group gels were able to produce. Safranin-O staining for sGAG was the most intense in these gels. Scale bar is in μm.

Biochemical Evaluation

GAG Assay. Glycosaminoglycan levels present in the alginate gels at 6 weeks were significantly lower than that of native cartilage (Figure 12). Swine chondrocytes encapsulated within alginate showed the greatest amounts of sGAG produced, at 10.58±3.85 μg/mg wet tissue. BMSCs cultured with chondrocytes produced the lowest amount of sGAG (4.83±.69 μg/mg wet tissue). Intrestingly, the 5 week samples of chondrocyte chonditioned media alginate gels yielded the most sGAG of all samples with BMSCs (6.13±2.19 μg/mg wet tissue), but still about 10.6% that of native cartilage.

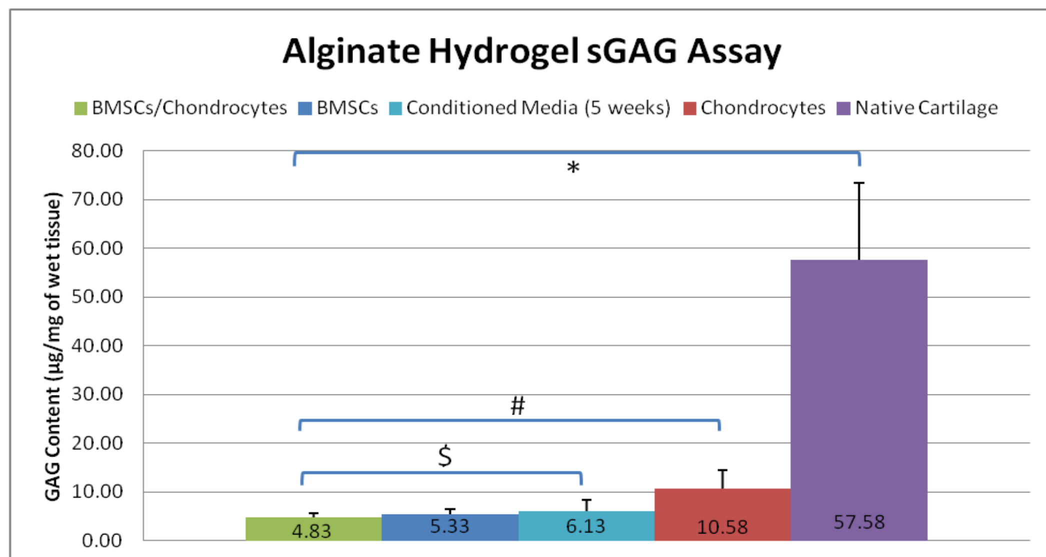

Figure 12: sGAG content in alginate hydrogels and native cartilage at 6 weeks *in vivo* unless otherwise noted (presented in μg/mg of wet tissue). Exact values are indicated within each group column. Bars indicate one standard deviation. There were no significant differences between the BMSCs/Chondrocytes, BMSCs, or conditioned media gels ($p>0.05$). Significant differences were found between the chondrocytes group and BMSCs/Chondrocytes, BMSCs and conditioned media groups ($p<0.05$). ($= $p>0.05$; #= $p<0.05$; *= $p<.001$).

Hydroxyproline Assay. Hydroxyproline content in the alginate gels was relatively low at 6 weeks, with all groups measuring less than 2 μg/mg wet tissue (Figure 13). Chondrocyte conditioned BMSCs showed the most hydroxyproline content of all groups with BMSCs (1.02±0.42 μg/mg wet tissue) despite spending one week less *in vivo*. Chondrocytes within alginate produced hydroxyproline levels similar to that of native cartilage (1.91±0.94 μg/mg wet tissue).

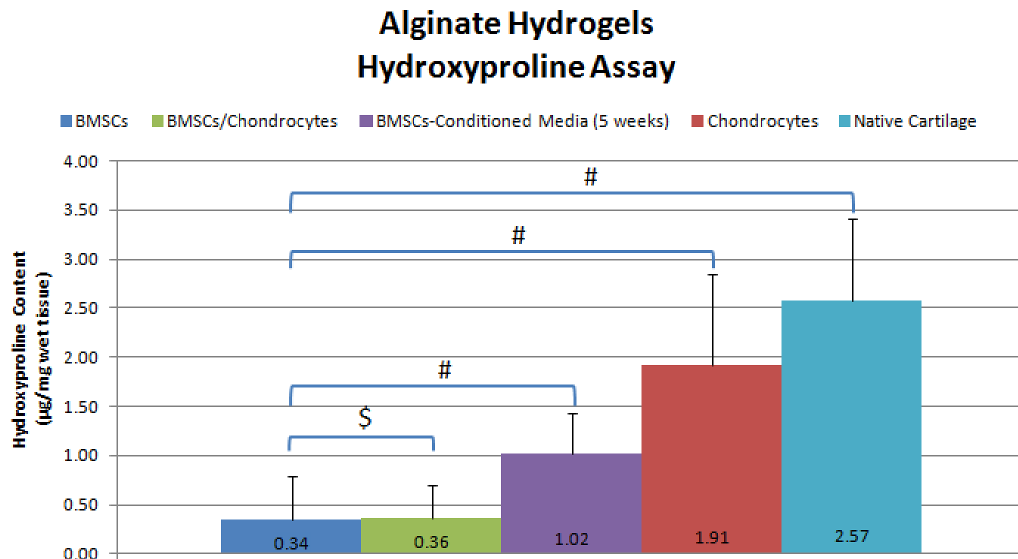

Figure 13: Hydroxyproline content in alginate hydrogels and native cartilage at 6 weeks *in vivo* unless otherwise noted (presented in μg/mg of wet tissue). Exact values are indicated within each group column. Bars indicate one standard deviation. The only measured values reported as insignificant ($p>0.05$) were those between the BMSCs and BMSC/chondrocyte groups. ($= $p>0.05$; #= $p<0.05$).

DISCUSSION

The current field of cartilage tissue engineering uses BMSCs and chondrocytes separately in a variety of scaffolds. While these two cell sources are not new, their utility has not yet been optimized. Many published works investigate one animal model and rarely do their results translate to other animal models. One of the benefits of working with swine as a large animal model is that the biomechanical features of the knee joint are similar to that in humans. The gap between swine and human knee joints and the application of translational research therapies into the clinical setting is more easily managed.

Comparison studies of cell sources have been carried out between swine and humans (Heino et al., 2012; Zhao et al., 2012). More specifically, as Zhao *et al*. investigated, most patients in need of articular cartilage therapies are middle-aged or older; thus facilitating the need for evaluation of elderly human articular chondrocytes. Yet, for tissue engineering purposes, knowledge on the cellular potential of each species to form new tissue is highly important. This will ultimately make the transition between research and clinical application quicker.

This study sought to deal specifically with the current shortfalls of AC therapy. ACI is the most direct comparison of current surgical treatments to the field of tissue engineering. Elderly human chondrocytes have been reported as capable of producing ECM (Zhao et al., 2012). In addition to other problems mentioned earlier, acquisition of sufficient chondrocytes is a major limiting factor.

By combining swine BMSCs and chondrocytes into one gel, most obviously, the strict requirement of chondrocytes as the lone cell source is relieved. Further, the potential for chondrocytes to direct the differentiation of BMSCs within the gel reduces the number of overall steps in the process for utilization of BMSCs as differentiated chondrocytes. It has been shown that cultured chondrocytes can release the growth factors TGF-β3 (transforming growth factor beta 3) and IGF-1 (insulin like growth factor), both of which have implications in directing BMSCs toward their chondrogenic lineage (Liu et al., 2012). Thus, one of the aims in performing this co-culture study was to investigate the direct effect mature chondrocytes have on the chondrogenic potential of BMSCs *in vivo*.

This lab has extensive experience with various scaffolds purposed for articular cartilage engineering (Bichara et al., 2011, 2012; Papadopoulos et al., 2011). Alginate serves as a suitable scaffold for chondrocytes because it allows the cells to maintain their chondrocyte phenotype and retard dedifferentiation (Chung & Burdick, 2008).

The results from this study indicate that BMSCs co-cultured with chondrocytes in alginate do not offer a significant advantage in engineering neocartilage over alginate gels with BMSCs or chondrocytes alone. Gross appearance showed some opaque formations in the BMSC and chondrocyte co-culture gel and BMSC only gels (Figure 6). The chondrocyte gel showed no visible opaque neocartilage formations macroscopically. Yet, not surprisingly, this gel produced about 100% and 500% more sGAG and hydroxyproline

respectively than the other gels in the co-culture experiment (Figures 8 & 9). Overall, the alginate gels constructed for this study were relatively inferior when comparing quantitative biochemical data with that of previous studies performed in this lab (Papadopoulos et al., 2011). Histological examination provided evidence of good ECM production, but could not be supported quantitatively when compared with native swine articular cartilage.

Histological evaluation of the BMSC and chondrocyte co-culture gel yielded no continuous ECM formation and many sections had to be cut before the presence of gel was detected. By comparison, the BMSCs gels appeared superior in ECM production and neocartilage formation was continuous throughout the gels. Chondrocyte gels showed pericellular ECM production only and limited continuous ECM formation (Figure 9). Safranin-O stained chondrocyte gel (Figure 9, right) shows the only continuous ECM formation within that particular sample. It is possible that the cell density for each of the different groups needs to be optimized before proper ECM production can occur. Prior studies in this lab have investigated the effect of different chondrocyte seeding densities within scaffolds, namely fibrin (Silverman et al., 1999). If the cell density is not high enough, pericellular ECM production dominates and never forms continuous neocartilage. This gives the neotissue a bubbly appearance as ECM islands form around the cell and never coalesce into a continuous ECM (Figure 9). Alternatively, if the cell density is high enough, continuous ECM forms neocartilage more similar to that of native cartilage.

The integration of the BMSC gel with the devitalized cartilage ring appeared to be superior to all other groups (Figure 8). Proper integration of engineered cartilage with native cartilage is important to prevent any proud edges that might be worn down through the normal shear stresses experienced by the articular surfaces in the knee. The integration in the chondrocyte gel did not follow the entire length of the devitalized cartilage ring contact points (Figure 9). Interestingly, there appears to be a thin, fibrous layer of cells between the chondrocyte gel and the devitalized ring, not present in the integration of the BMSC gel. No ECM accumulation directly around or within the thin layer of cells is evident. It might be concluded that the ECM integration shown in the BMSC gel (Figure 8) results in a more cohesive and seamless transition of neocartilage and native cartilage. One important point is that this study used devitalized cartilage rings and not living native cartilage. The repeated freeze-thaw cycles killed the chondrocytes and matrix production ceased. This allowed for a broader contrast when analyzing the alginate gels ECM production during histological evaluation. The fact that some of the gels showed integration with the devitalized ring lends credence to the thought that integration with living cartilage would be more complete. One of the innate capabilities of articular cartilage is the fact that they do not spontaneously heal and integration of engineered cartilage could make up for this.

The sGAG and hydroxyproline levels measured in the samples were significantly lower than that of native cartilage for the six week timepoint in this

study. Levels were expected to be about 5-6 times higher. According to previous papers in this lab, engineered cartilage constructs were capable of producing about 30μg sGAG/mg wet tissue and 5 μg hydroxyproline/mg wet tissue at 6 weeks *in vivo* (Papadopoulos et al., 2011). When the two week *in vitro* differentiation period is taken into account, the gels' lifespan, from creation to analysis, was 8 weeks. Still, the biochemical data did not yield expected results. Part of this may have been due to the fact that one-fourth of each sample was used for each biochemical test. Macroscopically, the neocartilage formation (Figure 6), was not evenly distributed throughout the gel and dispersion varied among quadrants of each gel. Since there were only four samples per group, it seemed prudent to gather all endpoint data from each sample in an attempt to normalize the results. This may have contributed to the lower than expected measurements of sGAG and hydroxyproline. When compared to the chondrocyte gel, BMSCs produced about 50% of the sGAG levels.

The conditioned media alginate gels produced the best results when compared with the three groups from the co-culture experiment. Histological comparison shows that the conditioned BMSCs produced the most continuous neocartilage. Conditioned media BMSCs produced similar levels of sGAG and hydroxyproline in one less week *in vivo*. During harvest of the samples, small calcifications were identified macroscopically, but could not be detected histologically. Despite the excellent ECM production of these samples, calcifications could be the cause of an overexpression of collagen type X,

indicating hypertrophic mRNA levels (Caron et al., 2012). It is possible that some of the effects are due to 2D monolayer culture of the BMSCs through three passages prior to suspension in a 3D alginate environment.

In summary, this study identified potential areas of interest in future pursuits of cartilage tissue engineering. Despite the study's limitations, it did identify specific strengths in the use of alginate as a scaffold for neocartilage formation. The chondrogenic properties and supportive bone-marrow derived mesenchymal stem cell niche of alginate provides a foundation for future studies to build upon. More specific aims need to be developed for future studies. In searching for alternative cell sources, BMSCs are capable of bearing some of these requirements which chondrocytes in current therapies such as autologous chondrocyte implantation exhibit. The transition to BMSCs as a replacement for chondrocytes is the ultimate goal and shown in this study to be an option worth pursuing.

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