ABSTRACT

Title of Document: ENGINEERING ZONAL CARTILAGE THROUGH UTILIZATION OF A MESENCHYMAL STEM CELL POPULATION

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Articular cartilage has a limited ability to repair itself after damage due to injury or disease. Regenerative therapies using chondrocytes, the primary cartilage cell population, result in poor quality repair tissue and often cause further damage at the donor site. Furthermore, there are no current therapies which aim to regenerate the zonal organization and function of the tissue. In an effort to address both cell source limitations and zonal tissue regeneration the goal of the presented work was to utilize a mesenchymal stem cell (MSC) population to generate abundant numbers of chondrocytes with zonal phenotypes. To this end, zonal subpopulations of articular chondrocytes were isolated, characterized for differences in gene and protein expression, and exposed to scaffold environments designed to aid in phenotype retention. From these results, and reports in the literature, it was clear a major functional difference between zones was the production of a lubricating protein, proteoglycan 4 (PRG4), in the superficial zone only. Middle and deep zone cells were found to be phenotypically similar and distinct from superficial zone cells. It was further found that gene expression of PRG4 by superficial

zone cells in alginate culture can be significantly enhanced by incorporation of matrix molecules hyaluronic acid (HA) and chondroitin sulfate (CS) to the scaffold environment. HA and CS also had favorable effects on MSC chondrogenesis by upregulating chondrogenic transcription factor Sox9 gene expression, and downregulating type I collagen (fibroblastic marker) gene expression. The potential of soluble signals derived from zonal (superficial or middle/deep) cartilage explants to drive MSC chondrogenesis was also investigated. Results show that signals derived from cartilage explants can induce chondrogenesis to varying degrees, with superficial zone explants inducing robust and sustained differentiation. This differentiation was found to be dependent on the proximity of the MSCs and tissue explants, implying that communication between MSCs and chondrocytes is necessary for chondrogenic induction. Coculture with superficial zone explants also upregulated MSC gene expression of PRG4. This research highlights the important functional differences between zonal chondrocyte populations and identifies MSCs as a progenitor population capable of differentiating into zone-specific chondrocytes.

ENGINEERING ZONAL CARTILAGE THROUGH UTILIZATION OF A MESENCHYMAL STEM CELL POPULATION

By

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Dedication

This work is dedicated to my grandmother, Doris Walsh.

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1 Introduction

Articular cartilage lines the surface of articulating joints to provide frictionless movement and resist loading. Cartilage tissue varies with depth and can be divided into three major zones: the superficial, middle, and deep zones. Each zone has differing structure and composition, and is designed to resist the load and stress particular to its location. Therefore, proper function of the entire tissue depends on its zonal construction. Chondrocyte phenotype varies considerably by zone, and it is the activity of these cells that help achieve structural organization. Despite zonal tissue structure and cellular phenotype, there is a distinct lack of articular cartilage regeneration treatments which aim to restore stratified tissue. Previous work in the field has investigated the response of primary zonal chondrocytes to a variety of culture systems, with the goal of retaining subpopulation phenotype. While these approaches have had success, they have largely ignored the role of cell source and its impact upon zonal chondrocyte phenotype. Cell source remains a major limitation in regenerative cartilage therapies, as culturing clinically relevant numbers of phenotypically stable chondrocytes is difficult without harvesting a damaging amount of donor tissue. Repair tissue generated from therapies utilizing primary chondrocytes is suboptimal fibrocartilage which lacks the robust mechanical properties of native tissue.

To address both the issue of cell source and regenerating zonally organized tissue this research is focused on indentifying differences in zonal cell populations and evaluating the potential of mesenchymal stem cells (MSCs) to differentiate into chondrocytes with zone-specific markers. Chondrocytes maintain their spherical

morphology in three dimensional pellet or hydrogel culture. Therefore, such culture is essential for retaining chondrocyte phenotype as well as differentiating progenitor cells down a chondrogenic lineage. Building on previous research in the laboratory, an alginate hydrogel was chosen for zonal chondrocyte and MSC culture. Additional molecules, such as extracellular matrix components, can be added to alginate prior to gelation, and it can also be modified for injectable delivery. Furthermore, the high water content and diffusive properties of alginate hydrogels provides a good system for studying the effects of soluble growth factors. In the presented work we utilize alginate hydrogel environments and soluble signaling molecules to study zonal chondrocyte phenotype retention and MSC chondrogenesis during *in vitro* culture. The objectives of the presented research are to:

1) Classify the zonal distribution in gene expression of major extracellular matrix components, insulin-like growth-factor (IGF)-1 and its extracellular binding protein, IGF-BP3, both with and without exogenous IGF-1 delivery.
2) Identify the potential of hyaluronic acid (HA) and chondroitin sulfate (CS) to influence zonal phenotype retention of chondrocyte subpopulations as well as zonal-differentiation of MSCs.
3) Establish the potential of zonal cartilage-derived soluble factors to drive zonal differentiation of MSCs.
4) Evaluate the viability of MSCs in photocrosslinked alginate, and MSC chondrogenesis by transforming growth factor (TGF)-β3 in photocrosslinked alginate with and without HA additive.

The presented objectives will further the understanding of the differences between cartilage zones and zonal populations of chondrocytes, and explore methods for retaining these differences in culture. Additionally, this work will identify the feasibility using MSCs as a clinically relevant and abundant cell source to aid in the production of zonally organized tissue.

2 Cartilage Engineering: Current Status and Future Trends*

2.1 Cartilage Tissue: Structure, Function, and Disease

2.1.1. Cellular and Extracellular Matrix Components

Articular cartilage, typically 2-5mm thick, is found on the surface of articulating joints throughout the body. Articular cartilage, along with the synovial fluid found inside the joint, provides frictionless movement between bones and absorbs loads during motion. The tissue is maintained by chondrocytes, which is the resident cell population. Chondrocytes are responsible for providing a balance between matrix synthesis and matrix breakdown, a process which is disrupted during disease or injury. The tissue is sparsely populated with cells; comprising less than 5% of the tissue volume. [1] Cartilage also lacks a lymphatic system, nerve fibers, or blood supply. As a result, all nutrient and waste exchange must occur through diffusion from the synovial fluid. Low cell density and the limited exchange of both waste and nutrients both play key roles in the limited ability of cartilage tissue to repair itself once injured.

2.1.2 Composition

Approximately 95% cartilage tissue is comprised of its extracellular matrix (ECM) – which the cells sustain. The ECM is a dense collagen and proteoglycan

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interconnected structure. Chondrocytes are linked to the ECM through cell-surface binding proteins. These connections allow cells to respond to the mechanical forced felt within the ECM. [2, 3]

Approximately 10-20% of the wet weight of the tissue is collagen. The collagen network is comprised mainly of type II collagen fibers; up to 90% of the total collagen content is type II collagen which is crosslinked by covalent bonds throughout the tissue. The type II collagen fiber is a triple helix of identical polypeptide α_1(II) chains, approximately 300nm in length. Minor collagen types make up the rest of the tissue's collagen content and include collagen type IX, XI, and X. Each type of collagen has a different function within the ECM. Type IX collagen is a short fibrillar collagen that helps connect the type II collagen network to proteoglycans. Type XI collagen is also a fiber formed with three distinct α-chains. Type XI forms co-polymers with type II collagen and acts to regulate fibril diameter, form bridges between fibrils, and even crosslinks to itself to increase the mechanical stability of the ECM. Type X collagen is a short helix molecule produced only by hypertrophic cells in the calcified tissue which divides articular cartilage from the underlying subchondral bone. [1, 3-5]

In addition to collagen, the cartilage ECM is comprised of minor and major proteoglycans. Minor proteoglycans within the ECM include decorin, biglycan, and fibromodulin. These small proteoglycans bind to other molecules and help the stabilized the overall matrix structure. Aggrecan is the major proteoglycan in the ECM. Aggrecan contains many branched glycosaminoglycans (GAGs) – primarily keratin sulfate (KS) and chondroitin sulfate (CS). The densely packed GAGs branch off of a central aggrecan backbone and give the molecule a molecular weight of 250,000 d. Each molecule

contains about 100 CS chains and 60 KS chains, and repeating sulfate groups in both give each aggrecan molecule a large negative charge. [1, 2, 4]

The aggrecan molecule is bound to a long unbranched hyaluronic acid (HA) chain via a link protein. Hyaluronic acid is polysaccharide chain with an average molecule weight of several million Daltons, with the addition of many aggrecan molecules linked to this backbone the aggregate molecular weight can reach up to several hundred million Daltons. The networks of HA chains linked to aggrecan molecules are entrapped within the collagen network to give cartilage an intricately organized ECM structure. [1, 2, 5]

The complex ECM is maintained by the chondrocyte cell population. Chondrocytes have limited cell-to-cell communication, and as a result each cell acts as somewhat of an individual - maintaining only the tissue immediately surrounding it. Cells receive information through both mechanical forces and interactions with growth factors and cytokines. The ECM directly surrounding a cell is called the pericellular or lacunar matrix. This area contains an abundance of proteoglycans and few collagen fibers. Directly outside this region is the territorial or capsular matrix – which encapsulates the cell or a group of cells. The chondrocyte exists in a low oxygen environment, and as a result its metabolism is driven by anaerobic pathways, mainly glycolysis. Although chondrocytes produce ECM components, they usually do not divide past adolescence. Low cell density and division both contribute to the tissue's limited repair capability. [6-8]

2.1.3 Structure

In addition to cellular components and a complex extracellular matrix articular cartilage also contains three distinct zones. Each zone has a distinct cellular phenotype and ECM organization. The superficial, or tangential zone, contains the articulating surface of the joint and extents to about 10% of the total tissue depth. The middle, or transitional zone, comprises approximately the middle 70% of the tissue depth and is followed by the deep, or basal, zone which is the bottom 20% of articular cartilage. Below the deep zone lies the tidemark – below which the tissue becomes calcified and eventually turns into subchondral bone. The calcified zone contains few blood vessels and effectively blocks the diffusion of nutrients and waste between the subchondral bone and the deep zone of the articular cartilage. [4, 9, 10]

The superficial zone is marked by cells and collagen fibers that are oriented parallel to the articulating surface. These cells are smaller than those of the other zones, thin, and disc shaped. The cell density is the highest in this zone, however the proteoglycan content is the lowest. The water content of the superficial zone is the lowest, with approximately 65% of the total water weight of cartilage found in the lower two zones. [2, 11, 12] The densely packed collagen fibers are small in diameter and packed in bundles parallel to the articulating surface. The tight organization of the superficial layer is thought to act as a boundary to block any large, unwanted molecules from the synovial fluid. [13] The superficial zone cells are the only cells to secret proteoglycan 4 (superficial zone protein); a lubricating protein secreted into the synovial fluid. [14]

The middle zone contains larger and more rounded chondrocytes. The cells, along with the collagen fibers are randomly oriented and can often be found in clusters. Middle zone chondrocytes produce higher levels of proteoglycans than superficial cells, and the cellular density here is lower than in the superficial zone. [11, 15]

Deep zone cells are oval in shape, and the cells along with collagen fibers are oriented in vertical columns perpendicular to the articulating surface. The deep zone cells produces elevated levels of collagen and proteoglycans compared to the superficial cells. This zone also has a lower cell density, approximately one third of that of the superficial zone. Figure 2.1 shows histological staining for primary bovine chondrocytes isolated from the three tissue zones. [2, 11]

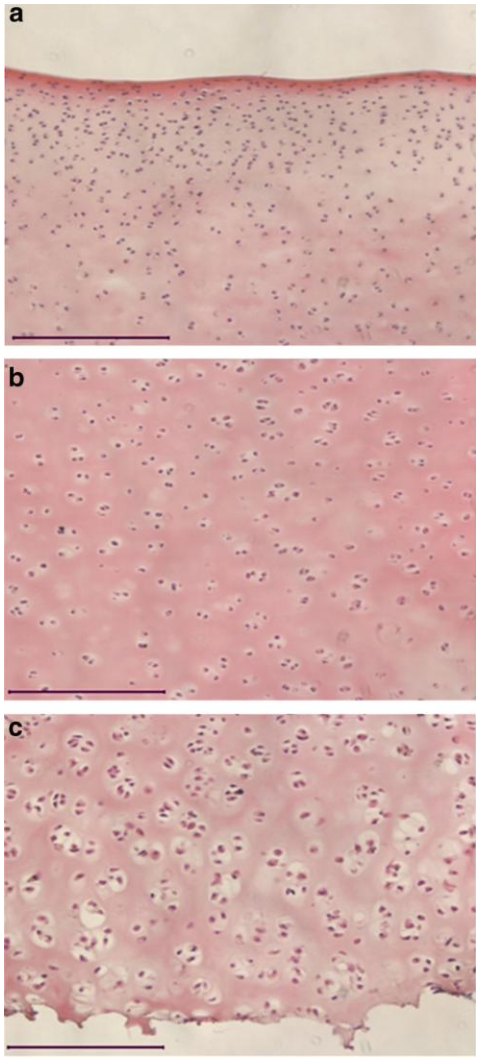

Figure 2.1. Hematoxylin and Eosin (H&E) staining of cells isolation from A) superifical, B) middle, and C) deep zones of bovine articular cartilage. Cell nuclei are stained dark violet, cell cytoplasm are stained light pink, and extracellular matrix is stained slightly darker pink. Scale bars all 100 μm.

2.1.4 Proper Tissue Function and Response to Stress

Cartilage can withstand large numbers of repetitive strains over many years. For the tissue to function properly many critical biological relationships must remain in balance. Some key processes include the metabolic activity of the chondrocytes (the balance between matrix synthesis and breakdown), proper cell secretion and concentration of hormones, production of growth factors and cytokines, and proper distribution of mechanical loading by the ECM.

The integrity of the proteoglycan and collagen networks is critical for proper function of cartilage tissue. The collagen network provides tensile strength, and the proteoglycans are critical for resisting compressive loading. The net negative charges on each proteoglycan, from the presence of the GAG groups, give the tissue a high osmolality. Negative charges attract cations, which further raises the osmolality, which in turn increase water uptake. The result is a high osmotic tissue pressure (350-450 mOsm), however the strong type II collagen matrix prevents the tissue from swelling. High osmostic pressure results in cartilage tissue being approximately 70% water. [2, 16, 17]

During joint loading this high tissue pressure resists load and deformation, however a small amount of water is pushed outside the tissue into the joint. Here, this liquid helps to further resist friction and assists in the smooth motion of the joint. In addition, this liquid absorbs nutrients in the synovial cavity, and when the load is released the liquid flows back into the tissue and delivers these nutrients. As a result, dynamic loading stimulates matrix production and is dependent on the amplitude and frequency of the load. Conversely, static loading decreases the synthesis of certain matrix proteins. Therefore, just as proper tissue structure is necessary for loading mechanics, healthy loading is also necessary for proper tissue homeostasis. [2, 16, 17]

2.1.5 Aged and Damaged Tissue

The natural aging process leaves cartilage less robust and with lower tensile strength as early as the third decade of life. With age the metabolic activity of the chondrocytes is altered; their ability to respond to growth factors and cytokines

decreases. Compromised mechanical properties and decreased activity of the chondrocytes leaves aged tissue more susceptible to damage. [13, 18]

Cartilage tissue can become damaged due to diseases such as arthritis or trauma which results tissue injury. The limited cell population and reliance on diffusion for nutrients and waste exchange make it difficult for chondrocytes to restore a damaged ECM. In unhealthy tissue the balance between matrix production and breakdown is disrupted and a cycle of tissue degradation ensues. Even minor tissue injuries usually do not fully repair, and leave the cartilage more susceptible to the onset of disease. [2, 19]

2.1.6 Disease

Arthritis is marked by degradation of cartilage and subchondral bone tissue which results in joint pain and loss of motion. Arthritis can be divided into two major classes: inflammatory rheumatoid arthritis (RA) and non-inflammatory osteoarthritis (OA). In both cases the complex structure and biochemistry of the tissue becomes disrupted. OA is much more common, and affects a large percentage of the elderly population. In fact, about 2 out of 3 people over the age of 65 show radiographic signs of OA. [20] In both diseases enzymes such as matrix metalloproteinases (MMPs) cleave the bonds that hold the matrix together. Inflammatory rheumatoid arthritis is an autoimmune disease, while osteoarthritis is a marked by degeneration of cartilage tissue. Due to its prevalence in society OA disease and repair strategies will be discussed. [6]

There is no uniform appearance or single pathogenic mechanism that marks OA. It can present itself in a variety of appearances and is caused by a number of different factors. Causes can include genetic defects, extended joint overloading or overuse, or

joint misalignment. OA can also onset as a result of trauma which results in direct injury to the joint or surrounding ligaments. OA can potentially affect any articulating joint, and is classified by pain, motion or gait problems, loss of ECM molecules into synovial fluid, loss of joint cartilage, and tissue remodeling in the subchondral bone. Factors such as alcohol abuse, obesity, and diabetes increase the risk for onset of OA. [1, 3, 18]

Loss of integrity of the type II collagen network is an early ECM change during OA. An increase in osmotic pressure results in swelling which causes proteoglycans to escape. A reduction in the proteoglycan concentration lowers the tissue's osmotic pressure, which compromises its ability to resist loading. Once the process of matrix degradation has begun it accelerates due to the tissues inherent limited ability to self-repair. [2, 21]

Disruption in production of ECM components, signaling molecules, and cytokines is also observed during disease. [21] In an attempt to combat matrix breakdown elevated levels of minor proteoglycans are usually observed in the early stages of osteoarthritis. Type X collagen, usually only found in the calcified zone, can be found throughout the various zones of articular cartilage with the progression of disease. Elevated levels of enzymes such as MMPs cleave critical bonds in the collagen and proteoglycan matrices. Chondrocytes begin producing a meta-stable form of type II collagen (type IIa collagen), which is degraded before it can be functionally incorporated into the matrix. [2, 13, 18]

Eventually the tissue becomes fragmented, with damaged areas alongside remaining healthy tissue. Failed repair events are noticeable throughout the tissue in the form of local accumulation of ECM precursor molecules (such as procollagen peptides), clumps of chondrocytes entrapped by bundles of minor collagens, and chondrocyte

dedifferentiation. Damaged cartilage is heterogeneous, and can manifest itself in a variety of structural disruptions. Loss of tissue height from the superficial and middle zones is a common manifestation of OA. [1, 3, 13, 18]

In the late states of the disease functional cartilage is gone, and areas of exposed bone-plate can be observed. Cracks in the subchondral plate and formation of subchondral bone cysts also occur. Gradually, bone marrow will make its way to the region and a layer of mechanically sub-optimal fibrocartilage will replace the once health cartilage tissue. [1, 3, 13]

2.1.7 Trauma

Trauma can occur due to a single excessive load, or repetitive joint overloading. Tissue damage can occur in the form of a microfracture, where the damage to the articular surface is not visible, or it can occur in the form of a visible tissue disruption of variable length. If the damage penetrates through the tidemark and into the subchondral bone it is called an osteochondral fracture. [2] Unfortunately, defects rarely repair themselves and only continue to grow worse with age. Most significant injuries to articular cartilage will result in the eventual onset of OA. The healing potential and severity of disease are dependent on the size and location of injury, as well as patient health and age. [19]

2.1.8 Need for Repair and Regeneration Strategies

Many obstacles make treating arthritis and cartilage injuries challenging. For one, it is difficult to repair a tissue lacking intrinsic repair mechanisms. Turnover in matrix

proteins is relatively low even in healthy tissue, in fact the half life of collagen and proteoglycans are approximately 100 and 3-24 years respectively. [22] Additionally, there is no single reason or way tissue degradation occurs – making treatment options hard to identify. Pain medication given to arthritic patients may relieve pain, but it does nothing to stop the tissue erosion cycle. Some pain medications – such as non-steroidal anti-inflammatory drugs (NSAIDS) - are even thought to hurt matrix production. Furthermore, therapies that target cell populations will be ineffective if the cells have already become phenotypically unstable and entered hypertrophy or fibroblastic lineages. [3] Currently, engineered cartilage therapies are not standard practice in treating cartilage defects. Standard of care still involves non-surgical interventions, or traditional surgical techniques. While these treatment methods have had some successes, they have several key disadvantages in restoring healthy tissue.

2.2 Current Standards of Care and Limitations

2.2.1 Current Treatments in Cartilage Repair

To date there are many approaches for treatment of cartilage defects and OA, however an ideal method is yet to be developed. The field has received much research attention, and many new products are in various stages of clinical trials. Despite this, there is a fairly limited range of treatments that are available on large scale. The estimated cost of OA and cartilage defects in the United States is between 10 [20] and 65 [23] billion dollars annually between loss of working days and medical treatments. Furthermore, more than one in eight Americans over the age of 25 are thought to be affected with some form of the disease. [24]

The main symptoms of OA are joint pain and loss of function. However, the disconnect often observed between radiographic evidence of cartilage damage and experienced pain presents a major challenge in patient care. In fact, more than half of patients with severe radiographic evidence of OA report no pain. [20] The heterogeneity of the disease both in its physical manifestations and in symptoms reported by patients make it hard to classify, treat, and prevent.

The American College of Rheumatology (ACR), European League Against Rheumatism (EULAR), and the Osteoarthritis Research Society International (OARSI) all recommend the following progression of treatments: non-pharmacological, pharmacological, and finally surgery. Patients should only move to the next treatment if the methods they are using are ineffective and pain persists. [20, 25]

2.2.2 Non-Surgical Treatments

Non-pharmacological treatments include: weight reduction if necessary, education and self management, physical therapy, aerobics, muscle strengthening, and acupuncture. Generally, light exercise helps to reduce pain. If none of these methods are successful pharmacological treatments should be used.

Drug administration can be divided into two groups: pain-reducing agents, and therapeutic agents. Pain reducing agents are used simply to manage patient pain in order to improve functionality. Therapeutic agents also help to relieve pain and additionally aim to stop matrix degradation and slow down disease progression.

The first line of pain-reducing agents are acetaminophen, NSAIDS, and cyclooxygenase-2 (Cox-2) inhibitors. Acetaminophen is safe for long-term use in small

doses however, it is relatively weak and can have adverse effects on the liver. While NSAIDS and Cox-2 inhibitors are anti-inflammatory drugs, they are used in OA management for their pain-relieving properties. If inflammation due to arthritis is present their use will be more effective. Neither should be used long-term or in high doses, as they can have adverse cardiovascular effects. Patients at risk for gastrointestinal complications should avoid NSAIDS and use a Cox-2 inhibitor, but both should be used in as low doses as possible. [20, 25]

More severe pain or flare-ups can be treated with corticosteroid injections or even opioids. Injections should be limited to every 3-4 months as they can have adverse metabolic effects and provide only short-term relief. [26] Opioids, which are effective pain relievers should be used in low doses and only for severe cases. [20, 25]

Therapeutic drugs which aim to retard matrix erosion include glucosamines, chondroitin sulfate, S-adenosylmethionine (SAM), and hyaluronic acid. Glucosamines are thought to have structural remodeling potential, but clinically have varying results. They are available in either chloride or sulfate formulations, and after absorption are converted to salts. It is recommended that patients try glucosamines for a few months and discontinue if no benefits are observed. They have almost no side effects. Chondroitin sulfate, SAM, and hyaluronic acid also have varying clinical reports success. SAM may increase GAG production in chondrocytes, and has been reported to decrease pain – but this may be due to the drug's anti-depressant effects. Hyaluronic acid injections are reported to decrease pain and improve functionality but are not effective in severe cases of matrix degradation or limb misalignment. Rarely are adverse effects observed, but pain and infection at the injection site has been reported. [20, 25, 27]

Several new compounds are in various stages of research investigation including inhibitors of MMPs, a new class of drugs called disease-modifying osteoarthritis drugs (DMOAD), and the use of growth factors. [25] However, there is currently no ideal drug, or cocktail of drugs for relieving pain, improving functionality, and stopping or reversing matrix destruction. The diverse nature of OA makes a single optimal treatment path difficult to identify.

2.2.3 Surgical Treatments

When non-pharmacological and pharmacological treatment methods prove ineffective the next step is surgical intervention. Surgical procedures can be broadly grouped into two classes: non-regenerative treatments and tissue replacement/regenerative treatments. Non-regenerative treatments aim to physically alter or remove the problem joint while regenerative treatments attempt to replace or regenerate the damaged tissue.

2.2.3.1 Non-Regenerative

Non-regenerative procedures include osteotomy, arthrodesis, and anthroplasty. Osteotomy is usually performed for joint misalignment and involves the removal of bone to redistribute loads to areas of healthy cartilage. Risk factors include hemorrhage, inflammation, and nerve damage. Anthroplasty refers to total joint replacement and is reserved for the most severe cases when all other treatments have failed. Although this is a fairly common surgery in the United States there is still a relatively large complication rate of 5.5%, most of which is associated with post-operative infection. Arthrodesis is

the induction of bone formation between two bones to immobilize a joint. This is usually performed on the small joints present in the hands and feet. [25, 28]

2.2.3.2 Tissue Replacement/Regenerative

Traditionally surgery has been used as a last resort option. However, one of the biggest risk factors for developing OA is the presence of cartilage defects. If these defects can been treated early and successfully with regenerative therapies, onset of disease may be slowed. Regenerative and replacement techniques can be subdivided into three groups: bone marrow stimulation techniques, osteochondral transfer or grafts, and cell-based therapies. [25, 29]

The most common bone marrow stimulation procedure is microfracture. During this operation micro-penetration of the subchondral bone plate fills the cartilage defect with blood cells that contain a population of mesenchymal stem cells (MSCs). MSCs can differentiate into chondrocytes, among other cell types. The result of MSCs populating the cartilage defect is the formation of fibro-cartilage tissue containing varying amounts of type II collagen. The procedure has several advantages and drawbacks. Advantages include limited invasiveness, low tissue morbidity, short recovery time, and cost-effectiveness. The greatest level of success is observed in young, athletic patients with early intervention. Drawbacks include formation of tissue lacking structure and function of healthy cartilage. The fibro-cartilage layer provides limited load-bearing capacity, is often much thinner than native tissue, does not fully integrate with surrounding tissue, and often includes overgrowth of the subchondral bone. A technique called enhanced

microfracture attempts to address these drawbacks by including growth factors which induce chondrogenesis of the MSC populations. [19, 25, 29, 30]

Osteochondral transfer includes both autografts and allografts. Autografts involve harvesting cartilage tissue from areas of low loading and transplanting to defects in weight bearing sites. There are several drawbacks of this procedures including difficulty in restoring proper joint architecture, pressure build up due to incongruity of restored surfaces, donor site morbidity, lack of integration of grafted tissue, and altered joint mechanics and load bearing capability. Allografts have the advantage of no donor site morbidity, however all the same disadvantages exists, plus potential immune response and transmission of disease. [31]

The most modern form of the osteochondral graft is a procedure called mosaicplasty. This uses several small grafts to fill a single defect. Moscaicplasty treatment is most successful in patients under 50 with no joint misalignment. [19, 25]

The first cell based therapy introduced was autologous chondrocyte implantation (ACI) in 1994. It has been used ever since with considerable success reported. The procedure has two steps. First, chondrocytes are harvested from the patient, isolated, and expanded in *in vitro* culture. Next, the expanded cell population is injected into a chondral defect. Despite positive surgical outcomes the procedure has many disadvantages. Donor site morbidity, the need for a second surgery, dislocation of cells implanted to defect, extended recovery, loss of chondrocyte phenotype in monolayer, and the formation and fibrous repair tissue are all limitations of the procedure. [19, 22, 25, 29, 30]

A more advanced form of ACI, characterized chondrocyte implantation, uses the same procedure but during *in vitro* culture identifies cells with genetic markers that indicate high levels of matrix production. This technique has limited approval in Europe and has not yet been approved in the United States. [29]

2.2.4 Limitations of Current Standard Practices and Need for Engineering Approaches

Despite the disadvantages of each, microfracture and mosaicplasty are currently the most popular choice of surgical interventions for repair of cartilage defects. [29] The ACI procedure is also popular, despite its challenges and potential complications. [32] The current standard of care treatments for cartilage defects and osteoarthritis leave much to be desired. There is no current treatment capable of thoroughly repairing cartilage defects and regenerating tissue that demonstrates chemical and physical properties similar to native cartilage. Tissue that is regenerated using current surgical methods is, at best, fibro-cartilage repair tissue that provides limited load-bearing capabilities and as a result will degrade over time. [22, 25, 29, 30]

The primary challenge of tissue engineering solutions is to regenerate cartilage tissue with composition, structure, and function comparable to that of native tissue. Tissue engineering can be defined as the interactions between biomaterials, growth factors, and cells to regenerate functional tissue. A major challenge for engineering articular cartilage is obtaining a sufficiently large chondrocyte population that is phenotypically stable and has not begun to de-differentiate down a fibroblastic lineage. [32] Many research efforts have investigated the ideal biomaterial to maintain a healthy and productive chondrocyte population. Due to these efforts the field has grown

considerably over the last decade. While current treatments do not usually involve tissue engineering approaches there are many products both abroad and in the United State in various stages of clinical trials. These new technologies may soon change the standard of cartilage repair procedures. [22, 29, 31]

2.3 Cartilage Engineering

2.3.1 Requirements of an Engineered Construct

A tissue engineering scaffold can be seeded with a desired cell population and implanted into a defect site. The scaffold provides both mechanical support and a three-dimensional environment for cells to attach and proliferate. The cell population will produce extracellular matrix components which will infiltrate the scaffold material and surrounding tissue. Slowly the scaffold material will degrade – leaving only cells and native tissue. There are many materials used for the scaffold component of an engineered construct. Scaffolds can be made out of naturally or synthetically derived components. The majority of cartilage scaffolds contain building blocks of either proteins or polysaccharides. Scaffolds can also come in a variety of physical forms, such as foams, viscous liquids, hydrogels, and porous matrices.

2.3.1.1 Required and Desired Construct Properties

Fundamental requirements of all cartilage engineering scaffolds are: lack of immune response and inflammation, adhesion of chondrocytes, maintenance of the chondrocytes phenotype, and initial mechanical stability within the defect. Beyond these

requirements there are many desirable, but not necessarily imperative, properties of a scaffold. These include: permeability to allow diffusion of signaling molecules and nutrients, adhesion to the defect site, controlled release of growth factors, injectable, minimally invasive, and biodegradable to allow growth of new ECM tissue to eventually fill the defect site. [32-34]

Depending on the nature of the defect the desired properties of the scaffold may change. An osteochondral defect which penetrates the subchrondal bone will be repaired differently than a chondral defect. Depending on the location and size of the chondral defect it may be repaired with different approaches as well. For example, a scaffold for an osteochondral defect may be biphasic – with a region for repair of the bone tissue and region for repair of the cartilage tissue. If the bone marrow has been penetrated and is entering the defect site this will also have to be addressed. Perhaps the bone marrow will be contained to the bone tissue, or factors to induce chondrogensis of the cell populations in the marrow will be added to the scaffold. Additionally, the source of cells could even change depending on the size and location of a chondral defect. If the defect is on the surface of the articulating surface, a population of superficial cell may be harvested for the cellular component of the scaffold. Similarly, deep zone chondrocytes maybe be harvested if the defect lies in the deep zone of chondral tissue. Because the structure and function of cartilage tissue varies throughout its depth and location, engineering approaches must be able to tackle a broad array of defects.

2.3.1.2 Current Model for Engineering Cartilage

There are two major approaches to cartilage tissue engineering. The first approach is to culture cells with or without growth factors *in vitro* for a brief period of time and then implant the construct into the defect site. This method allows the cells to mature and become active inside the body, where they will hopefully start production of a healthy ECM. The second, and more popular model, involves a much longer *in vitro* culture period before implantation. This allows the ECM to build up before the construct enters the defect site, with the intention of providing mechanical support immediately upon implantation. If the scaffold is mechanically and biologically mature and functional before introduction to the defect it will have a greater chance of remaining so while supporting loading regions. In both cases the model includes gradual resorption of the biomaterial as the ECM is produced, as well as integration of the new ECM with the surrounding native tissue. [17, 25]

An ideal current model for tissue engineering articular cartilage involves a multi-step procedure. First, an autologous cell population is obtained from the patient, either from cartilage tissue or tissue containing a population of MSCs (such as adipose tissue or bone marrow). Next, these cells are multiplied in monolayer culture, and then transferred to three dimensional culture on the scaffold material to help maintain the chondrocyte phenotype and re-differentiate cells if necessary. The scaffold is cultured for as long as desired, and then implanted into the defect site. [17, 32, 35]

Tissue engineering efforts focus on treating cartilage defects that can lead to OA, as designing a scaffold for treatment of advanced stages of cartilage disease is very

difficult. Through early intervention and treatment ideally the onset of OA can be delayed or avoided all together.

2.3.2 Biomaterials and Cells for Cartilage Engineering

Many materials have been developed for tissue engineering efforts. Among these there is a large range of chemical components, mechanical strengths, structure, surface topography, and biochemical properties. No ideal scaffold material has been developed, and each group of materials has their advantages and disadvantages. The major goal of the scaffold should be to ensure the retention of chondrocyte phenotype and provide mechanical stability. Hydrogels have received considerable attention in this area as they have properties similar to native tissue. [17, 32, 35] Table 2.1 includes a summary of materials that have been used in clinical or research settings for cartilage tissue engineering.

2.3.2.1 Scaffolds

Poly (L-lactic acid) (PLLA), poly(glycolic acid) (PGA), and the copolymer poly(lactic-co-glycolic acid) (PLGA) are some of the most popular synthetic materials investigated for cartilage engineering. Synthetic polymers usually have an open lattice and high porosity which is good for exchange of nutrients and molecules. Their degradation rates can be tailored through composition, and chondrocytes have been shown to adhere and maintain their signature rounded morphology on these materials. Animal models show some preliminary success with synthetic materials, but due to their limitations human trail data is largely unavailable. Some key limitations include:

difficulty to mold into complex shapes, hydrophobic – which generally means poor cell attachment and the need for very large chondrocytes populations, invasive implantation, and a strong foreign body reaction. [22, 33-35]

Table 2.1. Materials that have been used in cartilage engineering efforts in either clinical or research settings.

Materials Used in Cartilage Engineering			
Naturally Derived	**Reference**	**Synthetically Derived**	**Reference**
Fibrin	[36-39]	Poly(lactic acid)	[40, 41]
Collagen	[42-44]	Poly(glycolic acid)	[41, 45-48]
Chondroitin Sulphate	[44, 49]	Co-polymers of poly(lactic acid) and poly(glycolic acid)	[41, 50]
Alginate	[39, 51-57]	Poly(ethylene oxide)	[58, 59]
Agarose	[60-62]	Poly(ethylene glycol)	[63-66]
Silk	[67-69]	Ceramics	[70, 71]
Chitosan	[67, 72, 73]	Pluronic (copolymer of poly(ethylene oxide) and poly(propylene oxide)	[73, 74]
Hyaluronic Acid	[55, 74, 75]	Poly(urethane)	[36]
Cellulose	[76]	Poly(hydroxybutyrate)	[77, 78]
Gelatin	[79]	Poly(ethylene-terephtalate)	[80]
		Poly(tetrafluoroethylene)	[81]
		Poly(1,9-octanediol citrate)	[82, 83]
		Poly(caprolactone)	[84, 85]
		Poly(ether ester) co- polymer	[86]
		Carbon Fiber	[87, 88]
		Calcium Phosphate	[89]
		Poly(methacrylates)	[90, 91]

Naturally derived materials provide the advantages of usually being biocompatible and biodegradable. Due to its prevalence in the ECM collagen is one of the most popular natural biomaterials for cartilage regeneration. Porous collagen sponges have been made with and without GAGs and growth factors and show good cell attachment and maintenance of cellular phenotype. However, in some cases they have been shown to cause a foreign body reaction which interferes with tissue integration. Additionally, any porous natural material would also have to be delivered through an invasive open surgery. [22, 34, 35]

Hydrogels are popular in cartilage engineering due to their similarities to native tissue. Hydrogels are water-swollen polymer networks that can be chemically modified by crosslinks to form mechanically stable shapes. They are made by mixing a soluble polymer (natural or synthetic) in water and adding a crosslinking agent. They can be injectable and molded into desired shapes during gelation. This provides the potential for non-invasive delivery to a defect site. Their porosity can be adjusted by the network density, and their high water content and elastic properties make them similar to native tissue. Chondrocytes show strong attachment and retention of their phenotype in most hydrogels. Some natural hydrogels include alginate, agarose, chitosan, and fibrin. The main drawbacks of these materials include their lack of mechanical strength and difficulty controlling properties such as degradation rate. Synthetic hydrogels allow for somewhat more control over properties such as degradation rate. Some synthetic hydrogels used in cartilage engineering include: poly (ethylene oxide) (PEO), poly (propylene oxide) (PPO), poly (vinyl alcohol) (PVA), and poly(ethylene glycol)(PEG). Synthetic hydrogels often have more limited cell attachment properties than their

naturally-derived counterparts. Limitations of both natural and synthetic hydrogels include cellular encapsulation and formation of a uniform gel. Injection provides challenges in controlling gelation rate, and difficultly controlling the homogeneity of the formed gel. The use of photocrosslinking has been shown to provide more uniform gelation as the entire hydrogel crosslinks simultaneously upon ultraviolet light exposure. [8, 22, 33, 35, 92]

2.3.2.2 Cell Source

A major obstacle in tissue engineering articular cartilage is obtaining a sufficiently large, and phenotypically stable autologous cell population. Donor site morbidity makes a large cartilage harvest impractical and even dangerous. The low number of harvested chondrocytes creates the need for expansion culture in monolayer. Although chondrocytes maintain their phenotype better in three-dimensional culture their proliferation rates are much higher in monolayer. Monolayer culture causes chondrocytes to flatten, losing their rounded morphology and become more fibroblastic in nature. Three-dimensional culture following monolayer helps to re-differentiate the cells, however this process is relatively inefficient and the native phenotype is never fully restored. Quality and health of the harvested chondrocytes is also an issue of concern. Currently the mechanisms at play during chondrocyte differentiation and re-differentiation are not fully understood. Without this understanding the process will be difficult to control. [22, 25, 32]

Using MSC populations on their own or mixed with autologous chondrocytes can reduce the need for the invasive harvest procedure, however the optimal conditions for

chondrogenesis of a MSC are yet to be fully understood. Furthermore, bone marrow harvest of MSC populations is also an invasive procedure. The easiest place to harvest MSC is adipose tissue, where low donor site morbidity exists. However, MSCs derived from adipose tissue may be more difficult to differentiate into chondrocytes than those derived from bone marrow. [17, 22, 25, 32]

2.3.3 Engineered Constructs in Clinical Trials and Early Applications

Many new products have entered clinical trials or are already commercially available. Most of these products seek to improve the traditional surgical treatment through tissue engineering strategies. The majority of these clinical trials and products are not yet available in the United States and statistics on their long-term success in humans do not yet exist. [29]

2.3.3.1 Marrow Stimulation Techniques

A process called scaffold-guided microfracture uses a scaffold to help the bone marrow stay within the defect site following micofracture. The following products utilize this idea: BST-CarGel (Biosyntech Inc., Laval, Quebec, Canada), ChonDux (Biomet, Inc, Warsaw, Indiana), and Gelrin C (Regentis, Haifa, Israel). BST-CarGel is a biodegradable and injectable chitosan-glycerol phosphate based hydrogel. ChonDux is an injectable poly(ethylene glycol) hydrogel that contains an adhesive to stick to the defect site. Gelrin-C is a degradable and injectable copolymer of denatured fibrogen and poly(ethylene glycol). [29, 30]

2.3.3.2 Osteochondral Grafts

Scaffolds used in the place of tissue grafts can provide many benefits. Advantages include: biodegradability for new tissue to take its place, cost-effective, time-efficient, single procedure, no donor site morbidity, and the potential to include cell therapies. A drawback of using a substitute for a graft tissue, is of course, the lack of autologous, living tissue. Other potential complications include wear debris, inflammation, and friction between implanted material and tissue. Products developed for this use include: BST CarGel, Gelrin C, Salucartilage (Salumedica, Smyrna, GA), Chondromimetic (Ortho-mimetics, Cambridge, UK), TruFit Plug (OsteoBiologics/Smith & Nephew, Andover, MA), and OrthoGlide (Advanced Bio-Surfaces, Minnetonka, MN). SaluCartilage is another biodegradable and injectable hydrogel that solidifies *in vivo*. Chondromimetic is a dual-layer porous implant that has regions with properties similar to both subchondral bone and cartilage tissue. Tru-Fit and OrthoGlide are cylindrical-shaped polymers used for filling in circular drill holes where a defect site would lie. [29]

2.3.3.3 Cell-Based Therapies

The matrix-induced autologous chondrocyte implantation is very similar to the traditional ACI procedure, with the addition of a degradable matrix to support the transplanted chondrocytes until they form their own matrix. This helps keep the transplanted cells in the defect and provides much needed mechanical support. There is potential for growth factor incorporation to the scaffolds to aid in ECM production and retention of chondrocyte phenotype. [29] Developed products include: Carticel (Genzyme Inc, Cambridge, MA), ChondroGide (Geistlich Biomaterials, Wolhausen,

Switzerland), CaRes (Anthro-Kinetics, Essingen, Germany), Hyalograft-C (Fidia Advanced Biopolymers, Abano Terma, Italy, and Neocart (Histogenics, Waltham, MA). Carticel and Chondrogide are porcine-derived type I and type II collagen matrices, CaRes is a type I collagen matrix, Hyalograft-C is a hyaluronic acid based scaffold, and Neocart is made of a bovine collegen matrix. [22, 29, 93]

Fibrin based scaffolds are being developed on which minced harvested cartilage tissue is placed. The construct is then implanted into the defect site. A process called 'neocartilage implantation' is also being developed during which harvested cells are grown in a scaffold in a dynamic culture system to produce and ECM. The ECM is then isolated and implanted into a cartilage defect. [29] Table 2.2 lists product information for commercially developed cartilage engineering products.

Table 2.2. Name, company, and website for products which are commercially available or in/entering clinical trials for cartilage engineering.

Commercial Products in Cartilage Tissue Engineering		
Product Name	**Company**	**Website**
BST-CarGel	Biosyntech Inc., Laval, Quebec, Canada	www.biosyntech.com
ChonDux	Biomet Inc., Warsaw, IN, U.S.A.	www.biomet.com
Gelrin C	Regentis, Haifa, Israel	www.regentis.co.il
Salucartilage	SaluMedica, Smyrna, GA, U.S.A.	www.salumedia.com
Chondromimetic	Orthomimetics, Cambridge, UK	www.orthomimetics.com
TrueFit Plug	OsteoBiologics/Smith & Newphew, Andover, MA, U.S.A.	www.global.smith-nephew.com
OrthoGlide	Advanced Biosurfaces, Minnetonka, MN, U.S.A.	www.advbiosurf.com
Carticel	Genzyme Inc, Cambridge, MA, U.S.A.	www.genzyme.com
ChondroGide	Geistlich Biomaterials, Wolhausen, Switzerland	www.geistlich.ch
CaRes	Anthro Kinetics, Essingen, Germany	www.arthro-kinetics.com
Hyalograft-C	Fidia Advanced Biopolymers, Abano Terma, Italy	www.fidiapharma.com
NeoCart, VeriCart	Histogenics, Waltham, MA, U.S.A.	www.histogenics.com

2.3.4 Current Research Efforts

The development of cartilage tissue engineering products has been the result of decades of research efforts that span many natural and synthetic scaffold materials. Although not all of these materials have developed into usable constructs this research has, and continues to, contribute to the current understanding and knowledge base within the field.

2.3.4.1 Natural Scaffolds

Some of the most popular natural scaffolds used in cartilage engineering research include alginate, fibrin, agarose, hyaluronic acid, chitosan, and type I and II collagens. Studies using alginate, collagens, and fibrin are highlighted due to their prevalence in the literature.

Early studies using alginate to encapsulate chondrocytes were performed in the late 1980's, these studies demonstrated retention of the chondrocyte phenotype and proliferation of chondrocytes seeded in three-dimensions. [51] Following studies have established that chondrocytes remain phenotypically active and proliferate within alginate, even up to even 8 months in culture. [52-55] Markers for phenotype retention include gene expression or biochemical presence of ECM components such as type II collagen, aggrecan, and GAGs. High gene expression of type I collagen indicates cells have started to differentiate to a more fibroblastic lineage. Alginate has been investigated for its potential in re-differentiating cells that have started down a fibroblastic lineage due to expansion in monolayer. Results show encapsulation in alginate can aid in re-differentiating cells to express higher levels of matrix proteins and lower levels of type I collagen following two-dimensional culture. [53] Despite maintaining a healthy chondrocyte population alginate's drawbacks include limited mechanical stability and biodegradation. [37]

Studies using both type I and type II collagen matrices have also shown support of chondrocyte proliferation and maintenance of phenotype. [42-44] The incorporation of glycosaminoglycans, such as chondroitin sulfate, within the collagen scaffold have

shown to further improve expression of matrix proteins. [42, 44] Additionally, mechanical loading of chondrocytes seeded on collagen scaffolds has been shown to alter cellular gene expression. [43] Collagen scaffolds are biodegradable, however they can be expensive and fairly difficult to produce.

Fibrin glue is made by mixing fibrinogen and thrombin to form a biodegradable, injectable material. It has been studied and classified for mixing with chondrocytes and injecting into cartilage defect sites. Animal trials with this method show significant wound healing and integration with native tissue. [37] Fibrin biodegradation can be tailored and it can be mixed with other polymers to improve its relatively weak mechanical strength. Various models of fibrin-alginate scaffolds have been show to support proliferation and the chondrocyte phenotype. [38, 39]

2.3.4.2 Synthetic Scaffolds

Popular synthetically derived materials used in cartilage research include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly (lactic-co-glycolic acid) (PLGA), poly (caprolactone) (PCL), and poly (ethylene glycol) (PEG). As a result of their prevalence in the literature, efforts using PGA and PEG will be covered in more detail.

PGA is an alpha polyester that degrades within months into products the body can readily absorb, making it biocompatible. As it degrades a loss of mechanical strength is observed, however in vitro culture and formation of ECM may strengthen its mechanical properties. [94] PGA scaffolds for cartilage engineering are usually made in the form of porous meshes that allow for nutrient and molecule transfer. Production of GAGs,

aggrecan, and type II collagen are all observed in chondrocytes cultured *in vitro* for up to 40 days on PGA scaffolds. [46, 47] Porous PGA scaffolds seeded with bone marrow stromal cells and implanted subcutaneously into mice show formation of mature cartilage after 8 weeks. [48] PLA is another alpha polyester with similar mechanical and biological properties shown to support chondrocyte adherence and proliferation. A copolymer of PGA and PLA (PLGA), whose properties are similar and proportional to the proportion of each polymer, is also used in cartilage engineering efforts. [41, 94]

PEG can be formed in to an injectable hydrogel with properties similar to native cartilage tissue. It is biocompatible, but not biodegradable on it own. Therefore, it must to copolymerized to achieve *in vivo* degradation. PEG-based polymers can be photopolymerized with addition of a photoinitiator. In this model the polymer and cell solution would be injected to the defect site as a liquid to fill the exact shape of the defect, the polymer would then be photocrosslinked forming a solid matrix. Copolymers of PEG and PLA as well as PEG and poly(vinyl alcohol) are biodegradable and promote chondrocyte adhesion and matrix molecule production. [63-65] Additionally, incorporation of matrix molecules such as chondroitin sulfate has been shown to increase mechanical properties as well as gene expression of matrix molecules. [66] Poly(ethylene oxide) (PEO), a higher molecular weight form of PEG, has also been photopolymerized into hydrogels for cartilage applications. PEO-based research shows that cells remain viable and produce significant levels of GAG and collagen *in vitro* during encapsulation in hydrogel scaffolds.[58] Copolymers with PEG have also shown favorable mechanical and biochemical properties and chondrocyte activity. [59]

2.3.4.3 Growth Factors

Growth factors known to promote chondrocyte activity are often incorporated into scaffolds, or delivered to culture media to stimulate the cell population. Although many studies have investigated growth factor use, many of their effects – both alone and in combination- remain to be fully understood. The most prominent growth factors used in cartilage engineering studies include; insulin-like growth factor – I (IGF-I), basic fibroblast growth factor (bFGF), and transforming growth factor-β1 (TGF-β1). [5, 56, 95-97] All of these have demonstrated anabolic cellular effects and increased production of matrix molecules. Although the effects of these factors are generally understood, the ideal combination of growth factors and delivery mechanism remains to be established.

2.4 Future Directions

Many advances have been made over the past few decades in understanding cartilage engineering, however major hurdles still exist within the field. Cell source, maintenance of the chondrocyte phenotype *in vitro*, and recreation of tissue with the structure and properties of native cartilage are today's major challenges. Research which address these challenges include zonal cartilage engineering, the use of stem cells, and utilization of dynamic *in vitro* culture systems. Together these fields are likely to have a major impact on cartilage regeneration in coming years.

2.4.1 Zonal Cartilage Engineering

Recreation of the zonal complexities present in native cartilage tissue has become a focus of many cartilage engineering efforts. Initial studies, and most currently available

engineering solutions, attempt to remodel cartilage as a homogenous tissue. As the cellular and structural differences between cartilage zones are more fully understood, the need to recreate this complex tissue architecture is becoming more apparent. Articular cartilage is intricately organized and heterogeneous. It is unlikely that a homogenous tissue, based on a homogenous scaffold, can functionally replace this structure. Furthermore, it is likely that through formation of zonal organization there will be better integration with host tissue, and a more fluid transmission of stress between native and novel cartilage. Depth dependent variations in scaffold design (pore size, porosity, mechanical properties, and addition of growth factors, etc) and the origin of the seeded cells (super zone, middle zone, or deep zone) can be used as tools in designing zonal scaffolds. [33, 98]

While there is no current model for regenerating zonally organized tissue *in vitro*, several studies have attempted to establish the difference in phenotype between zonal cell populations and create culture systems which more closely mimic the native environment. These studies are paving the way for biomaterials which will help to restore defects in a zone-specific manner. For example, research has shown the shear modulus to vary by up 2 orders of magnitude through the depth of a single articular cartilage sample. [99] Additionally, studies show differences in matrix deposition, morphology, and gene expression between cultured populations isolated from distinct cartilage zones. [100, 101] Further studies have developed layered culture systems based on materials such as PEG, PEO, agarose, and alginate. [15, 57, 60, 102] These studies show both depth-dependent mechanical properties of the scaffolds and changes in metabolic activity of subpopulations cultured in layers. [15, 61] The continuation of such

studies and the development of zonally-engineered cartilage tissue could potentially be very influential the next generation of cartilage repair solutions.

2.4.2 Stem Cells

A major challenge in cartilage engineering is obtaining a sufficiently large chondrocyte population to seed onto the scaffold material. Both maintaining the chondrocyte phenotype during culture and injury at the harvest site are significant challenges in this approach. An alternative to autologous chondrocytes harvest is the use of MSCs. MSC use also has significant challenges that are yet to be met. Harvesting the MSC population is the first challenge. The most classified and understood MSC population lies in bone marrow. However, bone marrow harvest is both painful and a potentially risky procedure. Adipose tissue also contains a MSC population and is much easier to harvest, however it is more challenging to induce chondrogenesis in adipose-derived MSCs. Other tissues with MSC populations include the synovial membrane, muscle, periostium, and umbilical cord. [22, 103] Once harvested, the next major challenge is inducing chondrogenesis in the stem cells. Various growth factors have been identified and studied for inducing the chondrocyte phenotype, however an ideal growth factor or combination is yet to be discovered. Furthermore, *in vitro* culture often leads to production of fibro-cartilage features and hypertrophy in the stem cell population. [22, 103] Current animal and human models that have used MSCs for cartilage repair have shown mixed results, often plagued by fibro-cartilage formation. [17, 103, 104]

A biomaterial and proper incorporation or delivery of growth factors is needed which successfully differentiates MSCs into healthy articular chondrocytes. Several

attempts to design such scaffold have been met with preliminary success. PEG based hydrogels with decorin moieties are reported to promote *in vitro* chondrogenesis of MSCs, marked by deposition of ECM components such as type II collagen and aggrecan. [105] Additionally, PEO based hydrogels with hyaluronic acid and TGF-β3 are reported to induce chondrogenesis of MSC in *in vivo* animal models. [75] The future of MSCs in cartilage engineering will rely on development of a practical harvest method and production of a reliable chondrocyte phenotype. Eliminating the need for harmful autologous chondrocyte harvests will be a significant advancement for cell-based cartilage engineering strategies.

Embryonic stem cells for cartilage engineering have recently received considerable attention, and may hold promise for the future. These cells have the advantage of large cell source numbers and the ability to proliferate significantly. Their drawbacks include potential immune response, and differentiation challenges. [104] Animal models show varying reports of success depending on where the embryonic stem cells are injected. Mouse models show chondral defects treated with undifferentiated embryonic stem cells result in the formations of teratomas. However, embryonic stem cells injected to osteochondral defects in the same animal model show restoration of healthy tissue. [106, 107] Control of the differentiation process to form functional chondrocytes is essential to establish for the use of embryonic stem cells. Studies report chondrogenesis of embryonic stem cells through the use of growth factors such as bone morphogenic proteins, transforming growth factor-β1, and insulin-like growth factor-1. [108-110] Additional studies have investigated the differentiation of embryonic stem cells to mesenchymal-like stem cells and have reported success. [111, 112] The ability of

these cells to undergo chondrogenesis has been studied using a modified PEG-based hydrogel. Results indicate promise for the use of embryonic stem cells in cartilage tissue engineering. [112] While these studies indicate great potential for chondrogenesis of embryonic stem cells, research in this field has yet to establish precise cellular mechanisms at work during this process.

Induced pluripotent stem cells may also hold promise for cartilage regeneration. Advantages of induced pluripotent stem cell use include production of an autologous cell population and elimination of harmful cartilage or bone marrow harvests. [113] However, there is limited research investigating the chondrogenic potential of these cells. Use of induced pluripotent stem cells in cartilage engineering will require research efforts to clearly establish differentiation parameters.

2.4.3 Dynamic Culture Systems

Healthy loading is essential for the maintenance of cartilage in the body. To understand the important relationship between loading and chondrocyte metabolism many studies have investigated the effects of both static and cyclic loading on chondrocyte activity. Reports show mixed inhibitory and simulative effects depending on load magnitude, size, and which zone the chondrocytes originated from. [62, 114, 115] To create a culture system which mimics the dynamic *in vivo* environment many groups have designed bioreactor systems. Culturing engineered cartilage scaffolds in dynamic bioreactor systems is not the current standard, but this model holds great promise for maintaining healthier, and more phenotypically stable cell populations *in vitro*.

Results from bioreactor studies show increases in production of ECM molecules, cell proliferation, and mechanical properties. [116, 117] For example, PGA scaffolds in a perfusion system showed increases in both DNA and GAG content compared to controls. [118] Chondrocytes in PEG-based hydrogels exposed to dynamic laminar fluid flow showed increased levels of GAG and collagen production and better mechanical properties compared to controls. [119, 120] Studies have also added growth factors to dynamic culture conditions and observed even more favorable outcomes. [95, 97] The ideal combination of scaffold material, growth factors, and dynamic culture system are yet to be established for *in vitro* culture. Understanding how these factors work together to affect the chondrocyte phenotype is essential for the success of cartilage engineering strategies. Successful three-dimensional scaffold culture will create a plethora of stable chondrocytes producing ECM that can then be transplanted into cartilage defects. Current research strategies need to establish these culture conditions for practical implementation of engineering solutions.

3 Phenotypic Variations in Chondrocyte Subpopulations and Their Response to In Vitro Culture and External Stimuli*

3.1 Introduction

Articular cartilage is complex in its extracellular matrix (ECM) organization as well as cellular phenotype. The tissue is comprised of predominately type II collagen, proteoglycans, and chondrocytes. However, the morphology and metabolic activity of the cells as well as the structure of the ECM components vary greatly throughout the tissue depth. This intricate tissue organization allows cartilage to optimally resist loading and provide low-friction joint movement throughout a lifetime.

Cartilage tissue has a low cell density, with chondrocytes comprising only 5% of the total tissue volume. [1] Furthermore, after adulthood is reached chondrocytes rarely divide to provide the tissue with a new cell population. Articular cartilage lacks both a blood supply and direct access to the lymph system - leaving most nutrient, gas, and waste exchange to occur through diffusion. All of these factors contribute to the tissue's limited ability to self-heal. Cartilage defects rarely repair themselves and as a result often lead to complications later in life, or even disease. The most prevalent disease affecting articular cartilage is Osteoarthritis (OA). The inability of cartilage to self-repair, and the growing cost of OA to society (current estimates at $60 billion dollars annually in the United States [23]), have made cartilage engineering the focus of many research efforts.

Approximately 95% of cartilage tissue volume is the extracellular matrix. The

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matrix is comprised predominately of two interconnected networks: a type II network and a hyaluronic acid and proteoglycan network. Chondrocytes are linked to these networks through proteins on the cell surface which allow them to sense, and respond to, mechanical force. [2] Collagen content makes up about 10-20% of the wet weight of the tissue, and 90% of the collagen content is the type II collagen network. Type II collagen is a 300 nm long fiber with three identical polypeptides alpha helixes. The collagen fibers are linked by strong covalent bonds and provide much of the tensile strength of the tissue. [3, 4]

Aggrecan is the major proteoglycan in the tissue and contains many branched glycosaminoglycans (GAGs) originating from a central backbone. The GAGs are predominately keratin sulfate (KS) and chondroitin sulfate (CS), and each aggrecan molecule contains from 50-100 of each. The repeating sulfate groups give the molecule a large net negative charge. Each aggrecan unit is connected via a link protein to a long, unbranched hyaluronic acid polysacharride chain. The negative charges on the aggrecan molecules provide a high osmotic tissue pressure, which acts to resist compression during loading. The collagen network keeps the tissue from swelling as the proteoglycans retain water – which provides the tissue with further compressive strength. [2, 4, 5, 17] However, during loading a small amount of liquid is forced out of the tissue into the synovial cavity of the joint. Here the liquid will absorb nutrients which will be delivered to the tissue as the load is released and the liquid flows back into the cartilage. Thus, a healthy loading regime is essential for proper cartilage function. [16]

The average height of human articular cartilage on the femoral condyle has been measured at 2.4 mm, which includes superficial, middle and deep zones but not calcified

tissue. [121] In comparison, the average reported height of knee articular cartilage reported in rabbits is 0.4 mm, [122] sheep tissue is 0.7 mm, [123] and bovine cartilage is 1.7 mm. [124] Below the articulating surface the tissue has been divided into three zones: superficial or tangential zone, the middle or transitional zone, and the deep or basel zone. Each zone has distinct ECM organization, cell morphology, and metabolic activity. Many studies use slightly different definitions of zone depth. As a general rule, the superficial zone is defined as approximately the top 10-15% of the tissue and contains the articulating surface. The middle zone is the approximately the middle 60% of the tissue and the deep zone contains the remaining 30% of tissue depth. Following the deep zone is the tidemark – below which the tissue becomes calcified and eventually turns into subchondral bone. This calcified region effectively blocks any diffusion from the subchondral bone, and anchors the articular cartilage to the bone tissue below. This review will focus on cartilage engineering of the superficial, middle, and deep zones. [4, 9, 10, 98]

Extracellular matrix composition and structure vary greatly between zones. While the collagen content per weight does not change significantly with depth its orientation and number of crosslinks are both depth-dependent. The number of lysylpyridinoline crosslinks decreases with depth in mature cartilage, while the number of hydroxylysine and hydroxylysylpyridinoline crosslinks increase with depth. It is hypothesized that this, along with collagen fiber orientation, accounts for the differences in tensile strength and stiffness throughout the tissue depth. [125, 126] The tensile strength and stiffness of the tissue are highest in superficial zone and decrease into the middle and deep zone. [126] Collagen fibers in the superficial zone are orientated

parallel to the articulating surface in tight bundles. As well as providing tensile strength these fibers are thought to block any unwanted molecules from the synovial fluid in the joint. [13] The collagen fibers of the middle zone are randomly orientated, and those of the deep zone are oriented perpendicular to the articulating surface. During ageing and deformation the tissue will split parallel to the direction of the collagen fibers. That is, the split lines coincide with the collagen network orientation. [127, 128] Differences in proteoglycan content are also observed throughout the tissue depth. Proteoglycan content increases with distance from the articulating surface, and with it so does the compressive modulus of the tissue. [129] Consequently, the water content is lowest in the superficial zone, with approximately 65% of the water content of the tissue residing in the middle and deep zones. Furthermore, as a result of diffusion from the synovial fluid the oxygen concentration within the tissue is highest in the superficial zone and decreases through the middle and deep zones. [2, 98] Figure 3.1 shows the distribution of water content, oxygen, and compressive and tensile strengths though the depth of the tissue.

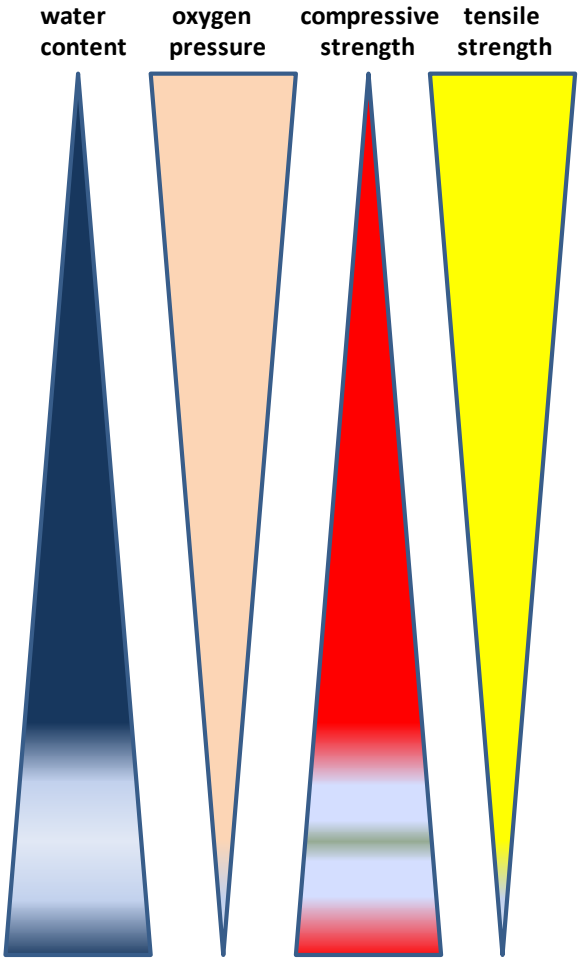

Figure 3.1 Schematic demonstrating the distribution of water, oxygen, compressive strength, and tensile strength through the depth of articular cartilage.

Zonal differences in matrix organization and content are largely due to variations in cellular activity. [11, 12] Among zones cells display differences in morphology, density, and metabolic activity. Superficial zone cells are the smallest and the most densely populated; they are elongated, thin, and oriented parallel to the articulating surface. These the major cells within articular cartilage responsible for producing proteoglycan 4 (PRG4), a large glycoprotein that aides in lubrication in the synovial fluid. [14] Proteoglycan 4 is also commonly referred to as superficial zone protein or lubricin. Middle zone cells are larger, less densely populated, and do not have a

particular orientation. Deep zone cells are also larger than superficial cells and are oriented in columns perpendicular to the articulating surface which serve to anchor the articular cartilage to the calcified layer below. While superficial and middle zone chondrocytes usually exist on their own, deep zone cells are often found in clusters of 5-8 cells. [13, 121] Several secreted proteins also exists as markers for cells of various zones, however their functions are not fully understood. [130-132] Relative maintenance of subpopulation phenotypes *in vitro* has been demonstrated in many studies throughout the past two decades, however comprehensive knowledge of the cellular mechanisms behind these differences has not been achieved. Furthermore, differentiation of chondrocyte progenitor cells into distinct chondrocyte subpopulations is yet to be demonstrated or of practical use.

While many cartilage engineering products are beginning to make their way into the clinical settings, there is a lack of commercial products which attempt to reconstruct the zonal organization of articular cartilage. Since zonal organization is integral to the proper function of the tissue, it is unlikely that a homogenous approach to tissue repair can adequately regenerate cartilage tissue. For the success of zonal cartilage engineering efforts maintaining and/or differentiating the subpopulation phenotype must be achieved both *in vitro* and *in vivo*. As maintaining the chondrocyte phenotype is a major challenge in all cartilage engineering efforts, adding variations within this phenotype provides an even greater obstacle. [22, 32] The first step in achieving this goal is thorough understanding and classification of the cellular mechanisms which make these cells different. The goal of this review is to facilitate this process thorough a search of the literature.

This review provides a comprehensive analysis of the variations in zonal chondrocyte phenotype. In an effort to better understand the cellular differences among zones we have reviewed the literature for studies which attempt to establish and classify the zonal variations in cell activity. Complete understanding of chondrocyte function will aid cartilage engineers in creation of zonally organized tissue. Our aim is to aid in this understanding and help to clearly identify cellular and structural properties which must be achieved for proper tissue restoration. We will investigate explant studies, monolayer and three-dimensional culture models, dynamic culture models, growth factor delivery, mechanical stimulation models, and multi-layer culture systems as they pertain to chondrocyte subpopulation variations.

3.1 Explant Culture

Studies which investigate cell populations immediately following harvest with limited culture time provide understanding as to the native function of the cells. Such studies show variations in metabolic, mechanical, and morphological properties of zonal cell populations. A study in 1994 which examined explants from different zones of 12-18 month-old bovine articular cartilage was one of the earliest works to identify a novel proteoglycan synthesized and secreted only in the superficial zone. [133] The proteoglycan was termed superficial zone protein (SZP) and monoclonal antibodies were raised against the protein to confirm its presence in the superficial zone and in the synovial lining in adult and fetal human articular cartilage. The same study also showed the protein was not present in the deep zone of articular cartilage, nasel septal cartilage, or synovial stromal cells. Analysis using flow cytometry showed the following

percentages for immunopositve cells isolated from full-thickness articular cartilage, superficial zone, deep zone and synovial cells respectively: 37.4, 52.5, 3.4, and 7.5. [134] Gene expression analysis has identified elevated levels of expression of the superficial zone protein in the superficial layer of articular cartilage. [135] It has been established that SZP is encoded by the proteoglycan 4 (PRG4) gene, which also is responsible for other proteins with very similar structure and function to SZP. Taken together, these group of proteins are sometimes referred to as PRG4. [136]

Additional zonal protein markers include clusterin, developmental endothelial locus-1 (Del1) protein, cartilage intermediate layer protein (CILP), and potentially cartilage oligomeric matrix protein (COMP). Clusterin is a glycoprotein whose exact function in cartilage tissue is yet to be identified. Its mRNA and protein are reported at elevated levels in diseased tissue, and in healthy tissue it is thought to play a role in a plethora of important biological functions. Gene expression for clusterin has localized it to the superficial zone only, [137, 138] identifying it as a marker for that zone. Del1 is thought to play a role in vascularization regulation and restricts endothelial cells during early development. When the Del1 receptor $\alpha v\beta 3$ was antibody-bound it was shown to inhibit angiogenesis. [131] This protein has been reported in the cell-associated matrix of isolated superficial chondrocytes and is enriched in tissue explants from the superficial zone versus the deep zone. [132] CILP, a protein thought to be unique to articular cartilage, is found only the middle zone of the tissue. Its exact function is yet to be identified, however it is thought to have a role in the progression of diseases such as osteoarthritis. [130] COMP is a large extracellular glycoprotein thought to stabilize matrix bonds and found in the matrix surrounding a chondrocyte. Studies have identified

its upregulation as a marker for osteoarthritis and rheumatoid arthritis, [139, 140] however it is also thought to be a marker for deep zone cartilage. [141, 142]

The Notch family receptors and their ligands also have zonal distributions. Cell-cell Notch receptor signaling is important for a variety of cell functions including proliferation, differentiation, and apoptosis. Four Notch receptors have been identified in humans (Notch-1,2,3, and 4), which are bound by the ligands Delta, Jagged-1, and Jagged-2. Murine studies show Notch-1 elevated in the superficial zone during development, but found Notch-1 distribution limited to deeper zones in mature tissue. Similarly, Notch-2,4, Delta, and Jagged-2 were found throughout the tissue during development, but the receptors were observed only in the deep zone of mature samples. The same model reported Notch-3 and Jagged-1 absent from all zones of developing cartilage but present in deep, mature tissue. [143] Human models show Notch-1 concentrated in superficial and deep zones, Notch-2 in all zones, Jagged-1 in deep zones, and Delta in only the superficial zone. While the exact reason and function of these distributions are not known, it is hypothesized they play a role in development of the zonal organization of the tissue, and that the Notch-1 receptor may be a marker for mesenchymal progenitor cells. [144, 145]

Yet another biochemical difference among zones includes the processes by which pH is regulated in the tissue. Chondrocytes exists in a low-oxygen environment and as a result their metabolism relies mostly on glycolysis, the products of which are acidic. This, along with a high concentration of cations gives the tissue a fairly low pH. However, at pH values below 6.8 proteoglycan synthesis is hindered. It is important for the pH of the tissue to be carefully maintained. Maintenance of pH in cartilage was

previously hypothesized to be dependent solely on the Na+/H+ exchanger, however it was recently demonstrated that superficial zone chondrocytes use a HCO_3^- dependent regulation system which is not found in the middle or deep zones.

Variations in mechanical properties of explanted tissues and cells have also been observed among zones. Single cell analysis of porcine chondrocytes using atomic force microscopy (AFM) indentation and micropipette aspiration show higher instantaneous modulus, relaxed modulus, and apparent viscosity for superficial cells versus middle/deep zone cells. [146] Results from fetal and newborn cartilage show average increases in compressive modulus by a factor of 4-5 from the top 0.1 mm (28+/-13 kPa) to 1 mm into the tissue (141+/-10 kPa). These increases correlated with an increase in GAG content. A weak correlation between collagen content and compressive modulus and an inverse correlation between modulus and cellular content are also observed. [147] However, investigations of the canine pericellular matrix demonstrated no difference in the Young's modulus between the pericellular matrix of superficial and middle/deep zone cells. [148]

Morphological and structural variations among zonal subpopulations have also been found to exist. In addition to the shape and size differences that have been well classified, variations in cellular density, cell-cell communication abilities, and cellular grouping have been identified. Using rabbit articular chondrocytes four different cell groups were isolated through centrifugation and a percoll density gradient. Of these four groups two displayed differing properties following a brief culture period. The cells of lowest density were large with low proliferation, maintained phenotype, and secreted large amounts of proteoglycan; it was hypothesized these cells originated in the deep

zone. The cells of highest density were small with large nuclei, proliferated slowly, expressed less ECM molecules, and produced larger amounts of interleukin 1-induced nitric oxide; it was hypothesized these cells were from the superficial zone. [149] It is commonly thought that chondrocytes have little cell-cell communication and function mainly in isolation. [6] However, a study examining cells from rabbit articular cartilage showed populations from the superficial zone may function in pairs, with rapid communication possible between the two cells. [150] Cells of the superficial zone also exist in patterned groups such as lines, clusters, and pairs. [151]

3.3 Monolayer Culture

In two dimensional culture gradual trends towards homogenization of subpopulations and loss of the chondrocyte phenotype are observed. The initial differences that are maintained show large variations in chondrocyte metabolism and further highlight the differences between zonal cells. Due to similarities in native tissue structure and cellular activity most studies pool middle and deep zone chondrocytes and study the superficial zone separately. Traditional monolayer culture studies demonstrate the inadequacy of two-dimensional culture techniques for zonal phenotype retention.

Differences in production of matrix components have been observed between populations in two-dimensions. Cells isolated from the middle and deep zones produce significantly thicker tissue with higher compressive modulus and substantially more glycosaminoglycans, large aggregating proteoglycans, and collagen than their superficial zone counterparts. [101, 152-154] Superficial zone cells also showed weaker and slower

cell attachment, and formation of clusters that were mainly cellular with little matrix. [101, 153]

Testing following a brief culture period showed that cells also retain their differences in mechanical properties. That is, cells isolated from the superficial zone demonstrated significantly higher relaxed and instantaneous moduli. [155] These differences were measured after only 18 hours in culture, so it is possible that cell populations had not yet been fully influenced by monolayer culture. A longer study over 7 days showed a loss in the differences in mechanical properties between superficial and middle/deep zone cells. Culture micropatterned surfaces aimed to restore spherical morphology only partially restored differences in mechanical properties. [156]

Many investigations report a loss of zonal phenotype with increasing culture time. A study which cultured 8 month old goat subpopulations in monolayer and transferred them to alginate beads after passages 1 to 4 concluded loss of cellar phenotype was rapid upon two-dimensional plating and was not restored with culture in alginate. Initially, superficial cells showed gene expression levels of proteoglycan 4 more than twice that of cells from middle/deep zones. The middle/deep zone population expressed 20 times more collagen than superficial cells. After three passages these differences were no longer detected and after four passages gene expression of type I collagen had increased 1200 fold and 8000 fold for the superficial and middle/deep zone cells respectively. Furthermore, suspension in alginate did not restore gene expression levels to initial values. Overall, monolayer culture resulted in conversion of subpopulations to a homogenous population and rapid loss of cellular phenotype. [157] Loss of subpopulation phenotype results in a cell population producing type I collagen and little

proteoglycan or type II collagen. [152] Additionally, the smaller size of the superficial zone cells disappears in two dimensional culture. [101]

Zonal equine chondrocytes lose their subpopulation phenotype in two-dimensional culture, however upon encapsulation in alginate hydrogels some zonal differences reappeared after four weeks. These zonal differences included expression of clusterin in the superficial zone, COMP in the deep zone, and increased GAG production in the deep zone. [158] Non-adhesive culture over agarose also helped in retaining cellular phenotype, and showed middle and deep zone cells to be significantly more active in producing ECM components. [152, 159]

Table 3.1 provides a summary of monolayer studies on zonal chondrocytes and highlights key findings. Monolayer culture results indicate two important factors; middle/deep zone chondrocytes are more active in production of matrix proteins that superficial cells, and eventually the subpopulations will converge to a homogenous population which does not retain the chondrocytes phenotype. Transferring cells to a three-dimensional environment or culturing on the surface of biomaterials such as agarose aids in phenotype retention, indicating environments closer to native tissue are favorable.

Table 3.1. Summary of monolayer studies on zonal chondrocytes including zone definitions, species and age of cell population, culture time, and key results found.

Monolayer Culture of Zonal Chondrocyte Populations

Ref	Zone Definition	Species, Age	Culture Time	Key Results Observed
[152]	*Superficial zone:* top 15% of tissue *Deep zone:* remaining tissue	Human, adult	32 days	*Initially:* deep cells synthesize more keratin sulphate (KS), with time HA and type II collagen (t2c) synthesis decrease *After 14 days:* morphological differences between populations disappear, both fibroblastic *After 32 days:* both populations producing predominantly small proteoglycans unable to form aggregates with HA
[157]	*Superficial zone:* top 10-20% of tissue *Growth zone:* remaining tissue	Goat, 8 months	Passages 0-4	*Initially:* superficial cells express 2.3 times more SZP, growth zone expresses 20 times more t2c *1st passage:* dramatic changes in ECM expression observed *3rd passage:* no differences between populations detected, t1c expression increased 1200 fold and 8000 fold for superficial and growth zones respectively
[154]	Enzymatic digestion to remove superficial zone. Isolated middle/deep (MD) zone and deep zone populations	Bovine, 6-9 months	On porous ceramic substrate for 8 weeks	*Deep zone cells:* produce the thickest tissue *MD cells:* produce most proteoglycans, and tissue with highest compressive modulus
[153]	*Superficial zone: top 20-30% of tissue*	Porcine	21 days	*Initially:* Deep zone cells produce KS, superficial cells do not

	Deep zone: remaining tissue			*With culture:* superficial cells produce KS, remain more rounded with reduced substrate adhesiveness than deep cells
[101]	*Upper cells:* top third of tissue (100-200μm) *Lower cells:* bottom third of tissue (200-350μm)	Porcine	32 days	*Initially:* upper cells smaller and produce less KS than lower cells *With Culture:* cell size differences not maintained, differences in KS production lost after several days, deep zone cells produce larger numbers of proteoglycans and higher percentage of aggregating proteoglycans
[155]	*Superficial zone:* top 10-20% of tissue *Middle/Deep zone:* remaining tissue	Bovine	3 and 18 hours	Superficial cells exhibit significantly higher relaxed and instantaneous modulus at both time points

3.4 Three Dimensional Culture

3.4.1 Scaffoldless Culture

With regard to recreating zonal differences, studies which attempt to layer high density chondrocytes subpopulations or culture subpopulations in micromass have shown differing results. Bovine superficial and middle zone cells cultured in alginate beads for one week and then seeded in high density constructs reported results similar to those observed in monolayer. That is, constructs of only superficial cells produced less matrix and had lower compressive moduli than those made with cells from the middle zone. Hybrid constructs made of a layer of superficial cells a top a layer of middle cells showed properties in between the two controls and production of SZP was limited to the superficial region. [160] Seemingly in contrast, another group which cultured both

superficial and middle bovine chondrocytes in micromass reported lower type I to type II collagen ratio (indicating phenotypic stability) and higher gene expression of aggrecan and SZP in superficial zone micromass cultures than middle zone cultures. Interestingly, greater differences were observed in immature samples (1-4 months in age) versus adult samples (18-36 months in age). [100] Furthermore, high density scaffoldless cultures of superficial cells atop middle cells failed to produce constructs which mimicked the mechanical properties of native tissue. That is, the trends in depth-dependent mechanical properties observed in native cartilage were not observed in the layered high density cultures. [147] Layered high density cultures of superficial and middle cells also failed to retain zonal organization or produce significant amounts of matrix when implanted into mini-pigs. [161] Results from scaffoldless constructs are varying at best, and usually do not maintain or mimic the zonal organization of articular cartilage. Despite challenges in creating zonal organization, it is important to note that scaffoldless cultures are not necessarily undesirable. Scaffoldless constructs treated with growth factors can display mechanical properties close to those of native cartilage tissue. [162] These constructs were formed in a process called 'self-assembly,' and formed tissue with biochemical and mechanical tissue properties similar to native tissue during non-adhesive culture over agarose. [163]

3.4.2 Scaffold-Based Culture

Constructs which support chondrocytes in a three-dimensional environment have shown further success in retention of phenotype and zonal properties. Table 3.2 provides a summary of scaffold-supported zonal chondrocyte studies. Culture of bovine

subpopulations in agarose demonstrated deep zone cells proliferating at the greatest rate, producing the most extracellular matrix, and highest amounts of aggregating proteoglycans. Superficial zone cells produced smaller non-aggregating proteoglycans that were degraded before they could be used in matrix assembly. [11, 12] Incorporation of matrix components to three-dimensional scaffolds can further aid in cell stability and activity. Chondroitin sulfate, type I collagen, and hyaluronic acid all had varying effects on bovine subpopulations encapsulated within poly(ethylene glycol) (PEG) gels. PEG containing chondroitin sulfate or type I collagen saw the greatest matrix accumulation by deep zone cells, while PEG containing chondroitin sulfate or hyaluronic acid saw the greatest production of matrix by superficial zone cells. Over all, the chondroitin sulphate group had the highest gene expression and production of ECM proteins, and in all cases the deep zone cells produced more GAG and matrix accumulation than the superficial zone group. [164]

Scaffold-based culture models have demonstrated retention of zonal markers such as clusterin and proteoglycan 4. Equine subpopulations were tested for clusterin immnuostaining in two culture groups. One group had immediately been encapsulated in a hydrogel and the other group had been cultured in monolayer to first passage and then transferred to alginate beads. Results showed monolayer culture resulted in loss of the chondrocytes phenotype and no clusterin staining. Superficial cells which were immediately encapsulated in an alginate hydrogel continued to express clusterin, while those of the lower zones did not. [165] Similarly, bovine chondrocytes in decelluarized cartilage constructs showed increased production of PRG4 in superficial cells over middle and deep cells, a difference which was maintained throughout culture. [136]

Variations in cytoskeletal organization of subpopulations in agarose culture have also been reported. A marked increase in organization of cytoskeletal elements (actin microfilaments, microtubules, and vimentin intermediate filaments) was observed over culture time for both superficial and deep zone cells. Additionally, deep zone cells were measured to have more organized cytoskeletal components than the superficial population. [166] An interesting study published in 1994 reported the self-organization of two distinct chondrocytes subpopulations as a result of a homogenous population cultured for 8 months in alginate beads. The authors report a stable chondrocytes phenotype and a layer 1-3 cells thick on the surface of the beads which were flattened, elongated, and sparse in matrix production. The second cell population, present throughout the rest of the bead, was larger, rounded, and surrounded by a matrix rich in proteoglycans and collagen. [52]

Scaffold-supported three dimensional culture aids in retention of phenotype and in differences among zonal chondrocytes populations. It is likely that dynamic culture systems which help to further mimic the three-dimensional environment may provide additional benefits. There is little data on subpopulation phenotype retention in dynamic cultures, however one recent study helps to shed light on the potential of such systems. Three dimensional fibrin-polyurethane scaffolds were seeded with full thickness and zonal chondrocytes populations and tested in a bioreactor that approximates kinematics and surface motion of joints. One hour of surface motion with cyclic compression was delivered twice a day for 3 days. Gene expression of hyaluronan sythases 1 and 2 (HAS1 and HAS2) and PRG4 as well as protein production of hyaluronic acid and PRG4 were tested and compared to both initial levels and static three-dimensional culture values.

HAS2 is thought to be responsible for production of hyaluronic acid in chondrocytes. Initially, PRG4 expression was elevated in superficial cells and HAS2 was elevated in middle and deep zone cells. Static three dimensional culture resulted in increased PRG4 expression at the surface of deep and full thickness chondrocytes scaffolds as well as increased HAS2 expression in superficial constructs. Dynamic culture increased PRG4 expression in superficial zone constructs and further increased expression in the top sections of deep and full thickness scaffolds. Interestingly, the PRG4 expression was highest in the top section all constructs compared with the bottom sections. Dynamic culture also increased HAS2 expression in all top sections of scaffolds, with the highest expression in the top section of superficial constructs. The increases in the gene expression in the top sections of the scaffold may be attributed to oxygen levels, access to nutrients, and proximity to loading force. Release studies indicated increases in hyaluronic acid in all groups, and no measureable amount of PRG4 were found in media from deep zone cells. [116] Dynamic culture clearly resulted in increased matrix production across populations, and demonstrated variations in response based on both population and scaffold location.

Table 3.2. Summary of scaffold-supported zonal chondrocyte culture including zone definition, species and age of cell population, culture time, biomaterial used, and key findings

Scaffold-Based Culture of Zonal Chondrocyte Populations.

Ref	Zone Definition	Species, Age	Culture Time, Biomaterial	Key Results Observed
[164]	*Superficial zone:* top 10% of tissue *Deep zone:* bottom 10-15% of tissue	Bovine, 5-8 months	3 weeks, PEG + HA, PEG + t1c, PEG + chondroitin sulfate (CS), PEGDA	*PEG + CS:* highest gene expression for matrix molecules and matrix accumulation in both cell groups *PEG + CS, PEG + t1c:* highest matrix accumulation in deep cells *PEG + CS, PEG + HA:* highest matrix accumulation in superficial cells *Overall:* Deep cells accumulate more matrix than superficial
[166]	*Superficial zone:* top 15% of tissue *Deep zone:* remaining tissue	Bovine, 18 months	21 days, agarose	Deep zone cells show greater organization of cytoskeletal components than superficial cells (actin filaments, microtubules, vimentin intermediate filaments)
[11, 12]	*Superficial zone*: 20-40 μm *Middle zone:* half of remaining tissue *Deep zone:* remaining tissue	Bovine, 15 weeks and 18-20 months	12 days, agarose	Deep zone cell populations produce most proteoglycans, higher ratio of aggregating : non-aggregating proteoglycans, superficial cells produce proteoglycans which are quickly degraded

[165]	*Superficial zone:* top 200 μm *Deep zone:* bottom 10% of tissue *Middle zone:* remaining tissue	Equine, 7-11 years	Alginate for 28 days, monolayer for 10 days followed by alginate for 28 days	*Initially:* faint clusterin staining in superficial populations, none in other populations *Monolayer expansion:* All differences disappear, no clusterin anywhere Re-differentiation in alginate: Clusterin stain reappears, mostly in superficial cells
[136]	*Superficial zone:* top 250 μm *Middle zone*: 500-1000 μm *Deep zone:* 1250 to 1750 μm	Bovine, 1-3 weeks old and 1-2 years old	9 days, devitalized cartilage substrate	Superficial cells excrete significantly more PRG4 than other populations, this is stimulated by ascorbic acid
[102]	*Superficial zone:* top 200 μm *Deep zone:* bottom 20% of tissue	Bovine, 5-8 week	6 weeks, PEODA bilayers and homogenous controls	*Bilayers:* Deep zone cells produce more matrix than superficial, deep zone cells in bilayer produce more matrix than controls, bilayers demonstrate greater shear and compressive strengths than controls
[15]	*Superficial zone:* top 10% of tissue *Middle zone*: middle 10% of tissue *Deep zone:* bottom 10% of tissue	Bovine, 5-8 weeks	3 weeks, PEDGA bilayer and homogenous controls	*Bilayers:* Show similar histological findings to native tissue: cell morphology and increases in proteoglycans and t2c with depth
[61]	*Superficial zone:* top 10% of tissue *Middle/Deep zone:* discarded bottom 15% of tissue and used bottom 50% of remainder	Bovine, calf	42 days, single layer and bilayer agarose of 2 and 3 %	*Bilayer constructs:* matrix production and zonal markers increase in both cell populations when layered next to the other. Bilayers show depth dependent mechanical properties similar to that of native tissue

3.5 Mechanical Stimulation

Additional studies have attempted to classify the distinct responses of subpopulations to mechanical stimulation. Generally, dynamic compressive strains appear to stimulate GAG production in middle/deep zone cells while dynamic tensile strains stimulate superficial zone cells. Bovine chondrocytes seeded in agarose and tested under static compressive strain (15%) and dynamic compressive strain (0.3, 1, 3 Hz at 15% strain) produced varying results based on subpopulation. Initially, deep zone cells underwent greater deformation, but after the 72 hours of testing this was reversed due to matrix accumulation. Deep zone cells produced significantly more GAG at all time points, and GAG production in these cells was unaffected by static loading or dynamic loading at 3 Hz. However, loading at 0.3 Hz reduced GAG accumulation and loading at 1 Hz stimulated GAG production. Production of GAG by superficial cells was inhibited by all loading regimes and in general cell proliferation was stimulated by dynamic strain and reduced by static strain. [62] Another investigation using the same testing parameters reported similar results; GAG production by deep zone cells was greater than superficial cells, and dynamic strain at 1 Hz significantly stimulated GAG production in deep zone cells. [167] A study using bovine subpopulations seeded in a fibrin hydrogel demonstrated that oscillatory tensile loading (1 Hz, 5% strain) stimulated proteoglycan synthesis in superficial cells only. Furthermore, proteins secreted by the deep zone became altered to more closely resemble the molecular characteristics of proteins present in the superficial zone. [114] These results are intuitive as the superficial zone typically resists higher tensile loads than the deeper cartilage zones. Therefore, middle and deep zone cells may be stimulated by experienced compressive strain, and

superficial zone cells may be stimulated by experienced tensile strain. In general, dynamic loading appears simulative and static loading inhibitory. Further studies which investigate the effects of long-term loading and bioreactor culture will provide more accurate picture of the importance of mechanical loading for long-term phenotype retention. Table 3.3 provides an overview of mechanical stimulation studies on chondrocyte subpopulations.

Table 3.3. Summary of mechanical stimulation studies on zonal chondrocytes including zone definition, species and age of cell population, biomaterial used, culture time, load delivered, and key findings.

Mechanical Stimulation of Zonal Chondrocyte Populations

Ref	Zone Definition	Species, Age, Biomaterial	Culture Time, Mech Load	Key Results Observed
[114]	*Superficial zone:* Top 200 μm of tissue *Middle zone:* 500-1000 μm of tissue *Deep zone:* 1250 – 2000 μm of tissue	Bovine, 2-4 weeks, fibrin hydrogel	7 days preculture, 3 days tensile loading: 1Hz at 5% strain amplitude for 12 hrs followed by 12 hr recovery	Tensile loading stimulated proteoglycan synthesis in superficial cells only, proteins excreted by deep cells after loading were altered in molecular structure to resemble those of the superficial zone
[62, 167]	*Superficial zone:* top 15-20% of tissue *Deep zone:* remaining tissue	Bovine, 18 months, agarose	72 hours, static and dynamic compressive loading: 0.3, 1, 3 Hz at 15% strain amplitude	*Initially:* deep zone cells deform more than superficial cells. *After loading*: deep zone cells deform less than superficial zone cells due to matrix accumulation, deep zone cells have higher proliferation and matrix accumulation at all time points than superficial, 1 Hz stimulates GAG production in deep cells, inhibits superficial GAG production but stimulates superficial proliferation
[116]	*Superficial zone*: top 10-20% of tissue *Deep zone:* bottom third of remaining tissue	Bovine, 10 months, polyurethane	8 days preculture, 3 days surface motion plus cyclic compressive loading: 2x a day for 1 hr for 3 days: 0.1 Hz	*Stimulation:* Increases PRG4 gene expression in superficial constructs and top sections of deep constructs, increases gene expression for HAS1 and HAS2 in all groups, more pronounced at top surface of constructs, HA synthesis increased in all groups by loading

	at 10-20% strain amplitude

3.6 Layered Culture Systems

An approach that aims to mimic the *in vivo* environment is a layered cell culture construct. These systems attempt to recreate more realistic environments by culturing chondrocytes in layers corresponding to their native arrangement. While only a handful of such systems have attempted to classify the behavior of layered chondrocyte subpopulations, results indicate that cell activity is significantly influenced by the presence of another cell population.

An agarose system has demonstrated varying mechanical properties and cellular activity between construct layers. Constructs seeded with a mixed chondrocyte population containing a layer of 2 weight percent agarose atop of a layer of 3 weight percent agarose contained two regions with distinct mechanical properties. Initially, the 3% agarose region displayed stiffer compressive properties; however after 28 days in culture this difference become less noticeable and the scaffold properties became more homogenous. [60] When this system was used to layer chondrocyte subpopulations modulations in cell activity depending both on weight percent agarose and the surrounding cell population were observed. After 42 days in culture it was found that superficial zone cells produced the highest levels of collagen and GAGs with higher agarose concentrations and when layered next to a population of middle/deep zone cells. Similarly, middle/deep zone cells produced more GAGs and had higher proliferation rates when layered next to a superficial zone population. Furthermore, bilayered constructs seeded with a superficial zone cell population and a middle/deep zone cell

population displayed depth-dependant compressive properties similar to those of native tissue. [61]

Culture systems based on photopolymerizable poly(ethylene oxide) diacrylate (PEODA) and poly(ethylene glycol) (PEGDA) have also been used to culture layers of chondrocyte subpopulations. In PEODA hydrogels it was reported that culturing deep zone cells next to a layer of superficial zone cells lowered their cell proliferation rate but increased production of matrix components. [102] Additionally, a PEG-based system which layered superficial, middle, and deep zone cells demonstrated histological staining similar to that of native tissue after 3 weeks in culture. Cells in the upper layer remained small and flattened, while those in the middle and deep layers were more rounded and larger. Furthermore, the upper layer contained little matrix, and collagen and proteoglycan staining increased with construct depth. [15] A layered system based on the popular hydrogel alginate has also been reported. This system has demonstrated mechanical properties similar to those of non-layered constructs, and production of matrix components over several weeks of culture with a mixed chondrocyte population. [57, 168]

There are fairly limited results for layered culture systems. The few existing models demonstrate increased matrix production, especially in middle/deep zone cells, when cells are cultured in a zonally organized fashion. Layered hydrogels show much potential for *in vitro* production of tissue with depth-dependent mechanical properties which are on the same scale of native tissue. Current results seem promising for creating zonally organized tissue *in vitro* and it is likely that a zonally organized culture method will aid in subpopulation retention.

3.7 Growth Factors and Cytokines

3.7.1 Growth Factors

Major growth factors used to stimulate *in vitro* matrix production in chondrocytes include insulin-like growth factor 1 (IGF-1), transforming growth factor-β1 (TFG-β1), basic fibroblast growth factor (bFGF), and bone morphogenetic proteins (BMPs). While these growth factors are generally understood to stimulate synthesis of ECM proteins, the mechanisms behind their varying effects on subpopulations are not yet fully understood. Identification of optimal growth factor delivery for each zonal population will further aid in phenotype retention *in vitro* and zonal engineering efforts. Table 3.4 provides a summary of key finding and experimental parameters for growth factors delivered to zonal cell populations.

Several studies have shed light on the effects of growth factors delivered to chondrocyte subpopulations. Delivery IGF-1 (10, 100 ng/mL), bFGF (10, 100 ng/mL), and TGF-β1 (5, 30 ng/mL) over three weeks resulted in distinct effects on superficial versus middle/deep zone cells. All concentrations of IGF-1 increased gene expression for aggrecan and type II collagen in the middle/deep zone populations, while all concentrations of TGF-β1 decreased expression in the same cells. The lower concentration of bFGF was found to increase aggrecan expression in the growth zone, while the higher concentration increased type II collagen expression. Superficial zone cells displayed lower expression for matrix proteins in all conditions, and were found to increase proteoglycan 4 expression for both concentrations of TGF-β1 and 100ng/mL IGF-1. [96] Results indicate that IGF-1 may be optimal for middle and deep zone cells to promote matrix production and reduce type I collagen, and TGF-β1 may be important for

superficial cells to aid in production of proteoglycan 4 and matrix components. Delivery of IGF-1 increases expression of aggrecan and type II collagen, but also decreases expression of tissue inhibitor of metalloproteinases-1(TIMP-1). This suggests that IGF-1 stimulates chondrocytes to produce elevated levels of matrix, but does not protect against the activity of MMPs which degrade existing ECM. [169] Additional results have demonstrated that TFG-β1 and IGF-1 stimulate production of the proteoglycan 4 in superficial cells, while interleukin-1β (IL-1β) and IL-1α both act to inhibit production. [170, 171]

Growth factors have also been shown to effect the cytoskeletal organization and mechanical properties of chondrocyte subpopulations. Unconfined creep compression testing of single chondrocytes showed that delivery of 5 ng/mL TGF-β1 or 100 ng/mL IGF-1 over 18 hours increased stiffness in both superficial and middle/deep cells. Furthermore, superficial cells showed higher stiffness values for both control and experimental groups. Similarly, staining for cytoskeletal F-actin was stronger in all groups with growth factor delivery. [172]

Bone morphogenetic proteins (BMPs) stimulate matrix production in chondrocytes, [173-175] however their effects on zonal cell populations are less documented. A recent study reported higher endogenous BMP activation in the deep zone cells versus superficial zone cells. Furthermore, adenovirus-mediated delivery of both BMP 2 and 7 resulted in increased matrix accumulation in superficial cell culture pellets with no change in cell diameter. Conversely, deep zone cells in culture pellets experienced an increase in diameter and no increase in matrix production. The BMP antagonist noggin decreased both matrix accumulation and cell diameter in both

superficial and deep zone cells. [176] These results indicate that BMPs may more appropriate for delivery to superficial zone cell population.

Trends in growth factor delivery in two-dimensional culture indicate TGF-β1 and BMPs may by influential in stimulating superficial zone chondrocytes while IGF-1 may be important for middle/deep zone chondrocytes. While these results provide much insight the majority of these models have examined chondrocytes in monolayer. It has been well-documented that chondrocytes and zonal phenotype are unstable in such environments. Several studies report that even with growth factor delivery morphological differences among zonal populations in two dimensional culture are not maintained. [96, 172] Further studies which utilize three dimensional culture will provide a more accurate picture of growth factor effects on zonal chondrocytes.

Table 3.4. Summary of growth factor delivery to zonal chondrocytes including zone definition, species and age of cell population, culture time, delivered growth factor, and key findings.

Growth Factor Delivery to Zonal Chondrocyte Populations

Ref	Zone Definition	Species, Age, Culture Method	Culture Time, Factor	Key Results Observed
[172]	*Superficial zone:* Top 200 μm of tissue *Growth zone:* remaining tissue	Bovine, 18 months, monolayer	3 and 18 hrs, TGF-β1 (5ng/mL), IGF-1 (100ng/mL), or both	Single cell testing showed all growth factor exposed groups were stiffer, superficial cells were stiffer than growth in all groups, more intense staining for actin filaments in growth factor groups, all cells exposed to growth factors were more rounded and less spread, no morphological differences between cell populations
[96]	*Superficial zone:* top 10-20% of tissue *Growth zone:* remaining tissue	Goat, 8 months, monolayer	8 days, IGF-1 (10,100ng/mL), or bFGF (10,100ng/mL), or TGF-β1 (5, 30ng/mL)	Superficial groups lower gene expression of aggrecan, t1c, and t2c in all groups and lower production of GAG. Both IGF-1 concentrations increase aggrecan and t2c gene expression in growth zone, both TGF-β1 concentrations decrease same expression. Lower concentration of bFGF increases aggrecan expression in growth zone and higher concentration increases t2c expression. Both TGF-β1 concentrations and higher IGF-1 concentration increase SZP in superficial cells.
[170]	*Superficial zone*: top 100 μm of tissue	Bovine, 3 months, monolayer	24 hrs and 3 days, TGF-β1	TGF-β1 increased production and gene expression of SZP, IL-1

			(10ng/mL), or IL-1β (10ng/mL)	decreased production and gene expression of SZP
[171]	*Superficial zone*: top 20 -40 μm of tissue *Deep zone:* remaining tissue	Bovine, 7 days and 18-24 months, human adult, agarose	10 days, IL-1α (10ng/mL), TGF-β (2ng/mL), IGF-1 (50ng/mL)	Elevated gene expression of SZP in all superficial zone groups compared to deep. IL-1 decreases SZP synthesis in superficial cells, TGF-β and IGF-1 increase SZP synthesis in superficial cells
[176]	*Superficial zone:* top 100 μm of tissue *Deep zone:* near calcified region	Bovine, 18-36 months, scaffold-free culture pellets	7 days, with and without adenovirus-mediated over expression of BMP-2,7 and BMP antagonist, Noggin	*BMP:* superficial cells increased matrix accumulation without increasing cell diameter, deep cell increased cell diameter without increasing matrix accumulation *Noggin:* decreased matrix accumulation and cell diameters in both groups

3.7.2 Catabolic Cytokines

In damaged or diseased cartilage elevated levels of cytokines such as interleukins (ILs) and tumor necrosis factors (TNFs) are often present. These cytokines have been shown to increase MMPs, cell death, and production of nitric oxide (NO). NO is involved in inhibition of proteoglycan synthesis and further contributes to the diseased state of the tissue. The production of cytokine-induced NO and subsequent metabolic inhibitions take place in a zone-dependent manner. Superficial zone cells produce significantly higher amounts of nitric oxide in response to IL-1 delivery than deep zone cells. Higher levels of NO production correspond to more severe inhibition of proteoglycan synthesis. Conversely, NO production can be inhibited by dynamic compressive loading regimes.

Cultures of superficial zone cells produced 2-3 times as much NO in response to IL-1 delivery. [177, 178] Delivery of TNFα and bacterial lipopolysaccharide (LPS) also stimulated superficial zone cells to produce more NO than deep zone cells. [179] Superficial zone cultures respond to lower concentrations of delivered IL-1 in terms of inhibition of aggrecan synthesis. One study indentified the minimum concentrations of delivered IL-1 for a 50% reduction in aggrecan synthesis to be 0.7 ng/mL and 4.5 ng/mL for superficial and deep zone cultures respectively. [178] Superficial zone cells were also less responsive to the therapeutic effects of the IL-1 receptor antagonist protein (IRAP), showed twice as many high affinity IL-1 binding sites, and produce elevated levels of MMPs in response to delivered cytokines. [180, 181] However, dynamic compression loading can inhibit NO release in IL-1 stimulated superficial zone cells and in unstimulated mixed zone cells. [182, 183]

Together these studies once again highlight the distinct metabolic activities of zonal chondrocyte populations. They demonstrate the elevated susceptibility of superficial zone cells to cytokines present in tissue, and identify dynamic loading as a possible mechanism for NO inhibition.

3.8 Progenitor Cells

A population of cells with characteristics of mesenchymal progenitor cells (MPC) has recently been identified in osteoarthritic human articular cartilage. Fluorescence-activated cell sorting (FACS) was used to identify a cell population of 2-12% MPC following harvest and 24-48% MPC after several passages in monolayer. These MPCs demonstrated chondrogenic, osteogenic, and adipogenic differentiation potential.[184]

Further studies using FACS have demonstrated that a population of progenitor cells can be isolated to the superficial zone only. These progenitor cells show a high affinity for fibronectin, colony forming, and expression of the Notch-1 gene. [145] Furthermore, stimulation of the progenitor population in the superficial zone with BMP-7 enhanced SZP expression, while stimulation with TGF-β1 upregulated type II collagen expression. [185]

3.9 Conclusion

Recreating the complex organization of articular cartilage following disease or injury is optimal for reestablishing tissue functionality. Subpopulations of chondrocytes which remain phenotypically stable may be able to produce a zonally organized matrix *in vivo*. Zonal tissue engineering has not yet achieved this goal, or become clinically practical. For this to happen, the differences among cell populations should be clearly identified, understood, and engineered to produce functional tissue. To aid in this process this review has classified the known differences among chondrocyte subpopulations and their responses to *in vitro* culture, mechanical stimulation, and growth factor delivery. Explant studies identify PRG4, Del1, clusterin, CILP, and COMP as zonal protein makers, as well as a zonal distribution in Notch-receptors and their binding ligands. Single cell analysis show superficial cells to have higher moduli and apparent viscosities than their counterparts from lower zones, while the compressive modulus of the tissue increases with tissue depth and GAG content. Further studies indicate that superficial zone cells may communicate in pairs and exists in groups of different sizes.

Monolayer culture of subpopulations highlights distinct metabolic activates between cell populations, but also shows a trend towards homogenization and loss of phenotype. Such culture studies show middle/deep zone cells producing significantly more ECM components and tissue with a higher compressive modulus than that of the superficial cells. However, a large number of reports show loss of differences and convergence to a non-chondrogenic phenotype following several passages in two-dimensional culture. Studies in three-dimensional culture provide a more native environment and help to retain both chondrocytes and subpopulation phenotypes. Encapsulation in alginate, agarose, devitalized cartilage constructs, PEG, and fibrin-polyurethane all show retention of zonal differences and increased ECM molecule production in middle/deep zone cells.

Studies which attempt to further mimic the native environment involve mechanical loading and layering of zonal populations. Results from mechanical loading indicate that dynamic compressive loading helps to stimulate middle/deep zone cells, while dynamic tensile loading stimulates superficial zone cells. Layered systems clearly demonstrate increased metabolic activity in zonally organized cells, as well as creation of tissue which histology indicates is zonally organized.

While some three-dimensional culture systems have achieved mechanical properties approaching native values, creating engineered cartilage with the desired mechanical properties remains a challenge. As the functionally of the tissue is greatly dependent on these properties, this issue is critical for load-bearing capacity and success of the engineered cartilage.

Growth factors such as IGF-1 may be important for stimulating matrix production in middle/deep zone cells and TGF-β1 and BMPS may serve to stimulate superficial zone cells. Superficial zone cells appear more susceptible to the catabolic influences of cytokines, and the superficial zone is likely to contain a population of mesenchymal progenitor cells.

3.10 Future Directions

While all these results are important and demonstrate the distinct activity between chondrocytes populations, tissue engineers have still not been able to manipulate these cells to produce functional tissues or clinically relevant solutions. For this to happen clear methods for retaining and/or creating phenotypically stable zonal cell populations must be established. Filling in existing knowledge gaps will facilitate this process. For example, identification of an optimal culture method is needed. While three-dimensional culture has proven beneficial it is likely that dynamic culture systems such as bioreactors may help further to retain zonal phenotype. There are very limited studies investigating this area, which could potentially be of great use. Mechanical loading studies also need further investigation. While it has been demonstrated that compressive loading benefits middle/deep zone cells and tensile loading stimulates superficial cells, perhaps there is a combination of these two loading regimes which can stimulate both groups. As the tissue experiences both compressive and tensile loading *in vivo*, such systems may help retain or organize zonal tissue *in vitro*. The role of mechanical loading (*in vivo* and *in vitro*) in both maintaining zonal phenotype and production of zonally organized tissue is yet to be

fully established. Understanding the role mechanical loading plays in cell activity and tissue formation will aid in identification of an optimal tissue engineering strategy.

Layered cell systems show much potential, but provide many questions. Current studies demonstrate increased productivity of layered cells, but do not clearly demonstrate that each population's phenotype is maintained. If this can be established, then a practical and simple method for *in vitro* culture and phenotype retention could be possible. Optimal growth factor delivery for subpopulation in three-dimensional culture also needs to be defined. While several growth factors have shown positive results in subpopulation maintenance, few of these studies have been conducted in three-dimensions, and optimal growth factors and delivery mechanisms per zone are yet to be established. The progenitor cells located within articular cartilage hold potential. Preliminary studies have indicated that it may be possibly to zonally-differentiate these cells, but no clear trends have been established. If these, or other progenitor cell populations, could be used to create populations of zonal chondrocytes *in vitro*, then they could be used for implantation to produce zonally organized tissue *in vivo*.

As mentioned, establishing culture methods to retain subpopulation differences is important for both studying these cells and potentially for creating zonally organized tissue. As superficial zone cells appear the least robust in both phenotype retention and matrix production, the retention of this cell type may be a challenge. Furthermore, for use of zonal cell populations in cartilage engineering strategies a clinically relevant method of maintaining or differentiating cell subpopulations should be established. Future studies utilizing progenitor cell populations could address this issue.

Yet to be investigated are studies which directly compare uniform scaffolds to those with zonal design. *In vivo* loading may have the potential to zonally organize a homogenous scaffold. Studies which compare organized scaffold and homogenous scaffold in the native loading environment may help to define the importance scaffolds designed with zonal organization.

4 Gene Expression of Alginate Embedded Chondrocyte Subpopulations and their Response to Exogenous IGF-1 Delivery*

4.1 Introduction

Articular cartilage is an alymphatic and avascular tissue whose extracellular matrix is maintained by the resident chondrocyte cell population. Nutrition and waste exchange occur through diffusion, lending to the tissue's limited ability to repair. [1, 2, 6] While initial research strategies attempted to model cartilage as homogenous, more recent work recognizes cartilage as a complex tissue comprised of three major zones with distinct extracellular matrix organization and cellular phenotype. [98, 102, 161, 186] Regeneration of healthy articular cartilage can only be complete with formation of all cartilage zones. In order for proper restoration of zonal structure to occur phenotypical differences between chondrocyte populations must be fully understood. If the response of chondrocyte subpopulations to growth factors and external stimuli can be classified, then potentially they can be engineered to recreate native cartilage tissue structure.

The three zones of articular cartilage are the superficial, middle, and deep zones. Below the deep zone is the tidemark – where the tissue starts to become calcified and eventually turns into subchondral bone. The superficial zone is approximately the top 10 percent of articular cartilage. [2, 9, 10] Here the cells are smaller than the chondrocytes of other zones, thin, and disc shaped. The cells, along with the extracellular matrix

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collagen fibers are oriented in bundles parallel to the articulating surface. The orientation of the collagen fibers give the superficial zone the highest tensile stiffness and strength, a property that decreases with tissue depth. [125, 126] The superficial cells secrete relatively low levels of extracellular matrix proteoglycans, [11, 12] but are the only chondrocyte population that produce proteoglycan 4; a lubricant secreted into the synovial fluid of articulating joints. [14] The superficial zone is also the only region of the tissue thought to contain a population of mesenchymal progenitor cells (MPC). [145, 185]

The middle, or transitional zone, of articular cartilage comprises approximately 60-70 percent of the tissue and contains larger and rounder chondrocytes. [2, 9, 10] The chondrocytes and the collagen fibers are randomly oriented, and sometimes found in small groups or clusters. Middle zone chondrocytes produce significantly more proteoglycans than those of the superficial zone, as well as increased levels of type II collagen. Higher concentrations of proteoglycans give the middle zone a higher compressive modulus than that of the superficial zone. [12, 15, 129]

The deep zone is approximately the bottom 10-15 percent of tissue located before the tidemark of the calcified region. [2, 9, 10] Deep zone chondrocytes also produce elevated levels of both proteoglycan and type II collagen compared to those of the superficial zone and are oval in shape. Consequently, the compressive modulus is highest here. The cells along with the type II collagen fibers are oriented in vertical columns which are perpendicular to the articulating surface. [12]

4.1.1 Growth Factors and IGF-1

Articular chondrocytes produce and secrete growth factors which modulate cell activity. Many cartilage engineering efforts have focused on delivery of growth factors to chondrocyte populations to stimulate extracellular matrix component production. The most widely investigated anabolic growth factors for cartilage include; insulin-like growth factor-1 (IGF-1), transforming growth factor –β (TGF-β), and bone morphogenetic proteins (BMPs).

IFG-1 was chosen for this study based on its importance in tissue homeostasis, and its well-documented effects on chondrocytes found both by our laboratory in the literature. [5, 56, 187, 188] IGFs are small, soluble proteins found in tissues such placenta, heart, lung, bone, and cartilage. IGF-1 belongs to a family of peptide hormones that has a single polypeptide structure similar to insulin. IGF-1 has been shown in enhance chondrocyte proliferation and differentiation as well as stimulate production of extracellular matrix components such as proteoglycans and type II collagen. [7, 188] Extracellulary, IFG-1 remains stable through interaction with one of its binding proteins. There are six known IGF-1 binding proteins (IGF-BPs), of which IGF-BP3 has the highest binding affinity. For cellular interaction IGF-1 must dissociate from its binding protein and bind to the IGF-1 receptor found on the cell surface. Surface receptor binding initiates several intracellular pathways including the PI3K and MAPK pathways. The PI3K pathway has been implicated in the production of both proteoglycans and type II collagen. [5]

While the mechanical and biochemical response of heterogeneous chondrocyte populations to IGF-1 delivery has been reported, [56, 97] there is limited information on

the responses of chondrocyte sub-populations to IGF-1 delivery. Previous sub-population studies report the response to growth factors of zonal chondrocytes in monolayer. These works show distinct responses based on the chondrocytes' original location within the tissue. Most notably, chondrocytes isolated from the middle and deep zones show increases in type II collagen and proteoglycan expression following IGF-1 delivery. However, IGF-1 delivery does not appear to have the same stimulatory effect on chondrocytes isolated from the superficial zone. [96, 169] Furthermore, despite IGF-1 delivery type I collagen expression continues to increase for all zones with time. [96] Additionally, IGF-1 has been shown to increase proteoglycan 4 accumulation in chondrocytes isolated from the superficial zone. [14] These results indicate significant differences in response of chondrocyte sub-populations to delivered growth factors. As chondrocytes are known to maintain their phenotype better in three-dimensional cultures than monolayer, [189] establishing sub-population behavior in three dimensions is the next logical step in this work. To this end, we have chosen an alginate bead hydrogel model to culture primary bovine chondrocyte sub-populations in three dimensions.

Encapsulation in alginate allows chondrocytes to maintain their spherical morphology, and has been shown to support chondrocyte proliferation and differentiation. [190-192] Alginate beads have been used for chondrocytes culture both in our laboratory's work [54, 56] and many other studies, [192-194] and results clearly demonstrate a favorable environment for chondrocytes. Previous studies in our laboratory have determined an optimum alginate concentration of 2.0% w/v and an optimum cell seeding density of 100,000 cells per bead. [54]

To the best of our knowledge, this work is the first to classify the zonal distribution in gene expression of endogenous IGF-1 and IGF-BP3 both with and without exogenous IGF-1 delivery. Furthermore, it is the first known study to investigate the effects if IGF-1 on ECM molecule gene expression of zonal chondrocytes encapsulated in three-dimensions. Through classification of the zonal effects of delivered growth factors the ideal culture method for retaining zonal phenotypes can be established. Culture of stable chondrocyte subpopulations will be a step forward engineering zonally organized articular cartilage.

4.2 Materials and Methods

4.2.1 Superficial, Middle, and Deep Zone Chondrocyte Isolation

Zonal chondrocyte sub-populations were isolated using a procedure similar to our lab's protocol for isolating full-thickness chondrocytes. [54, 56] Specifically, 4 mm diameter cartilage plugs were harvested from the femoral condyles of 20 week old calves using a Sklar Tru-Punch disposable biopsy punch (Sklar Instruments, West Chester, PA). The cartilage plugs ranged from 3 - 6 mm in depth and contained all cartilage tissue up to the subchondral bone. The top 10% (0.3 - 0.6 mm) was removed using a razor blade and taken as the superficial zone. The middle 70% of the plug (2.1 – 4.2 mm) was removed using a razor blade and labeled as the middle zone. Finally, the bottom 20% (0.6 – 1.2 mm) was labeled as the deep zone. Once the tissue zones were separated they were minced into smaller pieces and rinsed three times in Dulbecco's Modified Eagle Medium/Nutrient Mixtures F-12 Ham (DMEM/F12) media (Gibco/Invitrogen, Carlsbad, CA) with the following additives: 50 μg/mL ascorbic-2-phosphate (Sigma-Aldrich, St.

Louis, MO), 1 mg/mL bovine serum albumin (Sigma-Aldrich), 1.2 mg/mL sodium bicarbonate (Sigma-Aldrich), 0.1% penicillin/streptomycin (Gibco/Invitrogen), and 0.1% sodium pyruvate (Gibco/Invitrogen). The cells of each zone were isolated by digesting the cartilage in 0.2 % collagenase P (Roche, Basel Switzerland) for 24 hours at 37°C and 5% CO_2, filtering through a 40 µm mesh, and washing again in supplemented DMEM/F12 to remove any undigested tissue. The final cell solution was suspended in supplemented DMEM/F12 with 10% fetal bovine serum, and cell counts were performed using a hemacytometer (Hausser Scientific, Horsham, PA).

4.2.2 Viable Cell Density Per Cartilage Zone

Following harvest, cell counts were taken of each chondrocyte sub-population (superficial, middle, and deep), and the cellular density for each zone was determined using the dimensions of the explanted cartilage plugs. Trypan blue stain 0.4% (Gibco/Invitrogen) was used to determine viable cells. Viable cells appeared round and clear while non-viable cells absorbed the dye and appeared blue. Cell count per volume of explanted tissue was determined for each cartilage zone; average cell densities and associated standard deviations are reported (n=3).

4.2.3 Histological Preparation

Explants of full thickness cartilages were taken in 4mm diameter cartilage plugs as described above. Following isolation the samples were fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) and then decalcified in a solution of 0.24 M ethylenediamine tetraacetic acid (EDTA) tetrasodium salt (Fisher Scientific, Pittsburgh,

PA) and 0.24 M EDTA disodium salt (Fisher Scientific) for 10 days at 4°C. The EDTA solution served to remove ions such as calcium that might have been in the explanted samples. Following decalcification the explants were placed in histological cassettes and dehydrated through a series of ethanol washes (40%, 50%, 60%, 70%, 95%, and 100% x 3) for 15 minutes each followed by two 15 minute washes in Citrisolv (Fisher Scientific). The samples were embedded in paraffin (Paraplat X-tra, Fisher Scientific) and cut into 15 µm sections and mounted on a glass slide (Superfrost, Fisher Scientific).

4.2.4 Histochemical Staining

Samples were dried at 64°C for two hours, deparaffinized using Citrisolv in two washes for 3 minutes each, and rehydrated in 100% and 95% ethanol for one minute each. The samples were then rinsed in distilled water and stained using either Masson's Trichrome, Alcian blue, or Safranin O, Fast Green, and Weigert's iron hematoxylin staining solutions. (Poly Scientific, Bay Shore, NY). All samples were viewed under an Axiovert 40CFL light optical microscope (Zeiss, Thornwood, NY) and images were captured using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI) imaging software.

4.2.5 Immunohistochemistry

Samples were dried at 64°C for one hour, deparaffinized using Citrisolv in two washes for 5 minutes each, and rehydrated in 100%, 95%, and 70% ethanol. The samples were then rinsed in tap water and deionized water. Samples were then incubated with PEROXI*DAZED1* (Biocare, Concord, CA), an endogenous peroxidase blocker, and

BackgroundSNIPER1 (Biocare), a blocking reagent. Samples were then stained with antibodies to detect either IGF-1 or type II collagen. Primary antibodies used were anti-hIGF-1 (goat IgG antibody, AF-2910NA; R&D Systems) and anti-type II collagen (rabbit polyclonal antibody, ab300; Abcam, Cambridge, MA). Both antibodies were diluted 200x. The HISTOSTAIN®-SP kit (Zymed, San Francisco, CA) was used to visualize type II collagen presence by using horseradish peroxidase (HRP)-streptavidin-biotin system. The complex formation was then detected by a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. For IGF-1 visualization, a HRP-conjugated secondary antibody (goat IgG antibody, ab6885; Abcam) was used a 500x dilution and detected by a histochemical substrate (Liquid DAB Substrate Kit, Zymed). All samples were counterstained with hematoxylin, dehydrated in 95% and 100% ethanol twice for 1 minute, cleared in Citrisolv, and covered with a glass coverslip using Permount (Fisher Scientific).

4.2.6 Chondrocyte Encapsulation in Alginate and Culture

Chondrocyte encapsulation and culture techniques were done using protocols previously established by our lab. [54, 56] Briefly, 2.0% w/v alginate solution was prepared by mixing and heating alginic acid sodium salt from brown algae (Sigma-Aldrich), 0.15 M sodium chloride (Sigma-Aldrich), and 0.025 M HEPES, sodium salt (J.T. Baker, Phillipsburg, NJ) into deionized water (pH 7.4), and then sterile filtered, using a 0.22μm sterile filter. The alginate solution was mixed with the desired chondrocyte population and injected through a 18-gauge syringe into continuously stirred 0.1 M calcium chloride ($CaCl_2$) (Sigma-Aldrich). The resulting cellular density was

approximately 100,000 cells per bead, and each spherical bead had a diameter of approximately 5mm. The beads were incubated in $CaCl_2$ and supplemented DMEM/F12 for 15 minutes each. Five beads were then transferred into each well of a six-well plate and cultured in supplemented DMEM/F12 media and 10% fetal bovine serum (FBS) (Gibco/Invitrogen) at 37°C for 2 days to stabilize the chondrocytes within the alginate hydrogel. [51] The media was changed on day 3 with supplemented DMEM/F12 and relevant experimental groups received human insulin-like growth factor (IGF-1; R&D Systems, Minneapolis, MN) at a concentration of 100 ng/mL. Media was changed every other day and IGF-1 was delivered daily. At days 1, 4, and 8 chondrocytes were isolated from the alginate beads by addition of 4 mL of 0.1 M EDTA for 25 min at 37°C. The solution was then centrifuged to form a cell pellet, which was resuspended in phosphate-buffered saline and then used for RNA isolation.

4.2.7 RNA Isolation

Following isolation of chondrocytes from alginate beads, RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA was eluted into 30 µL of RNase free water and detected using a NanoDrop ND-1000 Spectophotometer (NanoDrop Technologies, Wilmington, DE). RNA concentrations at 1, 4, and 8 days were approximately 20, 50, and 200 ng/µL respectively.

4.2.8 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

All isolated RNA was reverse transcribed using a cDNA Archive Kit (Applied Biosystems, Foster City, CA), which can convert up to 10µg of RNA to cDNA. The

cDNA was normalized to the lowest concentration (day 1), and then combined with Universal Master Mix (Applied Biosystems) and oligonucleotide primers and Taqman probes (Applied Biosystems) for the genes of interest as well as a control gene. The genes of interest were type I collagen, type II collagen, aggrecan, IGF-1, and IGF-BP3 and the endogenous control gene was glyceraldehyde 3 phosphate dehydrogenase, GAPDH). Table 4.1 shows the sequences for all forward primers, reverse primers, and probes used. The reaction volume was 20 μl, the reaction was performed in technical triplicates, and the final concentration of cDNA per reaction well was approximately 5ng/μl. The reaction was conducted on a 7900HT Fast Real-Time PCR System Prism 7000 sequence detector (Applied Biosystems). The thermal profile followed was 2min at 50°C, 10min at 95°C, 40 cycles of 15s at 95°C, and 1 min at 60°C. Gene expressions were analyzed using the comparative C_t method, with glyceraldehydes 3-phosphate dehydrogenase (GAPDH) used as the endogenous control gene. The superficial zone samples were used as calibrators in all analysis since there metabolic activity is known to be the lowest of the chondrocyte subpopulations, and would serve as a consistent calibrator. [12, 152, 160] Fold changes in gene expression were calculated and are reported as the mean RQ values with associated standard deviations (n=3), in accordance with methods previously described by our laboratory: [55, 195]

$$RQ = 2^{-\Delta\Delta Ct} \text{ , where } \Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,ref}$$

where $\Delta C_{t,sample}$ is the C_t value for the sample normalized to the endogenous control gene, and $\Delta C_{t,ref}$ is the C_t value for the calibrator normalized to the endogenous control gene.

4.2.9 Statistical Analysis

Each experiment was performed at minimum in triplicate. All data was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test to determine statistical differences. A confidence interval of 95% ($\alpha = 0.05$) was used for all analysis and means and standard deviations are shown on each figure.

Table 4.1. Forward primer, reverse primer, and probe sequences used for GAPDH, Type II Collagen, Type I Collagen, Aggrecan, IGF-1, and IGF-BP3 used for qRT-PCR.

Primer and Probe Sequences used for qRT-PCR

Protein		**Sequence**
GAPDH	Forward Primer	TGCCGCCTGGAGAAACC
	Reverse Primer	CGCCTGCTTCACCACCTT
	Probe	CCAAGTATGATGAGATCAA
Type II Collagen	Forward Primer	CGGGCTGAGGGCAACA
	Reverse Primer	CGTGCAGCCATCCTTCAGA
	Probe	CAGGTTCACATATACCG
Type I Collagen	Forward Primer	AGAACCCAGCTCGCACATG
	Reverse Primer	CAGTAGTAACCACTGCTCCATTCTG
	Probe	AGACTTGAGACTCAGCC
Aggrecan	Forward Primer	GGGAGGAGACGACTGCAATC
	Reverse Primer	CCCATTCCGTCTTGTTTTCTG
	Probe	CAGGCTTCACCGTTGAG
IGF-1	Forward Primer	CCCAGACAGGAATCGTGGAT
	Reverse Primer	ACATCTCCAGCCTCCTCAGATC
	Probe	CTGCTTCCGGAGCTG
IGF-BP3	Forward Primer	CGCCTGCGCCCTTACC
	Reverse Primer	TTCTTCCGACTCACTGCCATT
	Probe	CTACCGTCCGCGTCAG

4.3 Results

4.3.1 Chondrocyte Subpopulation Isolation

Both cellular density and histological images confirm isolation of zonal chondrocyte populations. The average cellular density is the highest in the superficial zone, followed by the deep zone, and then the middle zone (Figure 4.1). Histological images show varying cell density, cell size, and extracellular matrix content throughout a sample of full thickness cartilage (Figures 4.2 and 4.3). Figure 4.2 depicts a full-thickness sample stained with Safranin-O, which stains negatively charged proteoglycans orange, and Iron Hematoxylin, which stains the cell nuclei black. Figure 4.3 depicts a full-thickness sample stained with Masson's Trichrome where all tissue elements are stained red, collagen fibers are stained blue, and the cell nuclei are stained black. The cells near the articulating surface are the smallest and have the highest density. The cells grow larger as the distance from the articulating surface increases until the deep zone is reached where the cells appear largest (Figures 4.2 and 4.3). The cells in the superficial zone appear small and oriented along the articulating surface, the middle zone cells appear round and without particular orientation, and the deep zone cells appear slightly elongated and columnar perpendicular to the surface. (Figure 4.2 B, C, and D as well as Figure 4.3 B, C, and D). Staining intensity varies throughout the sample. For both proteoglycan and collagen content the stain is most intense at the base of the sample, followed by the middle region, and finally the superficial layer, which appears the least stained.

4.3.2 Aggrecan mRNA Expression

Figure 4.4 shows aggrecan mRNA expression fold change for superficial, middle, and deep zone cells over the 8 day culture period. Gene expression remains elevated over the 8 days, but decreases marginally by day 8. Middle and deep zone cell expression is significantly higher than superficial zone cells throughout the study, with the exception of the deep zone group which received IGF-1 delivery at days 4 and 8. Addition of IGF-1 does not stimulate cell to express significantly more aggrecan mRNA, and in the deep zone on days 4 and 8 addition of the growth factor reduces gene expression to levels comparable to the superficial zone. The exception is the superficial zone on day 8, whose aggrecan expression is increased significantly by the addition of IGF-1.

4.3.3 Type I Collagen mRNA Expression

Figure 4.5 depicts type I collagen fold change for chondrocytes isolated from the superficial, middle, and deep zones over 8 days. All zones show an increase in expression from days 1 to 8, and expression between zones varies. A general trend of increasing type I collagen expression with time is observed throughout the study. Initially, cells isolated from the superficial zone show significantly higher expression of type I collagen than the middle or deep zone cells. Statistically higher expression of type I collagen by the deep zone cells versus the middle zone cells is also observed at day 1. At day 4 middle zone cells express significantly lower type I collagen, and by day 8 this trend has changed and the superficial cells express significantly lower levels of type I collagen. IGF-1 delivery generally increases type I collagen expression in superficial zone cells, and by day 8 it has also increased expression in the middle zone cells. Deep

zone cells however, express significantly lower levels of type I collagen on both days 4 and 8 when exposed to IGF-1 delivery.

4.3.4 Type II Collagen mRNA Expression

Figure 4.6 shows the gene expression profiles for type II collagen for the zonal chondrocytes over 8 days. A trend of decreasing expression is observed throughout the study, and the superficial zone cell activity is distinct from the middle and deep zone cell activity. Throughout the study middle and deep zone cells have significantly higher expression compared to superficial cells, with the exception of the middle zone group with IGF-1 delivery on day 1. On days 1 and 4 the middle zone control group has the highest expression, and on day 4 this group is significantly higher than all other groups. By day 8 however it is the deep zone group with IGF-1 delivery which statistically has the highest expression. Again, IGF-1 delivery does not appear to significantly stimulate type II collagen expression with the exception of the deep zone on day 8 and the superficial zone on day 1.

4.3.5 IGF-1 mRNA Expression

Figure 4.7 depicts expression of endogenous IGF-1 by zone over the course of the study. A trend of decreasing expression by zone and with time is observed. The changes in expression which occur in the deep zone appear the least severe over the length of the study. IGF-1 delivery generally decreases cell expression of endogenous IGF-1, with the exception of the superficial zone on day 1, the middle zone on day 4, and the deep zone on day 8.

4.3.6 IGF-BP3 mRNA Expression

Figure 4.8 shows the gene expression profiles for IGF-BP3 throughout the study. A trend of decreasing expression with time is observed, as well as lower expression by the superficial zone population. On days 1 and 4 similar profiles are observed; superficial cells display lowest expression and the middle zone control group has the highest expression. By day 8 this pattern has changed and expression increases from superficial to middle to deep zone cells. Delivery of IGF-1 affects different zones in distinct ways and is time-point dependent. At days 1 and 4, there is statistically no differences between superficial groups, the middle zone experimental group is significantly higher than the control group and the deep zone experimental group is significantly lower than the control group. However, by day 8 each experimental group is significantly higher than the control with the exception of the deep zone. Furthermore, changes in expression levels of the middle zone cells appear the most dramatic over the course of the study, and deep zone cells have significantly higher expression levels than superficial or middle zone cells by the last time point.

4.3.7 Histology

Figures 4.9 and 4.10 show histological staining of both control and experimental groups at days 1 and 8. It is difficult to visually identify differences between control and experimental groups, or between zones. However, images show that all groups have formed cell clusters by day 8, and these clusters are observed in higher concentrations at the periphery of the alginate beads. Figures 4.11 and 4.12 show additional staining using Alcian blue. In these images the cell nuclei is stained pink to red, the cell cytoplasm is

stained pink and acidic proteoglycans such as chondroitin residues, sulphated residues, and hyaluronic residues are stained blue (not be confused with the alginate bead which also stains blue). The cell clusters are again seen at day 8, with a build-up of acidic proteoglycans seen in the center of the clusters. In the day 1 images, the deep zone cells appear slightly larger than the superficial zone cells.

4.3.8 Immunohistochemistry

Protein production of IGF-1 and type II collagen by encapsulated chondrocytes is confirmed by immunohistochemistry for each protein. Figure 4.13 shows staining for IGF-1, both with and without exogenous IGF-1 delivery and Figure 4.14 shows staining for type II collagen, both with and without exogenous IGF-1 delivery. For both groups the protein presence is observed directly around the single cells on day 1, and in between and around the cell clusters on day 8. In all cases protein presence increases from day 1 to day 8, and indicate that both proteins are present throughout the study. Again, most cell cultures are observed around the periphery of the beads.

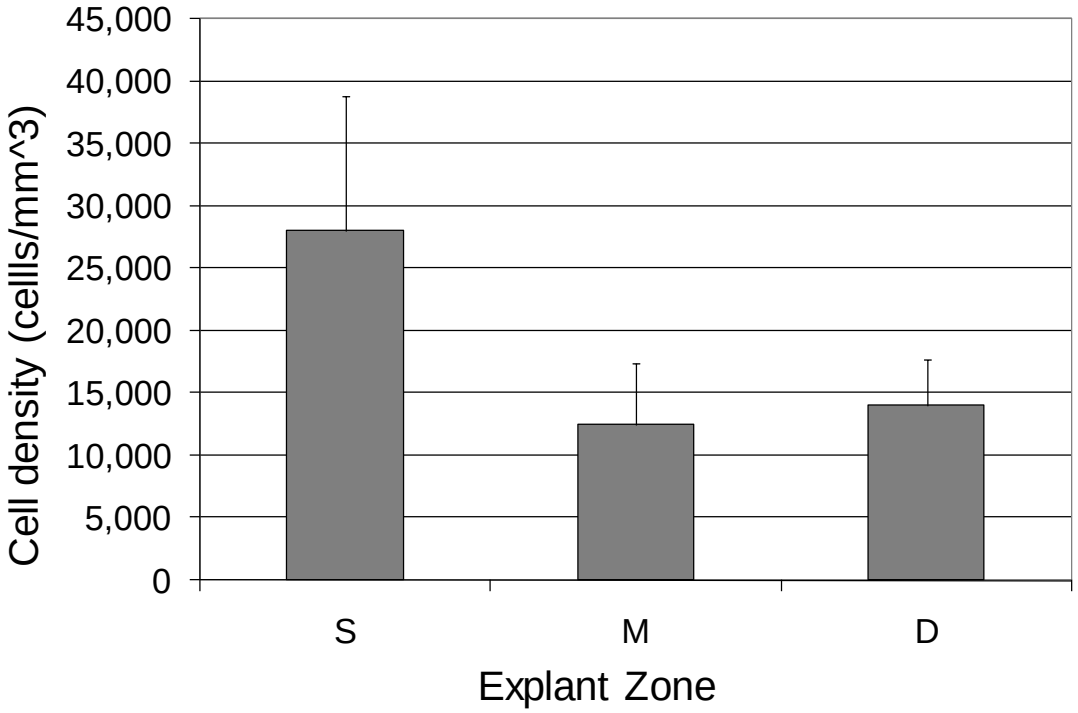


Figure 4.1. Average cellular density in cells/mm^3 from explanted cartilage tissue samples from bovine femoral condyles. The superficial (S), middle (M), and deep (D) zones are all represented.

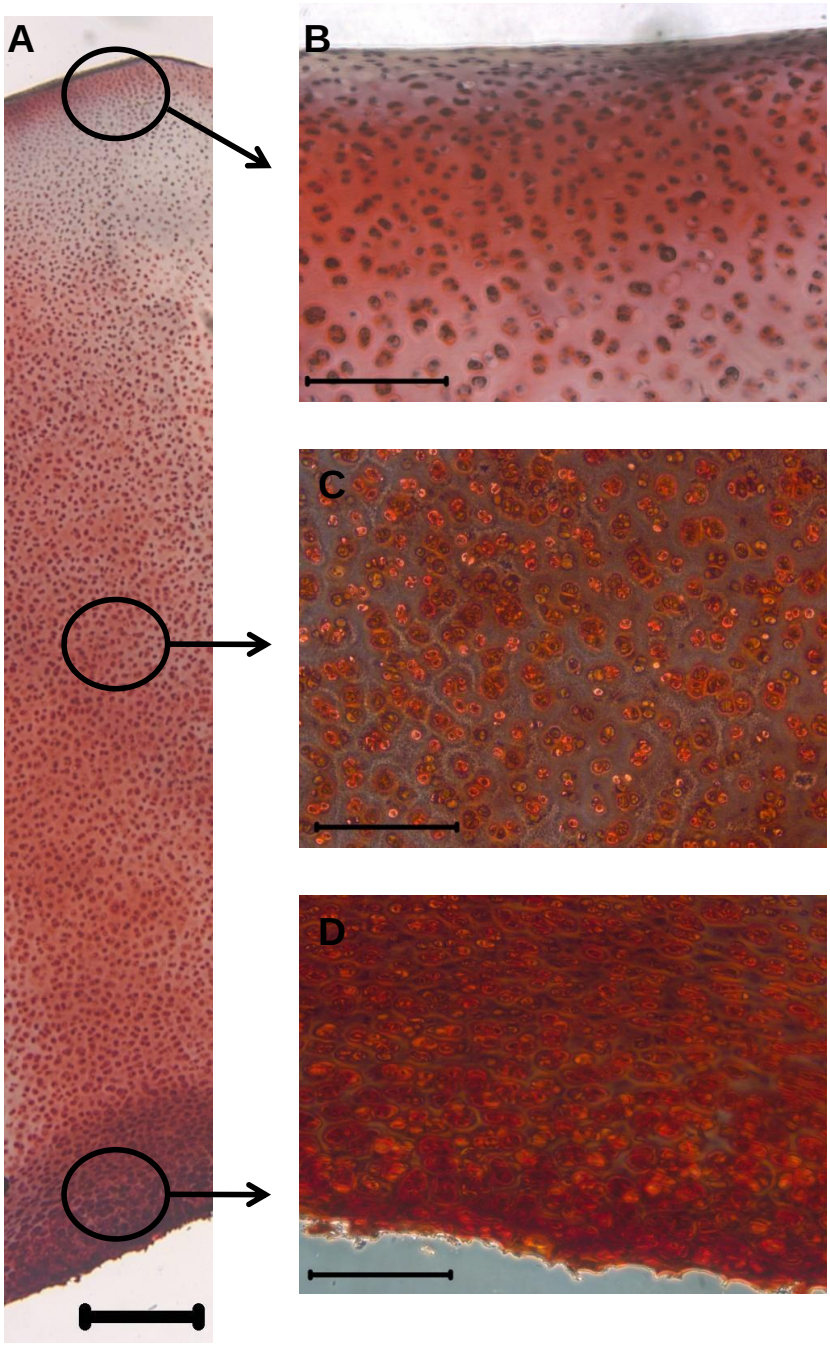


Figure 4.2. Histology of cartilage explants from femoral condyles of 20 week old calves stained with safranin-o, fast green, and iron hematoxylin. Cell nuclei are stained black, and red staining indicates proteoglycan content. A) Full thickness sample showing superficial zone, middle zone, and deep zone. Scale bar 500 μm. B) Magnification of superficial zone. Scale bar 100 μm. C) Magnification of middle zone. Scale bar 200 μm. D) Magnification of deep zone. Scale bar 200 μm.

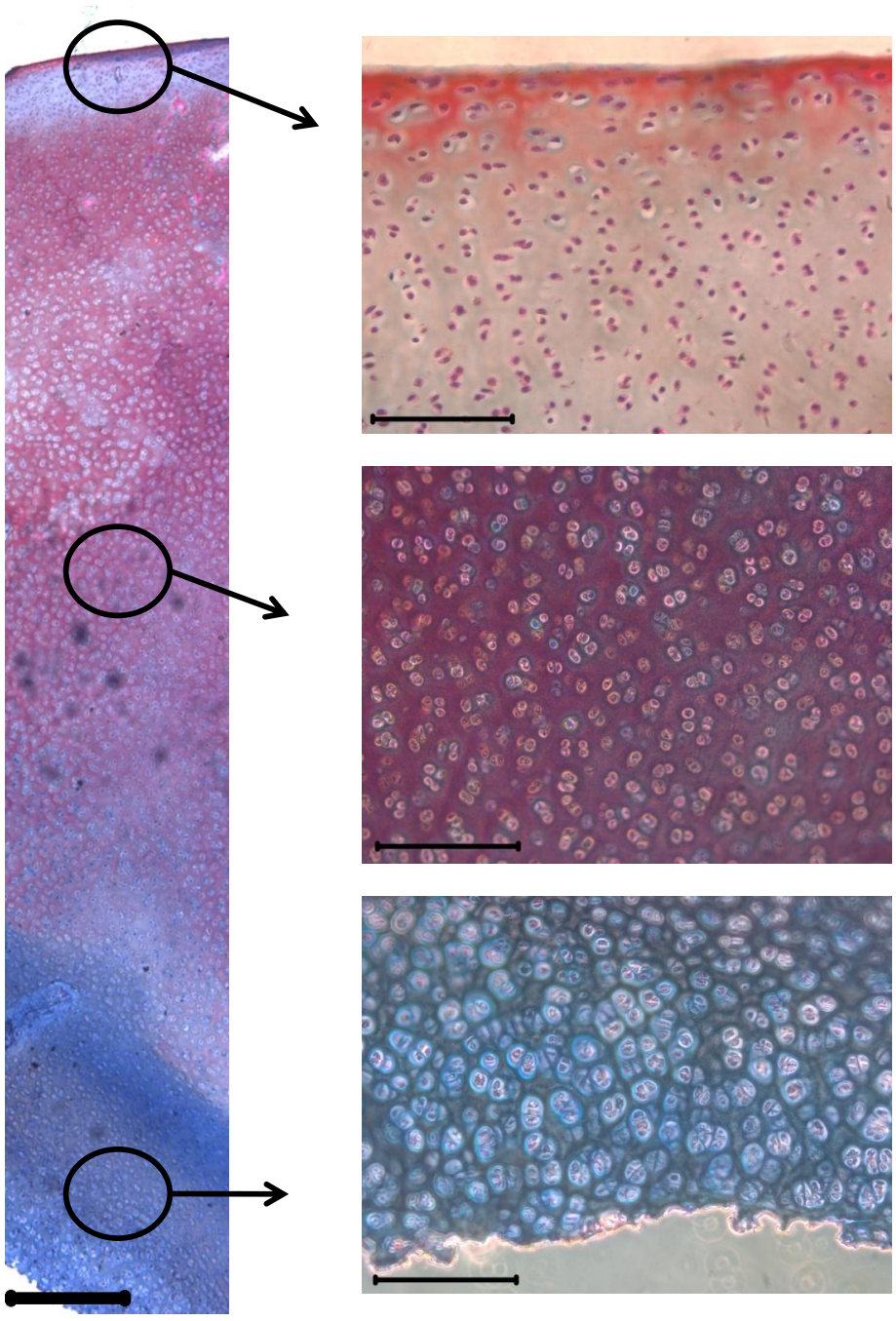


Figure 4.3. Histology of cartilage explants from femoral condyles of 20 week old calves stained with masson's trichchrome. Cell nuclei are stained black, all tissue elements are stained red, and collagen fibers are stained blue. A) Full thickness sample showing superficial zone, middle zone, and deep zone. Scale bar 500 μm. B) Magnification of superficial zone. Scale bar 100 μm. C) Magnification of middle zone. Scale bar 200 μm. D) Magnification of deep zone. Scale bar 200 μm.

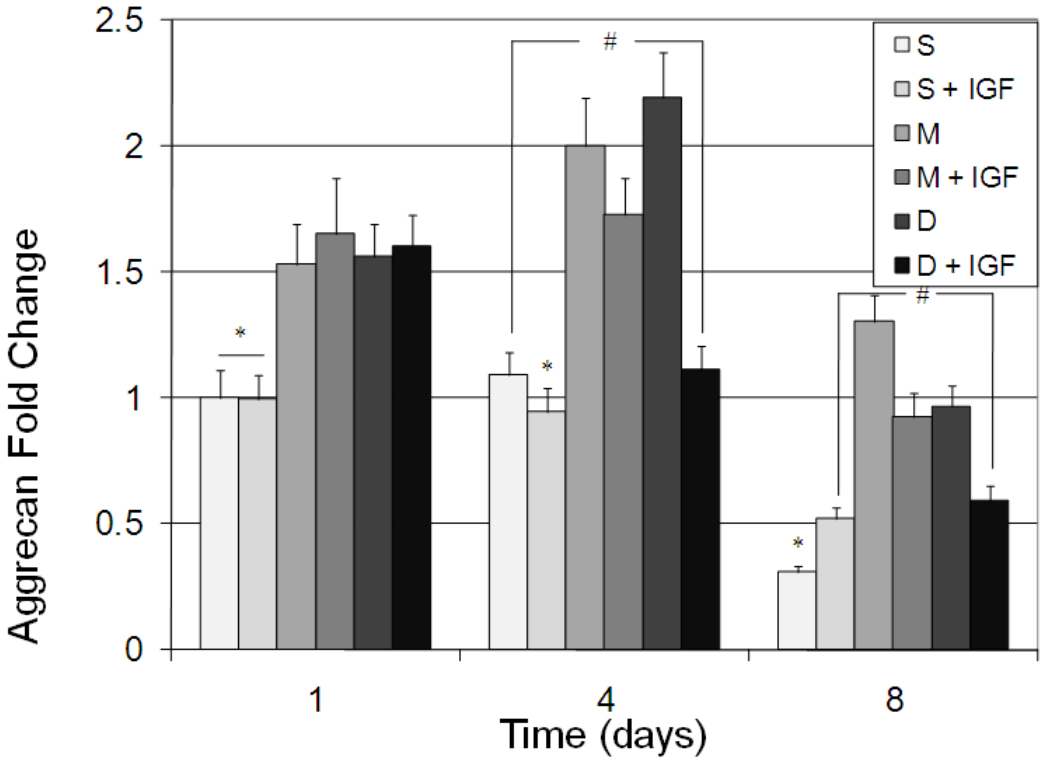


Figure 4.4. Aggrecan mRNA expression for chondrocytes isolated from superficial (S), middle (M), and deep (D) zones and cultured in alginate beads over 8 days. Two groups are represented for each zone, a control with no growth factor delivery and a group which received 100 ng/mL IGF-1 daily. Day 1, superficial zone control is the calibrator. A symbol indicates that a group is statistically different from all other groups. Multiple groups marked with the same symbol indicates the groups are statically similar to each other, and statistically different from all others. Means and standard deviations are reported (n = 3).

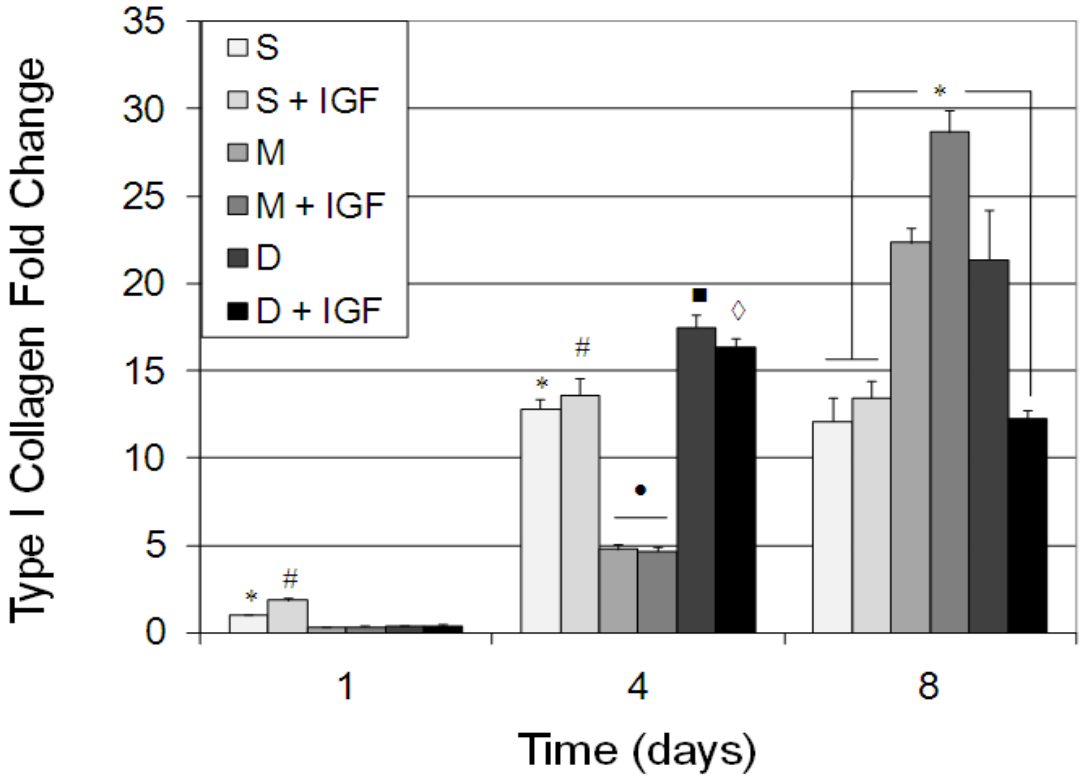


Figure 4.5. Type I Collagen mRNA expression for chondrocytes isolated from superficial (S), middle (M), and deep (D) zones and cultured in alginate beads over 8 days. Two groups are represented for each zone, a control with no growth factor delivery and a group which received 100 ng/mL IGF-1 daily. Day 1, superficial zone control is the calibrator. A symbol indicates that a group is statistically different from all other groups. Multiple groups marked with the same symbol indicates the groups are statically similar to each other, and statistically different from all others. Means and standard deviations are reported (n = 3).

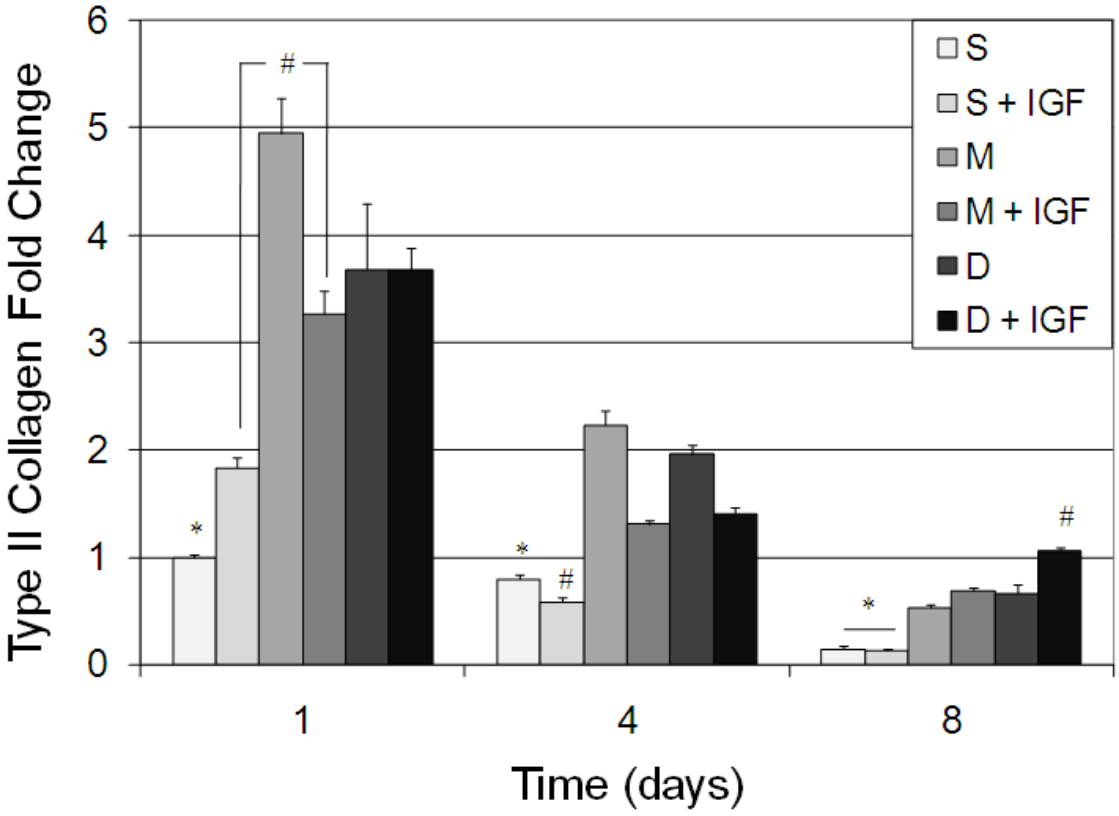


Figure 4.6. Type II Collagen mRNA expression for chondrocytes isolated from superficial (S), middle (M), and deep (D) zones and cultured in alginate beads over 8 days. Two groups are represented for each zone, a control with no growth factor delivery and a group which received 100 ng/mL IGF-1 daily. Day 1, superficial zone control is the calibrator. A symbol indicates that a group is statistically different from all other groups. Multiple groups marked with the same symbol indicates the groups are statically similar to each other, and statistically different from all others. Means and standard deviations are reported (n = 3).

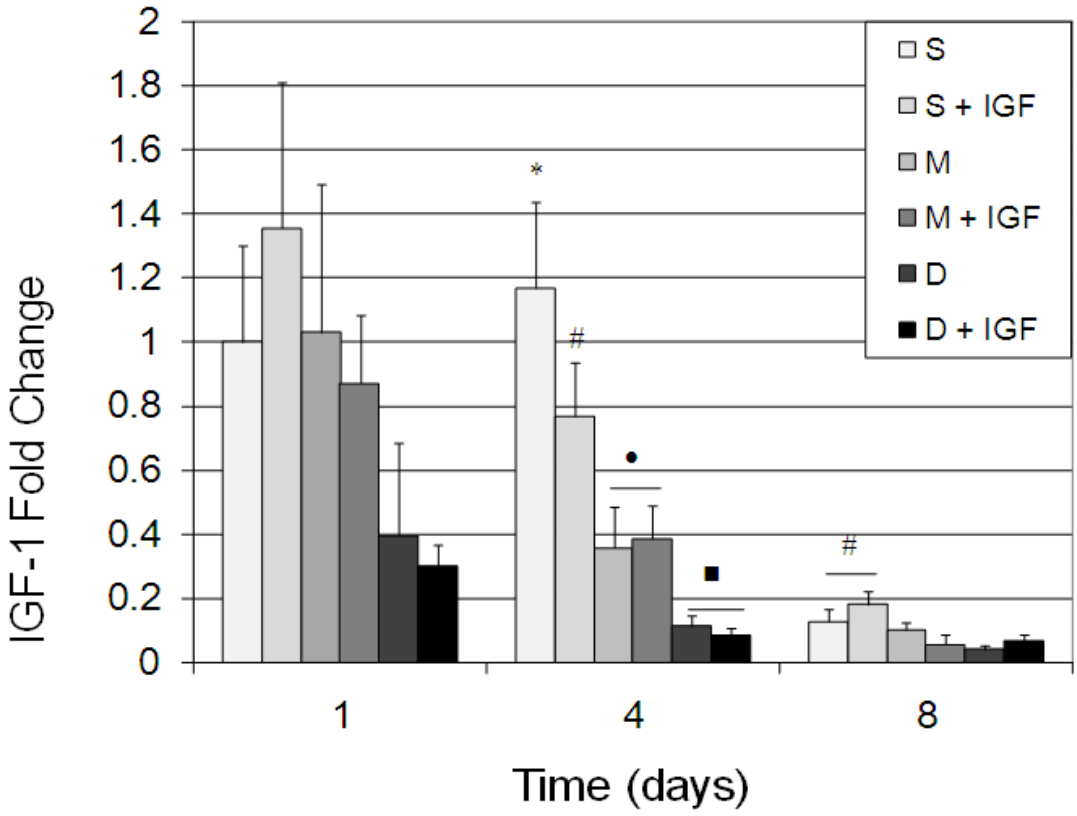


Figure 4.7. IGF-1 mRNA expression for chondrocytes isolated from superficial (S), middle (M), and deep (D) zones and cultured in alginate beads over 8 days. Two groups are represented for each zone, a control with no growth factor delivery and a group which received 100 ng/mL IGF-1 daily. Day 1, superficial zone control is the calibrator. A symbol indicates that a group is statistically different from all other groups. Multiple groups marked with the same symbol indicates the groups are statically similar to each other, and statistically different from all others. Means and standard deviations are reported (n = 3).

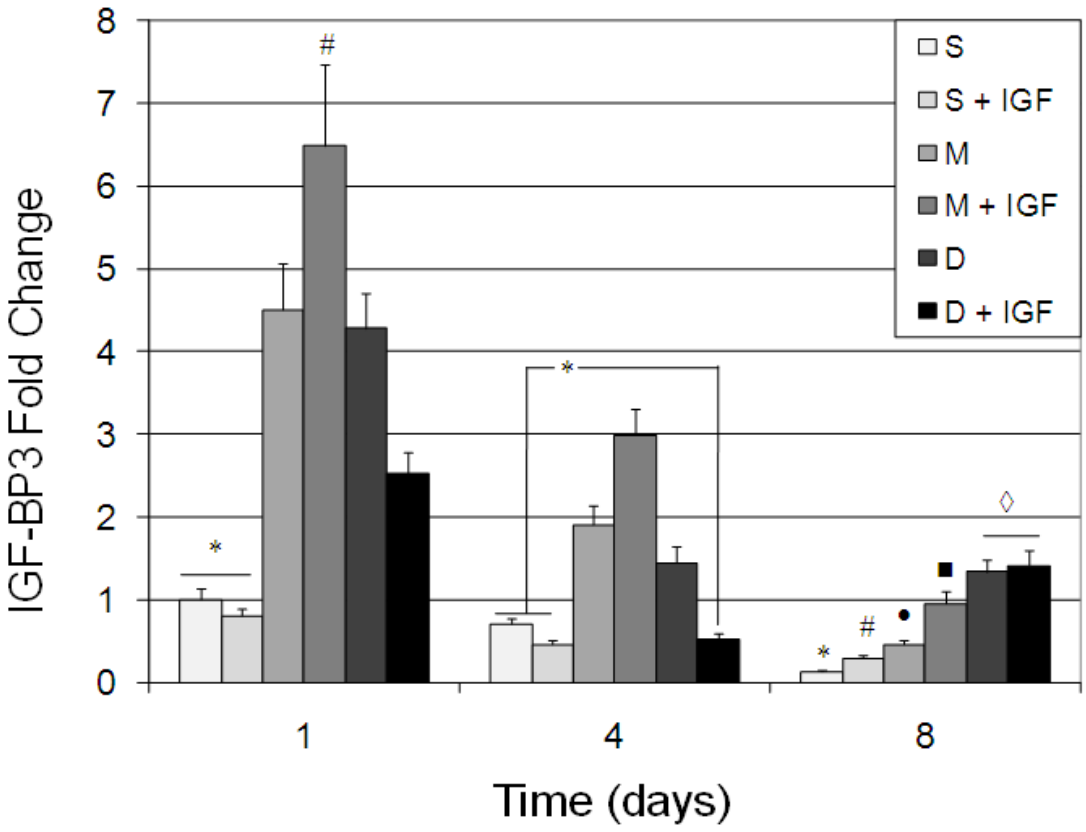


Figure 4.8. IGF-1 Binding Protein 3 mRNA expression for chondrocytes isolated from superficial (S), middle (M), and deep (D) zones and cultured in alginate beads over 8 days. Two groups are represented for each zone, a control with no growth factor delivery and a group which received 100 ng/mL IGF-1 daily. Day 1, superficial zone control is the calibrator. A symbol indicates that a group is statistically different from all other groups. Multiple groups marked with the same symbol indicates the groups are statically similar to each other, and statistically different from all others. Means and standard deviations are reported (n = 3).

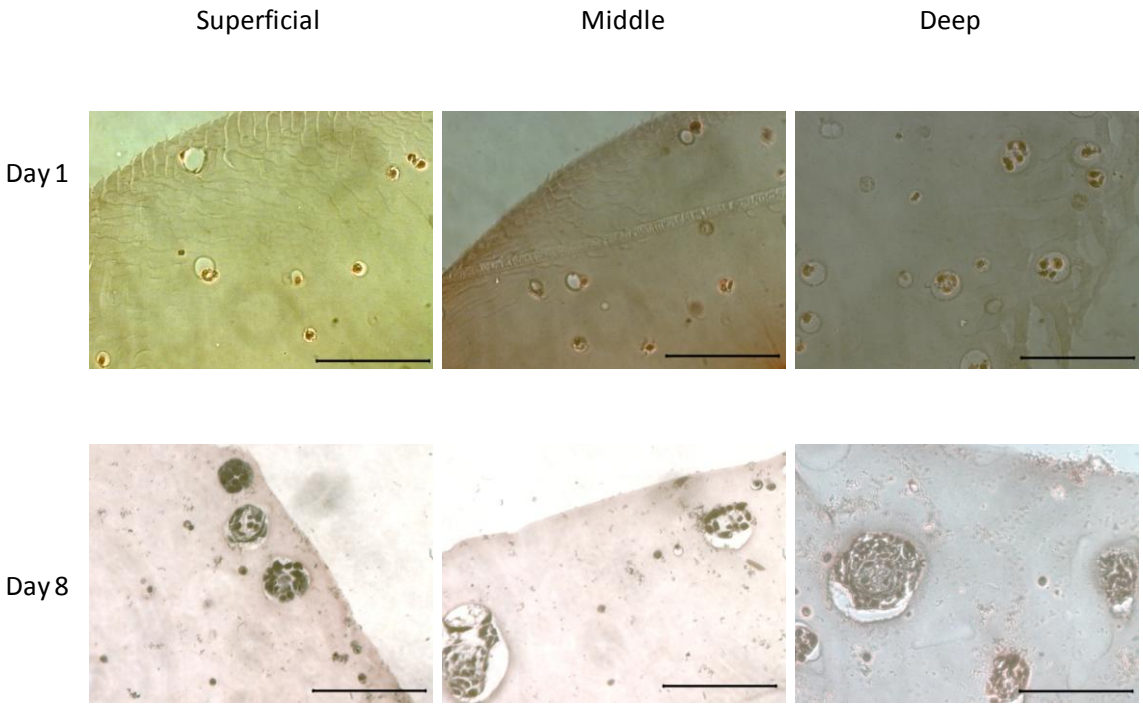


Figure 4.9. Histology staining with Safranin-O for control groups, all scale bars 100.

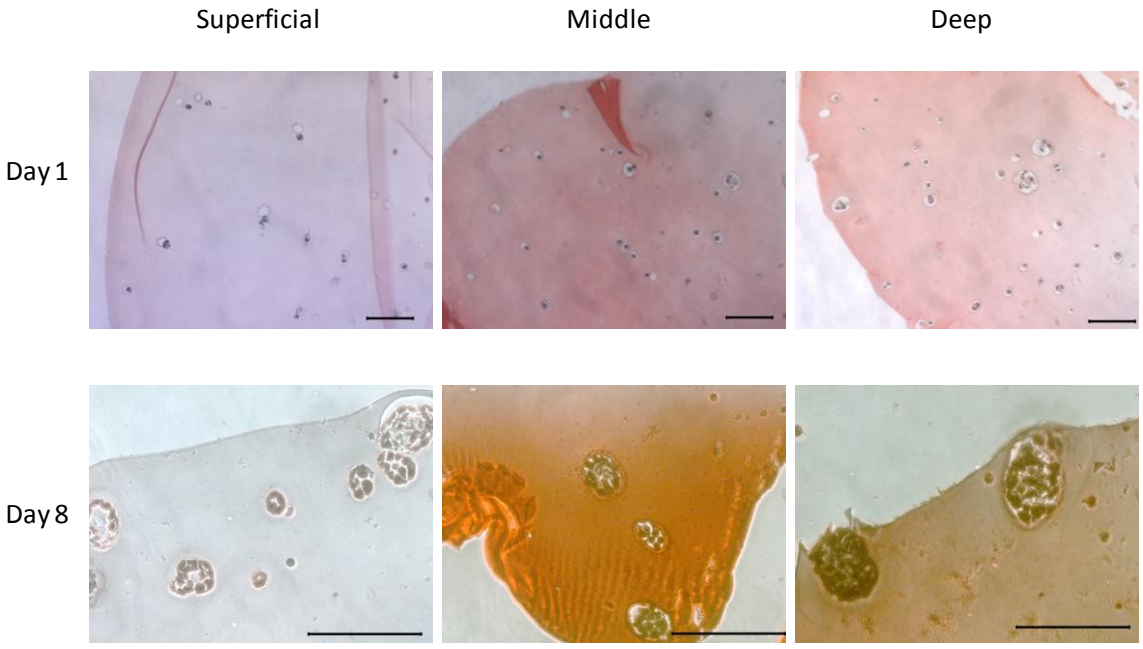


Figure 4.10. Histology staining with Safranin-O for IGF-1 delivered experimental groups, all scale bars 100 μm.

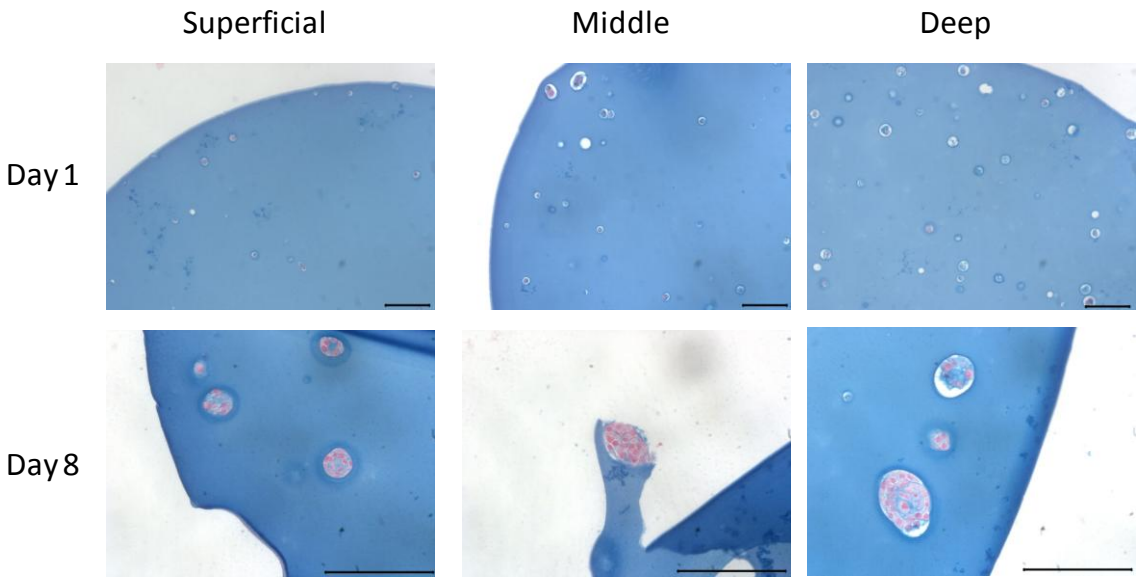


Figure 4.11. Histology staining with Alcian blue for control groups. Proteoglycans stained blue and cell nuclei and cytoplasm stained pink. Cell proliferation to form clusters with blue staining in between the cluster is observed between days 1 and 8. Note the alginate hydrogel stains a dark blue and proteoglycans around the cells a slightly lighter blue. Scale bars 100 μm.

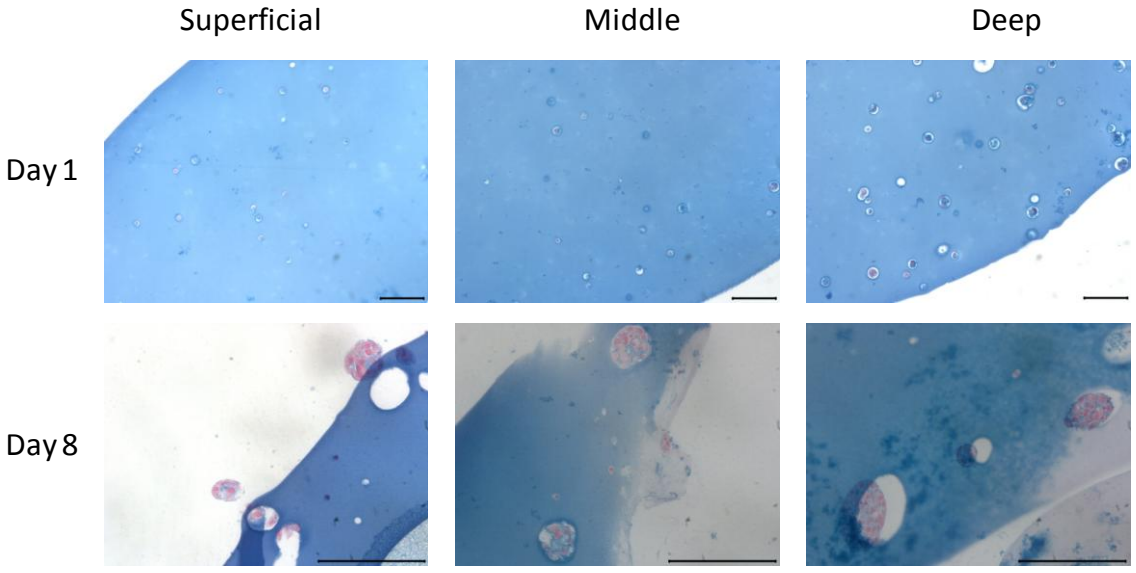


Figure 4.12. Histology staining with Alcian blue for IGF-1 delivered experimental groups. Proteoglycans stained blue and cell nuclei and cytoplasm stained pink. Cell proliferation to form clusters with blue staining in between the cluster is observed between days 1 and 8. Note the alginate hydrogel stains a dark blue and proteoglycans around the cells a slightly lighter blue. All scale bars 100 μm.

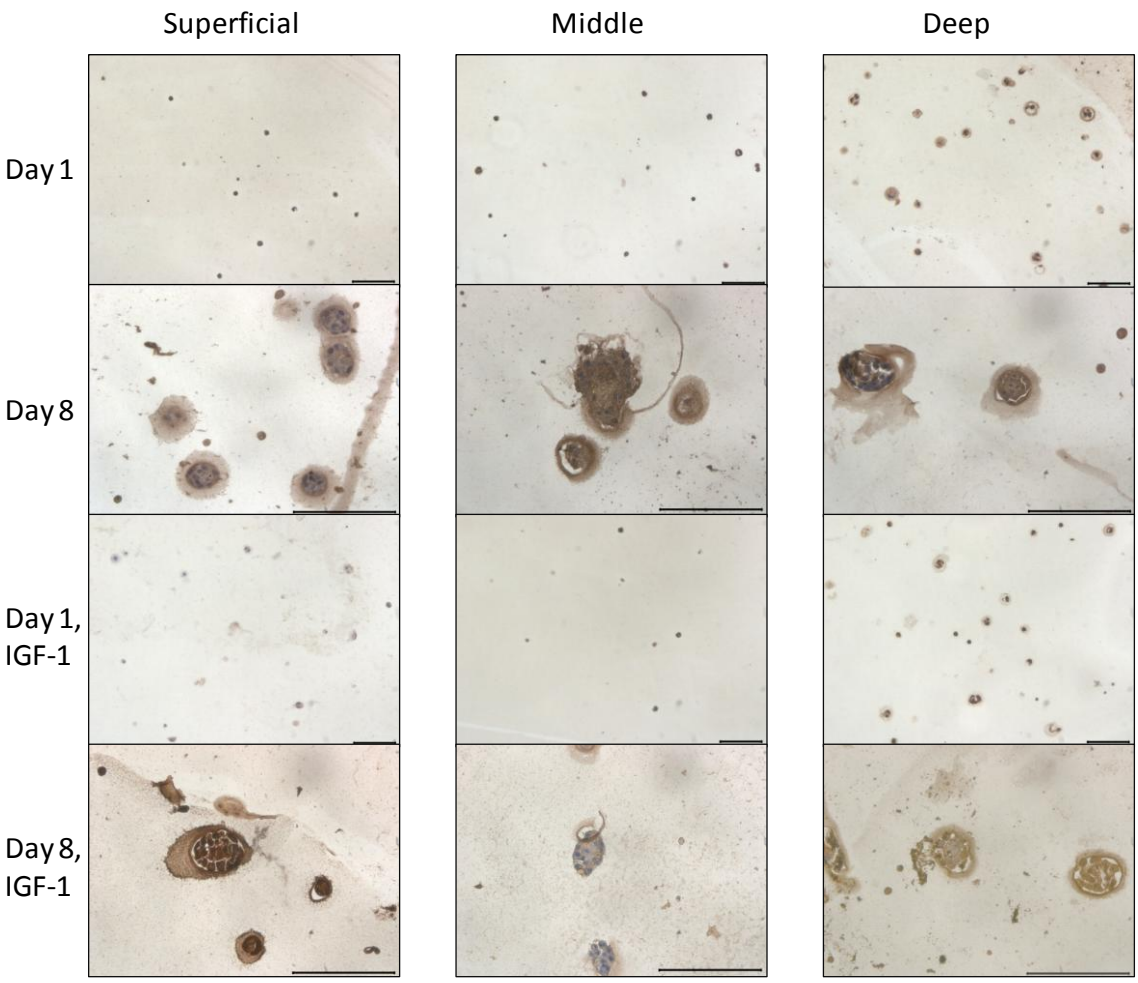


Figure 4.13. IGF-1 immunohistochemistry staining at days 1 and 8 for control and IGF-1 delivered experimental groups. Cell nuclei are stained dark violet, and IGF-1 staining is brown. Single cells are stained at day 1 and cell clusters with staining around them are observed at day 8. All scale bars 100 μm.

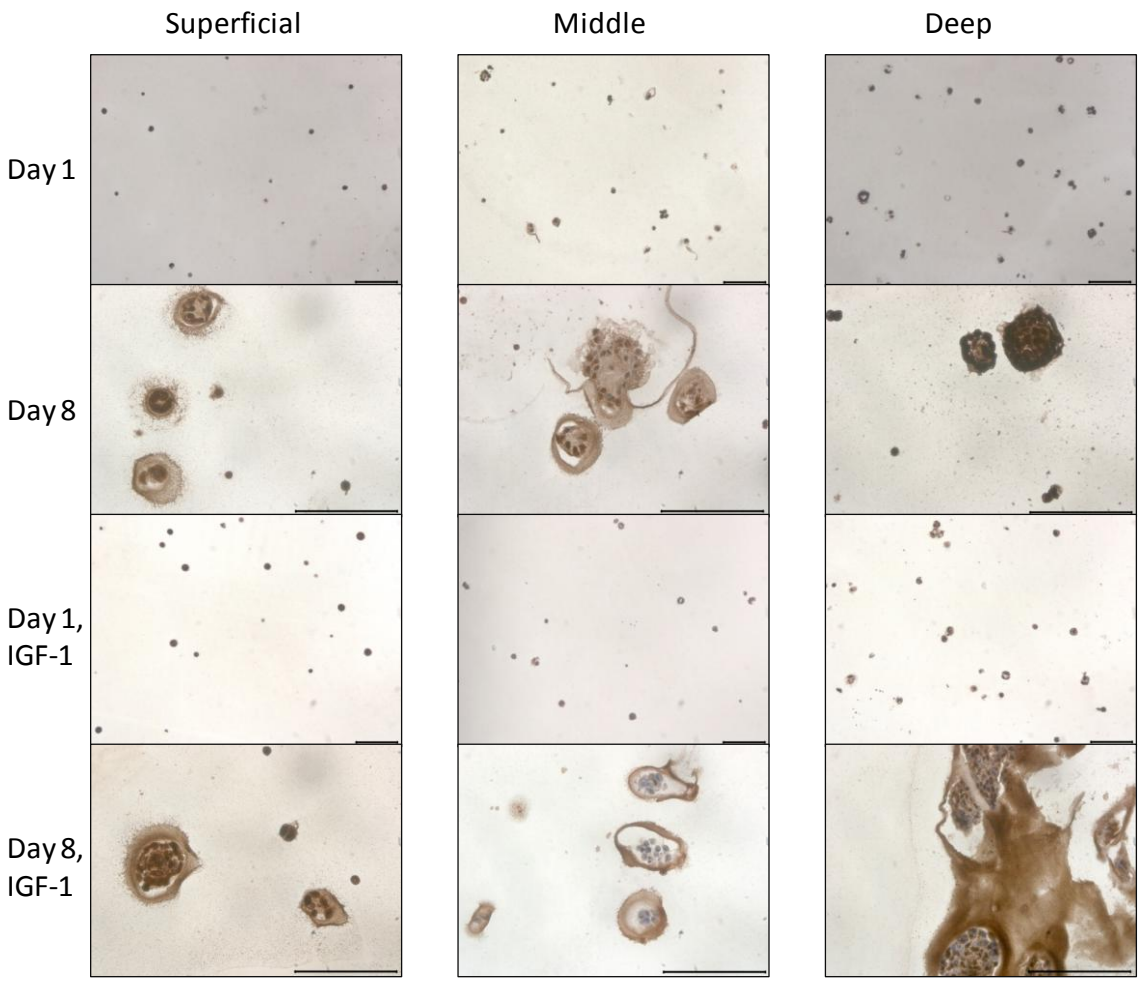


Figure 4.14. Type II collagen immunohistochemistry staining at days 1 and 8 for control and IGF-1 delivered experimental groups. Cell nuclei are stained dark violet, and Type II Collagen staining is brown. Deep zone staining at day 8 with IGF-1 delivery appears most intense. All scale bars 100 μm.

4.4 Discussion

To successfully engineer all three cartilage zones, the cellular phenotype and function of each chondrocyte subpopulation must be fully understood. Additionally, the response of subpopulations to delivered growth factors must be known if they are to be used to enhance extracellular matrix production. Only a handful of studies have investigated the gene expression of articular chondrocytes subpopulations with delivered growth factors. [96, 169-172, 176] These studies have identified trends in growth factor delivery, but have mainly been performed on chondrocytes in monolayer. It has been demonstrated that in two-dimensional culture chondrocyte subpopulations lose their phenotype and converge to a homogenous population expressing and producing little matrix proteins and high levels of type I collagen. [152, 157] Therefore, information about how growth factors affect zonal chondrocytes in three-dimensional culture is critical. Monolayer studies indicate that IGF-1 can improve mechanical properties of cells from both superficial and middle/deep zone populations, [172] increase gene expression for aggrecan and type II collagen in middle/deep zone populations, and increase SZP gene expression in superficial cell population. [96] The one known such study performed in three dimensional culture demonstrated that superficial cells encapsulated in agarose increased SZP synthesis in response to IGF-1 delivery. [171] The aim of this study was to determine subpopulation response to IGF-1 in terms of matrix component gene expression, and classify the distribution of IGF-1 and IGF-BP expression throughout cell populations. To meet these aims, gene expression of aggrecan, type I collagen, type II collagen, IGF-1, and IGF-BP3 by chondrocyte subpopulations both with and without delivery of IGF-1 was examined.

Isolation of subpopulations of bovine articular chondrocytes by methods similar to ours have been reported in the literature. [14, 15] Cell counts and histological evidence both support successful isolation of superficial, middle, and deep zone chondrocytes. Cell counts of chondrocytes located from each zone show the highest density of cells in the superficial zone, followed by the middle and deep zone, this trend is also reported in the literature [15] for explanted cell populations, as well as within native cartilage tissue. [2] Histological evidence also confirms isolation of all three cartilage zones, as seen in Figures 4.2 and 4.3.

The chondrocyte phenotype is indicated by cellular production of matrix components such type II collagen and aggrecan. Production of type I collagen indicates chondrocytes have become more fibroblastic in nature. While the increased levels of mRNA expression of type I collagen at day 8 for all culture groups indicate that cells are less phenotypically stable than on day 1, the expression of type II collagen and aggrecan at measurable levels throughout the study indicates chondrocytes are still active in production of matrix proteins.

Furthermore, differences in subpopulation gene expression are maintained throughout the study, indicating that stable zonal populations are retained. Elevated gene expression of matrix components by middle and deep zone cells compared to superficial cells is evident throughout the study as shown in Figures 4.4 and 4.6 (aggrecan and type II collagen fold change). Several studies have confirmed the elevated production of matrix proteins by middle and deep zone cells compared with superficial cells, [11, 12, 164] and these differences are supported by the tissue's native extracellular matrix composition. The differences observed between the cell groups are likely a result of both

chondrocyte phenotype and zonal chondrocyte phenotype remaining stable in the three-dimensional environment. Retention of differences observed in native tissue is encouraging for stable culture techniques and manipulation of these cells in tissue engineering applications.

Endogenous IGF-1 gene expression decreased by tissue depth and by time. Superficial cells continuously expressed the highest levels of endogenous IGF-1 followed by the middle zone and finally the deep zone. Conversely, IGF-BP3 expression was elevated in middle and deep zone cells compared to superficial zone cells throughout the study, and overall expression also decreased with time. To our knowledge, this is the first study to report such distributions. It is interesting to note that the cell populations which express the highest levels of mRNA for ECM components also express lowest amounts of growth factor mRNA and highest amounts of growth factor binding protein mRNA. Furthermore, even though superficial zone cells express elevated levels of IGF-1 mRNA, their matrix molecule mRNA expression is the lowest. These trends could be a result of a disconnect between mRNA expression of IGF-1 and IGF-1 protein available for cell binding. However, there also may be other growth factors or cellular signals at play which influencing matrix production. Quantified investigation of IGF-1 protein production, as well as endogenous expression of other major growth factors may provide further insight.

A concentration of 100ng/mL of IGF-1 was delivered to one group of cell isolated from each zone. The dose of 100ng/mL was chosen based on previous studies in our lab, [56] and doses commonly reported in articular chondrocytes studies. [96, 97, 169] Our goal was to investigate how the addition of IGF-1 may affect cells of distinct

subpopulations differently. Significant differences in subpopulation responses were observed. Overall, IGF-1 did not have the expected stimulative effect on expression of matrix component, with the exception of the deep zone group on 8 for expression of type II collagen. Within in the same group IGF-1 delivery lowered type I collagen expression on days 4 and 8, indicating a stabilizing effect on the chondrocytes phenotype. Therefore, it may be likely that IGF-1 is most appropriate for stimulating cell from deeper zones of articular cartilage during *in vitro* culture. It is unknown why IGF-1 would be a more effective at stimulating matrix production when acting on cells of one zone over another, but other literature appears to indicate this phenomenon as well. [96]

In general, IGF-1 delivery decreased or had no significant impact on endogenous expression of IGF-1. This is consistent with previous reports on mixed populations of chondrocytes, which report decreased endogenous IGF-1 expression with delivery. [56] We hypothesize that as cells sense delivered IGF-1 which is available for cell binding, they reduce their endogenous production of the protein. On day 1 the superficial zone cells do not follow this trend, nor do the deep zone cells on day 8, however neither of these groups were significantly different than their controls. Interestingly, the effects of IGF-1 delivery on IGF-BP3 expression vary by zone. Delivery consistently increases expression in the middle zone, while expression in the deep zone is decreased until day 8 where when it increases, and there are no significant effects observed in the superficial zone until day 8 where expression in increased. At day 8 there is an overall trend of increasing IGF-BP3 expression during IGF-1 delivery, a trend also supported by previous studies in our laboratory. [56] We hypothesize that with increased IGF-1 available, cellular production of IGF-BP3 increases in an effort to utilize the molecule.

Histological staining, Figures 4.9, 4.10, 4.11, and 4.12 do not visually demonstrate differences in proteoglycan production between cell groups, but confirm cell proliferation and production of proteoglycans. While aggrecan mRNA expression shows significant differences between superficial and middle/deep zone groups, visually these differences are not seen in proteoglycan staining. However, the histology serves to confirm proteoglycan production on the protein level.

Immunohistochemistry results confirm protein production of IGF-1 and type II collagen, as seen in Figures 4.13 and 4.14. On day 1, single chondrocytes with staining immediately surrounding the cells are observed. By day 8 cells have formed clusters with staining around and inside the cell clusters. For type II collagen on day 8 (Figure 4.14), staining appears most intense in the IGF-1 delivered deep zone group, the same group which expressed significantly higher type II collagen mRNA than all other groups at this time point. Here the stain for the protein connects cell clusters and is especially pronounced along the bead periphery.

The presented techniques inherently have their limitations, which can also affect the observed results. The $\Delta\Delta C_t$ method for quantifying PCR results presented here is depended on consistent expression of the endogenous control gene, GAPDH. It is also important to note that mRNA expression quantifies the gene expression activity of the cell, but does not always correlate to protein expression of that gene. Limitations in our hydrogel model potentially include unequal distribution of nutrients (oxygen, FBS, delivered IGF-1) to those cells on the periphery of the bead. Furthermore, use of FBS in our experimental media may limit the effects of delivered IGF-1. Serum-free studies could potentially give a more accurate picture of the effects of IGF-1 on cell populations.

4.5 Conclusion

Differences in gene expression of chondrocyte subpopulations are observed throughout the study. Middle and deep zone cells display similar behaviors in terms of matrix production and express significantly higher amount of aggrecan and type II collagen mRNA compared to superficial zone cells. IGF-1 expression decreases from superficial to middle to deep zone cells, a trend which is consistent throughout the study. IGF-BP3 expression is elevated in middle and deep zone cells throughout the study. Both IGF-1 and IGF-BP3 expression decrease with time. Exogenous delivery of IGF-1 did not have the simulative effects anticipated, but did positively affect phenotype retention in deep zone cells. Furthermore, delivery of IGF-1 generally decreased expression of endogenous IGF-1 and had varying effects by zone on IGF-BP3 expression. However, by day 8, IGF-1 delivery increased binding protein expression throughout all zones.

5 Matrix Molecule Influence on Chondrocyte Phenotype and Proteoglycan 4 Expression by Alginate-Embedded Zonal Chondrocytes and Mesenchymal Stem Cells

5.1 Introduction

Studies suggest that proteoglycan 4 (PRG4), a large glycoprotein encoded by the *PRG4* gene, is the critical boundary-lubrication mechanism in articular cartilage. [196] Superficial cells secrete elevated levels of PRG4 to provide lubrication at the articulating surface. [14] Middle and deep zone cells are more active in production of collagens and proteoglycans, and increased concentrations of matrix components are found with tissue depth to provide the strength needed to resist loading. [197, 198]

Despite advances, retaining both the chondrocyte phenotype and zonal phenotypes during *in vitro* culture remain challenges. Monolayer culture results in homogenization of subpopulations and loss of chondrocyte phenotype. [101, 152, 158] Constructs which support chondrocytes in a three-dimensional environment have shown success in retention of chondrocyte phenotype. [11, 12, 164, 199] However, robust retention of zonal chondrocyte phenotype for utilization in clinical treatments remains a major unmet challenge. [22, 25, 32] Cartilage tissue engineering research efforts focused on lubrication have resulted in several important findings which highlight the distinct role of superficial zone cells and the ability of progenitor populations to secret PRG4. Superficial zone cells in culture secrete elevated levels of PRG4 compared to chondrocytes of the middle and deep zones, and monolayer culture promotes PRG4 production in these cells compared to three dimensional culture. [136, 200] Surface motion [201] and ball oscillations [202] on engineering cartilage constructs seeded with a

mixed zone population can upregulate PRG4 mRNA expression as well as protein and secretion into media. Additionally, it has been demonstrated that bone marrow mensenchymal stem cells, articular chondrocytes (mixed zone population), meniscal fibrochondrocytes, [203] and mesenchymal progenitors from the synovium and infrapatellar fat pad [204] are all capable of secreting PRG4 in three dimensional culture. Bone marrow MSCs were shown to secret up to 10 times as much PRG4 in alginate culture than mixed zone chondrocytes or mensical fibrochondrocytes. However, MSC hydrogel constructs had poor ability to localize and utilize PRG4 to improve lubrication. [203] These results highlight the importance of engineering a construct with lubricating properties, however the optimal cell population and scaffold environment for achieving this goal is yet to be established.

Both hyaluronic acid (HA) and chondroitin sulfate (CS) are major components of the cartilage ECM and have been implicated in altering chondrocyte proliferation and metabolic activity. CS has been implicated in regulating chondrocyte phenotype, intracellular signaling, and cell surface connection to ECM components. In the literature, CS incorporation to collagen and poly(ethylene glycol) based scaffolds stimulated chondrocyte matrix production, [164, 196] and CS delivered in culture media increased mRNA expression of aggrecan and type II collagen. [205]

Chondrocytes bind to HA directly via the CD44 receptor on the cell surface. HA has been shown to affect cell proliferation, migration, and differentiation in various animal models. In chondrocyte culture, HA incorporation to poly(ethylene glycol) (PEG) based hydrogels stimulated matrix accumulation in superficial zone cells, [164] and when delivered in media increased cell proliferation and glycosaminoglycan production. [206]

A porous HA-collagen scaffold has been investigated for chondrocyte culture, [207] as well as composites of gelatin-HA-CS [208] and collagen-HA-CS. [209] Each of these systems indicate favorable effects on matrix production and chondrocyte phenotype. We hypothesis that CS and HA incorporation to our alginate scaffolds will have varying effects by chondrocyte subpopulation on matrix production, cell proliferation, and phenotype retention via PRG4 production.

We further hypothesize that HA and CS addition to alginate scaffolds will influence differentiation markers and zonal phenotype markers during stem cell chondrogenesis. While methods for inducing basic chondrogenesis of MSCs are fairly well established there is no established method of producing populations of chondrocytes with varying morphologies which mimic the superficial and middle/deep zone chondrocyte cell populations. MSC-derived chondrocytes are largely evaluated for their potential to secret structural ECM components such as collagens and proteoglycans. The work presented here aims to investigate HA and CS to influence zonal-differentiation of MSCs as well as zonal phenotype retention of primary chondrocytes. We focus on manipulating scaffold properties to influence the production of PRG4, a critical component of a functional superficial zone cartilage.

5.2 Methods

5.2.1 Superficial and Middle/ Deep Zone Chondrocyte Isolation

Zonal chondrocyte sub-populations were isolated according to a previously published laboratory protocol. [198] Briefly, cartilage plugs (4 mm diameter, 3-6 mm height) were harvested from the femoral condyles of 20 week old calves using a Sklar

Tru-Punch disposable biopsy punch (Sklar Instruments, West Chester, PA). The top 10% (0.3 - 0.6 mm) was taken as the superficial zone, and remaining tissue up was defined as the middle and deep tissue zones. Samples were minced, rinsed in Dulbecco's Modified Eagle Medium/Nutrient Mixtures F-12 Ham (DMEM/F12) media (Gibco/Invitrogen, Carlsbad, CA), digested in 0.2% collagenase P (Roche, Basel, Switzerland), filtered through a 40 μm mesh, and washing again in DMEM/F12 to remove any undigested tissue. A population of mixed zone chondrocytes was also obtained, omitting the zonal separation step.

5.2.2 Bone Marrow Stromal Cell Isolation

Primary bovine bone marrow tissue was harvested from the tibia 3 week old calves. The tissue was suspended in growth media (Minimal Essential Medium α, (Gibco/Invitrogen) supplemented with 0.1% penicillin/streptomycin antibiotics and 0.2 mM of ascorbic acid), filtered through a 70 μm mesh, and centrifuged to isolate the cell population. This population was then enriched for mesenchymal stem cells via plastic adhesion by plating in monolayer and culture in growth media with 10% fetal bovine serum. After two passages cells were trypsinized (Gibco/Invitrogen) and counted using Trypan blue staining and a hemacytometer.

5.2.3 Hyaluronic Acid and Chondroitin Sulfate Addition to Alginate Scaffolds

2.0 % control alginate was prepared using protocols previously established by our laboratory. [54, 56, 210] Briefly, alginate solution was prepared by mixing and heating alginic acid sodium salt from brown algae (Sigma-Aldrich, molecular weight 80,000-

120,000 Da, M:G ratio ~1.56, viscosity ≥ 2,000cP), 0.15 M sodium chloride (Sigma-Aldrich), and 0.025 M HEPES, sodium salt (J.T. Baker, Phillipsburg, NJ) into deionized water (pH 7.4), and then autoclaved for sterilization. Addition of HA (Sigma-Aldrich, MW 1.64x10^6 Da) and CS (Sigma-Aldrich, MW approximately 45,000 Da) to alginate scaffolds were done using protocols previously established by our laboratory. [55] Specifically, 2.0% w/v alginate was used as the base polymer, and various concentration of CS and HA were added to the liquid alginate solution. Concentrations were chosen based on previous results using HA from our laboratory. [55] as well as reports from the literature. [164, 205] HA and CS were added for the following concentrations: 0.1 mg/mL and 1 mg/mL CS and 0.1 mg/mL, 2 mg/mL, and 5 mg/mL HA. The solution was prepared by mixing and heating alginic acid, CS or HA, 0.15 M sodium chloride, and 0.025 M HEPES into deionized water. Final solutions were autoclaved for sterilization.

5.2.4 Cell Encapsulation and Culture

Alginate solutions were mixed with the desired cell population (superficial zone chondrocytes, middle/deep zone chondrocytes, MSCs) and injected through a 18-gauge syringe into continuously stirred 0.1 M calcium chloride ($CaCl_2$) (Sigma-Aldrich). The resulting cellular density was approximately 100,000 per bead, and each spherical bead had a diameter of approximately 5 mm (approximately 2 x 10^6 cells\mL). Zonal chondrocyte populations were cultured in supplemented DMEM/F12 + FBS for 14 days. At days 1, 7, and 14 chondrocytes were isolated from the alginate beads by addition of 4 mL of 0.1 M EDTA for 25 min at 37°C. The solution was then centrifuged to form a cell pellet, which was resuspended in phosphate-buffered saline (PBS) and then used for

RNA and DNA isolation. Culture time was chosen to assess changes in chondrocyte subpopulation phenotype, which can happen quickly upon isolation. [157] MSC populations were cultured in serum-free chondrogenic media for 21 days, following standard culture time for MSC chondrogenesis. [211] Chondrogenic media contained; high glucose αMEM (Gibco/Invitrogen) + 110 µg/mL sodium pyruvate, 40 µg/mL proline, 50 µg/mL ascorbate 2-phosphate, 0.1 µM dexamethasone, 1% ITS +premix (BD Biosciences, Bedford,MA), and 10 ng/mL TGF-β3 treatment (R&D systems, Minneapolis, MN). At days 1, 7, and 21 cells were isolated for RNA isolation, and at days 14 and 21 beads were fixed for histological preparation.

5.2.5 Histological Preparation

At desired time points, alginate beads were recovered and fixed for 3 hr at room temperature in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) with 0.1 M sodium cacodylate and 10 mM calcium chloride. Samples were then washed for 24 hr at room temperature in 0.1 M sodium cacodylate and 10 mM calcium chloride. Following washing, samples were placed in histological cassettes and dehydrated through a series of ethanol washes followed by two Citrisolv (Fisher Scientific) washes. The samples were embedded in paraffin (Paraplat X-tra, Fisher Scientific) and cut into 4 µm sections and mounted on a glass slide (Superfrost, Fisher Scientific).

5.2.6 Histochemical Staining

Samples were dried at 64°C for two hours, deparaffinized using Citrisolv, and rehydrated. The samples were then rinsed in distilled water and stained using Masson's

Trichrome, Alcian blue, and Sirius red staining solutions. (Poly Scientific, Bay Shore, NY). All samples were viewed under an Axiovert 40CFL light optical microscope (Zeiss, Thornwood, NY) and images were captured using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI) imaging software.

5.2.7 Immunohistochemistry

Samples were dried at 64°C for one hour, deparaffinized using Citrisolv and rehydrated. Samples were antigen retrieved using a Tris base and EDTA buffer (pH 8) containing TWEEN 20 steamed for 15 minutes in a Sunbeam® vegetable steamer. Samples were incubated with PEROXI*DAZED1* (Biocare, Concord, CA), an endogenous peroxidase blocker, and Background*SNIPER1* (Biocare), a blocking reagent. Samples were then stained with an antibody to detect PRG4. The primary antibody used was anti-lubricin/PRG4 (rabbit polyclonal antibody, ab28484; Abcam, Cambridge, MA), diluted to a working concentration of 4 µg/mL. The HISTOSTAIN®-SP kit (Zymed, San Francisco, CA) was used to visualize PRG4 presence by using the horseradish peroxidase (HRP)-streptavidin-biotin system. The complex formation was then detected by a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. Samples were counterstained with hematoxylin, dehydrated, cleared in Citrisolv, and covered. Negative control slides were stained using the same protocol, omitting the primary antibody.

5.2.8 Cell Proliferation Quantification

Cell proliferation was first measured by Trypan blue staining and cell count using a hemacytometer to establish general trends in the mixed zone chondrocyte population,

and then by more sensitive DNA quantification to identify differences between zonal chondrocyte populations.

5.2.9 DNA Isolation and Quantification

Total DNA was isolated using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) and a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) was used to quantify DNA content. Samples were excited at 480 nm and evaluated at an emission of 540 nm using a florescence microplate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). A standard curve was prepared using known quantities of DNA provided by the kit manufacturer, and used to determine DNA/alginate bead.

5.2.10 RNA Isolation

Following isolation of chondrocytes from alginate beads, RNA was isolated using the RNeasy Mini Kit (QIAGEN). Total RNA was eluted into 30 µL of RNase free water and detected using a NanoDrop ND-1000 Spectophotometer (NanoDrop Technologies, Wilmington, DE). RNA concentrations at 1, 7, 14, and 21 days were all diluted to approximately 10 ng/µL.

5.2.11 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

All isolated RNA was reverse transcribed using a cDNA Archive Kit (Applied Biosystems, Foster City, CA), which can convert up to 10µg of RNA to cDNA. cDNA was mixed with Universal Master Mix (Applied Biosystems) and oligonucleotide primers

and Taqman probes (Applied Biosystems) for the genes of interest as well as a control gene. Table 5.1 shows the sequences for all forward primers, reverse primers, and probes used. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was the endogenous control gene. The reaction volume was 20 μl, and the final concentration of cDNA per reaction well was approximately 0.5 ng/μL (10 ng per well). The reaction was conducted on a 7900HT Fast Real-Time PCR System Prism 7000 sequence detector (Applied Biosystems). The thermal profile followed was 2 min at 50°C, 10 min at 95°C, 40 cycles of 15s at 95°C, and 1 min at 60°C. Gene expressions were analyzed using the comparative C_t method. The day one control alginate samples were used as calibrators in all analysis. Fold changes in gene expression were calculated and are reported as the mean RQ values with associated standard deviations (n=3), in accordance with methods previously described by our laboratory. [55, 212]

5.2.12 Statistical Analysis

Each experiment was performed in triplicate (n=3). All data was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test to determine statistical differences. A confidence interval of 95% ($\alpha = 0.05$) was used for all analysis and means and standard deviations are shown on each figure.

Table 5.1. Forward primer, reverse primer, and probe sequences used for GAPDH, Type II Collagen, Type I Collagen, Aggrecan, Sox9, and Proteoglycan 4 (PRG4) used for qRT-PCR.

Primer and Probe sequences used for rRT-PCR

Protein		Sequence
GAPDH	Forward Primer	TGCCGCCTGGAGAAACC
	Reverse Primer	CGCCTGCTTCACCACCTT
	Probe	CCAAGTATGATGAGATCAA
COL2A1	Forward Primer	CGGGCTGAGGGCAACA
	Reverse Primer	CGTGCAGCCATCCTTCAGA
	Probe	CAGGTTCACATATACCG
COL1A1	Forward Primer	AGAACCCAGCTCGCACATG
	Reverse Primer	CAGTAGTAACCACTGCTCCATTCTG
	Probe	AGACTTGAGACTCAGCC
AGC	Forward Primer	GGGAGGAGACGACTGCAATC
	Reverse Primer	CCCATTCCGTCTTGTTTTCTG
	Probe	CAGGCTTCACCGTTGAG
SOX9	Forward Primer	AACGCCGAGCTCAGCAAG
	Reverse Primer	ACGAACGGCCGCTTCTC
	Probe	TTCAGCAGTCTCCAGAGCTTGCCCA
PRG4	Forward Primer	GAGCAGACCTGAATCCGTGTATT
	Reverse Primer	GGTGGGTTCCTGTTTGTAAGTGTA
	Probe	CTGAACGCTGCCACCTCTCTTGAAA

5.3 Results

5.3.1 Matrix Molecule Effect on Zonal Chondrocyte Populations

Cell proliferation is observed throughout the study, however no major differences are seen between zonal populations or experimental or control alginates. Figure 5.1A shows results from Trypan blue staining in a population of mixed zone chondrocytes, and Figures 5.1B and 5.1C show DNA quantification via picogreen assay in superficial and middle/deep zone cells respectively. Figure 5.1 shows proliferation throughout culture time for all groups, however no significant differences in proliferation rates between experimental and control groups are observed.

As shown in Figure 5.2, significant differences are seen between chondrocyte populations in expression of proteoglycan 4 mRNA. Figure 5.2A shows superficial and middle/deep zone chondrocyte expression of PRG4 mRNA over a 7 days culture period in control alginate. PRG4 expression remains elevated on days 1 and 7 by superficial zone cells, however middle/deep zone cells express significantly lower levels of PRG4 mRNA on day 1, (15 fold less than superficial zone cells) and no measureable expression by day 7. Superficial zone cell expression of PRG4 mRNA is significantly enhanced by addition of matrix molecules. Figure 5.2B shows PRG4 mRNA expression of superficial cells encapsulated in control alginate, and CS and HA-alginate. A general trend of increasing PRG4 mRNA expression with increasing matrix molecule concentration is observed. However, on day 14 expression peaks at 0.1 mg/mL in the CS group and 2 mg/mL in the HA group, neither of which are the maximum concentrations. By day 14, all but one experimental group are significantly greater than the control alginate group:

0.1 mg/mL CS is 3.5 fold greater, 0.1 mg/mL HA 6.5 fold, 2 mg/mL HA 15 fold, and 5 mg/mL HA 11 fold greater.

Expression of chondrocyte phenotype markers by superficial cells were also measured. Figure 5.3 depicts A) aggrecan B) type II collagen and C) type I collagen matrix molecule mRNA expression by superficial zone cells in control, HA, and CS-alginates throughout the culture period. Aggrecan and type II collagen are both major cartilage extracellular matrix components. Aggrecan expression remains elevated throughout the culture period, and on days 7 and 14 select experimental groups show greater expression than controls. On day 7, all groups other than the 2 mg/mL HA groups are upregulated over control alginate. On day 14, trends show increased aggrecan mRNA expression with increasing CS concentration. Although measureable at days 7 and 14, type II collagen mRNA expression decreases significantly throughout culture time, a consistent trend observed with culture of primary chondrocytes even during short culture periods. Type I collagen is a negative marker of chondrocyte phenotype, and its expression typically increases with *in vitro* culture time. Data shows increases in mRNA expression in all groups on days 7 and 14 compared to day 1.

Expression of chondrocyte phenotype markers by middle/deep zone cells in control, CS, and HA-alginate is seen in Figure 5.4. At each time point various experimental groups upregulate mRNA expression, however no clear trend is discernible. Aggrecan expression is seen in Figure 5.4A. On day 7, a general trend of increasing expression with matrix molecule concentration is observed, and 0.1 mg/mL HA and 2 mg/mL HA groups are 2 and 1.9 fold higher than the control, respectively. By day 14, only 0.1 mg/mL CS is significantly higher than the control (1.4 fold). In type II collagen

mRNA expression (Figure 5.4B) a similar phenomenon is observed. On day 1 the 2 mg/mL HA group is significantly higher than control (1.5 fold), on day 7 the 2 mg/mL HA group is again significantly greater than the control (3.5 fold), and by day 14 0.1 mg/mL HA is significantly higher than the control (1.3 fold). Figure 5.4C shows type I collagen mRNA expression of middle/deep zone cells. Here, trends appear to shift throughout the culture period. On days 1 and 7 there is significant upregulation in the higher concentration of HA groups, however by day 14 the 5 mg/mL HA group has significantly reduced expression compared to control (8.2 fold).

5.3.2 Matrix Molecule Effect on Chondrogenic Differentiation of Mesenchymal Stem Cells

Figure 5.5 shows mRNA expression of chondrogenic differentiation markers for MSCs encapsulated in control alginate, 1 mg/mL CS alginate, and 5 mg/mL HA alginate. The markers indicate chondrogenic differentiation, with early and elevated expression of transcription factor Sox9 (Figure 5.5A) and significantly elevated type II collagen expression by day 7 (Figure 5.5B). Type II collagen mRNA expression is upreguated significantly from day 1 to day 7 in all groups, indicating the chondrogenic lineage. By day 21, type II collagen expression is lower than day 7, but still significantly elevated over day 1. Matrix molecules have a simulative effect on Sox9 mRNA expression. On day 7 both experimental groups are significantly greater than control (CS group 4 fold, HA group 4.6 fold). On day 21, HA group is significantly great than control (3 fold increase). On day 21 the HA group is significantly elevated over the control with a 2 fold increase.

Type I collagen (Figure 5.5C) is a negative marker of the chondrocyte phenotype, and PRG4 (Figure 5.5D) is a marker for superficial zone chondrocytes. Increases in type I collagen throughout *in vitro* culture are often observed in primary chondrocytes, and reduction indicates a more stable chondrocyte phenotype. Here we see a clear trend in reduction of type I collagen mRNA in experimental groups. On day 1, the HA groups is 2 fold less than the control and the CS groups is 3.5 fold less than the control. On day 7 and 21 both experimental groups are statically similar and approximately 2.5 fold and 18 fold less than the control respectively. Trends in PRG4 mRNA expression (Figure 5.5D) show decreased expression with CS and HA presence. On day 7 the control group has significantly elevated expression (5 fold greater than HA and CS groups), and on days 1 and 21 the HA groups have significantly lower expressions, at 1.4 fold and 1.3 fold respectively. At every time point HA-alginate downregulates PRG4 mRNA expression.

Figures 5.6 and 5.7 show staining for cartilage matrix products by differentiating MSCs encapsulated in control and experimental alginates. Figure 5.6 shows staining by Alcian blue for sulfated proteoglycans and Figure 5.7 shows staining for collagen using both Masson's trichrome (Figure 5.7A) and Sirius red (Figure 5.7B). All stains show cell secretion of cartilage matrix products by days 14 and 21, further confirming differentiation down the chondrogenic lineage.

Finally, Figure 5.8 shows immunostaining for proteoglycan 4 on both days 14 (Figure 5.8A) and 21 (Figure 5.8B) of differentiating MSCs encapsulated in control and... experimental alginates. Results show negative control slides with only cell staining by the hematoxylin counter-stain, confirming all observed staining in other groups was antibody bound. On day 14 more intense staining in observed in both the HA-alginate

and CS-alginate groups compared to the control group, particularly around the encapsulated cell clusters. At day 21 similar results are observed, however the CS-alginate staining appears the most intense.

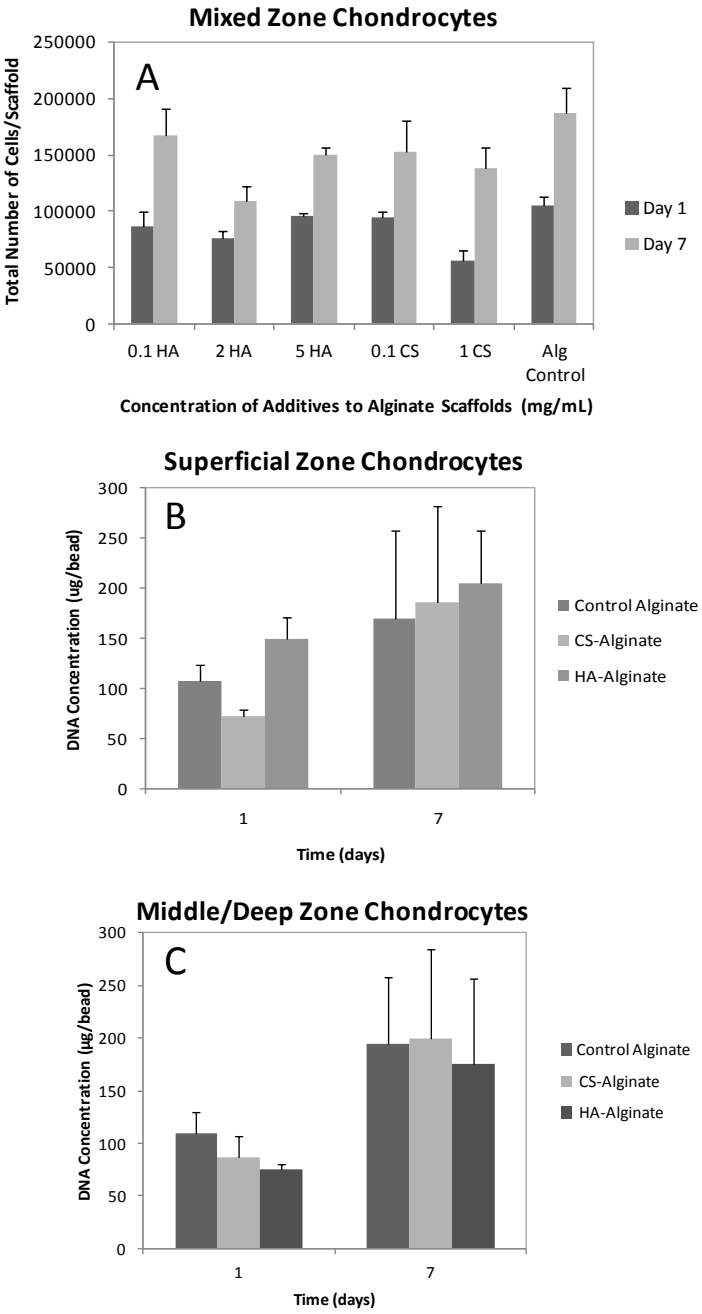


Figure 5.1. Proliferation by cell count per alginate scaffold via Trypan blue staining and hemacytometer count (A), and DNA concentration measured by picogreen assay (B,C). A) The cell population is of mixed zone chondrocytes. Proliferation is observed in all groups between days one and seven, and cell viability is high. There is no significant difference in proliferation rates between experimental and control groups. B) The cell population is superficial zone chondrocytes, and C) the cell population is middle/deep zone chondrocytes. A trend of increasing DNA per bead with culture time as cells proliferate is observed.

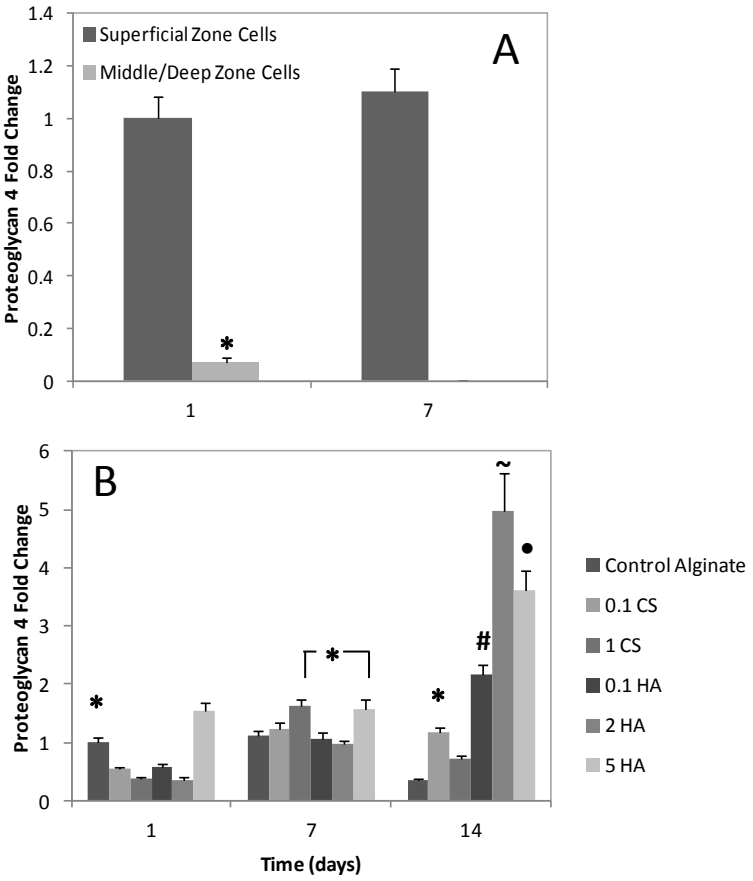


Figure 5.2. Expression of PRG4 mRNA by A) superficial zone and middle zone cells, and B) superficial zone cells only in experimental alginate groups. A) Superficial zone cells express significantly higher levels of PRG4 mRNA at days 1, and no measurable production by middle/deep zone cells is seen on day 7. Superficial day 1 is the calibrator. B) By day 14, a trend of increasing PRG4 expression with incorporated matrix molecules is observed. Control alginate day 1 is the calibrator. A symbol indicates a group is significantly different than other the group within the time point. Groups marked with the same symbol are statistically similar to each other, and different from others in the time point. Means and standard deviations are reported (n=3, α=0.05).

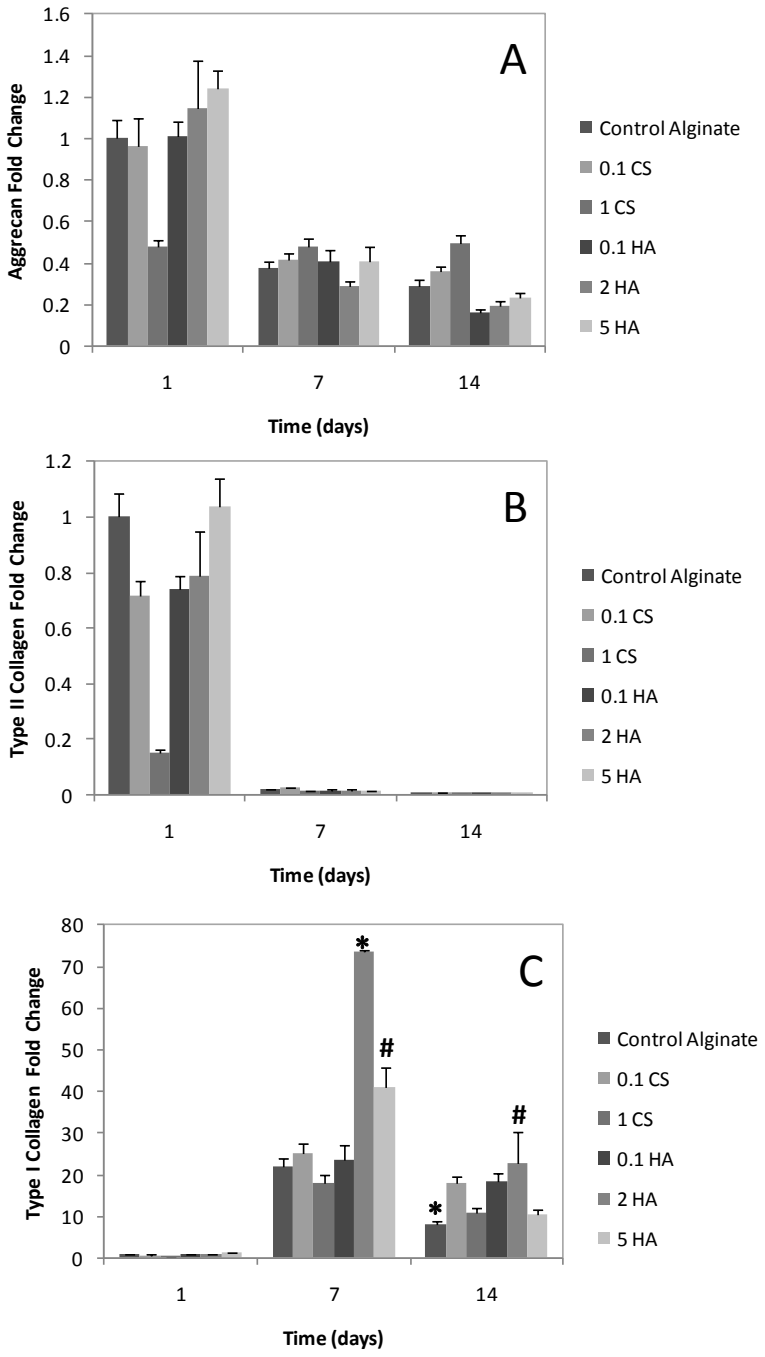


Figure 5.3. Expression of chondrocyte phenotype marker mRNA of superficial zone cells encapsulated in control alginate, CS-alginate, and HA-alginate. A) Aggrecan remains elevated, with slight increases in CS and HA-alginate groups on day 7, and CS-alginate groups on day 14. B) Type II collagen is downregulated significantly in all groups by day 7. C) Type I collagen increases with culture time. Control alginate day 1 is the calibrator. Star indicates a group is significantly different than all other groups in the time point. Means and standard deviations are reported (n=3, α=0.05).

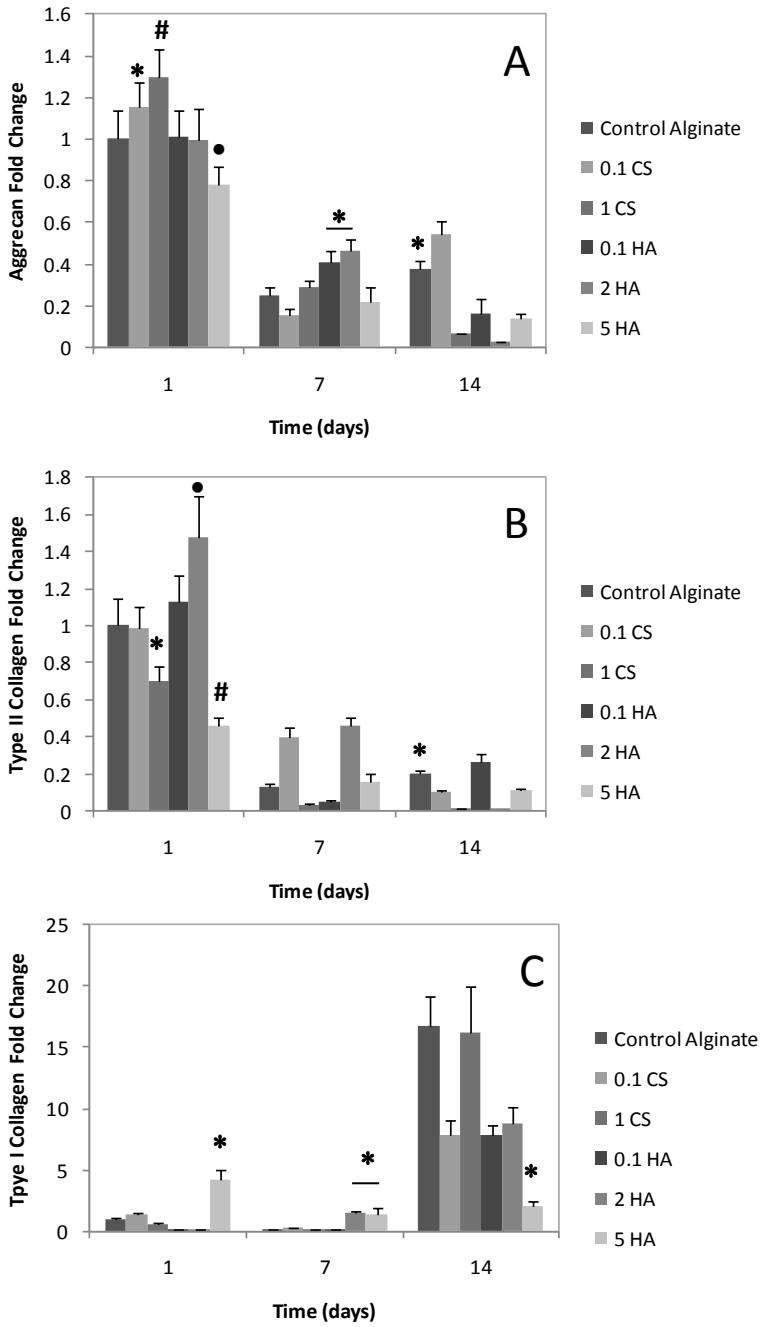


Figure 5.4. Expression of chondrocyte phenotype marker mRNA of middle/deep zone cells encapsulated in control alginate, CS-alginate, and HA-alginate. A) Aggrecan fold change, and B) type II collagen fold change. Varying concentrations of matrix molecules appear to upregulate expression profiles on days 7 and 14. C) Type I collagen increases with culture time. By day 14 experimental groups appear to have a stabilizing effect on chondrocyte phenotype, as observed by reduction in type I collagen expression. Control alginate day 1 is the calibrator. A symbol indicates a group is significantly different than all other groups in the time point, groups marked with the same symbol are statistically similar and different from others in the time point. Means and standard deviations are reported (n=3, α=0.05).

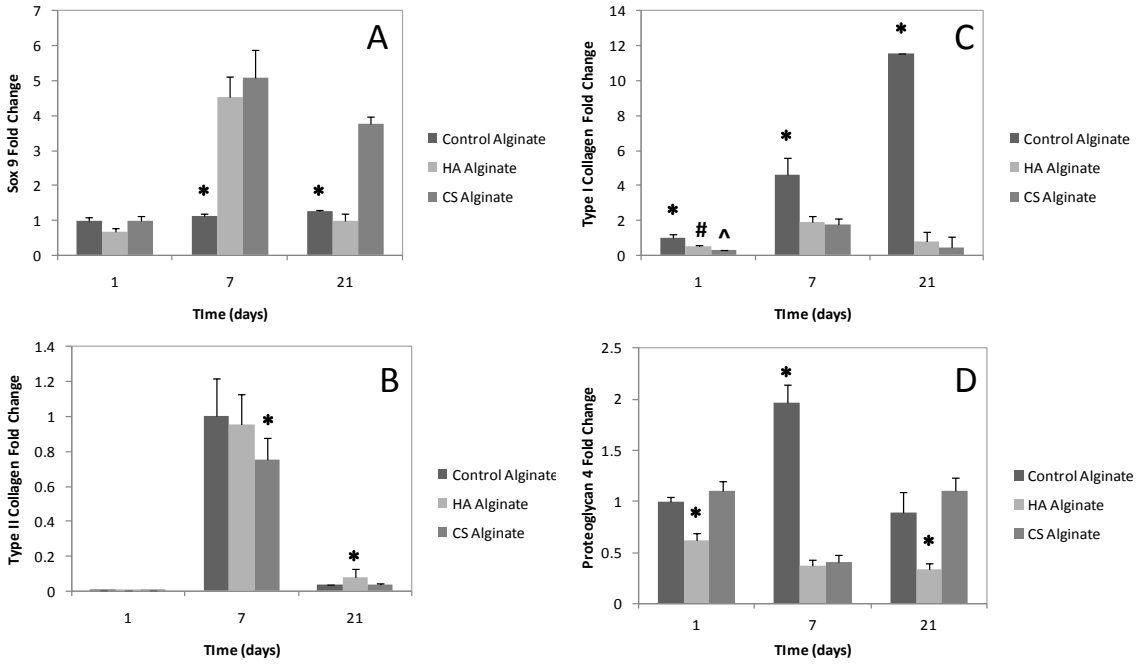


Figure 5.5. Expression of chondrogenic differentiation and phenotype markers by MSCs encapsulated in control alginate, 1 mg/mL CS-alginate, and 5 mg/mL HA-alginate. A) Experimental groups upregulate Sox9 expression on days 7 and 21, and type II collagen expression (B) on day 21. C) At all time points, experimental groups reduce type I collagen expression. D) At all time points HA-alginate significantly downregulates PRG4 expression. Control alginate day 1 is the calibrator. Star indicates a group is significantly different than all other groups in the time point. Means and standard deviations are reported (n=3, α=0.05).

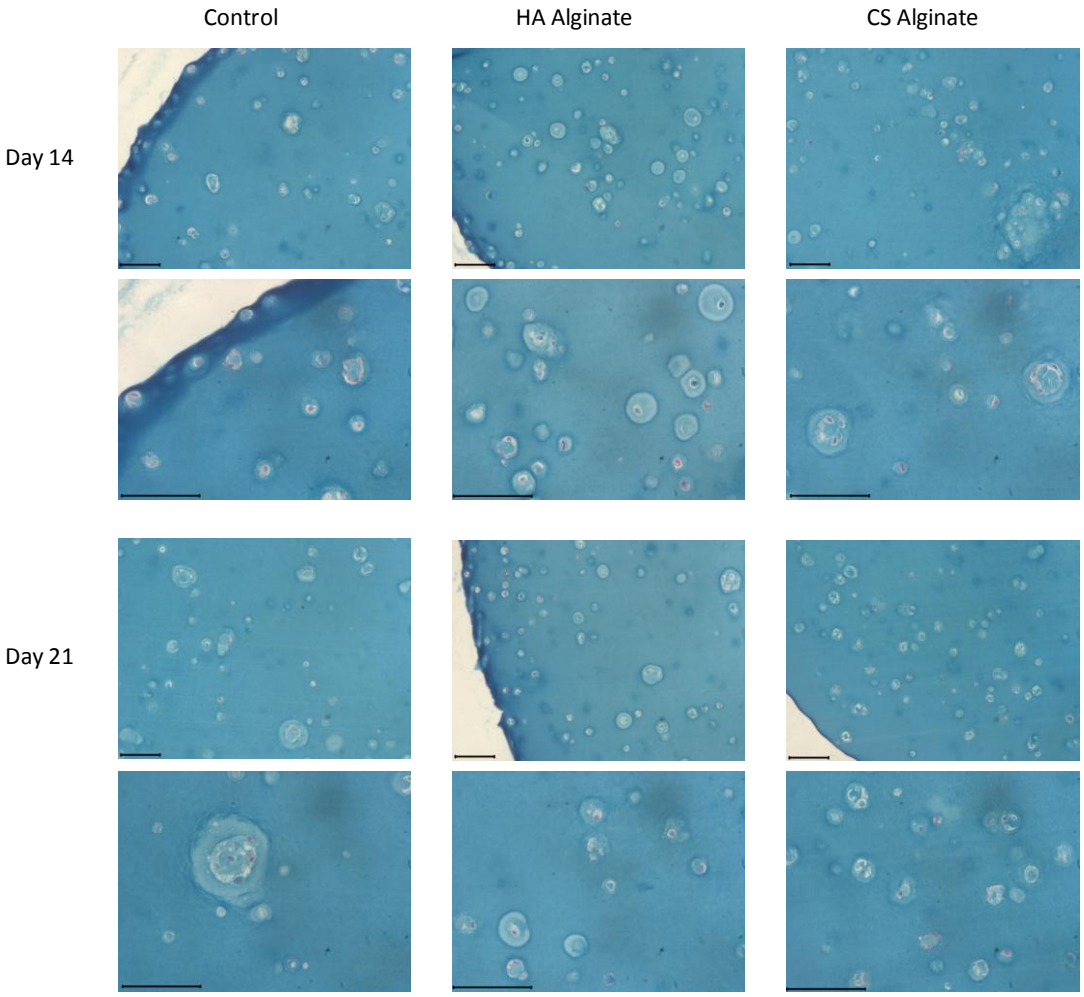


Figure 5.6. Histochemical staining of differentiating MSCs encapsulated in control, CS and HA alginate. Alcian blue stain for sulfated proteoglycans is shown. Cells appear as pink, cell nuclei darker pink, the alginate is stained a dark blue, and the sulfated proteoglycans secreted by the cells are stained lighter blue. Secreted molecules are observed around cells or cell cultures which have pushed back the surrounding. Scale bars 100μm.

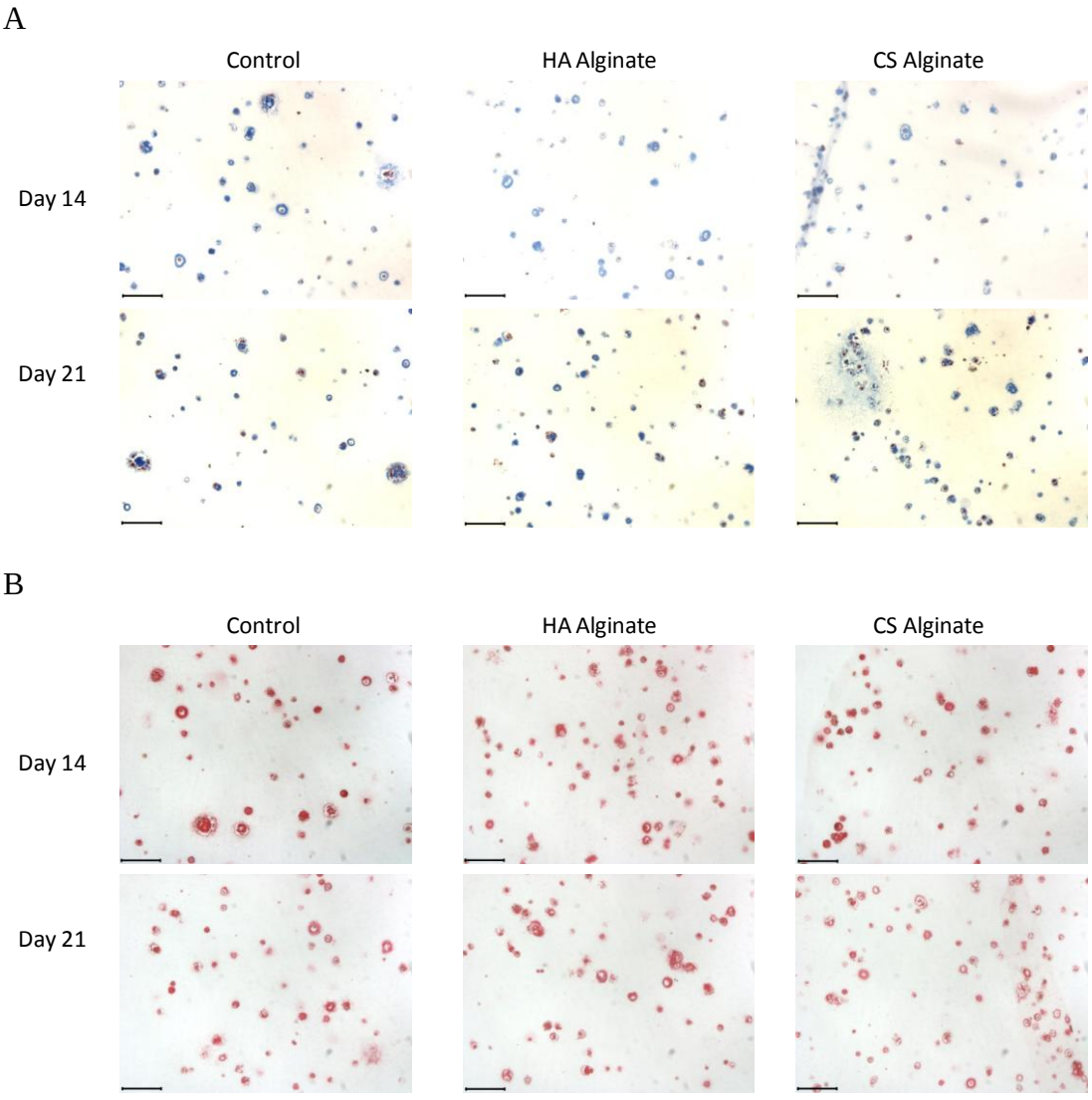
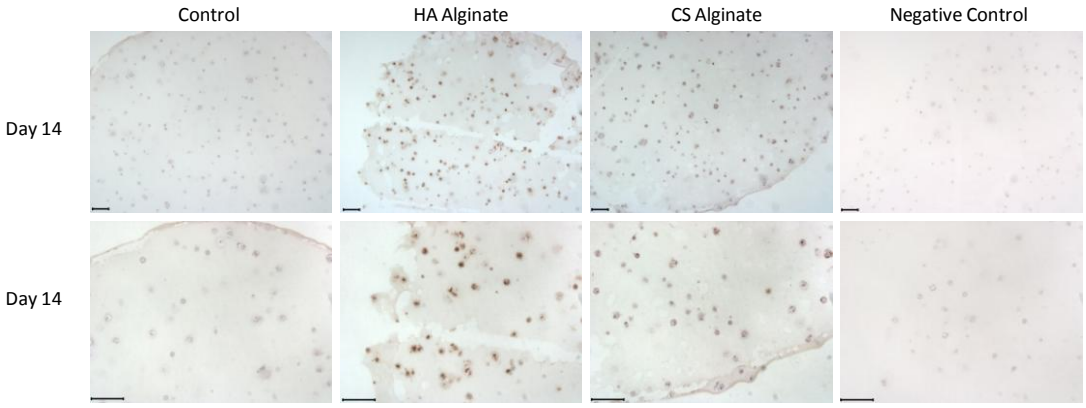


Figure 5.7. Histochemical staining of differentiating MSCs encapsulated in control, CS, and HA alginate. Both stains detect collagen. Figure 5.7A is Masson's trichrome stain (cells are stained dark violet and collagens are stained blue) and Figure 5.7B is Sirius red stain (cells are dark brown and collagen is red). Both stains show cell secretion of collagen. Scale bars 100μm.

A



B

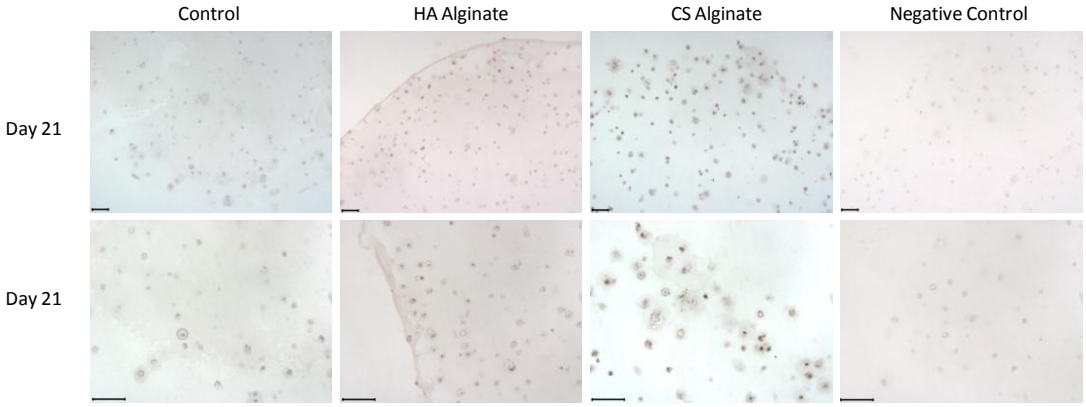


Figure 5.8. Immunostaining for proteoglycan 4 (PRG4) secreted by differentiating MSCs in control, CS, and HA alginate. Negative control group shows staining omitting primary antibody. Figure 5.8A shows day 14 images and Figure 5.8B shows day 21 images. PRG4-specific staining is observed, and appears most intense in HA and CS alginates. Scale bars 100μm.

5.4 Discussion

Proteoglycan 4 (PRG4), also called lubricin, [213] superficial zone protein, [171] megakaryocyte stimulating factor (MSF) precursor, [171] and camptodactyly-arthropathy-coxa vara-pericarditis (CAPC) protein [214] are homologous lubrication proteins which are encoded by the *PRG4* gene. Here we refer to these lubricating proteins as PRG4, recognizing that slight differences do exist. In the present study we confirm that alginate-encapsulated superficial zone chondrocytes produce PRG4 mRNA throughout the culture period, and present the novel finding that production can be upregulated by HA and CS. Middle/deep zone chondrocytes express limited PRG4 mRNA in culture, and none by day 7. Further, bone marrow derived MSCs express PRG4 mRNA during chondrogenic differentiation, and this expression is reduced by HA and CS. These findings have useful applications for regenerating functional superficial zone cartilage tissue and full thickness cartilage with zonal organization and cellular phenotype.

The protein encoded by the *PRG4 gene* plays a critical role in biochemical lubrication. [196] While boundary lubrication and the exact function of PRG4 are complex and not fully understood, it is known that PRG4 interacts with hyaluronic acid (HA) [215] both in synovial fluid [216] and on the surface of articular cartilage, [217] and that the complex significantly contributes to joint lubrication. [218, 219] The presented work examines the important relationship between HA and PRG4 mRNA and protein expression in the context of an engineered construct.

We demonstrate that both HA and CS can augment mRNA expression of PRG4 by superficial zone chondrocytes. By day 14, all experimental groups show greater

mRNA expression than the control alginate group. Peaks in mRNA expression are seen at 0.1 mg/mL CS and 2 mg/mL HA, indicating there is an optimum concentration for this upregulation which is below the maximum concentrations of 1 mg/mL CS and 5 mg/mL HA. Increased cell adhesion via HA and CS may contribute to changes in cell mRNA expression. Chondrocytes bind to HA via the CD44 cell surface receptor, and previous results from our laboratory confirm upregulation of CD44 mRNA and protein production by chondrocytes encapsulated in alginate containing HA. [55]

We also hypothesize that the PRG4 product will complex with encapsulated HA, and that some PRG4 will diffuse from the hydrogels into the culture media, a finding previously reported the literature. [203] A limitation of the presented study is that the amount of PRG4 lost to the media was not quantified. Enhancing the ability of the superficial zone to both produce and localize lubricating proteins may aid in reducing friction and help repair tissue function following injury or disease. Previous studies have indentified the ability of an engineered construct to localize PRG4 directly correlates with lubrication. [203] Therefore, identifying constructs which can retain the produced PRG4 will be critical for successful lubrication strategies.

Despite its critical importance in lubrication and cartilage's frictional properties, there is limited research investigating PRG4 production, regulation, function, and role in tissue engineering constructs. TGF-β's have been shown to stimulate PRG4 production in chondrocytes, [170, 171, 220, 221] as well as infrapateller fat pad progenitor cells. [222] MSCs in alginate culture secrete PRG4, [203] however the factors which can stimulate or regulate this production are unknown. The ability of TGF-βs to stimulate

PRG4 production may be linked to their role in intrinsic cartilage repair mechanisms, as they are found to be elevated in both injured and arthritic cartilage.

We show here that bone marrow MSCs express PRG4 in the presence of media containing TGF-β3. This stimulation can be further modified by incorporation of either HA or CS to our alginate scaffolds. We observed a significant decrease in PRG4 mRNA expression in HA-alginate groups at all time points, and a significant decrease in the CS-alginate group on day 7. As PRG4 is known to readily diffuse from hydrogels into solution, we speculate that entrapment of the protein by either HA or CS triggered the encapsulated cell population to reduce PRG4 production. HA and PRG4 interaction is highly likely, as this occurs in native tissue. Immunostaining results support this hypothesis, as more intense antibody-specific staining is observed in both HA and CS-alginate groups.

In addition to affecting superficial zone phenotype, HA and CS also influenced MSC chondrogenesis. We saw a significant reduction in type I collagen mRNA expression in both HA and CS groups throughout the study. These findings are consistent with others which indicate that during TGF-β induced MSC chondrogenesis additional signals such as growth factors, [223] or encapsulated matrix molecules [75], are necessary to reduce type I collagen expression. HA and CS groups also have elevated Sox9 mRNA expression throughout the study, again showing a beneficial effect on chondrogenesis. The large-scale use of HA and CS is considerably more economic than use of growth factors, and therefore may prove a powerful tool for stabilizing differentiating chondrocytes.

Interestingly, the inverse effect is observed in terms of PRG4 mRNA expression between MSCs and superficial zone chondrocyte populations. As mentioned, inclusion of the molecules generally increases mRNA expression in superficial populations and decreases expression in MSC populations. If the cause of reduced gene expression in the MSCs is due to entrapped protein presence, it is unclear why superficial cells respond differently. However, as distinct in their expression of PRG4 in normal cartilage tissue these cells may respond to matrix signals differently than their middle/deep counterparts or a progenitor population. Significant variation between chondrocyte and MSC PRG4 production in alginate cultures has previously been reported. [203]

Additional differences between cell populations include those observed between the superficial and middle/deep zone cultures. Most notably, PRG4 mRNA expression is significantly lower at day one in middle/deep zone cells compared to superficial zone cells, and by day 7 undetectable (Figure 5.2). This trend is consistent with reports in the literature of PRG4 production by zonal populations over culture time. [136] Aggrecan mRNA follows roughly the same expression trend in superficial and middle zone cultures, however, differences are observed in type I and type II collagen mRNA levels between populations. As seen in Figure 5.3, at day 7 there is a sharp decrease in superficial zone chondrocyte expression of type II collage mRNA, which correlates with a share increase in type I collagen expression. Comparing to Figure 5.4, middle and deep zone chondrocytes express higher levels of type II collagen mRNA throughout culture, and the sharp rise in type I collagen mRNA expression is not seen until day 14. As type I collagen is a negative marker for chondrocyte phenotype and type II collagen is a positive marker, these results indicate middle/deep zone cells have more robust phenotype

retention in hydrogel culture than superficial zone cells. These results are also consistent with reports of elevated mRNA and production of cartilage matrix markers in middle and deep zone cells versus superficial zone cells in three dimensional culture. [164, 198]

Diffusion, or degradation followed by diffusion, of the entrapped CS and HA particles out of the alginate hydrogels during culture is a possibility, as they are not covalently bonded to the alginate backbone. Changes in HA and CS concentration over time could account for changes in mRNA expression and PRG4 localization observed throughout time points. Staining for PRG4 appears most intense in the middle of the study for the HA group, at day 14 (Figure 5.8), but by day 21 has decreased and appears similar to the control. A reduction in the concentration of HA available for PRG4 interaction could play a role in this observed change. Conversely, staining for PRG4 appears the most intense in the CS group on day 21, suggesting differences between the entrapped HA and CS. Diffusion of acidic proteins out of alginate hydrogels has been reported as inversely proportional to their molecular weight. Acidic proteins are thought to minimally interact with the alginate matrix, and molecules which do interact with alginate would have longer diffusion times. [224] Due to its large size HA is unlikely to readily diffuse, however enzymatic degradation or breakdown of the alginate matrix over time would increase diffusivity. A similar study with a poly(ethylene glycol) diacryate polymer backbone utilized a similar method to successfully fabricate PEGDA-HA semi-interpenetrating networks. [164] Based on size, diffusion potential of CS is much higher. The high net negative charge of the molecule and interaction with cells in native tissue also make interactions with cells, cell secreted products, or ions in the hydrogel likely as well. [225]

5.5 Conclusion

In conclusion, superficial zone chondrocyte expression of PRG4 mRNA is significantly enhanced through addition of both HA and CS to alginate scaffolds. Conversely, PRG4 expression is downregulated by CS and HA in differentiating MSCs, possibly due to build up of entrapped protein as PRG4 complexes with HA or becomes entangled by CS. HA and CS induce favorable effects on chondrogenesis though upregulation of transcription factor Sox9, and downregulation of type I collagen. Taken together, these results indicate that HA and CS incorporation to alginate scaffolds can aid in production of critical lubricating protein mRNA, stabilize differentiating MSCs, and sequester lubricating proteins within the scaffold.

6 Zonal Cartilage and Mesenchymal Stem Cell Coculture and Conditioned Media Delivery

6.1 Introduction

Important difference exists between the zones of articular cartilage. It is becoming clear that the superficial zone is distinct from the middle and deep zones of the tissue both in matrix composition and cellular function. Here cells are smaller and more densely packed than in other zones, and are aligned parallel to the articulating surface. Their activity is directly involved in lubrication at the cartilage surface. Superficial zone chondrocytes are the only zonal population to secrete elevated levels of proteoglycan-4, [133, 135] a protein which can complex with hyaluronic acid, [215] and is a critical component of joint lubrication. [219]

Due to the important functional differences between cartilage zones, recent attention has focused on regeneration of stratified, zonally organized repair tissue. [204, 226, 227] However, cell source remains a major challenge for zonal cartilage engineering efforts. Bone marrow derived mesenchymal stem cells (MSCs) are a popular potential treatment population for articular cartilage defects. Typically, MSCs are cultured in a micromass or hydrogel and delivered chondrogenic media supplemented with transforming-growth factor βs (TGF-βs) and dexamethasone to induce chondrogenesis. Methods for inducing zone-specific chondryctes are yet to be established, but growth factor delivery [222] and cues from the scaffold environment [227] may aid in guiding progenitor populations to zone-specific cells.

Here we investigate a novel method of inducing zone-specific chondrocytes from MSCs; soluble signals derived from zonal cartilage tissue explants. While coculture of

these two populations has been reported, results have been mixed, and there are no reports of zonal coculture models. A clear trend has not been established on the exact influence or the mechanisms of influence between these two cell populations. Some studies report that chondrocyte secreted factors alone can influence the differentiation of MSCs. For example, it was reported that soluble secreted factors from mature cartilage explants were able to upregulate Sox9 gene expression and type II collagen synthesis in MSCs. However, type II collagen gene expression was not upregulated in the MSC population and the authors indicate the possibility that the increase in collagen synthesis could have been from the cartilage explants themselves. Cartilage explants were shown to secret TGF-β for up to 14 days, and upregulation of VEGF-164α, MMP-13, TIMP-1, and TIMP-2 was detected in the culture media, but it is not known which cell population they originated from. [228] Additional studies report that soluable factors derived from chondrocytes were able to upregulate chondrogenic markers and matrix production by MSCs, [229, 230] and reduce hypertrophic markers in MCSs. [228, 231] Growth factors such as IGF-1, BMPs, and TGF-βs were detected in chondrocyte conditioned media and indentified as necessary for the upregulation of type II collagen gene and protein expression. [229] Secreted factors from osteoarthritic chondrocytes were also able to induce chondrogenesis in MSCs and downregulate markers of hypertrophy. [232] However, there are also studies which report direct contact culture of MSCs and chondrocytes resulted in MSC differentiation, but secreted factors from chondrocytes did not. [233, 234]

Conflicting results are also reported on the effects of MSCs and chondrocytes cultured together in micromass or in hydrogels. Some studies report a direct benefit of

coculture on MSC chondrogeneis. [233-236] However, from these studies it is not always clear which population is upregulated in gene and protein expression of chondrogenic markers. Several studies which have cultured MSCs and chondrocytes of different species together noted that while there was upregulation of matrix products and gene markers in the recovered cell population, that these were the result of chondrocyte – not MSC activity. [237, 238] Similarly, micromass coculture of human MSCs and chondrocytes showed increases in matrix accumulation was likely from chondrocytes, and that the MSCs which did undergo chondrogenesis also underwent hypertrophy. [239] While the two cell populations definitely influence each other, from current reports it is unclear if MSCs are primarily responsible for upregulating the chondrocyte phenotype in the chondrocyte population, or if chondrocytes are primarily responsible for inducing chondrogenesis in the MSC population – or both. Also unclear, is the extent to which MSCs undergo hypertrophy in coculture models, and if chondrocyte secreted factors alone can guide MSC behavior.

In the presented work we aim to establish the potential of zonal cartilage-derived soluble factors to drive zonal differentiation of MSCs. We investigate both a coculture model and a conditioned media model to assess the impact of communication between cell populations. Due to the distinct phenotype and function of superficial zone chondrocytes, we hypothesize signals derived from this group will have a unique effect on the differentiation of MSCs. The goal of the presented study is to identify culture conditions which can promote chondrogeneis of zone-specific chondrocyte populations.

6.2 Materials and Methods

6.2.1 Superficial and Middle/ Deep Zone Explant Section Isolation

Zonal chondrocyte explants were isolated according to a previously published laboratory protocol. [198] Briefly, cartilage plugs (4 mm diameter, 3-6 mm height) were harvested from the femoral condyles of 20 week old calves using a Sklar Tru-Punch disposable biopsy punch (Sklar Instruments, West Chester, PA). The top 10% (0.3 - 0.6 mm) was taken as the superficial zone, and remaining tissue up was defined as the middle and deep tissue zones. Any subchondral bone was removed, samples were minced, rinsed twice in Dulbecco's Modified Eagle Medium/Nutrient Mixtures F-12 Ham (DMEM/F12) media (Gibco/Invitrogen, Carlsbad, CA), and placed in a six well plate for coculture.

6.2.2 Bone Marrow Stromal Cell Isolation

Primary bovine bone marrow tissue was harvested from the tibia 3 week old calves. The tissue was suspended in growth media (Minimal Essential Medium α, (Gibco/Invitrogen) supplemented with 0.1% penicillin/streptomycin antibiotics and 0.2 mM of ascorbic acid), filtered through a 70 μm mesh, and centrifuged to isolate the cell population. This population was then enriched for mesenchymal stem cells (MSCs) via plastic adhesion by plating in monolayer and culture in growth media with 10% fetal bovine serum. After two passages cells were trypsinized (Gibco/Invitrogen) and counted using Trypan blue staining and a hemacytometer.

6.2.3 Cell Encapsulation and Coculture

Alginate solution was mixed with isolated MSCs and injected through an 18-gauge syringe into continuously stirred 0.1 M calcium chloride ($CaCl_2$) (Sigma-Aldrich). The resulting cellular density was approximately 100,000 per bead, and each spherical bead had a diameter of approximately 5 mm (approximately 2 x 10^6 cells\mL). Control beads were cultured in serum-free chondrogenic media made of; high glucose αMEM (Gibco/Invitrogen) + 110 µg/mL sodium pyruvate, 40 µg/mL proline, 50 µg/mL ascorbate 2-phosphate, 0.1 µM dexamethasone, 1% ITS +premix (BD Biosciences, Bedford,MA), and 10ng/mL TGF-β3 treatment (R&D systems, Minneapolis, MN). Coculture groups were set up with either superficial or middle/deep explants chips in the bottom of a six well plate, a transwell membrane placed in the well, and alginate beads containing MSCs on top of the membrane. See Figure 6.1 for illustration of coculture experimental set up. Each group cocultured with superficial or middle/deep explants chips was delivered chondrogenic media with or without 10ng/mL TGF-β3. At days 1, 7, and 21 MSCs were isolated from the alginate beads by delivery of 0.1 M EDTA for 20 min at 37°C. The solution was then centrifuged to form a cell pellet, which was resuspended in phosphate-buffered saline (PBS) and used for RNA extraction. At days 7 and 21 beads were fixed for staining.

6.2.4 Conditioned Media Incubation and Delivery

To control for communicate between cell populations a conditioned media study was setup with the same experimental groups. In this setup, zonal explants chips and alginate encapsulated MSCs were cultured in separate wells. Growth factor free

chondrogenic media was delivered to zonal explant chips and allowed to incubate for 48 hours, after which the media was collected, relevant groups were supplemented with TGF-β3, and it was delivered to alginate encapsulated MSCs. See Figure 6.2 for illustration of conditioned media experimental set up. At days 1, 7, and 21 cells were isolated for RNA extraction, and at days 7 and 21 beads were fixed for staining.

6.2.5 Histological Preparation

At desired time points, alginate beads were recovered and fixed for 3 hours at room temperature in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) with 0.1 M sodium cacodylate and 10 mM calcium chloride. Samples were then washed for 24 hr at room temperature in 0.1 M sodium cacodylate and 10 mM calcium chloride. Following washing, samples were placed in histological cassettes and dehydrated through a series of ethanol washes followed by two Citrisolv (Fisher Scientific) washes. The samples were embedded in paraffin (Paraplat X-tra, Fisher Scientific) and cut into 4 µm sections and mounted on a glass slide (Superfrost, Fisher Scientific).

6.2.6 Histochemical Staining

Samples were dried at 64°C for two hours, deparaffinized using Citrisolv, and rehydrated. The samples were then rinsed in distilled water and stained using Alcian blue and Sirius red staining solutions (Poly Scientific, Bay Shore, NY). All samples were viewed under an Axiovert 40CFL light optical microscope (Zeiss, Thornwood, NY) and images were captured using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI) imaging software.

6.2.7 Immunohistochemistry

Samples were dried at 64°C for one hour, deparaffinized using Citrisolv and rehydrated. Samples were antigen retrieved using a Tris base and EDTA buffer (pH 8) containing TWEEN 20 steamed for 15 minutes in a vegetable steamer. Samples were incubated with PEROXI*DAZED1* (Biocare, Concord, CA), an endogenous peroxidase blocker, and Background*SNIPER1* (Biocare), a blocking reagent. Samples were then stained with primary antibody to detect PRG4 and type II collagen. The primary antibodies used were anti-lubricin/PRG4 (rabbit polyclonal antibody, ab28484; Abcam, Cambridge, MA) diluted to a working concentration of 4 µg/mL, and anti-collagen II (ab300, Abcam) diluted to a working concentration of 5 µg/mL . The HISTOSTAIN®-SP kit (Zymed, San Francisco, CA) was used to visualize PRG4 presence by using the horseradish peroxidase (HRP)-streptavidin-biotin system. The complex formation was then detected by a 3,3'-diaminobenzidine tetrahydrochloride (DAB) chromogen. Samples were counterstained with hematoxylin, dehydrated, cleared in Citrisolv, and covered. Negative control slides were stained using the same protocol, omitting the primary antibody.

6.2.8 RNA Isolation

Following isolation of MSCs from alginate beads, RNA was isolated using the RNeasy Mini Kit (QIAGEN). Total RNA was eluted into 30 µL of RNase free water and detected using a NanoDrop ND-1000 Spectophotometer (NanoDrop Technologies, Wilmington, DE). RNA concentrations at 1, 7, and 21 days were diluted to approximately 10 ng/µL.

6.2.9 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Isolated RNA was reverse transcribed using a cDNA Archive Kit (Applied Biosystems, Foster City, CA), which can convert up to 10μg of RNA to cDNA. cDNA was mixed with Universal Master Mix (Applied Biosystems) and oligonucleotide primers and Taqman probes (Applied Biosystems) for the genes of interest as well as a control gene. Table 6.1 shows the sequences for all forward primers, reverse primers, and probes used. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was the endogenous control gene. The reaction volume was 20 μl, and the final concentration of cDNA per reaction well was approximately 0.5 ng/μL (10 ng per well). The reaction was conducted on a 7900HT Fast Real-Time PCR System Prism 7000 sequence detector (Applied Biosystems). The thermal profile followed was 2 min at 50°C, 10 min at 95°C, 40 cycles of 15s at 95°C, and 1 min at 60°C. Gene expressions were analyzed using the comparative C_t method. The day one control alginate samples were used as calibrators in all analysis. Fold changes in gene expression were calculated and are reported as the mean RQ values with associated standard deviations (n=3), in accordance with methods previously described by our laboratory. [55, 212]

6.2.10 Statistical Analysis

Each experiment was performed in triplicate (n=3). All data was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test to determine statistical differences. A confidence interval of 95% ($\alpha = 0.05$) was used for all analysis and means and standard deviations are shown on each figure.

Table 6.1. Forward primer, reverse primer, and probe sequences used for GAPDH, Type II Collagen, Type I Collagen, Aggrecan, Sox9, and Superficial Zone Protein (SZP) used for qRT-PCR.

Primer and Probe Sequences used for qRT-PCR

Protein		Sequence
GAPDH	Forward Primer	TGCCGCCTGGAGAAACC
	Reverse Primer	CGCCTGCTTCACCACCTT
	Probe	CCAAGTATGATGAGATCAA
COL2A1	Forward Primer	CGGGCTGAGGGCAACA
	Reverse Primer	CGTGCAGCCATCCTTCAGA
	Probe	CAGGTTCACATATACCG
COL1A1	Forward Primer	AGAACCCAGCTCGCACATG
	Reverse Primer	CAGTAGTAACCACTGCTCCATTCTG
	Probe	AGACTTGAGACTCAGCC
AGC	Forward Primer	GGGAGGAGACGACTGCAATC
	Reverse Primer	CCCATTCCGTCTTGTTTTCTG
	Probe	CAGGCTTCACCGTTGAG
SOX9	Forward Primer	AACGCCGAGCTCAGCAAG
	Reverse Primer	ACGAACGGCCGCTTCTC
	Probe	TTCAGCAGTCTCCAGAGCTTGCCCA
PRG4	Forward Primer	GAGCAGACCTGAATCCGTGTATT
	Reverse Primer	GGTGGGTTCCTGTTTGTAAGTGTA
	Probe	CTGAACGCTGCCACCTCTCTTGAAA

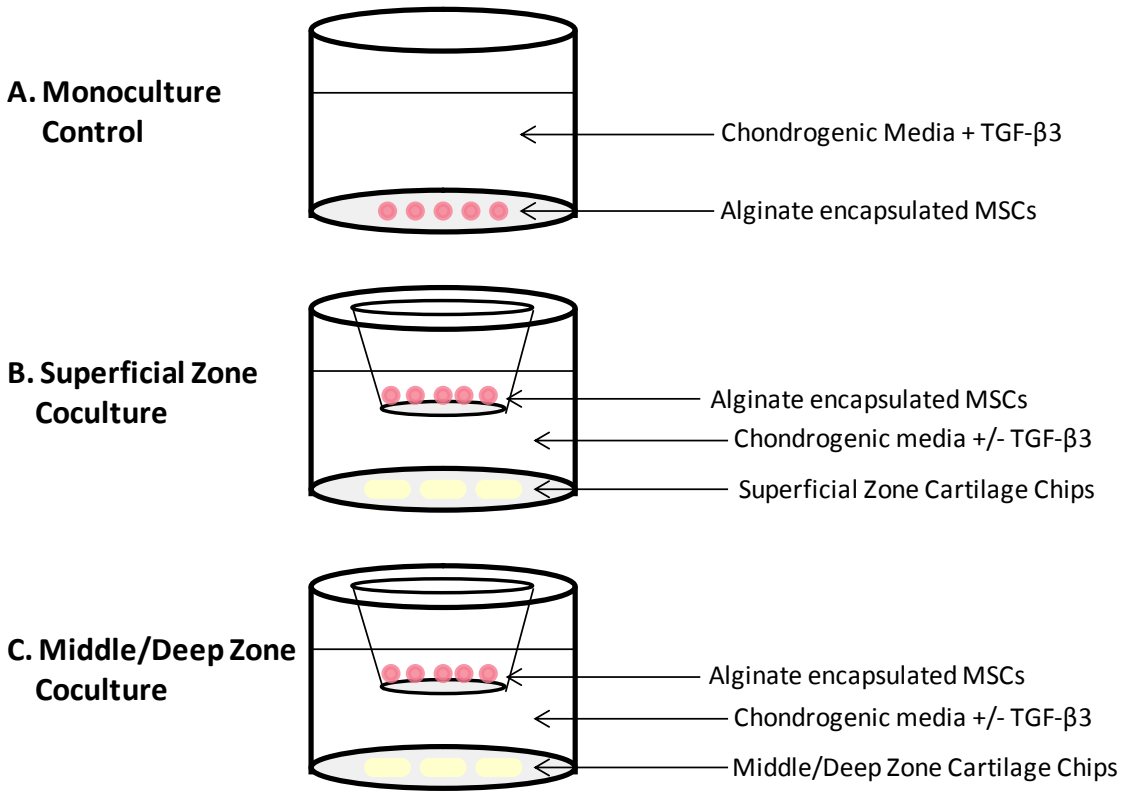


Figure 6.1. Schematic of coculture experimental set up. A) Monoculture MSC control, B) superficial zone coculture, and C) middle/deep zone coculture. Throughout the groups are referred to as control, S+, S-, M+, or M-. S/M denotes superficial or middle/deep zone coulture and +/- denotes with or without TGF-β3 supplemented media.

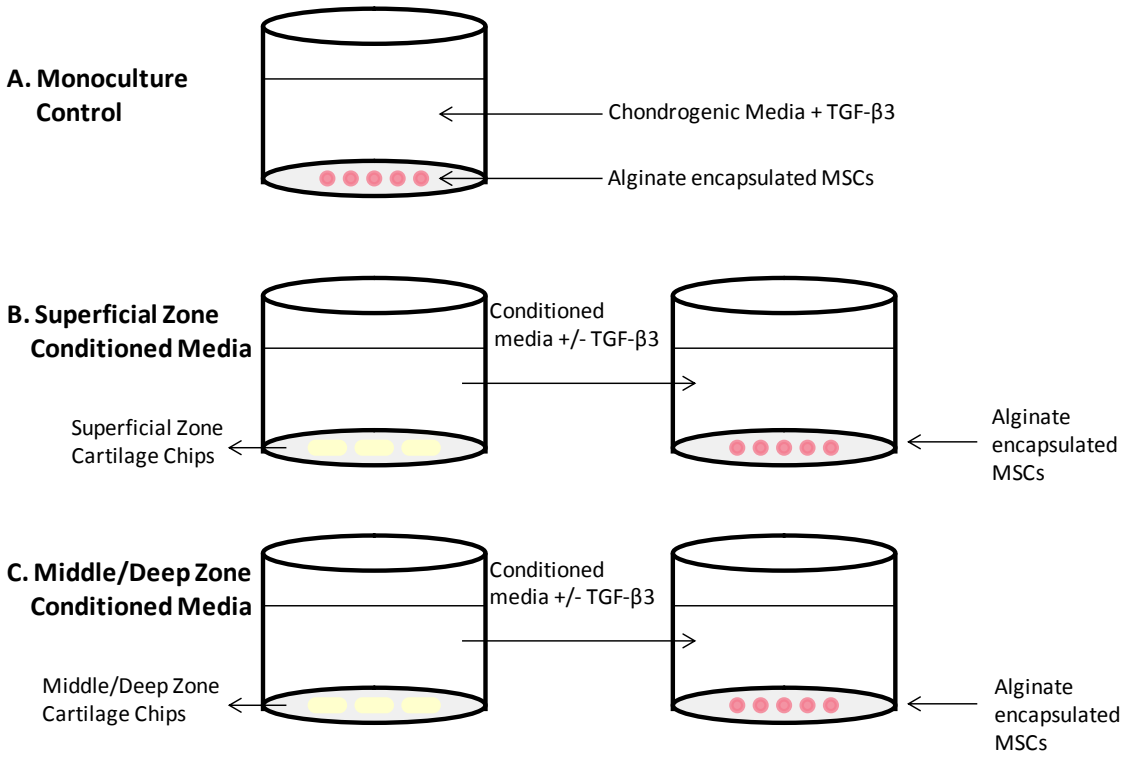


Figure 6.2. Schematic of the conditioned media experimental set up. A) Monoculture MSC control, B) superficial zone conditioned media, and c) middle/deep zone conditioned media. Throughout the groups are referred to as control, S+, S-, M+, or M-. S/M denotes superficial or middle/deep zone conditioned media and +/- denotes with or without TGF-β3 supplemented media.

6.3 Results

6.3.1 Zonal Coculture Differentiation of MSCs: Gene Expression

Figure 6.3 shows mRNA expression for chondrogenic markers during coculture chondrogenesis. Sox9, a transcription factor which regulates chondrogenesis, is expressed throughout the culture period by all groups, as seen in Figure 6.3A. At day 1 expression is similar among all groups, and at day 7 all groups are upregulated over day 1, with the middle/deep zone + TGFβ (M+) coculture group upregulated 1.4 fold over the control group. By day 21 a shift in pattern in observed. The control group remains elevated, however all experimental groups other than superficial zone – TGF-β3 (S-) are significantly downregulated compared to the control. The S- has the highest Sox9 mRNA expression, upregulated at 3.4 fold over the control.

Corresponding to this Sox9 upregulation in the S- group at day 21 is significant upregulation in chondrocyte phenotype markers. Type II collagen and aggrecan are major matrix components of articular cartilage and thus mark the chondrogenic lineage. Proteoglycan 4, unique to the superficial zone, marks the phenotype of these cells. In the S- group on day 21 type II collagen is upregulated 11.4 fold over the control (Figure 6.3B), aggrecan 5.6 fold over the control (Figure 6.3C), and proteoglycan 4 1.75 fold over the control (Figure 6.3E) - thus indicating that interactions between MSCs and superficial zone explants have a favorable effect on chondrogenic differentiation.

Superficial zone coculture groups at day 7 are also upreguated over control groups, again indicating that interactions between superficial zone explants and MSCs is beneficial. In the S+ group, Sox9 expression is upregulated 1.13 fold (not significant), type II collagen 50 fold (significant), and aggrecan 1.4 fold (significant) compared to

control groups on day 7. In the S-, type II collagen is significantly upregulated 1.6 fold over the control.

Type I collagen and proteoglycan 4 are markers for chondrocyte, and superficial zone chondrocyte phenotype respectively. At earlier time points (days 1 and 7) groups with TGF-β3 delivery show upregulated proteoglycan 4 expression, indicating the growth factor is playing a role in its expression. However, by day 21 the only group with elevated expression over the control is the S- group, indicating that exogenous TGF-β3 delivery is no longer as effective, or some influence of the coculture system has come into play. Type I collagen, a negative phenotype marker, is significantly elevated in all experimental groups on days 7 and 21, other than the S- group on day 21 for which it is significantly downregulated (1.14 fold compared to the control group), again indicating a favorable effect of the S- coculture group on chondrogenesis.

6.3.2 Zonal Coculture Differentiation of MSCs: Protein Production

Figures 6.4 and 6.5 show staining for extracellular matrix components at days 7 and 21 by alginate encapsulated MSCs in control and experimental groups. Results confirm gene expression data and indicate that coculture can cause upregulation of chondrogenic protein expression even in the absence of exogenous TGF-β3 delivery. Figure 6.4A shows staining by Alcian blue for sulfated proteoglycans. Here alginate is stained dark blue, secreted proteoglycans light blue, and cells pink. Increases in secreted proteoglycan staining are seen on day 21 in all groups. Figure 6.4B shows staining by Sirius red for collagens, again showing increases in staining from days 7 to day 21. Major differences between groups are not clear from histological staining, but both

Alcian blue and Sirius red appear to have more staining clusters in the control, M+, and M- groups compared to both the S+ and S- groups on day 21.

Figure 6.5A shows immunostaining for type II collagen and Figure 6.5B immunostaining for proteoglycan 4. Again, the only differences observed for type II collagen are in M+ and M- groups with some elevation in staining intensity. No apparent difference is observed between time points for proteoglycan 4 expression, perhaps due to the diffusion of protein and lack of accumulation within the hydrogel.

6.3.3 Conditioned Media Differentiation of MSCs

Gene and protein expression data demonstrate significantly less robust chondrogenesis in all experimental conditioned media groups as compared to a monoculture, TGF-β3 delivered control. This indicates the chondrogenic differentiation in experimental groups in the coculture model is dependent on some form of communication between the mature chondrocyte and MSC populations.

6.3.4 Conditioned Media Differentiation of MSCs: Gene Expression

Figure 6.6 shows chondrogenic mRNA markers over the course of the conditioned media study. All experimental groups are downregulated compared to a control delivered chondrogenic media supplemented with TGF-β3. There is decreased in Sox9 mRNA expression in all experimental groups at all time points compared to the control, and at day 21 the control is significantly greater than all experimental groups. There is also a trend of decreased Sox9 expression between groups which did not received TGF-β3 delivery. However, even in experimental groups supplemented with

TGF-β3 delivery Sox9 expression levels are still not restored to control values. Following the same trend, the expression of type II collagen is significantly reduced in all experiment groups as compared to the control at days 7 and 21, indicating a lack of the chondrocyte phenotype. At both later time points there are no significant differences between type II collagen experimental groups and the control is on average 236 fold and 47 fold greater than the experimental groups on days 7 and 21 respectively. Aggrecan expression follows the same trend, however, with a less dramatic decline in experimental groups.

Differences between coculture and conditioned media experiments are also observed in phenotype markers proteoglycan 4 and type I collagen expression, as seen in Figure 6.6D and 6.6E. On day one experimental groups with TGF-β3 delivery express similar or greater, levels of proteoglycan 4 mRNA. However, by days 7 and 21 proteoglycan 4 expression is reduced or not detected. In general type I collagen expression is low across experimental groups, with the exception of the superficial conditioned + TGF-β3 group at day 21.

6.3.5 Conditioned Media Differentiation of MSCs: Protein Production

Protein data supports the gene expression data and shows very little accumulation of cartilaginous matrix products – indicating lack of robust and sustained differentiation. Figure 6.7 shows histology staining for Alcain blue (6.7A) and Sirius red (6.7B), and very few differences are observed between days 7 and 21. The only group other than the control to show clusters of matrix accumulation is the middle/deep zone conditioned + TGF-β3 group. Figure 6.8 shows immunostaining for type II collagen (6.8A) and

proteoglycan 4 (6.8B), again very little matrix accumulation is seen and no observable differences between time points.

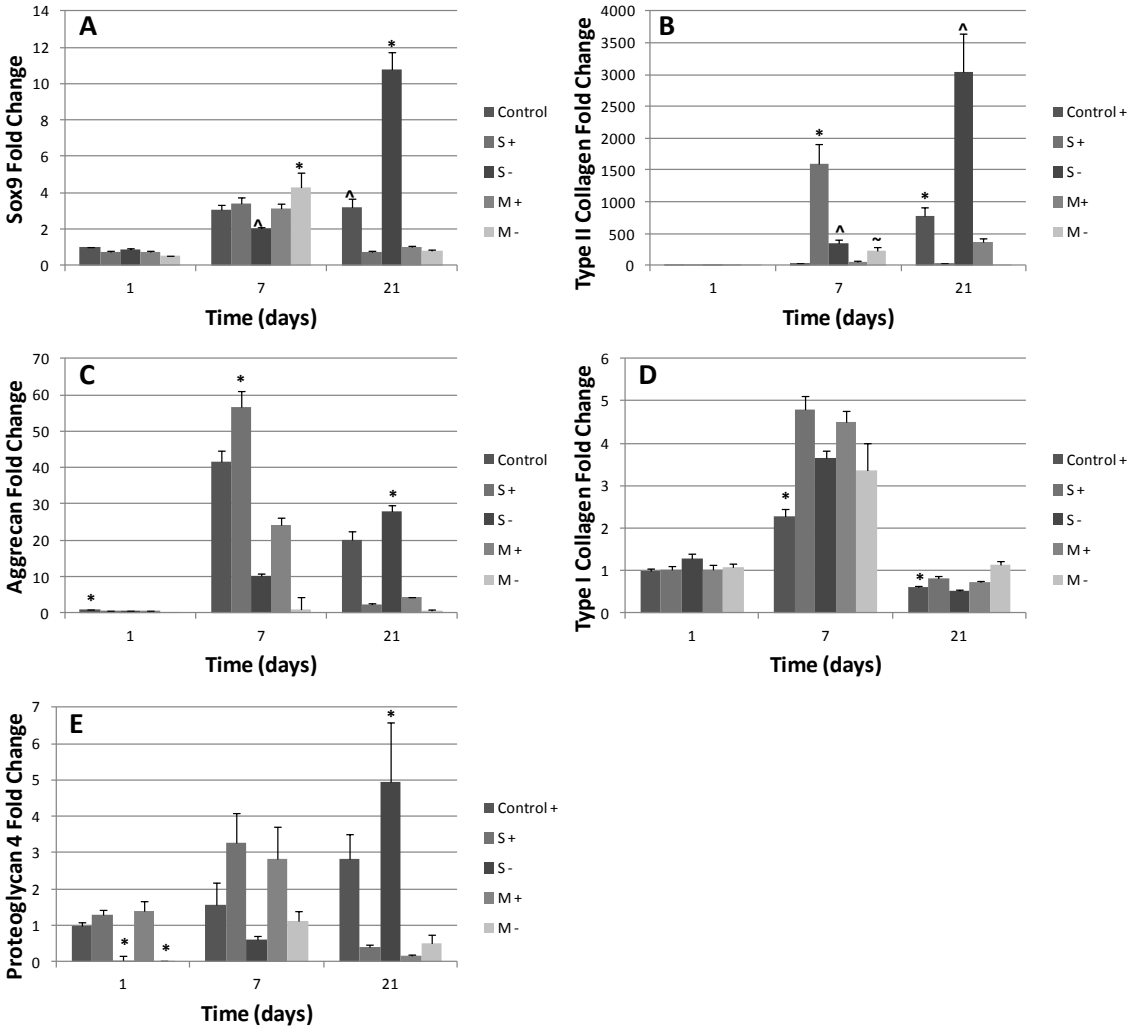


Figure 6.3. Chondrogenic differentiation markers by mRNA expression of MSCs cocultured with zonal cartilage explant chips. An "S" indicates coculture with superficial zone sections, an "M" with middle/deep zone sections, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A unique mark indicates a group is significantly different than all other groups within the time point. Means and standard deviations are reported (n=3, α=0.05).

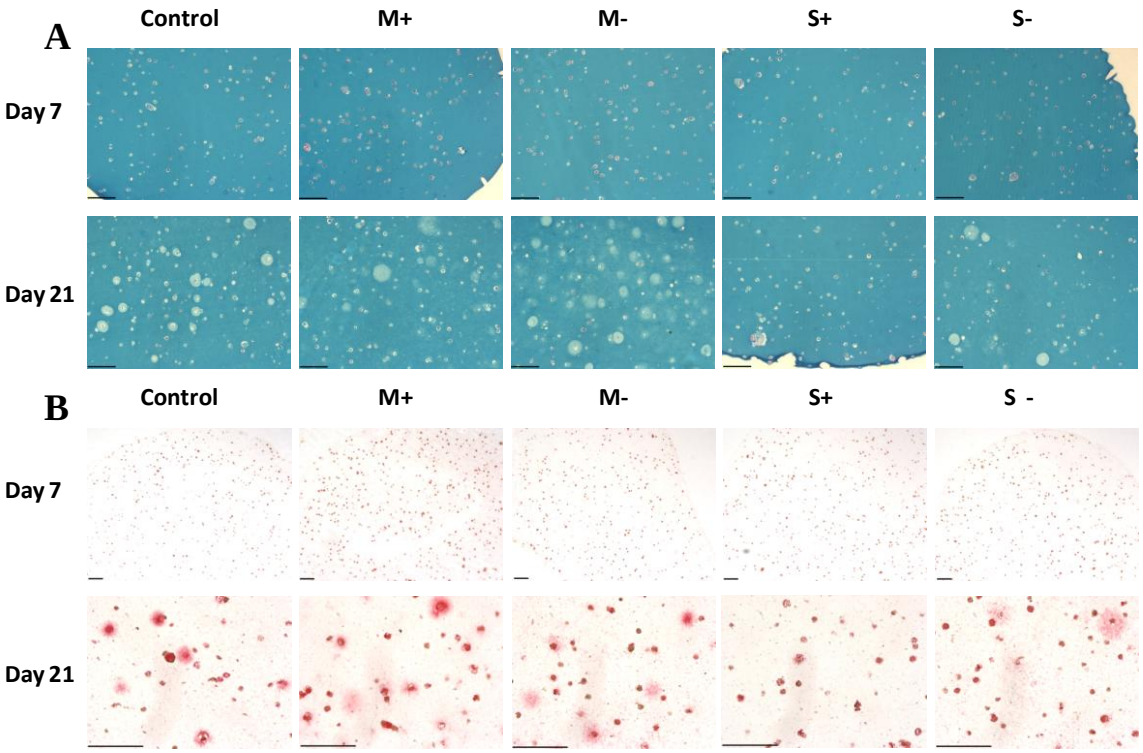


Figure 6.4. Histochemical staining of alginate encapsulated MSCs cocultred with zonal cartilage explant chips. An "S" indicates coculture with superficial zone sections, an "M" with middle/deep zone sections, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A) Alcian blue stain for sulfated proteoglycans. Cells appear as pink, the alginate is stained a dark blue, and the sulfated proteoglycans secreted by the cells are stained lighter blue. B) Sirius red stain, cells are dark brown and collagen is red. All scale bars 100 μm.

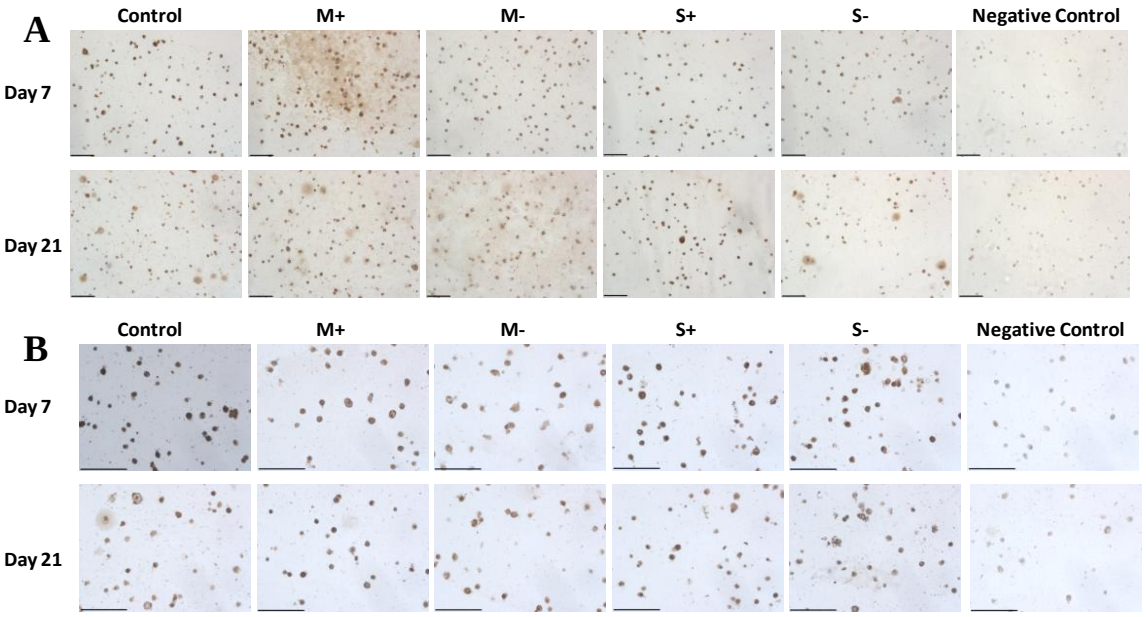


Figure 6.5. Immunostaining of alginate encapsulated MSCs cocultured with zonal cartilage explant chips. An "S" indicates coculture with superficial zone sections, an "M" with middle/deep zone sections, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A) Staining for type II collagen specific antibody, B) staining for proteoglycan 4 specific antibody. Negative control group shows staining omitting primary antibody. All scale bars 100 μm.

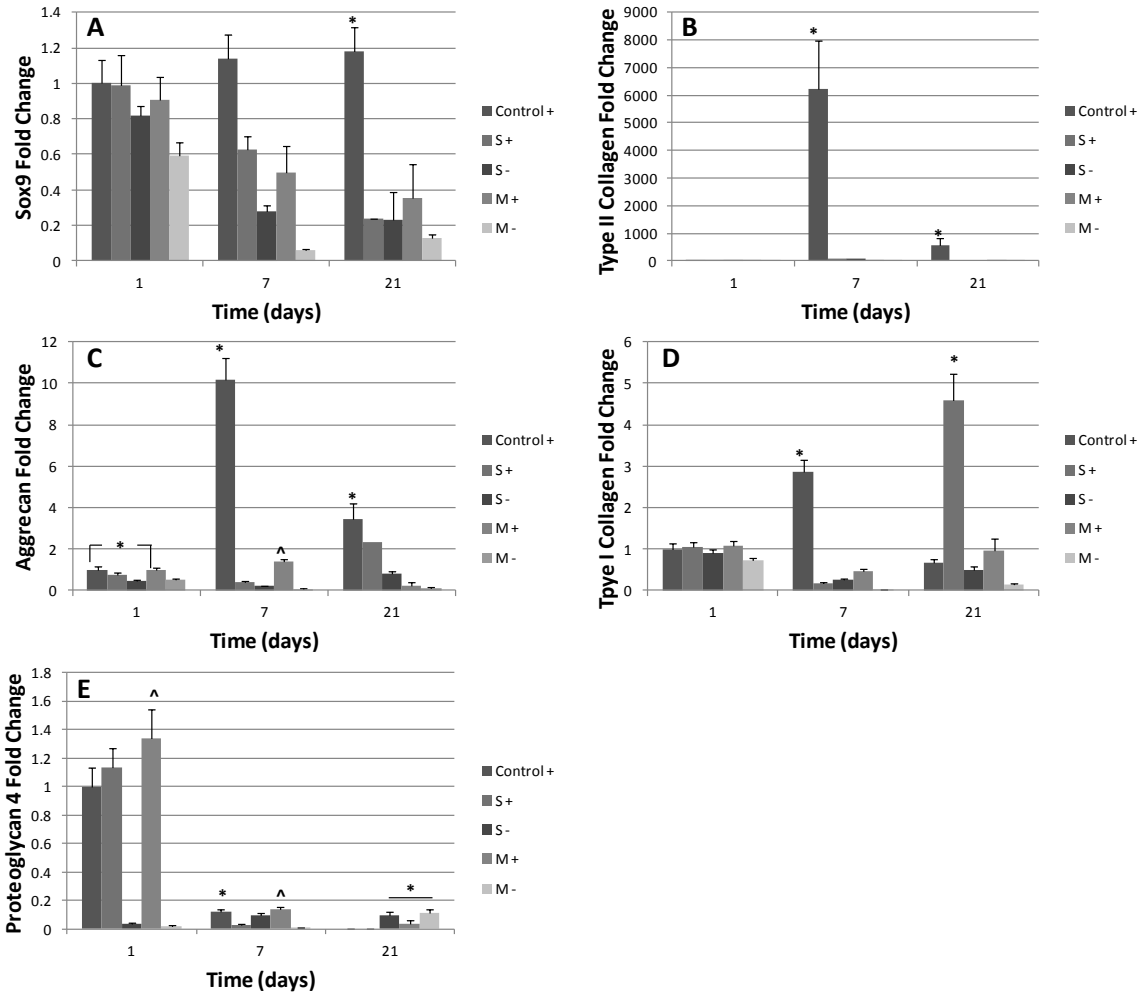


Figure 6.6. Chondrogenic differentiation markers by mRNA expression of MSCs cultured in zonal cartilage explant conditioned media. An "S" indicates delivery of superficial zone conditioned media, an "M" with middle/deep zone conditioned media, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A unique mark indicates a group is significantly different than all other groups within the time point, groups with the same mark are statistically similar. Means and standard deviations are reported (n=3, α=0.05).

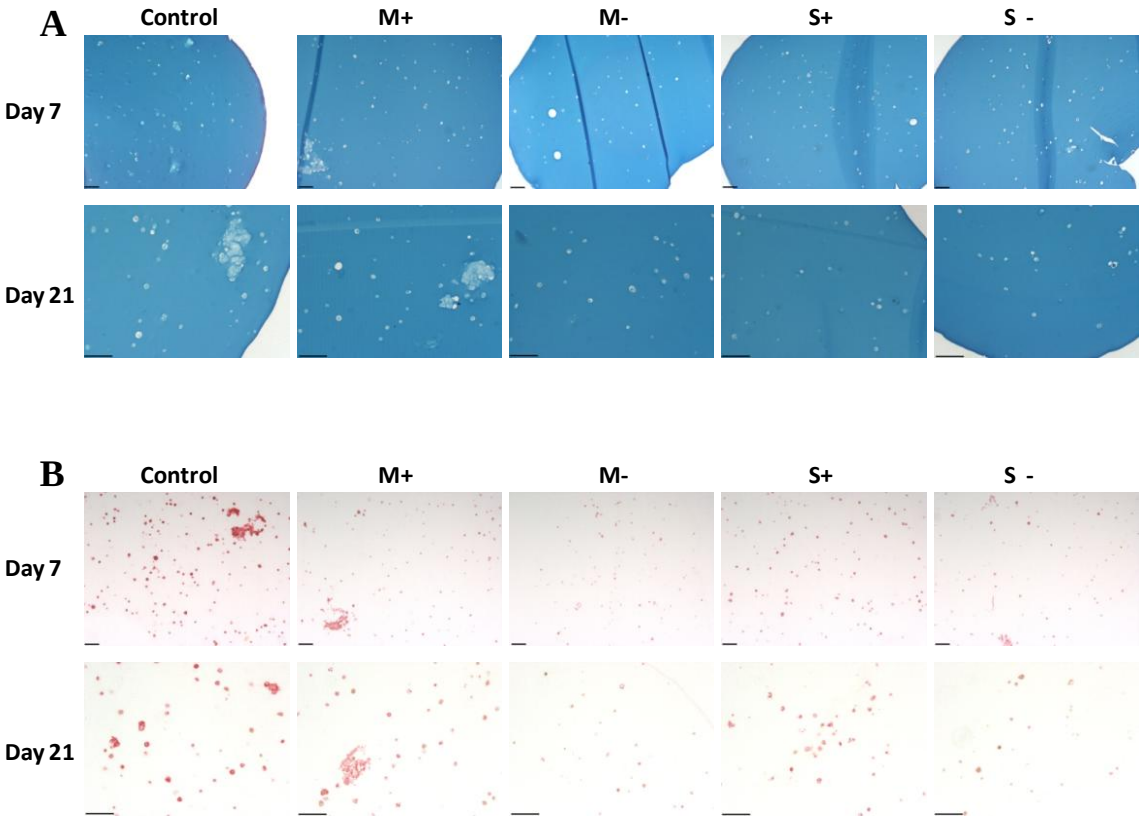


Figure 6.7. Histochemical staining of alginate encapsulated MSCs cultured in zonal cartilage explant conditioned media. An "S" indicates delivery of superficial zone conditioned media, an "M" with middle/deep zone conditioned media, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A) Alcian blue stain for sulfated proteoglycans. Cells appear as pink, the alginate is stained a dark blue, and the sulfated proteoglycans secreted by the cells are stained lighter blue. B) Sirius red stain, cells are dark brown and collagen is red. All scale bars 100 μm.

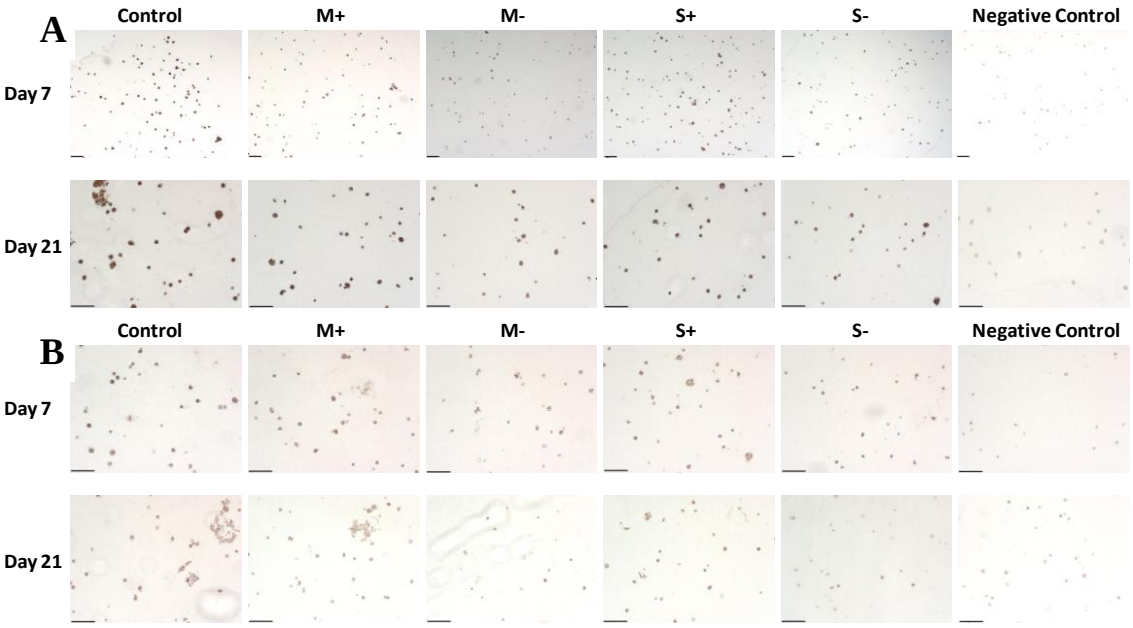


Figure 6.8. Immunostaining of alginate encapsulated MSCs cultured in zonal cartilage explant conditioned media. An "S" indicates delivery of superficial zone conditioned media, an "M" with middle/deep zone conditioned media, and a "+" indicates TGF-β3 delivery. Control groups are standard chondrogenic differentiation media with TGF-β3. A) Staining for type II collagen specific antibody, B) staining for proteoglycan 4 specific antibody. Negative control group shows staining omitting primary antibody. All scale bars 100 μm.

6.4 Discussion

Cuculture results show upregulation of chondrogenic markers thoughout the study. At the day 7 time point the superficial zone coculture group with TGF-β3 supplementation (S+) has higher mRNA expression than the control for all chondrogenic markers; Sox9 (1.1 fold greater), type II collagen (40 fold greater), aggrecan (1.4 fold greater), and proteoglycan 4 (2 fold greater). By day 21 the superficial zone coculture group without TGF-β3 supplementation (S-) has significantly greater expression of chonrogenic markers than all other groups, with Sox9 expression 3.3 fold greater than control, type II collagen 4 fold, aggrecan 1.4 fold, and proteoglycan 4 1.7 fold greater than control mRNA expression. These results show a clear trend of more robust chodrogenesis in groups cocultured with superficial zone cartilage explants. Although middle/deep zone coculture groups express similar levels of markers on days 1 and 7, by day 21 this expression has decreased considerably in all groups.

Cells of the superficial zone are metabolically distinct from those of the middle and deep zones, [152, 158, 198] so variation in their influence over a progenitor population is not surprising. The most established and understood difference between the superficial zone and deeper tissue zones is the production and presence of the lubricating protein proteoglycan 4, which is critical for boundary mode lubrication at the articulating surface. [133-136] Recent evidence also supports another major difference between the top layers of articular cartilage tissue and the lower layers; the presence of a progenitor cell population. [145, 185, 225, 240, 241] Traditionally cartilage was considered a homogenous tissue, with little to no cell turn over, no progenitor population, and no appositional growth. This view was challenged with identification of a Notch-1 positive

colony forming population, with high expansion potential and phenotype plasticity within the superficial zone. [145] Additional studies have identified a side population within the superficial zone that is Hoechst 3342 excreting, marking the stem cell phenotype. [185, 240]

While the presence of a progenitor population within articular cartilage has now been independently reported by several groups, the relative percentage of progenitor cells and the precise distribution has not been determined. It seems clear that the superficial zone is enriched for such a population, but to what extent in unclear. A 2009 study reported over 45% of cells throughout the tissue depth stained positive for the stem cell markers Notch-1, Stro-1, and VCAM (CD106), with increased staining for all in the superficial zone. However, when the authors used the Hoechst3342 dye and FACS analysis a side population of only 0.14% of the total population was found, [240] which is consistent with a previous side population report of 0.1% of the total cell population, and found only in the superficial zone. [185] A recent study in 2011 reported 16% of cartilage cells were positive for mesenchymal stem markers CD105 and CD166. The same study reported that 40% of superficial zone cells were positive for CD166, 39% of middle zone cells were positive for CD166, and 10% of deep zone cells were positive. [241] At present it is unclear what the relative percentage of stem/progenitor cells is and exactly where that population is zonally distributed. It has been hypothesized that both that the progenitor population is much higher than originally thought, and that mature chondrocytes may be positive for some stem cells markers and thus distort the detected progenitor numbers. In either case, results support that the superficial zone contains the highest relative percentage of a progenitor population as compared to other zones. A

stem cell niche within the superficial zone, with distinct chondoitin sulfate sulfation motifs which play a role in controlling signaling molecule availability, has also been reported which further supports this evidence. [225]

From the results of our coculture study it is also clear that the superficial zone is able to provide distinct signals to differentiating MSCs which result in expression of chondrogenic markers, and even the superficial zone chondrocyte marker, proteoglycan 4. The gene expression pattern of the superficial zone coculture induced chondrogenesis is more robust than the pattern observed for the standard chondrogenic control media. As evidence now supports a population of stem/progenitor cells within the superficial zone, stem cell differentiation may be a normal biological process within this zone, and thus signals derived from it provide favorable signals for chondrogenesis. What is not clear from our data is the source, or identity, of the soluable factors which induce chondrogenesis in the superficial zone coculture groups. As the explanted tissue would contain *both* a superficial zone chondrocyte population and the reported progenitor population it is not clear which population influenced alginate encapsulated MSC differentiation. In terms of responsible soluble factors, TGF-β1,2,3 are reported at elevated levels in superficial zone tissue, [242] and previous studies in our laboratory show superficial zone cells in alginate culture express significantly higher levels of IGF-1 mRNA as compared to cultured middle and deep zone cells. [243] Bone morphogenetic proteins (BMPs), along with other signaling molecules, are also likely to play a key role.

Our results also show that the chondrogenesis induced by the coculture groups is dependent on communication between the explanted tissue cell populations and the MSCs, as evidenced by the conditioned media study. Here we show that the

chondrogenesis seen the coculture study is not induced unless the groups are cultured in proximity to each other. This provides the hypothesis that there is critical communication between cell populations within the superficial zone that allows for progenitor cell maintenance and differentiation.

Also of note is the upregulation of proteoglycan 4 mRNA expression at early time points by groups which received TGF-β3 delivery in both coculture and conditioned media studies. This is consistent with other reports that TGF-β delivery, along with BMP delivery, can upregulate proteoglycan 4 (superficial zone protein) expression in bovine progenitor cell populations derived from the superficial zone of articular cartilage, [185, 244] from the infrapatellar fat pad of the knee, [204, 222] and from the synovial fluid. [204]

6.5 Conclusion

In the presented study we demonstrate that cartilage explants from the superficial and middle/deep zones of articular cartilage are able to induce MSC chondrogenesis through soluble signaling factors to varying degrees. The most robust differentiation is observed in the superficial zone coculture group, even without exogenous TGF-β3 delivery. Superficial zone explants were also able to provide signals which upregulated the superficial zone phenotype marker PRG4 in the encapsulated MSC population. Furthermore, we show that the coculture induced chondrogenesis is dependent on communication between the cell populations. Conditioned media from the same zonal explants was unable to induce chondrogenesis, even when supplemented with exogenous TGF-β3. We provide further evidence of important differences between the zones of

articular cartilage and show that signals derived from the superficial zone have a role in guiding progenitor cell fate.

7 Chondrogenic Differentiation of Mesenchymal Stem Cells Encapsulated in Photocrosslinked Alginate and Hyaluronic Acid

7.1 Introduction

Articular cartilage is a highly organized tissue maintained by the resident chondrocytes. Its function is to provide a low-friction, wear-resistant, and protective surface that allows the joint to withstand compressive forces and maintain smooth movement. [5, 245] Chondrocytes are embedded in a matrix of collagen, proteoglycans, and glycosaminoglycans. [2, 5] This network allows for cell adhesion, mechanical support, and the transduction of chemical and mechanical signals. [245]

Cartilage tissue, however, has minimal reparative capabilities due to the decrease in chondrocyte metabolism as a result of disease, injury, and age. This hinders the ability of the tissue to repair damage and maintain homeostasis, and can lead loss of tissue structure and the development of disease, such as osteoarthritis. Tissue engineering efforts aim to replace lost or damaged cartilage tissue. [2, 5, 33, 245] An important component of tissue engineering is the use of an appropriate biomaterial scaffold. This scaffold provides the framework for tissue to be regenerated and eventually implanted into a defect. [246]

Current cell based treatments use autologous chondrocytes, which present problems such as donor site morbidity, and inadequent numbers of recovered chondrocytes for transplantation. Adult mesenchymal stem cells (MSCs) are a potential alternative to autologous chondrocytes. MSCs are easily obtained in many tissues such as bone marrow, adipose tissue, and the synovial membrane. Additionally, they have a high proliferation capacity, so a small sample can be cultured into a large population.

Chondrogenesis can be induced by the exposing MSCs to transforming growth factor-β (TGF-β). This growth factor is found to upregulate expression of transcription factor Sox9 followed by matrix molecules such as type II collagen and aggrecan. [103]

Tissue engineering scaffolds should provide an environment for the maintenance of cell morphology as well as the growth of tissue. Scaffolds should be biodegradable to allow for the biological tissue to eventually replace the artificial scaffold. [247] Hydrogels are common scaffolds used for tissue engineering applications. Like natural cartilage tissue, hydrogels have extremely high water content, which allows for nutrient and waste transport. Alginate is a natural polysaccharide derived from brown algae containing repeating units of mannuronic and guluronic acid. In the presence of divalent cations, these alginate functional groups form crosslinks that solidify a liquid alginate-cell solution into a three-dimensional gel structure. Alginate hydrogels have been shown to support chondrocyte survival, maintain spherical morphology, and allow for cartilage matrix synthesis *in vitro*. [5, 56]

Alternative methods for crosslinking alginate hydrogels are being researched for more desirable clinical applications. In this study, the addition of methacrylate groups to the alginate polymer chains allows it to undergo free radical polymerization initiated by a photoinitiator during UV light exposure. This forms a covalent crosslink between methacrylate groups instead of the ionic crosslink formed by the calcium in non-modified alginate. [247] The main advantage to photocrosslinkable hydrogels is that the crosslinking process can be performed *in situ.* The liquid alginate-cell solution can be injected into the cartilage defect and crosslinked using UV light to fill the irregular shape of the injury. [248, 249] This eliminates the need to fabricate the chondrocyte-embedded

hydrogel *in vitro* and then implant it into the joint using a more invasive procedure. Additionally, the ability to match the shape of the defect allows for improved integration between the native tissue and the engineered scaffold. Furthermore, hydrogels formed by methacrylated alginate provide the capability to control the mechanical properties, swelling ratios, and degradation rates by altering the concentrations of substrate and photoinitiator, and the amount of UV exposure. [250, 251]

There are limitations to the photocrosslinking method. The procedure exposes cells to harmful UV light, chemical photoinitiators, and organic solvents. Therefore, minimal exposure to these elements is ideal for a viable cell system. A shorter UV exposure time and the use of low cytotoxicity photoinitiators can decrease harmful cellular effects. Irgacure 2959 is the primary photoinitiator used for photocrosslinking alginate, but it is found that the photoinitiator VA-086 has lower toxicity, and will therefore be used in this experimental procedure. [247] Previous studies on methacrylated alginate systems have reported on the mechanical properties of the photocrosslinked hydrogels, and the viability of encapsulated cell populations. There are no reports of effects on gene expression profiles, or stem cell differentiation in photocrosslinked alginate. In the presented work we aim to evaluate the potential of photocrosslinked alginate systems to support stem cell chondrogenesis as measured by gene and protein expression of chondrogenic markers.

Alginate provides a favorable environment for chondrocyte growth, however there are limited site for adhesion and thus may not provide adequate signals for the proper synthesis and organization of matrix proteins. In this experiment, methacrylated alginate constructs were created that incorporated hyaluronic acid, a highly prevalent

cartilage ECM component. Hyaluronic acid is a long, unbranched polysaccharide chain, which binds a large number of GAG chains, as well as chondrocytes via the CD44 cell surface receptor. Hyaluronic acid has been shown to contribute to the assembly of the cartilage matrix, binding growth factors, and controlling cell proliferation. Hyaluronic acid has been shown to cause an increase in cell proliferation and GAG expression by chondrocytes in hydrogel culture. [206] Composites of gelatin-HA-CS [208] and collagen-HA-CS [209] have also been demonstrated as favorable environments for chondrocyte growth.

In this work, we present methods for encapsulating mesenchymal stem cells into photocrosslinked methacrylated alginate with the addition of hyaluronic acid to the construct. We evaluate for the first time the viability of MSCs in photocrosslinked alginate, and also MSC chondrogenesis in photocrosslinked alginate with and without hyaluronic acid additive. We hypothesis photocrosslinked alginate will provide a favorable and nontoxic environment for chondrogenesis and that HA will influence gene and protein markers of the chondrocyte lineage.

7.2 Materials and Methods

7.2.1 Methacrylated Alginate Synthesis

Methacrylated alginate was synthesized based on the methods of previous studies. [246, 247] Alginic acid (Sigma-Aldrich, St. Louis, MO) was dissolved in deionized water to make a 2.5% w/v alginate solution. The same volume of methacrylic anhydride (Sigma-Aldrich) was added to the alginate solution. For the duration of the reaction, the solution was maintained at room temperature and a pH of 7 by adding 5N NaOH

dropwise every 3-4 hours. The reaction was run over a total of 72 hours. The mixture was then poured into ethanol (5x original volume of alginate solution) to precipitate out the methacrylated alginate product. Under the biohood, the ethanol and modified alginate solution was vacuum filtered using a Buchner funnel through 5 μm filter paper. The entire filter apparatus was previously sterilized in the autoclave prior to use. The precipitate was recovered wet from the filter and re-dissolved in sterile PBS. The product was precipitated out a second time, spread over a glass dish, and left to dry in the biohood for 24 hours.

7.2.2 Bovine Mesenchymal Stem Cell Isolation

Primary bovine bone marrow tissue was harvested from the tibia 3 week old calves. The tissue was suspended in growth media (α-minimal essential medium (Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% penicillin/streptomycin antibiotics (Gibco/Invitrogen) and 0.2 mM of ascorbic acid (Sigma-Aldrich)), filtered through a 70 μm mesh, and centrifuged to isolate the cell population. This population was then enriched for mesenchymal stem cells via plastic adhesion by plating in monolayer and culture in growth media with 10% fetal bovine serum. After two passages cells were trypsinized (Gibco/Invitrogen) and counted using Trypan blue staining and a hemacytometer.

7.2.3 Hyaluronic Acid Alginate

Hyaluronic acid (Sigma-Aldrich) was dissolved in PBS to make a 5% w/v solution. The solution was sterile filtered using a 0.22 μm syringe filter. Under the

biohood, previously sterilized methacrylated alginate powder was added to the HA solutions to make a 2% w/v alginate solution. The final solution was filtered through a 0.8 μm syringe filter.

7.2.4 Cell Encapsulation and Culture

The photoinitiator, VA-086 (Wako Chemicals), was dissolved in 70% ethanol and then added to each of the alginate solutions to make a 1.4% w/v solution. The MSCs were pelleted and mixed with each of the alginate solutions (with and without HA) to obtain a cell density of $3x10^6$ cells/mL. A syringe was used to transfer 2 mL of each cell-seeded alginate into a 6 well plate. The plate was then exposed to 5 minutes of UV light (365 nm longwave, 2 $\mu W/cm^2$). Cylindrical sections of the crosslinked alginate were cut using a 4 mm Sklar Tru-Punch disposable biopsy punch (Sklar Instruments, West Chester, PA). The constructs were then washed with PBS and suspended in αMEM with 10% FBS growth media for viability studies and chondrogenic media for differentiation studies.

For the cell viability tests, non-modified alginate was made using methods previously established by our laboratory. [54, 56, 210] Briefly, alginic acid was mixed with 0.15 M sodium chloride (Sigma-Aldrich) and 0.025 M HEPES sodium salt (J.T. Baker, Phillipsburg, NJ) in deionized water (pH 7.4) and then sterile filtered using a 0.22 μm syringe filter. The alginate solution was mixed into an isolated cell pellet for a resulting alginate-cell density of $3x10^6$ cells/mL. This solution was injected through an 18-gauge syringe into a 0.1 M calcium chloride (Sigma-Aldrich) bath to form beads. The

beads were stirred in the calcium chloride for 15 minutes and then transferred to αMEM with 10% FBS.

7.2.5 Cell Viability and Metabolism

Before conducting mRNA and histological analyses, cell viability was assessed using both LIVE/DEAD® Viability/Cytotoxicity kit (Invitrogen) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on days 1 and 10. The Live/Dead kit was used following the manufacturer's guidelines. Tests were performed on the methacrylated alginate constructs, the unmodified alginate beads, and a dead control. Samples were incubated with the PBS for 1 hour to diffuse out the media and proteins from the constructs. The PBS was aspirated off of the constructs and 1 mL of the Live/Dead solution was added to each well. The constructs were incubated at room temperature in the dark for 45 minutes and then observed under the microscope using fluorescent light.

To assess cell metabolism, MTT analysis was performed on the constructs. Live cells reduce MTT to pigmented formazan which can be solubilized and the absorbance can be read on microplate reader. The hydrogels were transferred to wells so that there were three biological replicate wells for each experimental group. The MTT solution (Sigma-Aldrich) equal to 10% of the volume of media was added to each well. The constructs were incubated for 3-4 hours. The MTT solvent (Sigma-Aldrich) was then added directly to the culture in an amount equal to the original culture volume. The constructs were then incubated overnight to allow all of the crystals to dissolve and diffuse out of the constructs. For each sample, 100 μL of the solution was plated into 3

wells (96 well plate) so that each group also had three technical replicates. The spectrophotometer was used to measure absorbance at a wavelength of 570 nm, and a background absorbance of wavelength 690 nm was subtracted from this value. The total absorbance was then divided by the construct volume to account for the difference in construct shape between the experimental and control groups.

7.2.6 Chondrogenic Differentiation

MSCs encapsulated in photocrosslinked alginate were cultured in serum-free chondrogenic media made of; high glucose αMEM (Gibco/Invitrogen) + 110 μg/mL sodium pyruvate, 40 μg/mL proline, 50 μg/mL ascorbate 2-phosphate, 0.1 μM dexamethasone, 1% ITS +premix (BD Biosciences, Bedford,MA), and 10ng/mL TGF-β3 treatment (R&D systems, Minneapolis, MN). At day 1, 7, 14, and 21 hydrogels were exposed to a solution of 20 mg/mL alginate lyase (Sigma-Aldrich) for 30 minutes and the cell population was recovered by centrifugation.

7.2.7 RNA Isolation

RNA was isolated from isolated cells constructs using the RNeasy Mini Kit (QIAGEN, Valencia, CA). Total RNA was eluted into 40 μL of RNase free water and detected using a NanoDrop ND-1000 Spectophotometer (NanoDrop Technologies, Wilmington, DE).

7.2.8 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR)

Isolated RNA was reverse transcribed using a cDNA Archive Kit (Applied Biosystems, Foster City, CA), which can convert up to 10μg of RNA to cDNA. cDNA was mixed with Universal Master Mix (Applied Biosystems) and oligonucleotide primers and Taqman probes (Applied Biosystems) for the genes of interest as well as a control gene. Table 7.1 shows the sequences for all forward primers, reverse primers, and probes used. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) was the endogenous control gene. The reaction volume was 20 μl, and the final concentration of cDNA per reaction well was approximately 0.5 ng/μL (10 ng per well). The reaction was conducted on a 7900HT Fast Real-Time PCR System Prism 7000 sequence detector (Applied Biosystems). The thermal profile followed was 2 min at 50°C, 10 min at 95°C, 40 cycles of 15s at 95°C, and 1 min at 60°C. Gene expressions were analyzed using the comparative Ct method. The day one control alginate samples were used as calibrators in all analysis. Fold changes in gene expression were calculated and are reported as the mean RQ values with associated standard deviations (n=3), in accordance with methods previously described by our laboratory. [55, 212]

7.2.9 Statistical Analysis

Each experiment was performed in triplicate (n=3). All data was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple-comparison test to determine statistical differences. A confidence interval of 95% ($\alpha = 0.05$) was used for all analysis and means and standard deviations are shown on each figure.

7.2.10 Histological Preparation

On days 1, 7, 14, 21, methacrylated alginate constructs were fixed for 4 hours at room temperature in 4% paraformaldehyde (Sigma-Aldrich) with 0.1 M sodium cacodylate (Sigma-Aldrich) and 10 mM calcium chloride (Sigma-Aldrich). Samples were placed in histological cassettes and then washed for 24 hr at room temperature in 0.1 M sodium cacodylate and 10 mM calcium chloride. Samples were dehydrated through a series of ethanol washes (40%, 50%, 60%, 70%, 95%, and 100% x 3) for 15 min each followed by two 15 min washes in Citrisolv (Fisher Scientific) and two 30 min washes in paraffin (Paraplat X-tra, Fisher Scientific). The samples were then embedded in paraffin blocks, cut into 4 µm sections, and mounted on a glass slide (Superfrost, Fisher Scientific).

7.2.11 Histology Staining

Samples were dried at 64°C for two hours, deparaffinized using Citrisolv, and rehydrated. The samples were then rinsed in distilled water and stained using Alcian blue, Safranin-O and Sirius red staining solutions (Poly Scientific, Bay Shore, NY). All samples were viewed under an Axiovert 40CFL light optical microscope (Zeiss, Thornwood, NY) and images were captured using SPOTSOFTWARE (Diagnostic Instruments, Inc., Sterling Heights, MI) imaging software.

Table 7.1. Forward primer, reverse primer, and probe sequences used for GAPDH, Type II Collagen, Type I Collagen, Aggrecan, Sox9, and Superficial Zone Protein (SZP) used for qRT-PCR.

Primer and Probe sequences used for qRT-PCR

Protein		Sequence
GAPDH	Forward Primer	TGCCGCCTGGAGAAACC
	Reverse Primer	CGCCTGCTTCACCACCTT
	Probe	CCAAGTATGATGAGATCAA
COL2A1	Forward Primer	CGGGCTGAGGGCAACA
	Reverse Primer	CGTGCAGCCATCCTTCAGA
	Probe	CAGGTTCACATATACCG
COL1A1	Forward Primer	AGAACCCAGCTCGCACATG
	Reverse Primer	CAGTAGTAACCACTGCTCCATTCTG
	Probe	AGACTTGAGACTCAGCC
AGC	Forward Primer	GGGAGGAGACGACTGCAATC
	Reverse Primer	CCCATTCCGTCTTGTTTTCTG
	Probe	CAGGCTTCACCGTTGAG
SOX9	Forward Primer	AACGCCGAGCTCAGCAAG
	Reverse Primer	ACGAACGGCCGCTTCTC
	Probe	TTCAGCAGTCTCCAGAGCTTGCCCA
PRG4	Forward Primer	GAGCAGACCTGAATCCGTGTATT
	Reverse Primer	GGTGGGTTCCTGTTTGTAAGTGTA
	Probe	CTGAACGCTGCCACCTCTCTTGAAA

7.3 Results

7.3.1 MSC Viability in Photocrosslinked Constructs

The Live/Dead Viability/Cytotoxicity Kit determines cell viability by detecting intracellular esterase activity (living) with green-fluorescent calcein-AM, and compromised plasma membrane integrity (dead) with red ethidium homodimer-1. With both a live (cells in calcium crosslinked alginate) and dead control (cells in calcium crosslinked alginate exposed to toxic levels of methanol) for each time point, we can confirm the methacrylated alginate constructs maintain living cells. Based on previous research, a methacrylated construct exposed to 5 minutes of UV light with 1.4% w/v VA-086 concentration can be expected to have 80% cell viability immediately after crosslinking. [247] As shown in Figure 7.1C, the cell viability of the methacrylated alginate on day 1 is similar to that of the positive control group with no signs of red fluorescence. The live control appears to have a brighter fluorescent stain due to the difference in thickness of the constructs (control is spherical where methacrylated is cylindrical). On day 10, the methacrylated alginate cell viability is not as high as in the control alginate group. Figure 7.1F shows that dead cells are present in the scaffold, indicated by the red halo, but the majority of the cells in the construct are still alive, indicated by the green.

MTT is able to assess cell viability by measuring relative cell respiratory levels. The optical density reading is proportional to the metabolic respiration, and can therefore be used as a comparative measurement tool. As the control construct is a different shape than the methacrylated alginate construct, the optical density reading was divided by volume to normalize the cell number. In Figure 7.2, we see that the amount of metabolic

respiration for each group is similar on day 1, and the methacrylated alginate group has slightly decreased respiration on day 10 as compared to the control group, however there is no statistical difference between groups. Absorbance increased in both groups from day 1 to day 10, indicating cell proliferation.

7.3.2 MSC Chondrogenesis in Photocrosslinked Constructs

Sox9 is a transcription factor which induces chondrogenesis and upregulates type II collagen expression. As seen in Figure 7.3A Sox9 mRNA expression is detected throughout the study, however it decreases with time. Robust chondrogenic induction is indicated by sustained and elevated Sox9 gene expression throughout the study period. [252, 253] Figure 7.3A indicates poor chondrogenesis, which is further supported by the mRNA expression profiles of type II collagen and aggrecan, as seen in Figures 7.3B and 7.3C. Type II collagen is upregulated on days 7 and 14, however not to extent that is normally observed during robust chondrogenesis. Aggrecan expression also decreases with time throughout the culture period.

Chondrocyte phenotype markers are shown in Figure 7.4. Proteoglycan 4 is a lubricating protein found at the joint surface and is a marker for the superficial zone chondrocyte phenotype. Type I collagen is a negative marker for the chondrocyte phenotype. Figure 7.4A shows mRNA expression of proteoglycan 4, which again decreases with culture time for both control and experimental groups. Type I collagen mRNA expression, Figure 7.4B, also decrease with culture time, a trend which usually indicates phenotypically stable chondrocytes. [253, 254]

Histological staining shows limited secretion of cartilage matrix products, as seen in Figures 7.5-7.7. Figure 7.5 shows Alcian blue staining where cells are stained light pink, and alginate matrix dark blue. Typically light blue stain indicates negatively charged cell-secreted proteoglycans, however here light blue appears to indicate a difference in alginate density as there is no matrix accumulation seen around cells or groups of cells. The addition of hyaluronic acid in particular appears to cause a change in the scaffolds absorption of Alcian blue stain – with large areas of the scaffold appearing lighter blue, indicating fewer negatively charged species. In Figure 7.6 and Figures 7.7 very little matrix accumulation is observed. Figure 7.6 shows Safranin-O staining again for negatively charged proteoglycans. Here the alginate is stained a faint pink, secreted proeglycans a darker pink, and cells dark brown. Figure 7.7 shows Sirius red staining for collagens, where cells are stained dark brown and collagen red. Minimal collagen is observed, with some faint staining directly surrounding encapsulated cells.

7.3.3 Hyaluronic Acid Influence

Little difference is observed between control photocrosslinked alginate groups and experimental photocrosslinked groups with hyaluronic acid. The only trend observed is an upregulation in gene markers on day 7 in the HA groups. Other than Sox9 all gene markers are significantly upregulated over the control in the HA group on day 7; type II collagen is 5.2 fold higher, aggrecan is 3.26 fold higher, proteoglycan 4 is 2.13 fold higher, and type I collagen is 11.77 fold higher. One day 1, proteoglycan 4 expression is also 2.58 fold higher in the HA group than the control. The only differences in

histological staining appear to be due to the presence of HA in the scaffold, as observed in the staining of Alcian blue (Figure 7.5), not due to differences in cellular activity.

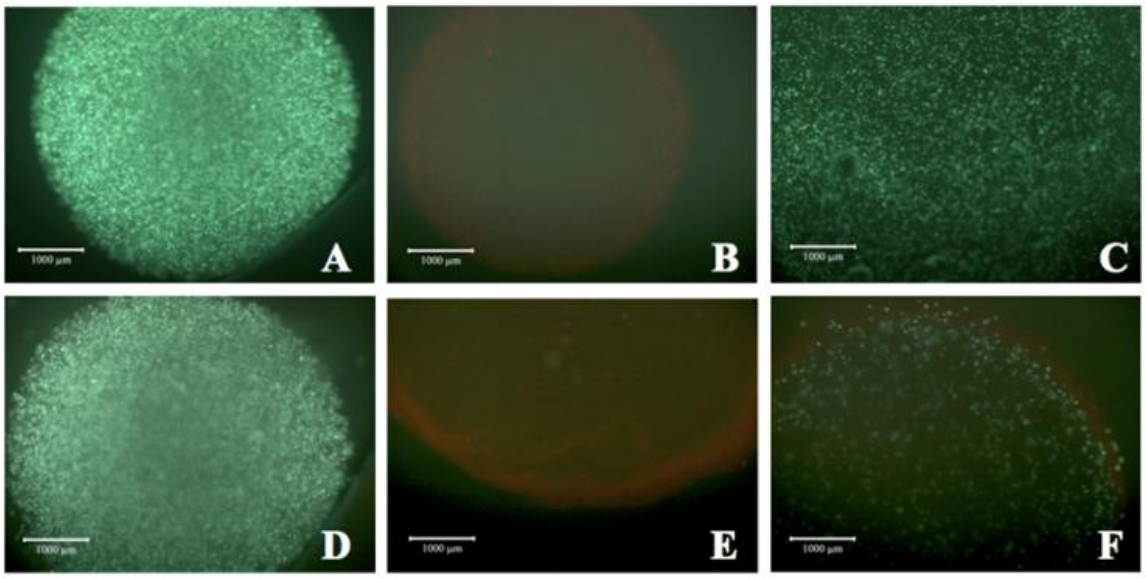


Figure 7.1. Live/dead fluorescent staining. Green fluorescence indicates live cells by calcein AM cleavage by cytoplasmic esterases. Red fluorescence indicates dead cells as ethidium homodimer-1 binds with nucleic acids of membrane-compromised cells. (A) Day 1 alginate control, (B) Day 1 alginate dead control, (C) Day 1 methacrylated alginate, (D) Day 10 alginate control, (E) Day 10 methacrylated alginate dead control, (F) Day 10 methacrylated alginate. After 10 days dead cells are observed in the photocrosslinked alginate, as indicated by the red glow, however most cells are alive, as indicated by the green staining. Note that control constructs are thicker and thus have more cells, as they appear to have more intense live staining. All scale bars 1000 μm.

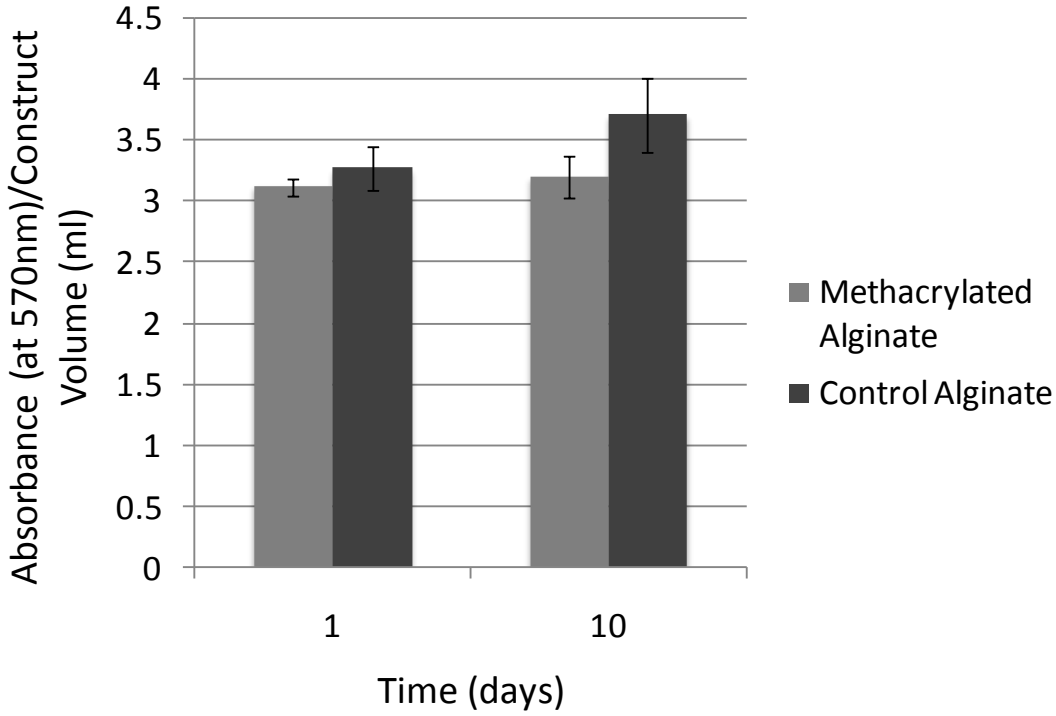


Figure 7.2. Cell metabolism by MTT analysis. Absorbance per volume is shows on the y-axis which corresponds to cell metabolic rates. Cells isolated from photocrosslinked methacrylated alginate and calcium crosslinked non-modified alginate control. Methacrylated and control alginate constructs vary in shape so the absorbance is normalized by the construct volume. Metabolic rates are comparable between control and experimental groups and no statistical differences are observed ($n=3$, $\alpha=0.05$).

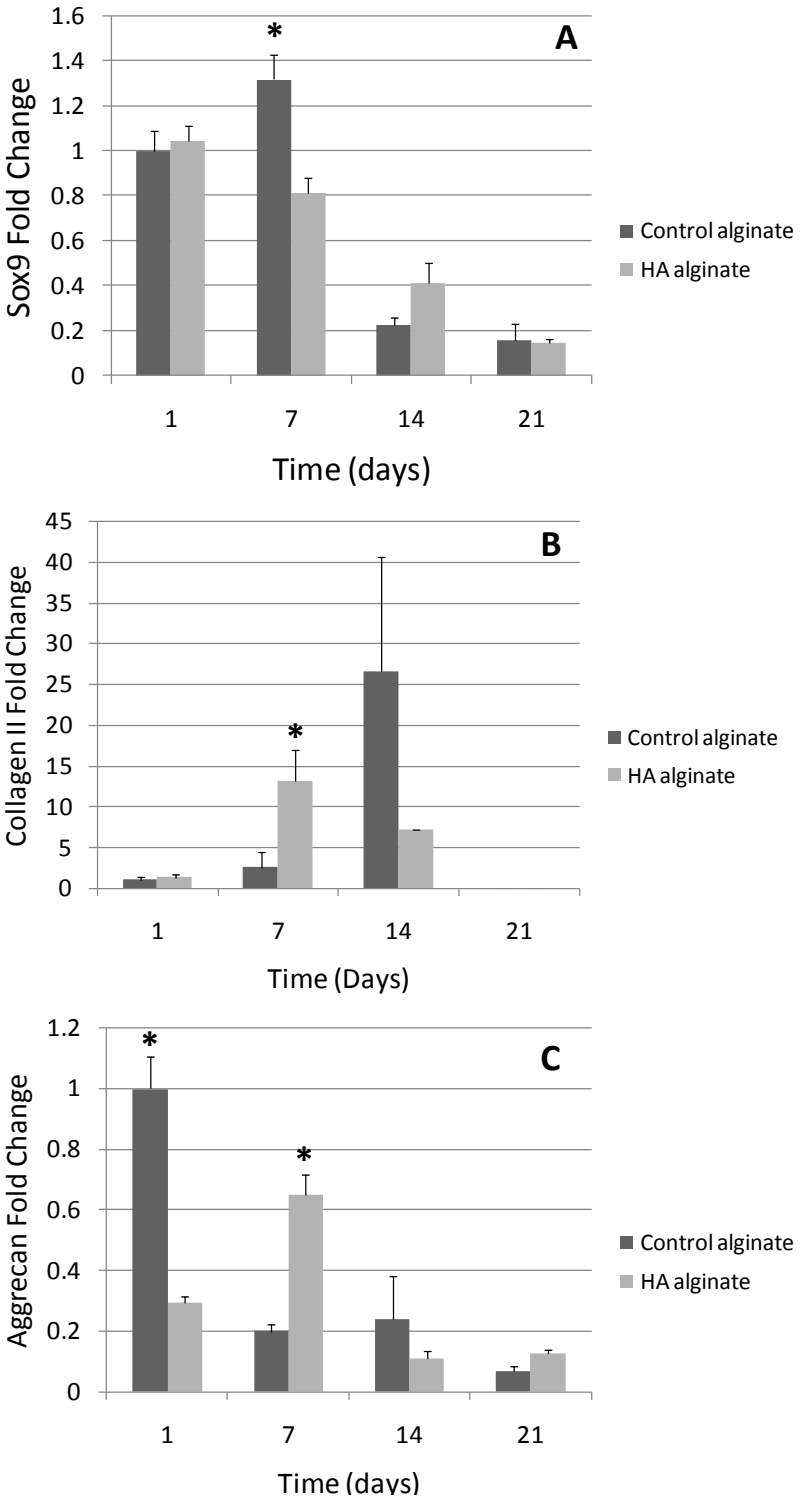


Figure 7.3. Chondrogenic differentiation markers by mRNA expression of MSCs encapsulated in photocrosslinked alginate (Control alginate) and photocrosslinked alginate with hyaluronic acid (HA alginate). Control alginate day one is used as the calibrator. Differentiation markers are expressed but fall off over culture time, and by day 21 are significantly reduced. A unique mark indicates a group is significantly different than all other groups within the time point. Means and standard deviations are reported (n=3, α=0.05).

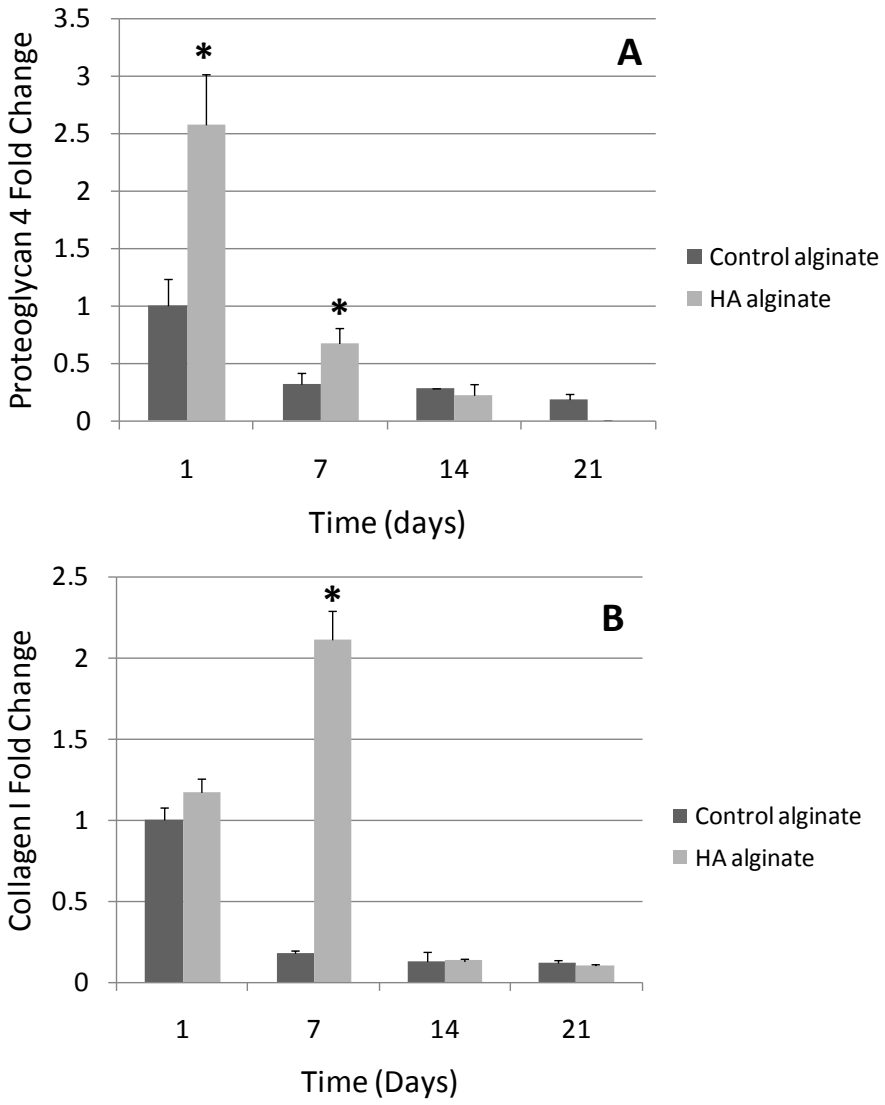


Figure 7.4. Chondrocyte phenotype markers of MSCs encapsulated in photocrosslinked alginate (Control alginate) and photocrosslinked alginate with hyaluronic acid (HA alginate). Control alginate day one is used as the calibrator. Both markers generally are reduced with culture time. A unique mark indicates a group is significantly different than all other groups within the time point. Means and standard deviations are reported (n=3, α=0.05).

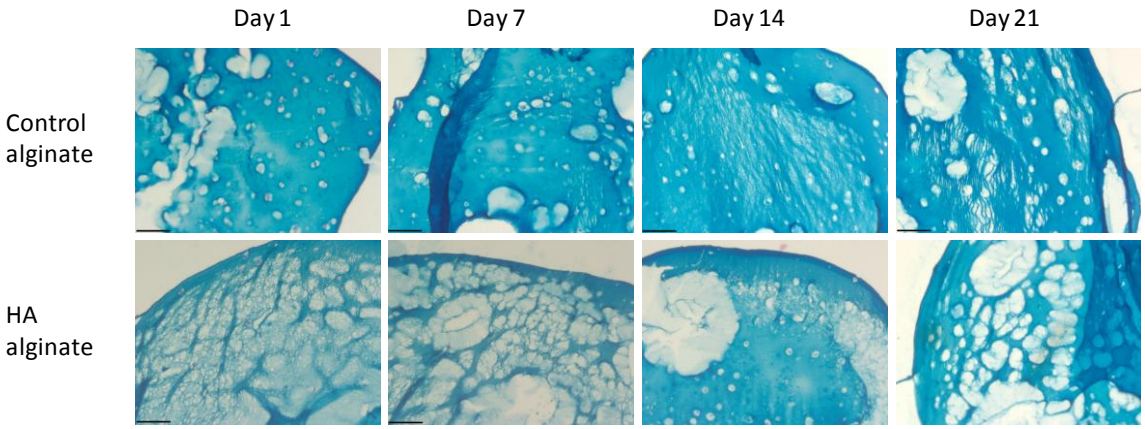


Figure 7.5. Alcian blue staining of MSCs encapsulated in control photocrosslinked alginate (Control alginate) and photocrosslinked alginate with hyaluronic acid (HA alginate). Cells appear are stained pink, negative charges stain blue, either alginate or sulfated proteoglycans. All scale bars 100 µm.

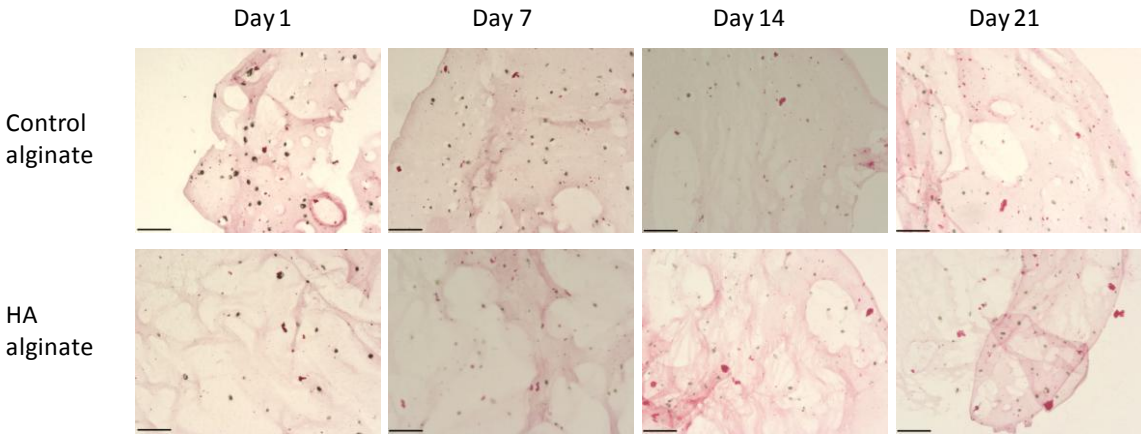


Figure 7.6. Safranin O staining of MSCs encapsulated in control photocrosslinked alginate (Control alginate) and photocrosslinked alginate with hyaluronic acid (HA alginate). Cells are stained dark brown, alginate is stained light pink, and sulfated proteoglycans are stained darker pink. All scale bars 100 µm.

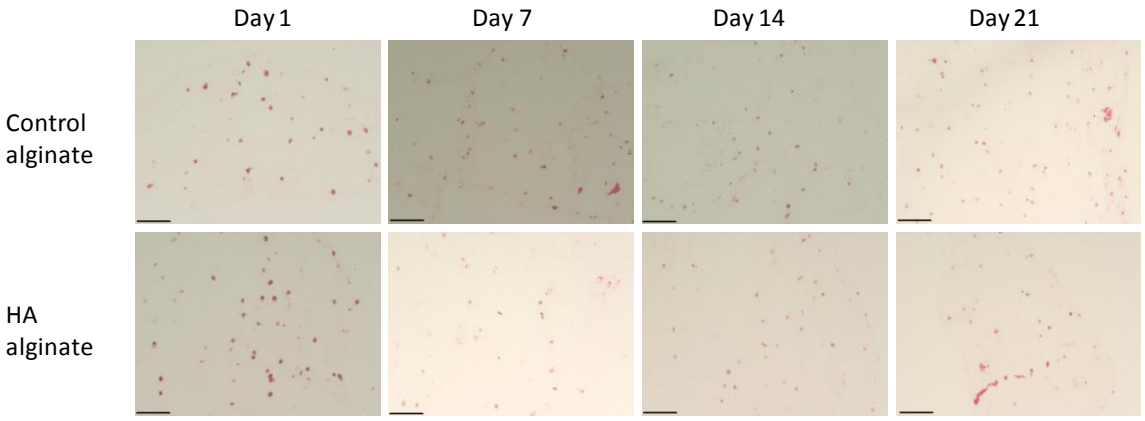


Figure 7.7. Sirius red staining of MSCs encapsulated in control photocrosslinked alginate (Control alginate) and photocrosslinked alginate with hyaluronic acid (HA alginate). Cells are stained dark brown and collagen is stained red. All scale bars 100 μm.

7.4 Discussion

In the presented work we have functionalized the alginate polymer with a methacrylate group to allow for crosslinking by UV light exposure. We report high encapsulated cell viability after 10 days, and no statistical difference between cell metabolism as measured by MTT assay between cells encapsulated in calcium crosslinked alginate and modified photocrosslinked alginate. However, MSC chondrogenesis in photocrosslinked alginate, both with and without HA additive, is limited and chondrogenic gene markers decrease with time throughout the culture period. For robust chondrogenesis Sox9 mRNA expression should remain elevated throughout the 21 day culture period, but as seen in Figure 7.3A expression decreases with each time point. Following this trend are decreases in aggrecan and proteoglycan 4 mRNA expression with time, and limited type II collagen mRNA upregulation. The maximum type II collagen expression is seen on day 14, where the control group is 26.6 fold greater than day one. This is an order of magnitude below the type II collagen upregulation normally observed in our laboratory during MSC chondrogenesis in calcium crosslinked alginate. Reports from the literature cite ranges from 1,000 to 150,000 for type II collagen upregulation over day 1 observed throughout differentiation culture – again far above what is observed here. [228, 255] Histological staining confirms limited production of cartilage extracellular matrix markers, with little if any observed staining for both proteoglycans and collagen. Taken together, results indicate that while cells remain viable in the photocrosslinked alginate, the scaffold does not provide a favorable environment for MSC chondrogenesis, even with the addition of hyaluronic acid.

A methacrylated form of alginate which could be crosslinked using a UV light source was first reported in 2001. Physical properties of the resulting hydrogel were characterized and reported to be dependent on the degree of methacrylate modification, and thus the number of covalent crosslinks. [256] Since then a handful of studies have evaluated cellular viability in methacrylated alginate hydrogels. A study in 2009 reported high viability by live/dead fluorescent staining of bovine chondrocytes encapsulated in methacralyated alginate crosslinked using photointiator Irgacure D2959 and cultured for 7 days. [250] A study using the same system reported high chondrocyte viability for up to 6 weeks and production of glycosaminoglycans (GAGs). Incorporation of the adhesion peptide sequence Arg-Gly-Asp to the methacrylated alginate stimulated both chondrocyte proliferation and GAG production. [251] Interestingly, a 2008 study using bovine nucleus pulposus (NP) cells reported that viability was dependent on the degree of alginate methacylation; the higher the degree of methacrylation, the lower the viability. Viability also decreased with time throughout the study in all methacrylated groups and cell encapsulated in groups with the highest degree of methacrylation did not secrete characteristic proteoglycans. [246]

Harmful effects of methacylated monomers on cell populations have also been reported in research for dental materials. [257] Dental materials are commonly polymers which are formed *in situ* from methacylate acid-based monomers. [258] However, the polymerization process is always incomplete and a considerable fraction of methacrylate monomers or co-monomers may be released through mechanical stress or enzymatic degradation via hydrolyzable ester bonds. [259] Methacylate monomers have been reported to induce both cytotoxic and genetoxic effects. [260] Specifically, they have

been shown to interact with DNA in human lymphocytes, causing single and double-strand breaks and alterations in DNA bases that were associated induction of apoptosis and changes in the cell cycle. [261-263] In the presented work we hypothesis that incomplete crosslinking, and/or esterase activity, may have increased the concentration of methacylate monomers over culture time, and resulted in adverse effects on the encapsulated cell population. We hypothesize that methacrylate monomers may have interacted with MSCs and caused cellular changes which resulted in limited chondrogenic differentiation. Future studies are necessary to identify the mechanisms of action involved in these changes, and potential threshold values below which methacrylate monomers may be tolerated by an encapsulated cell population.

7.5 Conclusion

In agreement with previous studies we report high cell viability and no statistical difference in metabolic activity between MSCs cultured in calcium crosslinked alginate and photocrosslinked alginate at day 10. Despite viability, chondrogenesis of the encapsulated MSC population was limited. While chondrogenic markers such as Sox9, type II collagen, proteoglycan 4, and aggrecan mRNA were detected during culture, most markers slowly fell off throughout the culture period. Type II collagen was upregulated to some extent on day 7 and 14, however values were an order of magnitude below what is observed in our studies of MSC chondrogenesis in calcium crosslinked alginate. Futhermore, by day 21 no type II collagen mRNA was detectable. Histological staining for secreted collagen and proteoglycan confirm poor chondrogenic induction. Harmful effects of the initiator system or photocrosslinking are unlikely to be responsible for the

observed cell behavior, as those changes should be seen immediately after the photocrosslinking reaction. Due to incomplete crosslinking and enzymatic activity, it is likely that the methacrylate monomer concentration may increase with culture time. We hypothesize that increasing concentrations of the monomer throughout the culture period resulted in the limited potential of encapsulated MSCs to undergo chondrogenic differentiation.

8 Summary

Functional articular cartilage is dependent on zonal tissue organization and the integrated activity of distinct chondrocyte subpopulations. Chondrocytes of the superficial zone maintain the extracellular matrix to resist tension and provide lubrication at the joint surface. The middle and deep zones provide the tissue's high compressive strength. Proteoglycan 4, a critical lubricating protein, is secreted largely by superficial zone cells and is one of the most classified zonal differences. Despite critical zonal differences, there are currently no clinical therapies which aim to regenerate stratified articular cartilage. The overall goal of the presented work was to classify major differences in gene and protein expression between isolated zonal chondrondrocyte subpopulations, and evaluate the potential of mesenchymal stem cells to differentiate into chondrocytes of zonal phenotypes.

The first objective was to classify the zonal distribution in gene expression of major extracellular matrix components, the growth factor IGF-1 and its extracellular binding protein, IGF-BP3, both with and without exogenous IGF-1 delivery. Chondrocyte populations of the superficial, middle, and deep zone were isolated and cultured separately in alginate hydrogels for 8 days. Differences in gene expression of subpopulations were observed throughout the study. Middle and deep zone cells were similar in terms of matrix production, and expressed significantly higher amounts of aggrecan and type II collagen mRNA compared to superficial zone cells. IGF-1 mRNA expression was elevated in the superficial zone, and IGF-BP3 expression was elevated in middle and deep zone cells throughout the study. Exogenous delivery of IGF-1 did not

have the simulative effects anticipated, perhaps due to the presence of FBS, but did positively affect phenotype retention in deep zone cells. Furthermore, delivery of IGF-1 generally decreased expression of endogenous IGF-1. By day 8, IGF-1 delivery increased binding protein expression throughout all zones. From these findings, middle and deep zone cells were determined to be phenotypically similar, and distinct from the superficial zone population. Future studies therefore utilized two isolated populations; superficial zone chondrocytes and middle/deep zone chondrocytes.

The second objective was to identify the potential of hyaluronic acid (HA) and chondroitin sulfate (CS) to influence zonal phenotype retention of chondrocyte subpopulations as well as zonal-differentiation of mesenchymal stem cells (MSCs). Superficial zone cells, middle/deep zone cells, and MSCs were isolated and cultured in control alginate, HA-alginate, and CS-alginate. Cell populations were evaluated for chondrocyte phenotype markers, including the lubricating protein proteoglycan 4 (PRG4). Superficial zone chondrocytes expressed significantly higher levels of PRG4 mRNA (by day 7 middle/deep zone cell expression was not detectable), and this expression was significantly enhanced through addition of both HA and CS to alginate scaffolds. Conversely, PRG4 mRNA expression was downregulated by CS and HA in differentiating MSCs, possibly due to build up of entrapped protein. HA and CS had favorable effects on chondrogenesis though upregulation of transcription factor Sox9, and downregulation of type I collagen. These results indicate that HA and CS incorporation to alginate scaffolds can aid in production of critical lubricating protein mRNA, stabilize differentiating MSCs, and sequester lubricating proteins within the scaffold. Results also highlight PRG4 as a marker for the superficial zone chondrocyte phenotype.

The third objective was to establish the potential of zonal cartilage-derived soluble factors to drive zonal differentiation of MSCs. We investigated both a coculture explant model and a conditioned media explant model to assess the impact of communication between cell populations. Superficial zone explants and middle/deep zone explants with and without TGF-β3 were used for both studies. Results demonstrated that cartilage explants from the superficial and middle/deep zones of articular cartilage are able to induce MSC chondrogenesis through soluble signaling factors to varying degrees. The most robust differentiation was observed in the superficial zone coculture group, even without exogenous TGF-β3 delivery. Superficial zone explants were also able to provide signals which upregulated PRG4 expression in the encapsulated MSC population. Furthermore, results showed that coculture induced chondrogenesis is dependent on communication between the cell populations. These results provide further evidence of important differences between the zones of articular cartilage and show that signals derived from the superficial zone have a role in guiding progenitor cell fate.

The fourth objective was to evaluate the viability of MSCs in photocrosslinked alginate, and MSC chondrogenesis by TGF-β3 in photocrosslinked alginate with and without hyaluronic acid additive. It was hypothesized that photocrosslinked alginate would provide a favorable and nontoxic environment for chondrogenesis and that hyaluronic acid would influence gene and protein markers of the chondrocyte lineage. Alginate was functionalized with a methacrylate group and photocrosslinked using an initiator and UV light source. Hyaluronic acid was added to experimental groups prior to photocrosslinking. In agreement with previous studies we report high cell viability and

no statistical difference in metabolic activity between MSCs cultured in calcium crosslinked alginate and photocrosslinked alginate at day 10. Despite viability, chondrogenesis of the encapsulated MSC population was limited. While chondrogenic markers such as Sox9, type II collagen, proteoglycan 4, and aggrecan mRNA were detected during culture, most markers slowly fell off throughout the culture period. By day 21 no type II collagen mRNA was detectable. We hypothesize that increasing concentrations of the monomer throughout the culture period resulted in the limited potential of encapsulated MSCs to undergo chondrogenic differentiation.

In conclusion, in an effort to move zonal cartilage engineering closer to clinical practice we have classified differences in gene expression of matrix and signaling molecules by tissue depth, confirmed PRG4 as a marker for superficial zone cells, evaluated scaffold environments to aid in PRG4 expression and localization, and demonstrated MSC express PRG4 during TGF-β3 induced chondrogenesis. We show hyaluronic acid and chondroitin sulfate can both enhance PRG4 mRNA expression in superficial zone cells during alginate culture, and aid in chondrocyte phenotype retention of differentiating MSCs. We hypothesize hyaluronic acid interacts with cell-secreted PRG4 and therefore may be a useful tool in localization of lubricating proteins. We also demonstrate that soluble signals derived from superficial zone explants can drive chondrogenesis of alginate encapsulated MSCs and upregulate PRG4 expression when the two populations are cultured in proximity. Taken together these studies highlight the importance of modeling the superficial zone as distinct both in function and cellular phenotype.

9 Future Directions

The completed work has provided insight to chondrocyte subpopulation phenotype and the ability of MSCs to express zone-specific markers. These studies have also generated new questions and inspiration for future works.

We observed that signals derived from superficial zone explants can drive MSC chondrogenesis. We also saw that IGF-1 is secreted at elevated levels by superficial zone cells in culture. The literature reports that the superficial zone contains a progenitor cell population. Many fundamental questions remain unanswered about this population and its potential role in tissue repair. Such as, is progenitor cell chondrogenesis a normal function within the superficial zone? What is the relative ratio of the progenitor population to the total cell population? Do superficial zone chondrocytes secrete signaling molecules to drive this differentiation, and if so what are they? Do the progenitor cells secrete molecules which influence chondrocyte activity? How can we stimulate the progenitor population to aid in native tissue repair capacity? Flow cytometry methods could be utilized to isolate and recover a progenitor population from superficial zone tissue which could then be studied *in vitro*.

Hyaluronic acid complexes with PRG4 in the body to aid in lubrication, and is also a major part of the cartilage extracellular matrix. We report it can affect gene expression of PRG4 in superficial zone cells and influence MSC chondrogenesis. However, in our model hyaluronic acid was not covalently bonded to the alginate polymer. Utilizing a scaffold made out of HA, or covalently binding it to the alginate network could control for any potential temporal effects of the molecule due to diffusion. Furthermore, greater control over the concentration of HA within the scaffold would

allow for its cellular effects to be studied with greater control. Alternate photocrosslinking chemistry or a reduction in alginate methacrylatation could also be studied to identify a more suitable environment for MSC chondrogenesis in photocrosslinked hydrogels. This will allow for injectable delivery and aid in clinical relevance.

Finally, there are limited studies investigating dynamic culture conditions for chondrogenic differentiation of MSCs. Our laboratory has recently developed a Tubular Perfusion System (TPS) bioreactor, in which a cell population encapsulated in alginate beads can easily be cultured in dynamic conditions. Evaluating the potential of this system to enhance chondrogenesis would be of value. Furthermore, identifying dynamic culture parameters which could upregulate zonal phenotype markers, such as PRG4, would directly contribute to the goals of the presented work.

Current treatment options are limited for damaged or diseased cartilage. Osteoarthritis alone affects a large percent of the population, particularly those over 60, and can cause chronic pain, severely impacting quality of life. Cell-based regenerative treatment options are limited, and largely result in inadequate fibrocartilage repair tissue. Adult stem cell populations hold promise for improving therapies, however their potential to date has not been realized. Any future work which can bring stem cell therapies closer to clinical practice is of value and importance.

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