

**ENHANCEMENT OF CHONDROGENESIS BY DIRECTING
CELLULAR CONDENSATION THROUGH CHONDROINDUCTIVE
MICROENVIRONMENTS
AND
DESIGNED SOLID FREEFORM FABRICATED SCAFFOLDS**

by

Elly Elisabeth Liao

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Doctoral Committee:

Professor Scott J. Hollister, Chair
Professor Paul H. Krebsbach
Assistant Professor Blake J. Roessler
Assistant Professor Kurt D. Hankenson, University of Pennsylvania

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To my grandparents and parents, for their love and support.

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TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGMENTS	iii
LIST OF TABLES.....	viii
LIST OF FIGURES.....	ix
LIST OF APPENDICES	xii

CHAPTER

1	INTRODUCTION	1
	1.1 PROBLEM STATEMENT.....	1
	1.2 GENERAL STRATEGY.....	2
2	ARTICULAR CARTILAGE	6
	2.1 INTRODUCTION	6
	2.2 MORPHOGENESIS OF CARTILAGE	7
	2.3 PROPERTIES AND COMPOSITION OF CARTILAGE.....	9
	2.4 DISEASE, DAMAGE, AND HEALING.....	11
	2.5 CLINICAL THERAPIES.....	14
	2.6 CARTILAGE TISSUE ENGINEERING.....	18
	2.6.1 Cells.....	19

2.6.2	Chondrogenic Growth Factors.....	21
2.6.3	Biomaterial Scaffolds	23
2.7	CONCLUSIONS.....	28
3	EFFECTS OF CHONDROINDUCTIVE BIOMOLECULE HYALURONIC ACID ON CHONDROGENESIS	42
3.1	INTRODUCTION	42
3.2	MATERIALS AND METHODS	45
3.3	RESULTS	51
3.4	DISCUSSION	57
3.5	CONCLUSIONS.....	60
4	EFFECTS OF CHONDROCONDUCTIVE PORE GEOMETRY ON CHONDROGENESIS	79
4.1	INTRODUCTION	79
4.2	MATERIALS AND METHODS	81
4.3	RESULTS	87
4.4	DISCUSSION	91
4.5	CONCLUSIONS.....	96
5	COMBINED EFFECTS OF HYALURONIC ACID AND PORE GEOMETRY ON CHONDROGENESIS.....	111
5.1	INTRODUCTION	111
5.2	MATERIALS AND METHODS	112
5.3	RESULTS	118
5.4	DISCUSSION	121

5.5	CONCLUSIONS.....	124
6	CONCLUSIONS AND FUTURE WORK.....	135
6.1	INTRODUCTION	135
6.2	EXPERIMENTAL CONCLUSIONS	136
6.2.1	Hyaluronic Acid Stimulated Chondrogenesis.....	136
6.2.2	Ellipsoid Pore Geometry Induced Chondrogenesis	136
6.2.3	Combined Effects of Hyaluronic Acid and Pore Geometry	136
6.2.4	Comprehensive Summary	138
6.3	SIGNIFICANCE AND IMPLICATIONS	139
6.4	FUTURE DIRECTIONS.....	145
6.4.1	Evaluation of constructs at an orthotopic site.....	145
6.4.2	Effects of pore size and cellular density	145
6.4.3	Non-invasive Methods of Cartilage Evaluation	146
6.5	CLOSING REMARKS.....	147
	APPENDICES.....	155

LIST OF TABLES

Table

4.1	Design parameters and calculated scaffold properties for SFF scaffolds with cubic and ellipsoid pore geometries.....	98
6.1	Summary of the histomorphometric analyses of all conducted experiments. The results are organized by scaffold biomaterial and pore geometry, cell type, hydrogel type, culture medium and culture period conditions.....	148
6.2	Designed scaffold parameters and physical properties. Comparison between small and large pore designs of the same cubic and ellipsoid pore geometries	149

LIST OF FIGURES

Figure

2.1	Skeletal condensations and micromass cultures stained with peanut agglutinin lectin, Alcian Blue, and Safranin O.....	30
2.2	The four zones of articular cartilage: superficial, middle, deep, and calcified cartilage.....	31
2.3	Autologous chondrocyte implantation is commonly used to repair single defects in articular cartilage	32
3.1	Schematic of hyaluronic acid mediated cellular aggregation.....	62
3.2	Images of SFF design files and fabricated cubic scaffold.....	63
3.3	BMSC aggregation within 1, 2, 5% HyA and collagen I only hydrogels.....	64
3.4	Chondrocyte aggregation within 1, 2, 5% HyA and collagen I only hydrogels.....	65
3.5	DMMB analysis of BMSC and chondrocyte seeded hydrogels with varying HyA concentrations	66
3.6	Cartilage formation in PCL scaffolds seeded with BMSC in collagen I and 5% HyA/collagen hydrogels and cultured in chondrogenic medium	68
3.6	Cartilage formation in PPF scaffolds seeded with chondrocytes in collagen I and 5% HyA/collagen hydrogels	69
3.7	Immunohistochemistry detecting collagen II in SFF scaffolds seeded with BMSC and chondrocytes	70
3.8	Immunohistochemistry detecting collagen I in SFF scaffolds seeded with BMSC and chondrocytes	71

3.9	Results from Safranin O histomorphometric image analysis of BMSC and chondrocytes seeded SFF scaffolds	72
3.10	Passaged chondrocytes seeded into SFF scaffolds using collagen I and composite 5% HyA/collagen hydrogels	73
3.11	Passaged chondrocytes seeded into SFF scaffolds using collagen I and composite 5% HyA/collagen hydrogels	74
4.1	Designed scaffold architectures and fabricated SFF scaffolds	97
4.2	Fluorescent microscope images of pre-chondrogenic aggregates in BMSC seeded scaffolds.....	99
4.3	BMSC seeded into PPF scaffolds using collagen I hydrogels and cultured in basal medium showed no areas of chondrogenesis	100
4.4	Cartilage formation in PPF scaffolds. BMSC seeded in cubic and ellipsoid scaffolds and cultured in chondrogenic medium	101
4.4	Cartilage formation in PPF scaffolds. Chondrocytes seeded in cubic and ellipsoid scaffolds and cultured in basal medium	102
4.4	Cartilage formation in PPF scaffolds. Chondrocytes seeded in cubic and ellipsoid scaffolds and cultured in chondrogenic medium.	103
4.5	Immunohistochemistry detecting collagen II and collagen I in SFF scaffolds seeded with BMSC and cultured in chondrogenic medium	104
4.6	Immunohistochemistry detecting collagen II and collagen I in PPF scaffolds seeded with chondrocytes and cultured in basal medium	105
4.7	Immunohistochemistry detecting collagen II and collagen I in PPF scaffolds seeded with chondrocytes and cultured in chondrogenic medium	106
4.8	Impact of scaffold architecture on chondrocytic activity using histomorphometric data. Analysis of Safranin O stain sections of cubic and ellipsoid pore scaffolds seeded with BMSC or chondrocytes and cultured in basal or chondrogenic medium	107

5.1	Designed scaffold architectures and fabricated PCLF scaffolds	125
5.2	Visualization of pre-chondrogenic cellular aggregates using rhodamine-conjugated peanut agglutinin	126
5.3	Cartilage formation in cubic and ellipsoid PCLF scaffolds seeded with BMSC using collagen hydrogels	127
5.3	Cartilage formation in cubic and ellipsoid PCLF scaffolds seeded with BMSC using 5% HyA/collagen hydrogels	128
5.4	Cartilage formation in cubic and ellipsoid PCLF scaffolds seeded with chondrocytes using collagen hydrogels	129
5.4	Cartilage formation in cubic and ellipsoid PCLF scaffolds seeded with chondrocytes using 5% HyA/collagen hydrogels	130
5.5	Evaluation of the impact of hydrogel type and pore geometry on BMSC activity using histomorphometric data	131
5.6	Evaluation of the impact of hydrogel type and pore geometry on chondrocytic activity using histomorphometric data	132
5.7	Immunohistochemistry detecting collagen II in PCLF scaffolds seeded with BMSC and chondrocytes	133
6.1	Cartilage formation in PCLF scaffolds with designed large pore cubic and ellipsoid geometries	150
E.1	Examples of Safranin O stain intensity correlation to sGAG concentration	180
E.2	Safranin O quantification by histomorphometrical analysis	181
F.1	Safranin O quantification by histomorphometrical analysis	187

LIST OF APPENDICES

Appendix

A	PRIMARY CHONDROCYTE AND BMSC ISOLATION.....	156
B	SCAFFOLD BIOMATERIAL AND SFF FABRICATION.....	161
C	COMPOSITE HYALURONIC ACID/COLLAGEN HYDROGEL FABRICATION AND SFF SCAFFOLD SEEDING.....	167
D	DMMB ASSAY FOR GAG QUANTIFICATION	171
E	SAFRANIN O QUANTIFICATION BY HISTOMORPHOMETRIC IMAGE ANALYSIS	175
F	IMMUNOHISTOCHEMISTRY	183

CHAPTER 1

INTRODUCTION

1.1 PROBLEM STATEMENT

The human body has remarkable regenerative and repair mechanisms, where regeneration of tissue and function are achievable in many parts of the body.^{1,2} But for many centuries, physicians and scientists have attempted to repair or regenerate articular cartilage. To this day, it is still widely accepted that ulcerated cartilage cannot be fully regenerated. Despite advances in science and clinical therapies, full restoration of the biochemical composition, structural organization and mechanical properties of cartilage have not yet been achieved.³

Articular cartilage is a very complex organ that is unique in its isolation from the body. Due to the absence of vasculature, lymphatic vessels, and nerves, the repair mechanisms that are usually elicited after an injury do not occur. Repair mechanisms are only activated when the subchondral bone is penetrated during an injury. The repair tissue that forms is a type of fibrocartilage that is composed of collagen I and substitute proteoglycans. This tissue does not have biochemical or mechanical properties comparable to native tissue, and will generally lead to degeneration of the repair tissue.

The difficulty with regeneration of articular cartilage is due to a deficit of three key elements: 1) a large number of responsive cells able to repopulate the defect, 2) growth factors and signaling molecules to promote new tissue formation, and 3) an extracellular matrix or framework that fills the defect space and helps

organize the cells spatially.⁴ The clinical therapies used currently for articular cartilage defects do address some of these concerns, but not all three at once. Thus, fibrocartilage formation is still the standard result and in many cases treatment only temporarily alleviates the pain and/or problem.⁵

The development of tissue engineering strategies has increased the potential to promote chondrogenesis in defects and enhance the quality of the repair tissue. Tissue engineering combines responsive cells with the appropriate growth factors and biomaterials matrices to address the three deficits together. For cartilage repair and regeneration, a combination of these three elements and an understanding of the defect microenvironment will lead to the generation of more hyaline-like tissues. Many scientists have investigated the formation of repair tissue stimulated by microenvironments that induce cellular condensation and chondrogenic differentiation. Some factors that initiate chondrogenesis such as growth factor cocktail^{6,7} and high cellular density^{8,9} have been established. Other aspects that will make these tissue-engineered constructs feasible for use in clinical defects continue to be unresolved. One such factor is the poor mechanical stability of the matrices currently being investigated for cartilage tissue engineering.¹⁰ Another factor that has not been examined readily is the physical properties of scaffolds that are used for cartilage tissue engineering and their impact on tissue regeneration.

1.2 GENERAL STRATEGY

Our approach to cartilage tissue engineering focuses on stimulating adult mesenchymal stem cells and chondrocytes to form new cartilage tissue within solid freeform (SFF) fabricated scaffolds. SFF technology is a powerful scaffold fabrication method that allows users to define many scaffold properties, such as the external geometries, porosity, pore architecture, pore size, and scaffold

permeability. Furthermore, the mechanical properties of the SFF scaffolds can also be controlled and adjusted to provide support to the developing tissue. Since many of these scaffold properties could not be manipulated or defined with the conventional scaffolds used in cartilage tissue engineering, the desired scaffold properties remain largely unknown. The advantage of SFF is the ability to design scaffolds that can isolate these properties, so that we can begin to understand how the physical properties of scaffolds influence cartilage tissue formation.

The work in this thesis examines chondroinductive and chondroconductive microenvironments that induce cellular condensation and evaluate the ability of these microenvironments to stimulate chondrogenesis by BMSC and chondrocytes within SFF scaffolds. First, chondroinductive hyaluronic acid (HyA) hydrogels that will serve as cell carriers for the SFF scaffolds are evaluated. The hydrogels increase cellular retention within the SFF scaffolds and HyA is an ubiquitous glycosaminoglycan that is present during mesenchymal condensation. It facilitates cellular migration, proliferation, and also aids in the formation of cellular aggregates. HyA is also found in the extracellular matrix of cartilage, where it also serves a mechanical role, as the backbone of the proteoglycan network. The second step evaluates the effect of two designed pore geometries, cubic and ellipsoid, on cartilage tissue formation. Previous research has shown that high cellular density, spherical cell morphology, and lower permeabilities are required for chondrogenic differentiation. The ellipsoid pore geometry mimics the shape of cellular condensations and increases cellular packing density, which will in turn increase chondrogenic differentiation. The last part combines the hyaluronic acid hydrogels with designed pore geometries to assess the synergistic effects of these factors on chondrogenesis.

The global hypothesis of this thesis is that the formation of high-density cellular condensations directed by biomolecules (hyaluronic acid) and designed scaffold architecture in the presence of chondroinductive growth factors will provide an environment that enhances chondrogenesis by bone marrow stromal cells (BMSC) and chondrocytes in SFF scaffolds.

After a short introduction to the morphogenesis of cartilage and the properties of articular cartilage, Chapter 2 reviews the current treatment methods for cartilage defects, relevant studies in cartilage tissue engineering, and the motivations behind the work in this thesis. Chapter 3 describes the assessment of hyaluronic acid hydrogels both *in vitro* and *in vivo* in SFF scaffolds. In Chapter 4, the effect of SFF scaffold pore geometry on chondrogenesis is investigated. The combined effects of HyA hydrogels and SFF scaffold pore geometry are detailed in Chapter 5. In Chapter 6, the conclusions and future directions are presented.

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CHAPTER 2

ARTICULAR CARTILAGE

2.1 INTRODUCTION

Articular cartilage is a complex organ that is unique in its isolation from the body due to the absence of vasculature, lymphatic vessels, and nerves. In its isolation, the repair mechanisms of the body that are usually elicited after an injury do not occur. A repair response is only generated when the underlying subchondral bone is penetrated. The infiltrating blood brings mesenchymal stem cells and growth factors required for growth and regeneration. It is through a combination of this response and the events of embryonic cartilage morphogenesis, that we model the research described in this thesis. The objective is to create conducive microenvironments for chondrogenic differentiation by using biochemical and biomaterial scaffolds that influence cellular condensation. Creating microenvironments that are present during limb morphogenesis will help us recreate a tissue construct that will be able to regenerate function and promote the growth of tissue that is similar to native tissue.

The field of tissue engineering has opened up new possibilities for repair and regeneration of cartilage by combining responsive cells, biological factors, and scaffold matrices.^{1,2} By using these three key elements, scientists hope to recreate an environment that initiates morphogenesis or a regenerative response after injury. For cartilage tissue engineering, this biomimetic approach can be recapitulated in the chondrogenic events of the embryo. During embryonic pre-

chondrogenesis, mesenchymal progenitor cells form cellular condensations, which become the template for cartilaginous and osseous formation. Through a series of cell-cell, cell-matrix interactions and other signaling mechanisms, the cells within these condensations differentiate into chondrocytes. Bone then replaces cartilage and in some areas, the cartilage becomes permanent. Understanding this process of chondrogenesis and endochondral ossification should elucidate fundamental factors that are required for chondrogenesis of hyaline-like repair tissue in adults.

2.2 MORPHOGENESIS OF CARTILAGE

The embryonic limb is considered the classic model for studying cartilage morphogenesis. The formation of a skeletal element that will develop into a limb occurs in a four phase process: 1) migration of mesenchymal cells to the site of skeletogenesis, 2) the interaction of the mesenchymal cells with epithelium and epithelial basement membrane, 3) these interactions with epithelial cell products initiate cellular condensation and 4) overt differentiation of cells into chondroblasts or osteoblasts.³ The first step of the process begins when undifferentiated mesenchymal cells start to produce an extracellular matrix that is rich in collagen I, hyaluronan, tenascin and fibronectin.⁴ These proteins in the matrix aid in the migration of dispersed MSC population to a central location.

The epithelial-mesenchyme interactions of the second phase localize the skeletogenic mesenchyme. The interactions also provide signals to initiate condensation of the mesenchyme, permit differentiation of cartilage (or bone, enamel), and set the number of progenitor cells required for the skeletal element.⁵

Condensation is the earliest stage during organ formation when tissue-specific genes are upregulated. It is a multistep process where initiation, establishment of boundary conditions, cell adhesion, proliferation, growth, and cessation of growth occur. There are a few ways in which condensations may arise: 1) a difference between the cells within the condensation and the cells surrounding the condensation, 2) the cells within the aggregate may have increased proliferation, have shorter cell cycle times, or decreased cell death, 3) the cells are part of a larger dividing cell population or 4) the cells are migrating towards a center or do not move away from a center. These cellular condensations lead to altered mitotic activity, changed cell density, and aggregation. The mesenchymal cells within these condensations have a round morphology, high nucleocytoplasmic ratio, large nucleoli, poorly developed endoplasmic reticulum, and small mitochondria. (FIGURE 2.1) The condensation is accompanied by an increase in gap junction formation and increased cell-to-cell communication.⁶

In order for the cells to proceed into the differentiation, they must attain a critical size. Umansky found that chondrogenesis was only initiated if the cell density exceeded 5000 cells/mm².⁵ Furthermore, the cells within the condensation must communicate and act together as a unit in order for differentiation to occur. All cells present must be of the same differentiation type and be at the same stage of differentiation. Chondrogenic differentiation of the cells starts with the downregulation of N-cadherin and NCAM to stop growth and decrease fibronectin expression.⁷ During overt differentiation, the expression of Hox genes, signaling of the BMP pathways, and the activation of transcription factor sox9 and its co-factors, L-sox5 and sox6, lead to the production of collagen II, IX, and XI, aggrecan and other cartilage proteins.^{8,9}

2.3 PROPERTIES AND COMPOSITION OF CARTILAGE

At first glance, articular cartilage appears to be a rather simple, inert tissue, lacking blood vessels, lymphatic vessels, and nerves. Compared with other tissues, cartilage seems to have a low metabolic activity (due to the low number of cells present, not due to the metabolic activity of the cells). Upon further examination, although only a few millimeters in thickness, its ability to distribute loads, its durability and functions indicate that cartilage is a highly complex organ with many interactions.

The cartilage tissue consists of cells, extracellular matrix, and extracellular fluid. The cells, chondrocytes, occupy roughly 1% of the tissue volume. Although there is only one cell type, the chondrocytes present in each zone of cartilage are different in size, shape, metabolic activity and mechanical function. The extracellular matrix adds another 20 to 40% of wet weight in cartilage. The structural macromolecules, such as collagens, proteoglycans, and non-collagenous proteins, give the cartilage tissue its form and stability. Water and other electrolytes found in the extracellular fluid comprise up to 80 percent of the wet tissue weight. The mechanical properties of the cartilage are dependent upon the flow of this extracellular fluid in and out of the tissue and the interaction of the negatively charged proteoglycans with the electrolytes in the fluid.

Cartilage is divided into four zones from the articular surface to the subchondral bone: superficial, middle, deep, and calcified cartilage. (FIGURE 2.2) Each zone has a different extracellular macromolecular composition and the cell morphology and function also vary accordingly. This zonal organization is very important to the overall mechanical function of the tissue.¹⁰

The superficial zone is the thinnest zone, but it serves as the first mode of protection from mechanical and biologic stresses. The chondrocytes in this zone are ellipsoid in shape and are oriented parallel to the articular surface. The extracellular matrix surrounding the cells is a dense network of collagen fibrils that are also oriented parallel to the surface. The orientation of the collagen fibrils increase the tensile stiffness and help resist shear and compressive forces. The dense network of collagen also serves as a barrier and filter for larger proteins, such as antibodies and other inflammatory proteins. This zone contains the highest concentration of collagen and water, but the lowest amount of proteoglycans (PG).

The middle zone is a transitional layer which has both elements of the superficial and the deep zones. The chondrocytes are spheroid and have higher proteoglycan synthesizing capabilities than chondrocytes in the superficial zone. The extracellular matrix consists of a lower concentration of water and collagen with larger fibril diameters and higher concentrations of proteoglycans.

The cells and collagen fibrils in the deep zone are oriented perpendicularly to the articular surface. The perpendicular orientation further enhances the compressive properties of the tissue. This region has the largest diameter collagens and the highest amounts of large aggregating proteoglycans, but the lowest amounts of water. The negatively charged proteoglycans also contribute repulsion forces to protect the matrix and cells from compressive stresses.

The tidemark is a curvy demarcation that delineates the separation between the non-calcified deep zone and calcified cartilage. The large perpendicular collagen fiber bundles from the deep zone cross past the tidemark and into the zone of calcified cartilage. These collagen fibers furcated to form an interlocking network that anchors the cartilage tissue to the subchondral bone.¹¹

One of the primary functions of cartilage is to absorb mechanical shocks and distribute high loads evenly across the underlying bone. The collagen and PG network, cells, and interstitial fluid are organized within the tissue to support a high compressive load with very low friction and wear. The complex interaction of cartilage tissue can be separated into three interacting phases: 1) a charged solid phase, that is composed of a collagen network that is reinforced by a hyaluronan mesh with many linked aggregating proteoglycans, such as chondroitin sulphate, keratan sulphate, and other negatively charged glycosaminoglycans, 2) the fluid phase, or the interstitial fluid, which is mostly water, and 3) the ionic electrolytes present in the interstitial fluid that balance out the negative charges of the glycosaminoglycans (GAG).¹² The capacity of articular cartilage to support load is dependent upon the interaction of these three phases. When a compressive force is exerted upon the tissue, the first response is the efflux of the interstitial fluid. The collagen and PG network is then redistributed until equilibrium is achieved between the PG aggregates and the remaining interstitial fluid. Some studies have shown that the interstitial fluid pressurization can sustain to 95% of the total load applied, while the remaining 5% is supported by the collagen and PG network.¹³

2.4 DISEASE, DAMAGE, AND HEALING

Deterioration of articular cartilage and alterations in the joint, which lead to loss of function and integrity of the articular cartilage, are among the most common causes of pain and loss in the quality of life amongst middle-aged and senior persons. The degeneration of articular cartilage is most commonly presented clinically as osteoarthritis (OA), where the progressive loss of normal structure and function result in the fraying of the superficial zone, decreased proteoglycans in both superficial and transitional zones, and the invasion of blood vessels from the underlying subchondral bone into the cartilage matrix.

Studies indicate that there is an increased prevalence of OA with age, although the cause is not due to the 'wear and tear' of age and lifelong mechanical stresses. Age related changes in chondrocyte function and activity may contribute to the disease progression. Other studies indicate that joint instability, abnormal joint anatomy, long term static loading and inadequate muscle strength show greater risks for degeneration. Specific activities, such as repetitive movements that cause joint stress, high impact, or torsional loading, may also increase the probability of disease.¹⁴

The primary reason for the limited ability of cartilage to regenerate is due to its isolation from the body. The only source of metabolites and nutrients for the cells is delivered by the interstitial fluid, which is further filtered by the dense extracellular matrix.¹⁵ Due to this sheltered environment, very few repair mechanisms are activated when damage occurs. Three types of injuries can occur: 1) damage to the extracellular matrix and cells without visible disruption to the surface of the cartilage, 2) partial thickness cartilage lesions with fractures and ruptures, and 3) full thickness osteochondral lesions.¹⁶

The first type of injury is damage to the cartilage matrix and cells without a visible disruption on the cartilage surface. The first response of damaged chondrocytes to a traumatic injury is to undergo apoptosis. The process of apoptosis is thought to help regeneration and stimulate the neighboring cells to increase proliferation and matrix production to account for the degeneration and apoptosis. Depending on the extent of the damage, the tissue may be regenerated or if the tissue loss is significant, then a lesion may develop and lead to further degeneration and possibly to the formation of a partial thickness defect.

The response to a partial thickness defect, where the subchondral bone is not penetrated is similar to the superficial injury. There is apoptosis, limited cell proliferation and a slight increase in matrix deposition. Synthesis of a new matrix and cellular proliferation will fill the defect partially. Depending upon the location and size of the lesion, the lesion may lead to further instability or if structural integrity is present the lesion may not deteriorate any further. In most cases, defects smaller than 2 mm² it will heal completely, but defects larger than 2 mm² will lead to further matrix degeneration and cell death.¹⁷

If the defect penetrates the subchondral bone, then a spontaneous healing response is observed. A blood clot forms as the foundation for the influx of growth factors and mesenchymal progenitor cells. Generally in mature cartilage, two to three months following the injury, repair tissue forms a new layer of bone and fibrocartilage. But upon further examination, the repair tissue has high amounts of type I collagen and substitute proteoglycans that are not usually found in articular cartilage. This fibrocartilage formed lacks the required mechanical properties and will degenerate over the next 6 – 12 months.¹⁸

The quality of repair tissue that is formed after injury to cartilage is dependent not only upon the size and depth of the defect, but also upon the age of the individual, as shown in a study conducted by Johnstone and Yoo.¹⁹ The repair response was compared between 5 week and 4 month old rabbits. Full thickness defects 2 mm in depth were created in the trochlear groove and the repair tissue was assessed after 12 weeks. The results showed that the younger rabbits were able to completely regenerate hyaline-like cartilage with a new tidemark distinctly dividing newly formed cartilage and bone. The new tissue was also fully integrated with the non-damaged tissue. At the time of injury, the cartilage extracellular matrix was still developing in the younger rabbits, so the number of pluripotent mesenchymal stem cells that infiltrated the defect site and the

biosynthetic activity of the immature cartilage cells were high enough to regenerate the tissue. In the older rabbit, however, fibrocartilage and scar tissue replaced the defect. The metabolic activity of chondrocytes decreases with age, as does the number of pluripotent mesenchymal stem cells present in the subchondral bone. The weak repair response in the older rabbits is due to the presence of fewer numbers of chondrocytes that are able to increase extracellular matrix production to repair the defect and decreased numbers of mesenchymal stem cells that infiltrate the defect from the subchondral bone that are able to differentiate chondrogenically. The ratio of proteoglycans also changes with age, and as the concentration of lower molecular weight proteoglycans increases, the mechanical strength of the tissue decreases and makes it more susceptible to damage.

2.5 CLINICAL THERAPIES

Many of the clinical approaches to repairing cartilage defects exposes the subchondral bone to initiate a spontaneous healing response that brings mesenchymal stem cells and appropriate growth factors. The outcome of these treatments depends upon many factors, such as the age of the patient, the post-operative rehabilitation, defect size, and the condition of the joint. Determining the success of a treatment is ultimately assessed by the quality of the repair tissue that is generated, the biochemical composition of the repair/regenerated tissue, the integration between the new and native cartilage, and its response to mechanical stresses. These factors must be taken into account when evaluating the repair tissue and also treatments for defects.

Current therapeutic approaches to cartilage injury include various types of shaving or drilling into the surface of the cartilage or transplanting autogenic chondrocytes to the defect (autologous chondrocyte implantation, ACI).

Methods that do not penetrate the subchondral bone, such as debridement and abrasion arthroplasty, are generally used when defects are less severe. For more degenerative conditions, microfracture or mosaicplasty are used to help repair the defect by puncturing the subchondral bone and promoting spontaneous healing.²⁰

For single defects located in the femoral condyle or patella, autologous chondrocyte implantations (ACI) may be used to repair the defect.²¹ (FIGURE 2.3) During an initial arthroscopy, the damaged area is assessed and 200 to 400 mg of articular cartilage is harvested from a non-loading bearing area, such as the trochlea or the intercondylar notch. Chondrocytes are isolated from this tissue and expanded by monolayer culture *in vitro*. Three to six weeks later, the patient returns for the second half of the ACI procedure. The expanded chondrocytes, usually between 10 – 20 million in number, are prepared for implantation by trypsinization and resuspension in DMEM. The damaged tissue is debrided down to the subchondral bone. A periosteal implant is harvested from the proximal tibia and sutured to the bottom of the defect. The cell suspension is then injected into the defect site. The periosteal implant serves as a barrier to retain the injected cells at the defect site. The periosteum also contains pluripotent cells that have the potential to differentiate into chondrocytes. Furthermore, endogenous expression of TGF- β by the cells in the periosteum provides a source of biofactors that encourage cartilage development.^{22,23}

Compared to the treatments mentioned previously, ACI have high success rates (89-92%) for single defects in the patella or condyle and patients under 35 seem to generate better repair tissue.²⁴ Defects ranging between 1 and 16 cm² and up to 8 mm in depth are reparable with good results, given that no other degenerative diseases or other changes distress the repair site.²⁵ A few randomized clinical studies have compared these techniques to assess the ability

to generate hyaline cartilage. Since these studies were only conducted one or two years prior, it is hard to compare the repair tissue generated after these treatments without longer-termed evaluations. In two studies, one comparing mosaicplasty to ACI and another comparing microfracture to ACI, found that there were no significant differences between the patient scores or the histology of the repair tissue.^{26,27}

Despite the high success rates, there are still major disadvantages to chondrocyte implantation. The use of ACI for multiple lesions, degenerative diseases, such as osteoarthritis, and repair of defects located at other cartilaginous surfaces is poor. Long term assessment of the repair tissue suggests that there are also complications attached to the use of periosteal grafts that may limit the quality of the repair tissue. The first is the sensitivity of the periosteal implant to drying; prolonged exposure (over 5 min) of the implant to air is very detrimental to the graft. Drying drastically inhibits neocartilage formation and collagen II expression of the explant.²⁸ Furthermore, between 18 – 25% of patients that have had ACI treatment develop fibrous and hypertrophic repair tissue, and in the worst cases, calcified tissue and ectopic bone may form at the defect site. The development of hypertrophic graft edges lead to poor graft integration and delamination. This leads to symptoms such as locking, instability, or pain that require further treatment and surgery.²⁹

In recent years, improvements upon the ACI technique have been made to make the surgery less invasive and reduce the risk of hypertrophic tissue development. The procedure is very similar to ACI, donor chondrocytes are still harvested, but the periosteal graft is replaced with a matrix seeded with the expanded chondrocytes. Currently, there are a few matrices undergoing clinical evaluation for use as matrix-based autologous chondrocyte implantation devices. The use of these implants seeks to decrease the cell morbidity, increase cell retention at

the defect site, reduce implant hypertrophy, and simplify the surgical procedure. The replacement of the periosteal graft with a collagen or a hyaluronan matrix allows the cells to be loaded into the scaffold and cultured *in vitro* prior to the implantation. Seeding the cells onto the implant prior to the surgery allows greater cell retention within the matrix and it also gives the cells time to adjust, redifferentiate, proliferate, and start depositing an extracellular matrix within the implant. Without the removal of a periosteal graft, the surgical time is reduced. Many of these implants can be fixed to the defect site using fibrin glue, which further reduces the surgical time.

Some examples of these implants used in conjunction with ACI are shown in Figure 2.3. Chondro-Gide is a mesh-like matrix made from a combination of collagen I and collagen III. The implant has a rough collagen surface on one side for cell seeding and on the other surface, a higher collagen density and smoother surface prevents fibroblast invasion into the implant. The short term results (1 – 3 years) of the matrix-induced autologous chondrocyte implantation (MACI®) show comparable tissue formation and graft hypertrophy was reduced to 6%.³⁰ Another implant that is being used is Hyalograft C, also a mesh-like matrix, that is made from hyaluronan ester.^{31,32} Hyaluronan is a natural biologic found in the joint and many studies have shown positive cell response to injections of hyaluronic acid into the joint space and as well as matrices made from hyaluronan.^{33,34} Instead of a mesh-like matrix, Ars Arthro is in clinical trials with a collagen I hydrogel (CaReS®) that reduces the *in vitro* expansion time period, which reduces dedifferentiation of the cells. The CaReS® implant also has some viscoelastic properties that are comparable to articular cartilage. With all of these new implants, it is still too early to determine if the pre-culture time *in vitro* is more beneficial for the long term repair, if the degradation of the implants affects the repair tissue and how the repair tissue compares with other treatments.

The incorporation of scaffold matrices with the ACI procedure to spatially organize the cells within the defect is currently being evaluated, but these meshes and hydrogels do not have the mechanical properties required to protect the developing tissue from joint stress. Since the clinical trials for these matrices are still in the early stages, it remains to be seen how stable the repair tissue will be within these matrices. Thus, this is an area where cartilage tissue engineering research can be applied to generate a better repair construct within load-bearing scaffolds.

2.6 CARTILAGE TISSUE ENGINEERING

The prevailing dilemma with cartilage regeneration is a lack of cells that are capable of repair, a lack of growth factor stimulation and a lack of spatial organization for the cells. Successful strategies for tissue engineering cartilage combine responsive cells in sufficient numbers, appropriate biological signaling factors, and biocompatible scaffold matrices to address this issue. A combination of these three factors is used to create microenvironments that simulate the cellular condensation stage of cartilage morphogenesis. Environmental conditions, such as high cellular density, low permeability, and chondroinductive biomolecules, have been found to increase chondrogenesis and enhance the quality of the repair tissue.⁵

The potential candidates for each of the three key elements and how they aid in the regeneration of cartilage are reviewed in this section, along with their advantages and disadvantages. First, there are two predominant cell sources being investigated for the regeneration of cartilage, chondrocytes and adult mesenchymal stem cells (MSC) derived from the bone marrow. Both of these cell types are responsive to an established growth factor cocktail, which induces chondrogenic differentiation and promotes extracellular matrix production. The

final factor discussed are the biomaterial scaffolds. The scaffolds can be classified into two major categories: 1) conventional sponge, mesh, and hydrogel matrices and 2) computer designed scaffolds made with solid freeform fabrication (SFF).

2.6.1 Cells

There are two cell sources that are currently under investigation for cartilage repair/regeneration: chondrocytes and adult mesenchymal stem cells (MSC). Sources for autologous chondrocytes are limited and the explant areas required to repopulate the defects are large. At many of these explant sites, the removal of cartilage results in fibrocartilage development and further cartilage degeneration. As described earlier, in order to attain the number of cells required for repairing defects, the chondrocytes are cultured *in vitro* in monolayer. Many studies have shown that monolayer culture and cell expansion alters the chondrocytic phenotype. The chondrocytes become fibroblastic and lose their ability to synthesize collagen II and other cartilage proteins.³⁵ The repair tissue formed using these expanded chondrocytes is weaker than the tissue regenerated by non-passaged chondrocytes.^{36,37}

Current research indicates that adult MSC may be a better regenerative cell source for cartilage repair. MSC are derived from bone marrow aspirates and are cultured as a heteropopulation of cells termed bone marrow stromal cells (BMSC) or a purified population with cell markers that are indicative of multipotency (MSC). Both populations of cells are pluripotent and retain the capacity to differentiate into osteoblasts, chondrocytes, adipocytes, myocytes, and even neurons when cultured with the appropriate differentiation factors.^{38,39} These cells are the adult versions of the cells that participate in the mesenchymal condensations during embryonic cartilage morphogenesis. The use of MSC or

BMSC alleviate the need for donor chondrocytes, since they can be isolated from most individuals and cultured as an autologous source of cells. Not only is the removal of these cells less taxing on the body, but some studies have shown that the repair tissue is more hyaline-like than the tissue formed by committed chondrocytes.⁴⁰

The extracellular environment also affects the capability of the BMSC to differentiate into chondrocytes. Both high density culture and the scaffold material affect the cell-cell and cell-matrix signaling mechanisms which influence BMSC differentiation. A key factor for inducing BMSC into chondrogenic differentiation *in vitro* is high density cell culture or minimizing cell attachment to the substrate. The cell shape and the maintenance of a spherical morphology is also an important factor to consider. An established method for chondrogenic differentiation was introduced by Yoo and Johnstone.^{41,42} The scientists developed a technique of culturing limb-bud mesenchymal cells in high density pellets *in vitro* to study the process of embryonic chondrogenesis and endochondral ossification. Recently, these cellular pellet cultures have been used to maintain cell phenotype and induce cell differentiation.^{43,44} High density cell pellet cultures help prevent dedifferentiation of chondrocytes *in vitro*^{45,46} and induce BMSC to differentiate into chondrocytes.^{47,48} The drawback with this method is that the maturation of the cells into hypertrophy is accelerated. After 1 week of culture, the presence of collagen X in the matrix is seen. The appearance of collagen X leads to vascular invasion of the extracellular matrix and eventually to bone formation. Furthermore, the fabrication of these high density cell pellets is time consuming and difficult to mass produce. Therefore, the discovery of new methods to create cellular aggregates that are less time intensive and inhibit maturation by combining cellular aggregates with growth factors will improve upon the current techniques used to stimulate chondrogenesis *in vitro*.

2.6.2 Chondrogenic Growth Factors

Biofactors will be used to stimulate the BMSC and chondrocytes into chondrogenic differentiation and extracellular matrix production *in vitro*. These biofactors will simulate the *in vivo* environment and give specific signals to the cells to activate the chondrogenic pathways. Previous experimentation has shown that without the addition of the chondrogenic biofactors, BMSC in cell pellets do not aggregate and do not differentiate.⁴²

The defined chondrogenic media used in the experiments for this thesis consists of high glucose Dulbecco's Modified Eagle's Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% Penicillin/ Streptomycin (PS) supplemented with 10 ng/mL transforming growth factor- β_1 (TGF- β_1), 5 $\mu\text{g}/\text{mL}$ insulin, 100 nM dexamethasone, 50 $\mu\text{g}/\text{mL}$ 2-phospho-L-ascorbic acid, 0.1 mM non-essential amino acids, and 0.4 mM L-proline. Using this defined media formulation, Martin *et al.* found that a combination of dexamethasone, insulin, and TGF- β_1 increased collagen, sulfated glycosaminoglycans (sGAG) and DNA content in poly-glycolic acid (PGA) meshes seeded with bovine BMSC.⁴⁹

Each of the elements in the chondrogenic media provides a signaling factor to induce differentiation or provide the nutrients required for extracellular matrix deposition and cellular metabolism. The essential biofactors in the media are TGF- β , dexamethasone and insulin. Their roles will be described further below. The other components, such as the proline and non-essential amino acids, are not found in the DMEM formulation, but are required for collagen morphogenesis. Ascorbic acid is an organic acid which also aids in extracellular matrix production. The addition of exogenous ascorbic acid to micromass cultures doubled the amount of sGAG when compared to cultures without ascorbic acid.⁵⁰

A combination of TGF- β , dexamethasone, and insulin significantly increased the amounts of DNA, GAG, and collagen over groups with dexamethasone alone or dexamethasone and insulin.⁴⁹ The combination of these three elements stimulates multiple signaling pathways that eventually lead to the chondrogenic differentiation of BMSC. The paragraphs below present an overview of the observed effects these biofactors have on BMSC differentiation.

The primary signaling factor in the chondrogenic media is TGF- β . Both TGF- β_1 and TGF- β_3 enhance BMSC differentiation into chondrocytes by increasing cellular proliferation presumably beyond a critical density that is required for chondrogenesis.^{51,52} TGF- β also helps initiate cellular condensation by regulating the expression of fibronectin.⁵ Increased expression of type II collagen mRNA and other extracellular matrix proteoglycans are also associated with TGF- β signaling. Cell aggregates cultured in a series of TGF- β_1 and then IGF-1 increased the markers of chondrocytic function, such as proteoglycan content and procollagen type II mRNA.⁵³

Chondrocytes and BMSC have cell receptors that have a high affinity for both insulin and IGF-1.⁵⁴ Insulin is intimately involved during the progression and the onset of chondrogenesis.⁵⁵ It stimulates both growth-promoting and metabolic activities in chondrocytes. It has been also shown to increase cell size and act synergistically with amino acids to promote sulfate incorporation in cartilage.⁵⁶

Dexamethasone functions to support cell viability and modulate cellular activities of differentiating cells.⁵⁵ In a study on the effects of dexamethasone and human BMSC formation of bone *in vivo*, dexamethasone increased the reproducibility of bone formation between patients and increased bone forming

ability of lower capacity BMSC.⁵⁷ In another study, the addition of dexamethasone induced chondrogenesis of mouse progenitor cells.⁴⁹

The addition of some growth factors that are in the defined chondrogenic media may cause dedifferentiation or alteration of the metabolic activity of the committed chondrocytes. The chondrogenic defined media for the committed chondrocytes used in this thesis will not contain TGF- β_1 and dexamethasone. Both have been shown to have a detrimental affect on the cellular metabolism of chondrocytes. An inverse proportional relationship was found between the concentration of dexamethasone and expression of type X collagen mRNA.⁵⁵ The effect of TGF- β_1 has been studied on cartilage explants, osteoarthritic tissue, and chondrocytes grown on both monolayer and in three-dimensional culture.⁵⁸ Most studies found that TGF- β is detrimental to DNA and proteoglycan synthesis.^{54,59} TGF- β also changed the cellular morphology of the chondrocytes, making them appear flattened and more fibroblast-like.⁶⁰ The combination of FBS and TGF- β decreased collagen II and proteoglycan synthesis in addition to the fibroblastic morphology in equine chondrocytes that were cultured in a fibrin gel.⁶¹ Other studies showed that interarticular injections of TGF- β caused osteoarthritic-like pathology and induced the formation of osteophytes in murine cartilage.⁶²

2.6.3 Biomaterial Scaffolds

Scaffold design, architecture and biomaterial are also important considerations in the tissue engineering of cartilage because the scaffold serves many purposes in aiding the regeneration of tissue. The main role of the scaffold is to provide a spatial framework that fills the defect space. Without a scaffold matrix to define the extents of the defect void, the repair tissue, even with the addition of cells and biofactors, would still incompletely fill the defect.⁶³ Other desirable scaffold

properties include increasing cell retention and serving as a template for the repair tissue. Ideally, the scaffold material should be biocompatible, biodegradable, and slowly degrade over time so that the generation of the repair tissue and the degradation of the scaffold are matched. Furthermore, the scaffold material should also facilitate cell attachment and aid in the integration of repair and native tissues. Cell interactions with scaffold biomaterial can influence BMSC differentiation and can promote extracellular matrix formation.^{64,65} Another important consideration are the biomechanical properties of the scaffold. The scaffold should provide mechanical support to protect the developing tissue.⁶⁶ Ideally the physical properties of the scaffold should match the native tissue. Some studies have shown that improperly matched scaffold constructs lead to tissue degeneration.²⁰ Mechanical assessment of articular cartilage taken from various joints show compressive moduli between 0.4 – 0.8 MPa, shear moduli between 0.2 – 2 MPa, and tensile values ranging between 0.32 – 10.2 MPa.⁶⁷

Conventional scaffolds for cartilage tissue engineering consist of porous non-woven meshes, sponges, and hydrogels made from a variety of poly(α -hydroxy) acids, poly(ethylene glycol), other synthetic polymers, and also natural biologics, such as collagen, hyaluronan, chitosan, and alginate.^{68,69,70,71,72} All of these scaffold matrices facilitate new tissue growth by providing spatial organization for the cells and aiding in cell retention. The porous matrices have high porosity and high surface areas for cell attachment. Often times the pores of these scaffolds are highly tortuous and small in size which hinders cell migration and diffusion. Another disadvantage of these matrices is their poor mechanical integrity. If implanted at an orthotopic site, many of these implant devices would ultimately fail because both the scaffold and developing tissue would not be able to withstand the forces. For example, autologous articular chondrocytes were seeded into type II-collagen sponges and cultured for 4 weeks *in vitro* prior to

implantation in chondral defect in a canine model. After 15 weeks, the reparative tissue filled 88% of original defect, of which 42% was hyaline tissue. Stiffness from indentation tests still showed that the reparative tissue had properties that were 20 times lower than that of articular cartilage.⁷³ In another study conducted with alginate hydrogels seeded with chondrocytes, the equilibrium tensile modulus measured after 2 weeks of *in vitro* culture was lower than 10 kPa.⁷⁴ This value is significantly lower than the tensile modulus for articular cartilage, which is around 25.5 MPa.⁷⁵ New technologies and advances in nanofibers and weaving may introduce methods to improve upon the mechanical properties of these conventional matrices. Moutos *et al.* developed a construct that is made from uniquely woven multifilament polyglycolic acid (PGA) fiber that has a diameter of 104 μm . Chondrocytes were seeded into these scaffolds and the mechanical properties were assessed. The biomechanical properties of the scaffolds were within the same order of magnitude for compressive, tensile and shear properties of articular cartilage.⁷⁶

An alternative method of scaffold fabrication has developed with the advancement of rapid prototyping systems, known as solid freeform fabrication (SFF). SFF has improved the physical properties of tissue engineering scaffolds and expanded the possibilities beyond traditional sponges and hydrogels. The number of controllable design inputs has greatly increased due to the ability to interface computers and the rapid prototyping machines. Computer programming and design allow the user to custom-design the shape of an implant, control the scaffold porosity, pore interconnectivity, and even design mechanical reinforcements to account for biomaterial degradation.⁷⁷ Another significant benefit is the load-bearing properties of most designed SFF scaffolds. The tested mechanical properties are within the range of or greater than native cartilage. Furthermore, SFF can be used with a variety of biomaterials. Many SFF scaffolds have been made from both synthetic and natural polymers.^{78,79,80,81}

There are a variety of rapid prototyping systems available currently, from fuse deposition modeling (FDM), solid laser sintering (SLS), solid freeform fabrication (SFF) to organ printing systems which print viable cells and matrices into three-dimensional constructs. The success of the scaffolds to promote new cartilage tissue formation has been shown by many studies, both *in vivo* and *in vitro*. Some studies show that these SFF scaffolds are better for cellular retention and for increasing extracellular matrix formation. In a study conducted by Miot *et al.*, the ability of chondrocytes to redifferentiate and deposit extracellular matrix was compared between compression molded scaffolds (which are comparable to conventional sponge scaffolds) and FDM scaffolds. They found that the larger pore volumes of the FDM scaffolds were better for chondrocyte activity, showing increased sGAG and collagen II production. It is assumed that the difference was due to higher permeability, lower connective density, and lower tortuosity of the FDM scaffolds.⁸² In another study, similar compression molded and 3D fiber deposition (3DF) scaffolds were used to evaluate cell migration into the center of the scaffolds over time. The results showed significantly greater numbers of cells present at the center of the 3DF scaffolds.⁸³

Many novel scaffold designs have been created using these rapid prototyping systems. For example, a scaffold with the anisotropic zonal organization that is present in articular cartilage was created using 3D fiber deposition. The scaffold was divided into 3 zones: the deep with 2 mm spacing between fibers, the middle with 1 mm spacing, and the superficial layer with 0.5 mm spacing. When seeded with chondrocytes, the scaffold promoted anisotropic cell distribution that correlated with three zonal spacings. Although the level of GAG/DNA was similar across the zones, the amount of collagen II and GAG content was greater in the 'deep' zone where there was larger zonal spacing.⁸⁴ Other examples include scaffold designs with honey-comb like pore structures and auricle shaped constructs.^{35,85}

There are infinite possibilities for scaffold design due to the various types of rapid prototyping systems available and the number of possible design inputs. Many of the controllable scaffold properties, such as porosity, pore size, and pore shape and scaffold permeability have not been investigated to determine which design inputs are more effective in stimulating chondrogenesis. Through computer design and manipulation of these inputs, isolation of scaffold design variables can be used to evaluate their influence on cell behaviour.

For example, scaffold permeability is one such parameter that has not been investigated. Scaffold permeability affects the diffusion of nutrients and oxygen into the scaffold. The diffusion of nutrients ultimately affects the cell metabolism and extracellular matrix formation. Although studies have been conducted on how oxygen tensions affect chondrocytic behaviour in both BMSC and chondrocytes, there are no studies on how scaffold permeability may influence chondrocytic activity or even any studies relating scaffold permeability to oxygen tensions. Research that have been conducted show that periosteal implants cultured in lower oxygen tensions (12-15%) produced the most GAG and collagen compared to other groups (1-5%, 12-45%, and 90% O₂).⁸⁶ Rat mesenchymal stem cells cultured at low oxygen tensions (5%) had greater number of colonies and proliferated more rapidly than cells cultured at 20% oxygen.⁸⁷ There is also evidence that at lower oxygen concentrations, the cells do not 'age' as quickly presumably due to the reduction of free radicals.⁸⁸

In a study of oxygen tension levels within SFF scaffolds, Malda *et al.* found that an oxygen gradient exists between the edge and the center of the scaffolds. The oxygen tension levels out to a non-zero plateau, which indicates that oxygen is not the limiting factor at the center of these scaffolds. It is known that in the deep zone of cartilage, chondrocytes mainly use glycolysis to generate energy, so glucose may be the limiting factor.⁸³ Heywood found that culturing

chondrocytes in media with low glucose levels upregulates oxygen consumption and inhibits glycolysis. This finding further indicates that glucose is a key factor in regulating aerobic respiration within tissue-engineered constructs.⁸⁹ Since the scaffold permeability and diffusivity will affect both the glucose concentration and oxygen tension, it will be important to investigate the relationship between these factors.

Another scaffold design property that can be easily changed with SFF design is the scaffold pore size. The effects of scaffold pore size on the regeneration of cartilage have not been studied extensively, although changes in pore size will affect the cellular densities. A few studies have been conducted in conventional sponge matrices, where the pore sizes are smaller than 250 μm .^{90,91} Yamane *et al.* examined the pore sizes ranging between 100 and 400 μm in chitosan-hyaluronic acid woven fiber scaffolds. They found that the 400 μm pore showed greater areas of cartilage and higher numbers of chondrocytes.⁹² Specific studies looking at SFF scaffold pore sizes and cartilage regeneration have not been conducted. It is suggested that SFF scaffolds with pore interconnection sizes between 300 – 500 μm can help facilitate diffusion into the center of the SFF scaffolds that were cultured in a bioreactor.⁹³

2.7 CONCLUSIONS

Conventional cartilage tissue engineering scaffolds have been able to support chondrogenesis, but do not have adequate mechanical properties. The use of SFF scaffolds will not only provide mechanical support to the developing tissue, but the ability to design and manipulate scaffold properties will increase the knowledge of how physical scaffold properties affect cartilage formation. From the studies reviewed above, it has been shown that simulation of the microenvironment that is present during the cellular condensation step of

cartilage morphogenesis can enhance the chondrogenic activities of BMSC and chondrocytes. The studies in this thesis will investigate how to enhance chondrogenesis within SFF scaffolds by directing cellular condensation through chondroinductive biomolecule, hyaluronic acid, and chondroconductive pore geometries that mimic micromass cultures. The effects of these factors will be tested on both BMSC and chondrocytes.

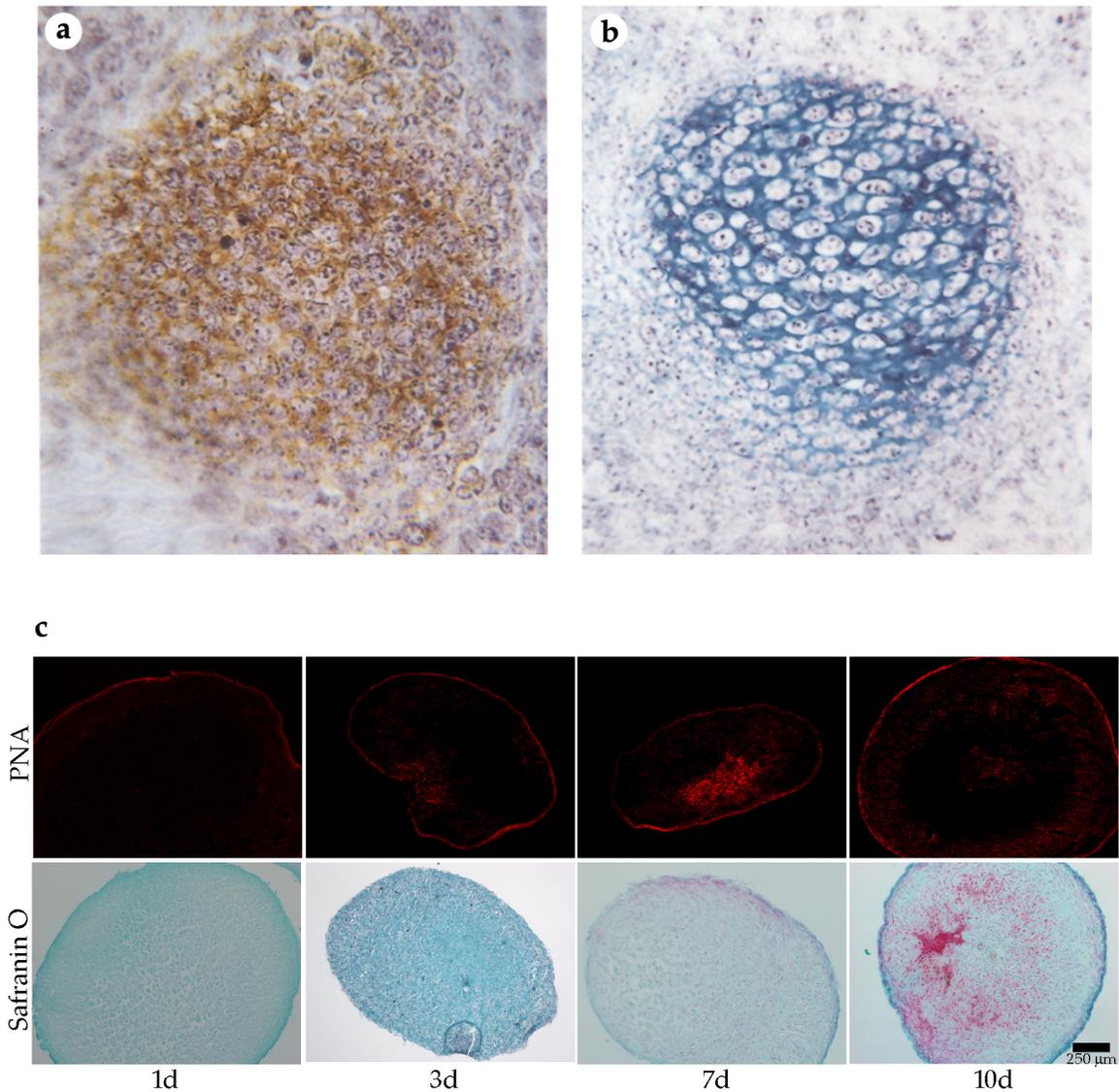


Figure 2.1: Skeletal condensations and micromass cultures stained with peanut agglutinin lectin (PNA), Alcian Blue, and Safranin O. a) Condensation of hyoid cartilage from mouse embryo at stage 21 visualized with peroxidase staining of PNA. b) Hyoid cartilage from mouse embryo at stage 22 showing glycosaminoglycan (GAG) staining with Alcian Blue. c) Temporal staining using rhodamine-conjugated PNA and Safranin O at 1, 3, 7 and 10 day timepoints of porcine BMSC micromasses cultured in defined chondrogenic media. As the cells within the micromass differentiate, the ability to bind PNA is lost and gradually sGAG deposition in the extracellular matrix increases. [(a) and (b) from Hall and Miyake, 1992]

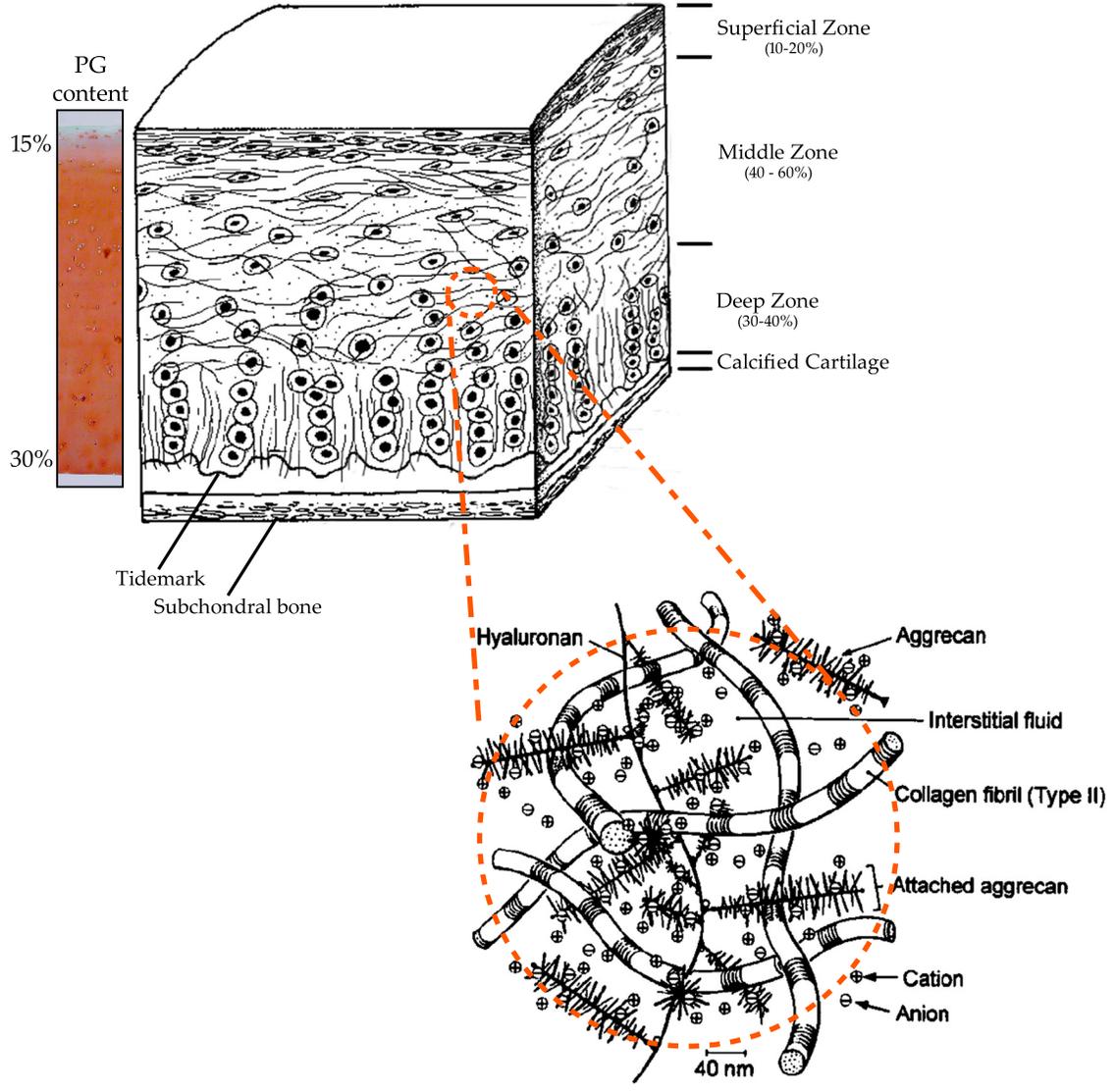


Figure 2.2: The four zones of articular cartilage: superficial, middle, deep and calcified cartilage. Each zone has a different macromolecular composition and the cell morphology also varies according to zonal function. The superficial layer acts as a barrier towards larger proteins and the parallel orientation of the collagen fibers increase tensile stiffness and resist shear and compressive forces. The middle and deep zones have higher proteoglycan (PG) concentrations as shown by the cartilage section stained with Safranin O; greater red staining intensity indicates greater concentrations of GAGs. The collagen fibrils in the deep zone are also oriented perpendicular to the articular surface to further enhance the compressive properties of the tissue. The capacity of the articular cartilage to support loads is dependent upon the interaction of the charged solid network made of collagen and proteoglycans, the interstitial fluid, and the electrolytes in the interstitial fluid. A magnified cross-section depicts the dynamic between these three phases. [partially adapted from Buckwalter et al, 1994 and Mow et al, 1999]

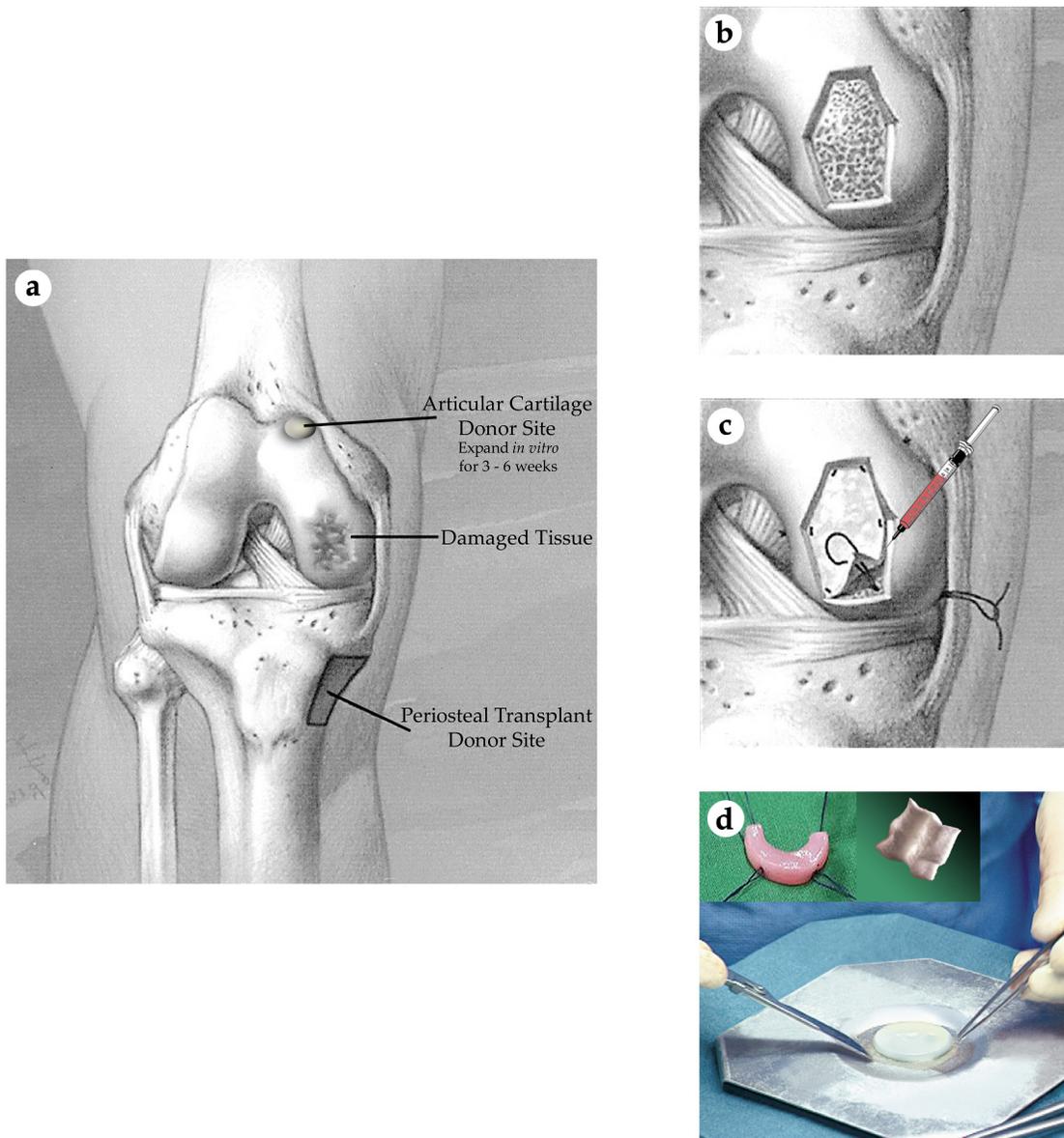


Figure 2.3: Autologous chondrocyte implantation (ACI) is commonly used to repair single defects in articular cartilage. a) During an initial arthroscopy, healthy donor cartilage is taken from a non-load bearing area. The chondrocytes are isolated from this tissue and cultured *in vitro* to expand the number of cells. b) Three to six weeks later, the patient returns for the ACI procedure. The damaged tissue is debrided and the base is abraded down to the subchondral bone. c) The periosteal graft is harvested from the proximal tibia and is used to help retain the cells at the defect site. The expanded chondrocytes are resuspended and injected into the site. d) Complications that arise due to the periosteal implant have led to improvements to the ACI procedure which replace it with collagen hydrogels or matrices made hyaluronan or a mixture of collagen I/collagen III (matrix-associated autologous chondrocyte implantation, MACI). [partially adapted from (O'Driscoll et al., 2001), CaReS[®] implant (Ars Arthro), and Fidia Advanced Biopolymers]

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CHAPTER 3

EFFECTS OF CHONDROINDUCTIVE BIOMOLECULE, HYALURONIC ACID, ON CHONDROGENESIS

3.1 INTRODUCTION

Many types of hydrogels have been explored for providing a three-dimensional environment that maintains the chondrocytic phenotype and promotes new matrix formation. These hydrogels alone, as previously stated, have weak mechanical properties and are incapable of withstanding forces normally exerted at clinically relevant defect sites.^{1,2} However, combining hydrogels and solid freeform fabricated (SFF) scaffolds creates a synergistic construct for tissue engineering cartilage. The hydrogel will stimulate and enhance extracellular matrix production and other chondrocytic cellular activities, while the SFF scaffold provides the mechanical support to the developing tissue. The hydrogel can increase the cellular retention within the scaffold and also provide a template to guide the regeneration of new cartilage tissue.

Collagen is an ideal substrate for cartilage tissue engineering because it can be formed *in situ*, is easily manipulated, and can serve as a natural, biologic delivery vehicle for cells and growth factors. Collagen hydrogels have been explored in numerous applications, including regeneration of vocal chords³ and repair of spinal cord conduits⁴ and cartilage defects.^{5,6} However, collagen I fibrils have been shown to destabilize the cellular phenotype, where the chondrocytes acquire fibroblast-like morphologies and produce collagen I and collagen X instead of collagens II, IX, and XI.^{7,8} Chondrocytes cultured in collagen I hydrogels exhibit reduced proliferation and decreased biosynthetic activity.^{9,10}

The addition of hyaluronic acid (HyA) to collagen I hydrogels may help stabilize chondrocyte phenotype and increase proteoglycan synthesis presenting a possible solution to mitigate the effect of collagen I fibrils. Hyaluronic acid is a ubiquitous glycosaminoglycan found in many tissues of the body. HyA is synthesized in large quantities during wound healing to facilitate cell motility and in fetal healing where it reduces scar formation.¹¹ As described in Chapter 2, HyA is also present during the mesenchymal condensation period, where it aids the migration of the mesenchymal stem cells to a 'central' location and in the formation of cellular condensations. This effect has also been observed *in vitro*, where studies show that the exogenous addition of low concentrations of hyaluronic acid (0.1 mg/mL or less added to culture medium) prompted chondroprogenitors to form three dimensional clusters and induced chondrocytes to produce more collagen II and aggrecan.^{12,13} It will be important to investigate the concentration of HyA required to promote cellular aggregation because the amount of HyA-cell receptor interaction directs the formation of cellular condensations. At low concentrations of HyA, the cells have free receptors that are able to bind to HyA that is already bound to a neighboring cell (FIGURE 3.1). This creates a cross-bridge between the cells and mediates cellular aggregation. At higher concentrations of HyA, the receptors become saturated because they bind to individual HyA molecules. The cross-bridging is inhibited due to the lack of free receptors, so aggregate formation does not occur.

Furthermore, HyA is an essential mucopolysaccharide that serves as the backbone of the proteoglycan network in the extracellular matrix of cartilage. It is also found in the synovial fluid where it acts as a lubricating agent. HyA is also required for maintenance of the pericellular matrix and is partially responsible for the characteristic ability of cartilage to withstand compression due to its organization and retention of aggrecan and sulfated glycosaminoglycans (sGAG) within the extracellular matrix. Both chondrocytes

and BMSC are able to bind to HyA through cell surface receptors, such as CD44 and RHAMM. Cellular binding to HyA initiates signals that modulate cell proliferation, migration, and differentiation.^{14, 15} The benefits of HyA in facilitating chondrogenesis have been shown in several studies. Bovine chondrocytes cultured in gelatin sponges with exogenous high molecular weight HyA increase cell retention and proteoglycan synthesis.¹⁶ Chondrocytes embedded in collagen I hydrogels cultured with exogenous HyA increase cell proliferation and chondroitin sulfate synthesis at 0.1 and 1 mg/mL concentrations.¹⁷ Furthermore, chondrocytes cultured in composite HyA/alginate beads showed increased sGAG deposition and enhanced synthesis of both DNA and hydroxyproline.¹⁸

In this study, we evaluated the effects of HyA on BMSC and chondrocyte behavior using a combination of composite hyaluronic acid/collagen I hydrogels and designed porous SFF scaffolds. Our hypothesis is that addition of low concentrations of high molecular weight HyA to collagen I hydrogels will have a beneficial effect on matrix production and phenotype stability. First, we evaluated the metabolism and proteoglycan synthesis of BMSC and chondrocytes embedded in composite hyaluronic acid/collagen I hydrogels *in vitro* to determine HyA concentrations with the greatest benefit. In a separate *in vivo* phase, composite HyA hydrogels were used in combination with designed poly(propylene fumarate) (PPF) or poly(ϵ -caprolactone) (PCL) scaffolds with cubic architecture. Composite HyA hydrogels were used to seed the scaffolds with BMSC or chondrocytes and then implanted in mice to evaluate both the effects of the hydrogels on maintaining the chondrocytic phenotype and extracellular matrix production.

3.2 MATERIALS AND METHODS

3.2.1 Cell isolation

Primary porcine chondrocytes were isolated from the metacarpophalangeal joints of domestic pigs. Cartilage slices were extracted aseptically within four hours of slaughter. Chondrocytes were isolated from the tissue using a solution of 1 mg/mL collagenase II (Sigma, St. Louis, MO) and antibiotics. The isolated cells were filtered and were allowed to adhere for 24 hours. The adherent chondrocyte population was used for experimentation.

Porcine BMSC were isolated from the bone marrow aspirate of Yucatan minipigs (Seguin Animal Hospital, Seguin, TX). The aspirate was collected in a solution of heparin and antibiotics. The whole bone marrow was cultured for weeks and the entire adherent cell population was used for these studies. The cells were not passaged or passaged once prior to use.

3.2.2 *In vitro* experimentation

In vitro cell culture

Cells were encapsulated in either collagen I or composite HyA/collagen I gels and cultured in either basal or chondrogenic media to determine the combination most favorable for chondrogenesis. After trypsinization, cells were resuspended at 3.5×10^6 cells/mL in solubilized collagen I (BD Bioscience Discovery Labs, San Jose, CA) with 200 μ L of culture medium. The pH of the collagen/cell suspension was increased with the addition of 20 μ L of 0.5 M sodium hydroxide solution (Appendix C). The mixture was pipetted in 70 μ L aliquots into 96-well tissue culture plates. The resulting collagen I hydrogels were 2.2 mm in height and 6.35 mm in diameter. To create the composite HyA/collagen I gels,

hyaluronic acid (stock concentration: 2.7 mg/mL in 0.8 M NaCl, MW: 3×10^6 Da; Hyalogic LLC, Edwardsville, KS) was added to the collagen I cell suspension to produce 1, 2, 5, and 10% (w/w) HyA/collagen I gels. The HyA concentrations for the corresponding weight percent gels were 0.05, 0.09, 0.23, and 0.47 mg/mL, respectively. The pH was raised to physiologic levels with NaOH as described above. (The amount of collagen I used to resuspend the cells in composite hydrogels was adjusted so that the final volumes of the HyA/collagen cell suspension was similar to collagen I only hydrogels.) All hydrogels were placed in an incubator at 37°C and allowed to solidify for 30 minutes. Either basal medium or chondrogenic medium (basal medium supplemented with 10 ng/mL TGF- β_1 , 100 nM dexamethasone, 50 μ g/mL 2-phospho-L-ascorbic acid, 0.4 mM proline (Sigma), 5 μ g/mL insulin (Gibco) and 0.1 mM non-essential amino acids (Gibco)) was then added to the tissue culture wells. The defined chondrogenic medium for the chondrocytes did not contain TGF- β_1 and dexamethasone. The hydrogels were cultured for up to two weeks under gentle agitation on an orbital shaker and with the media replaced every other day. Hydrogels were assessed at one week and two weeks for glycosaminoglycan content, cell viability and cell number. Three replicates of each group from three separate experiments were used for analysis.

DMMB assay for GAG quantification

Cultured hydrogels were rinsed with Hank's Balanced Salt Solution (HBSS, Gibco) and then digested with proteinase K at 65°C for 2 hours (Appendix D). Samples spiked with known amounts of chondroitin sulphate and empty samples with just proteinase K digest solution were used as positive and negative controls, respectively. After the gels were completely digested, the temperature was increased to inactivate the enzyme. The digested samples were centrifuged through a filter for 10 minutes at 14,000 rpm and the supernatant kept. Dimethyl-methylene blue (DMMB) assay was conducted as described by

Farndale.¹⁹ Since HyA is a non-sulphated glycosaminoglycan, it does not bind to DMMB and will not interfere with the sGAG detection.²⁰ Five microliters of supernatant from each sample was mixed with 200 μ L of DMMB solution and 525 nm absorbance was read in a spectrophotometer. Chondroitin sulphate from shark cartilage (Sigma) was used to create a calibration curve to correlate the measured absorbance to known amounts of sGAG. Data from the DMMB analyses were assessed using the student's t-test with $\alpha = 0.05$. The data between the groups was compared using analysis of variance (ANOVA) using hydrogel composition and culture medium as the factors for the Tukey post hoc test.

Cell viability and count

Cell viability and count were determined using the Cell Titer 96[®] AQueous One non-radioactive cell proliferation assay (Promega, Madison, WI). Cell viability was determined by reading the 490 nm absorbance of a formazan product bio-reduced from a tetrazolium compound. Cell count was also determined using this assay via a linear cell standard correlating the amount of formazan product formed in a given amount of time and number of cells present. A hemacytometer and coulter counter was used to verify the cell count from digested hydrogels and validate the cell standard. The amount of DNA present was calculated from the cell number, assuming 7.7 picograms of DNA per cell.

3.2.3 *In vivo* experimentation

Scaffold design and fabrication

Image-based design (IBD) techniques²¹ were used to create cylindrical scaffolds with defined internal pore architectures. The external dimensions of all designed scaffolds were 3.5 mm in height and 5 mm in diameter. Unitcells, the basic repeating unit of a 3D periodic structure, for cubic pore designs were [0.7 mm, 0.7 mm, 0.7 mm] (FIGURE 3.2a). The strut size of the cubic scaffolds was

designed to be 350 μm thick, which corresponds to pore space dimensions of 350 μm in length, width and height. The designed porosity of the scaffold was 50 percent. Repeating unitcells were used to create the interconnected pore architecture of the scaffolds shown in Figure 3.2b.

Wax molds were printed from design files using a three dimensional rapid prototyping system (Patternmaster™, Solidscape, Inc., Merrimack, NH). The wax molds were cast into poly(propylene fumarate) using indirect SFF fabrication techniques described previously.²² Briefly, one gram of PPF (MW: 1500 Da) was dissolved in one milliliter of N-vinyl-pyrrolidone (NVP). After homogenization, 0.02 grams of benzoyl peroxide was added to the PPF/NVP mixture. Then, two microliters of N, N-dimethyl-*p*-toluidene was added to induce crosslinking immediately before casting.²³ A custom polytetrafluoroethylene (PTFE) mold (diameter = 6 mm) was used to force the polymer solution through the pore spaces of the wax molds. The PTFE mold was placed in a nitrogen chamber at room temperature for 24 hours until the polymer cured. The wax was then removed with acetone, and the remaining PPF scaffolds were rinsed in distilled deionized water and sterilized in 70% ethanol.

Scaffold Design Verification and Permeability Calculations

Scaffolds cast in PPF from the wax molds were scanned using a micro-computed tomography machine (MS-130, GE Medical Systems, Toronto, Canada) to determine the accuracy of the fabrication process. The scaffolds were scanned in air at a resolution of 16 μm . The measured features were within 5% of the designed specifications.

In vitro pre-culture

In preparation for cell seeding, the PPF and PCL scaffolds were rinsed twice with sterile HBSS and once with culture medium. The scaffolds were then placed in a

custom made PTFE mold (diameter = 5.1mm). Cells were seeded into the scaffold pores using a collagen I or 5% HyA/collagen I hydrogel similar to those described above. For these hydrogels, 40×10^6 cells/mL were resuspended in a solution containing either ~3.8 mg/mL collagen I or 5% (w/w) HyA/collagen I after trypsinization. The pH of the solution was increased, and the cell/hydrogel suspension was pipetted into the scaffold pores (~60 μ L) immediately after sodium hydroxide addition. The gels were allowed to solidify within the scaffolds in a humidified chamber at 37°C for 30 minutes. The PPF scaffolds were then removed from the PTFE mold and placed in a 24-well tissue culture plate. Basal culture medium or defined chondrogenic medium was added to the wells, and then the plate was placed in the incubator and gently agitated on an orbital shaker. The chondrocyte-seeded scaffolds were pre-cultured for 2 weeks with the culture media changed every other day during this period.

In vivo implantation

After pre-culture, 8 PPF scaffolds seeded with chondrocytes and 16 PCL scaffolds seeded with BMSC or fibroblast-like chondrocytes (passage 6) (n = 4 for all groups) using either collagen I or 5% HyA/collagen I hydrogels and were implanted subcutaneously in 5- to 8-week-old immunocompromised mice (N:NIH-bg-nu-xid; Charles River, Wilmington, MA). The animals were anesthetized with intramuscular injections of ketamine (50 μ g/g) and xylazine (5 μ g/g) in saline. Four dorsal subcutaneous pockets were created by blunt dissection and one PPF or PCL scaffold from each experimental group was placed in each pouch and in a different pouch location in each mouse. The implantation sites were closed with surgical wound clips. The animals were housed in groups for four weeks with free access to food and water.

Evaluation of implants

The mice were sacrificed after 4 weeks. The scaffolds were harvested and fixed in Z-FIX (Anatech, Battle Creek, MI) overnight. The scaffolds were then dehydrated and processed for histology. The specimens were embedded in paraffin and sectioned at 7 μm . Sections were affixed to slides and stained with hematoxylin and eosin (H&E) or Safranin O counterstained with fast green FCF.

Histomorphometric image analysis

Histological sections taken from four separate planes (~500 μm difference) of each scaffold and were used for analyses. Low magnification (2X objective) images of Safranin O stained cross-sections were taken using CCD camera (RT Color 2.2.1, Diagnostic Instruments, Sterling Heights, MI) mounted on an inverted microscope (E600, Eclipse, Nikon, Japan, ETC). Histomorphometric image analysis (Appendix E) was used to quantify the average sGAG concentration and the amount of cartilage-like matrix of each plane. Negative controls, composite HyA/collagen hydrogels without cells were also stained with Safranin O/Fast Green to verify that the HyA does not stain positively with Safranin O. The histomorphometric data was statistically analyzed using ANOVA to determine if hydrogels containing 5% HyA promoted more chondrogenesis than hydrogels with collagen I alone. All statistical tests were performed using SPSS.

Immunohistochemistry (IHC) was used to detect the presence of collagen I and collagen II (Appendix F) in the extracellular matrix. Paraffin sections were used for the IHC staining. Primary antibodies raised in rabbits against human-collagen I and human-collagen II were used and have been shown to cross-react with porcine collagen I and II. Sections of porcine cartilage were used as positive controls and to determine the optimal concentrations for the primary antibodies. For negative controls, the primary antibodies were not added.

3.3 RESULTS

3.3.1 *In vitro* evaluation of HyA hydrogels

After two weeks of *in vitro* culture in either basal or chondrogenic medium, 97% – 99% of all cells in all hydrogels remained viable. Additionally, the number of viable cells after 2 weeks in culture was greater compared to that after 1 week in culture. The number of cells increased with increasing concentrations of HyA and was also greater in hydrogels cultured in chondrogenic medium compared to basal medium. These results verify that all combinations of medium and hydrogel studied are capable of supporting BMSC and chondrocyte activity.

The addition of HyA up to the 5% HyA concentration promoted cellular aggregation within the BMSC and chondrocyte seeded hydrogels. The number of aggregates did not increase dramatically from one timepoint to the next. After the initial condensation or aggregation, which appeared over the course of the first week, few additional aggregates were seen at the second week timepoint. Once the aggregates were formed, however, the cells remained condensed together and did not disperse over time. Culture in the chondrogenic defined media also seemed to increase the size of the aggregates present. Phase contrast images of the aggregates within the hydrogels were taken at 1 and 2 week timepoints (FIGURE 3.3). BMSC aggregates of various sizes were seen within the composite HyA hydrogels at 1%, 2%, and 5% concentrations. Numerous small aggregates were observed in the 1% HyA hydrogels and as the HyA concentration increased, the size of the aggregates increased while the number of aggregates decreased. The largest aggregates were seen within the composite 5% HyA hydrogels cultured in chondrogenic media. Safranin O staining of all hydrogels showed active chondrogenesis and matrix deposition in the areas surrounding the cellular aggregates. A limited number of aggregates were seen

in 10% HyA/collagen hydrogels or hydrogels with collagen I only, although some areas of chondrogenesis stained positive with Safranin O. Cellular aggregates were also observed in the chondrocyte-seeded hydrogels (FIGURE 3.4). The 2% and 5% HyA concentrations showed some aggregate formation, although the response to the HyA was not as widespread and overt as the BMSC aggregates.

The results from the DMMB quantification of the sGAG content within the HyA hydrogels further confirmed the increase in extracellular matrix production by both BMSC and chondrocytes. A trend was observed, where increasing amounts of HyA (up to 5% HyA) resulted in increased amounts of sGAG/DNA ratios measured. The 10% HyA hydrogels seeded with BMSC and chondrocytes exhibited mixed behavior that was in some instances greater than hydrogels with collagen I only (controls) and in other cases lower than expected.

Analysis of the sGAG/DNA data for BMSC seeded hydrogels indicated that some concentrations of HyA may be better for aiding chondrocytic differentiation and extracellular matrix formation. The sGAG/DNA HyA hydrogels for the first week of culture showed low amounts of sGAG production (FIGURE 3.5a), although all additions of HyA increased the sGAG/DNA content over the control hydrogels ($p < 0.01$). Culture in the chondrogenic defined media increased amounts of sGAG over control hydrogels, but of greater interest is the larger increase in sGAG/DNA in the 5% and 10% HyA hydrogels. The sGAG/DNA ratio of the 5% HyA hydrogels cultured in chondrogenic media is significantly greater than all other groups ($p < 0.05$). After two weeks of *in vitro* culture, the sGAG/DNA ratios of all the hydrogel groups increased by at least a factor of two over the one week data, except for the 10% HyA group that was cultured in basal medium (FIGURE 3.5b). Once again, the 5% HyA hydrogel group showed highest sGAG/DNA ratio compared to all groups ($p < 0.01$). This

result occurred regardless of the culture media used, thus indicating that the amount of HyA maybe able to stimulate chondrogenesis without the addition of exogenous growth factors.

The addition of HyA to chondrocyte-seeded hydrogels generally resulted in increased sGAG deposition compared to hydrogels with collagen I alone, irrespective of the culture medium or the culture duration. After one week of culture, the 1%, 2%, and 5% HyA hydrogels cultured in basal medium increased the sGAG/DNA ratios over the controls and the 2%, 5% and 10% HyA hydrogels cultured in chondrogenic media had greater sGAG/DNA ratios over controls (Figure 3.5c). The 5% HyA hydrogels had the greatest sGAG/DNA ratio over all other groups ($p < 0.05$). The sGAG/DNA ratios of chondrocyte seeded hydrogels after two weeks of culture are shown in Figure 3.5d. At two weeks, sGAG quantification of the hydrogels containing HyA that were cultured in basal medium were significantly increased over controls ($p < 0.05$). Of the HyA hydrogels cultured in chondrogenic media, only the 5% HyA group was greater than the controls ($p < 0.01$), while all others were similar to control values. Comparison of the one week and two week results showed that sGAG/DNA increased significantly for all hydrogels (composite and controls) cultured in basal medium and conversely decreased, though not significantly, for hydrogels in the chondrogenic medium. The results for the chondrocytes are not as clearly resolved as the BMSC data, although the 5% HyA group seems to increase GAG synthesis in chondrocytes as well.

Among all combinations of hydrogel and culture medium, the highest amount of matrix formation, as quantified by the amount of sGAG normalized by the amount of DNA, was obtained with a 5% composite hydrogel cultured in chondrogenic medium for BMSC and a 5% composite hydrogel cultured in basal medium for chondrocytes. These combinations were thus chosen to seed BMSC

and chondrocytes, respectively, into PCL or PPF scaffolds that subsequently were implanted into mice in *in vivo* experiments.

3.3.2 *In vivo* comparison of HyA hydrogels

Two studies were conducted to assess the effects of 5% HyA on cartilage formation during *in vivo* implantation. In the first study, PCL scaffolds were seeded with BMSC using either 5% HyA/collagen or collagen I hydrogels and cultured for 2 weeks *in vitro* in defined chondrogenic medium. In the second study, PPF scaffolds were seeded with chondrocytes using either 5% HyA/collagen or collagen only hydrogels and cultured for 2 weeks *in vitro* in basal medium. All scaffolds were implanted and the mice survived surgery without complication. All mice lived 4 weeks without signs of abnormality. All implanted scaffolds were extracted, found intact, and processed for histological analysis.

Microscopic examination of the tissue/scaffold sections showed widespread positive Safranin O staining of the pore spaces confirming the presence of sGAG and viable chondrocytes. Chondrogenically differentiated BMSC and chondrocytes filled the pore spaces of the scaffolds and were surrounded by dense extracellular matrix, which stained positive with Safranin O. The general morphology of the cells was round and chondrocytic in appearance with established lacunae, even at the center of the scaffolds. (FIGURE 3.6:a4, b4, c4, d4) Within these same sections some areas counterstained with fast green indicating the presence of non-cartilaginous matrix. Spindle-shaped fibroblast-like cells and endothelial cells were found in these areas, which were primarily at the edges of the scaffolds and in scaffold pores where cells were embedded in collagen I. A greater number of cells within the scaffolds with collagen I only hydrogels stained positively with fast green, indicating that extracellular matrix

surrounding the cells did not contain sGAG and that the cells may have lost the chondrocytic phenotype and the ability to produce sGAG. Greater areas of cells that stained positively with fast green were seen within the scaffolds with collagen only hydrogels than the scaffolds containing HyA hydrogels.

Immunohistochemistry verified the presence of both collagen II and collagen I in the extracellular matrix. The prior confirms the presence of chondrocytes and new cartilage matrix formation, while the latter shows fibroblast invasion as well as dedifferentiated chondrocytes. Areas of detected collagen II correlated well with the areas of positive Safranin O staining, confirming the presence of cartilage-like matrix for both BMSC and chondrocyte-seeded constructs. (FIGURE 3.7) Collagen I was detected in areas of non-cartilaginous connective tissue and in some regions of weak Safranin O staining, such as the edges of the scaffolds and in pores with fibroblastic cells or where vascularization was observed. (FIGURE 3.8)

Positive Safranin O staining was found in all specimens, but more robust and intense staining was seen in scaffolds with composite 5% HyA/collagen hydrogels than with collagen I only hydrogels. Histomorphometrical analysis of the Safranin O stained slides confirmed these observations (Figure 3.9a). BMSC seeded in PCL scaffolds using 5% HyA composite hydrogels showed greater areas of chondrogenic differentiation compared to scaffolds with collagen I hydrogels. The positively stained area for the BMSC seeded scaffolds was $3.5 \pm 1\%$ and $10 \pm 5\%$ for collagen I and 5% HyA hydrogels, respectively. For chondrocytes seeded in PCL scaffolds, the percentage of pore area stained positive with Safranin O for scaffolds seeded with composite 5% HyA hydrogels was 63 ± 6 . The percentage of pore area stained positive with Safranin O for scaffolds seeded with collagen I only hydrogels was 29 ± 4 . Statistical analysis

showed that the chondrocytes cultured in composite 5% HyA/collagen I hydrogels had significantly greater areas of positive Safranin O staining.

The average intensity of the Safranin O staining was converted to sGAG concentration using a calibration curve. The intensity of the Safranin O stain correlates directly to the amount of sGAG present in the matrix.²⁵ The average GAG concentration for BMSC seeded scaffolds was 3.2 ± 0.3 and 3.6 ± 0.2 $\mu\text{g}/\text{mm}^2$ for collagen and 5% HyA hydrogels, respectively (FIGURE 3.9b). The average sGAG concentration for chondrocyte-seeded scaffolds with collagen I hydrogels was 7.1 ± 2 $\mu\text{g}/\text{mm}^2$. The average sGAG concentration for the scaffolds with 5% HyA/collagen I hydrogels alone was 9.5 ± 0.9 $\mu\text{g}/\text{mm}^2$. The results showed significantly greater amounts of sGAG in the extracellular matrix of the scaffolds with 5% HyA hydrogels for chondrocytes only.

The extracellular matrix forming capability of fibroblast-like chondrocytes was also evaluated within composite 5% HyA and within collagen hydrogels. These chondrocytes had been cultured on monolayer for 5 weeks and had been passaged six times. After the long culture period, the cells had lost their round morphology and had become cuboidal and fibroblast-like in shape. Previous studies have shown that passaging chondrocytes and extended monolayer culture reduce the cells ability to produce collagen II and other cartilage proteoglycans.²⁴ Even when the cells are returned to a three-dimensional environment, the ability of the chondrocytes to 'redifferentiate' and regain the chondrocytic phenotype is still greatly reduced. After the two week *in vitro* culture period and four week implantation, the fibroblastic chondrocytes with reduced chondrocytic capacities showed qualitatively greater areas of Safranin O staining with the 5% HyA hydrogels (FIGURE 3.10), while almost no Safranin O staining was observed within the scaffolds with collagen hydrogels. The presence of the HyA may help the fibroblastic chondrocytes redifferentiate back

into chondrocytes or help maintain their chondrocytic phenotype and aid in the formation of cartilage-like matrix.

The results from the histomorphometric image analysis indicate that more chondrocytes are present in the 5% HyA hydrogel than the collagen I hydrogels. We conclude that HyA stimulates chondrogenic differentiation of BMSC, promotes proteoglycan synthesis of chondrocytes, and increases phenotype stability of chondrocytes at an ectopic site.

3.4 DISCUSSION

The aim of this study was to evaluate the effects of composite HyA/collagen I hydrogels on BMSC and chondrocyte activity *in vitro* and to analyze the feasibility of using HyA as a chondroconductive cell carrier for use with SFF scaffolds *in vivo*. Currently, most clinical applications of hyaluronic acid are administered as interarticular injections, where the residence time of the HyA is limited to days.²⁵ The incorporation of HyA into collagen I hydrogels may increase the lifetime *in vivo* and extend the timeframe of its chondroprotective properties.

Hyaluronic acid, at low concentrations, modulates cell proliferation, matrix synthesis, and cellular condensation. Our results are consistent with previous studies, where increases in sGAG production and cell proliferation were seen with the addition of HyA concentrations between 0.1 to 0.5 mg/mL.^{14,26} The HyA concentrations studied in these experiments increased aggregate formation, sGAG production and increased cell proliferation. The increase in sGAG/DNA ratios in the BMSC seeded hydrogels that contained 5% HyA after one week of culture indicate the ability of HyA to initiate cellular condensation at a critical level that stimulates early chondrogenesis. This effect is even more pronounced

in the two week data, which show the BMSC within the 5% HyA hydrogels cultured in basal media were able to differentiate into chondrocytes without the addition of exogenous growth factors. The sGAG/DNA ratio of these hydrogels is comparable to the ratios found in chondrocytes that were cultured in the control hydrogels. We believe that the HyA is able to induce chondrogenesis by helping the cells attain the critical condensation size required for differentiation.

At a favorable concentration, an increased number of cells are able to bind to HyA that is in the environment to promote cell movement, proliferation, aggregation, degradation of HyA, and mediate the formation of pericellular matrices.²⁷ Beyond this concentration; the aggregation effect is inhibited due to the saturation of cell receptors for HyA.²⁸ This effect was seen with the cells in the 10% HyA hydrogels, where cellular aggregate formation was limited. Furthermore, at high concentrations of HyA, the individual HyA domains overlap and may form entanglements through self association and steric interactions.^{29,30} These steric interactions and hydrophobic patches may deter proteins and other molecules with large hydrodynamic sizes from diffusing through the composite HyA hydrogels. This may explain some of the mixed results that were seen in the 10% HyA hydrogels, where the sGAG production and cellular metabolism may have been affected.

The culture medium is also a contributing factor in cellular metabolism and matrix synthesis. The BMSC were the most influenced by the defined chondrogenic medium, while the effects on chondrocytes were less implicit. Our results show that the chondrogenic medium increased sGAG/DNA at both timepoints for hydrogels seeded with BMSC at all concentrations of HyA. Hydrogels seeded with chondrocytes containing collagen I and the 10% HyA hydrogel at one week, although mainly small differences were seen between the other composite HyA hydrogels cultured in basal and chondrogenic medium.

This difference may also be attributed to the steric inhibitions and entanglements of the HyA molecules preventing the proteins in the chondrogenic medium from diffusing into the hydrogels. Due to the hydrophobic patches that are created by the HyA domain (FIGURE 3.11), molecules with larger hydrodynamic sizes will diffuse slower through the hydrogels.³¹ This indicates that the interactions of HyA molecules may be influencing nutrient diffusion into the hydrogels, particularly at the higher HyA concentrations. Furthermore, contrary to our expectations, the increase in cell proliferation seen with cells cultured in chondrogenic medium was not accompanied by a proportionate increase in sGAG production. This may explain the decrease of the sGAG/DNA ratio from week one to week two. Further analysis of the quantitative gene expression and assessment of the collagen production of these cells may help clarify these results.

The beneficial effects of the HyA are also seen after four weeks of *in vivo* implantation. Larger areas of cartilage-like matrix and higher concentrations of sGAG show that the pre-culture step is important in attaining hyaline tissue *in vivo*. The initial culture conditions are critical in determining the type of tissue that is formed. The two week pre-culture period is essential in organizing the cells and helping them establish pericellular matrices and other extracellular support within the SFF scaffolds.^{32,33} The pre-culture period is also favorable for cellular retention.^{34,35} The three-dimensional environment provided by the scaffold stimulates ECM production in chondrocytes and provides an environment that is conducive for chondrogenic differentiation of the BMSC. The addition of HyA to the collagen hydrogels significantly increased the quality of generated tissue. The presence of HyA mediated the formation of pericellular matrices by the chondrocytes and helped them reestablish their niches within the SFF scaffolds.³⁶ The chondrocytes cultured in collagen hydrogels would have required more time to restore chondrons and pericellular matrices.³⁷ Furthermore, the presence of HyA in the hydrogels was able to increase the

production of cartilage-like extracellular matrix by passage 6 chondrocytes. (FIGURE 3.10) Greater areas of Safranin O staining were seen within the scaffolds with the 5% HyA/collagen hydrogels compared to scaffolds with collagen only hydrogels.

Overall, our results show that the addition of HyA to collagen I hydrogels created an environment that may be more conducive for BMSC differentiation, cellular proliferation and increased extracellular matrix production. Both BMSC and chondrocytes are able to bind to HyA through surface receptors such as CD44 and RHAMM.³⁸ The binding of the cells to HyA may not only increase cellular retention within the hydrogels, but also reduce morphological changes due to interactions with collagen I fibrils.⁵ The presence of HyA may also increase the number of attachment sites for chondroitin sulphate, keratan sulphate, and other sGAG. The increases in sGAG retention within the hydrogels can be seen with the increase of sGAG measured in the composite gels with the DMMB assay. The increase of sGAG is also seen *in vivo*, where the area of positive Safranin O staining and the intensity of the stain was significantly higher than in the collagen I hydrogels. Furthermore, the increase of sGAG binding to HyA may enhance the compressive properties of the hydrogel by recreating the negative charge repulsions present in native cartilage.

3.5 CONCLUSION

In conclusion, the beneficial effects of hyaluronic acid make it a good candidate substrate for use in cartilage tissue engineering applications, but there are limits to this effect depending on the concentration of HyA. At high concentrations, the effects of steric inhibition, self association, and chain entanglement become important factors influencing nutrient diffusion and cellular metabolism. Our results show that the addition of hyaluronic acid to collagen hydrogels is better

than collagen I alone for both BMSC and chondrocytes. The most beneficial concentration studied in these experiments was a 5% HyA hydrogel, which was shown by the increased cellular condensation, cell proliferation, and sGAG content over all groups tested. These composite HyA hydrogels are compatible with SFF scaffolds and helped to increase the areas of cartilage matrix formation within the scaffolds and increase sGAG concentration in the extracellular matrix.

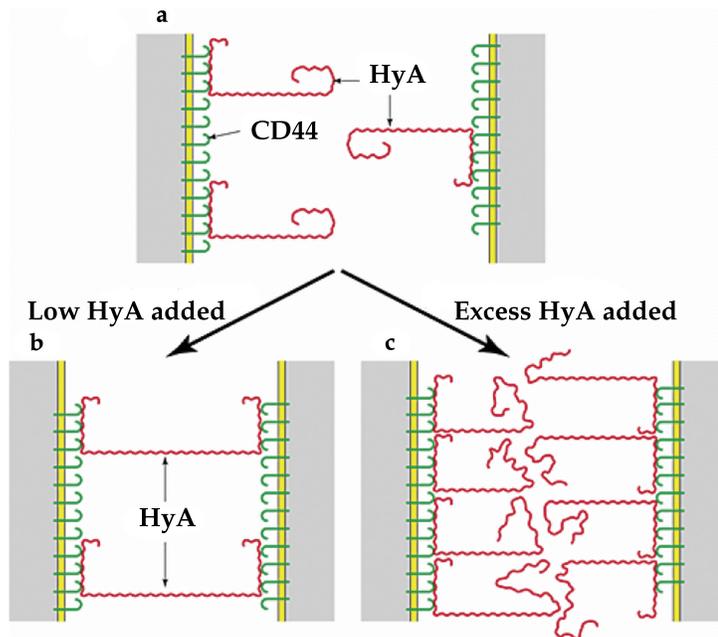


Figure 3.1: Schematic of hyaluronic acid (HyA) mediated cellular aggregation. a) Both BMSC and chondrocytes have CD44 surface receptors that bind to HyA. b) If a low concentration of HyA is added, the cells will bind to the same molecule of HyA and create a cross-bridge between them which mediates aggregation of the cells. c) If higher concentrations of HyA are added, then the cells bind to many HyA molecules and the CD44 receptors become saturated. The HyA cross-bridges are not formed and this inhibits cellular aggregation. [adapted from Toole, 1998]

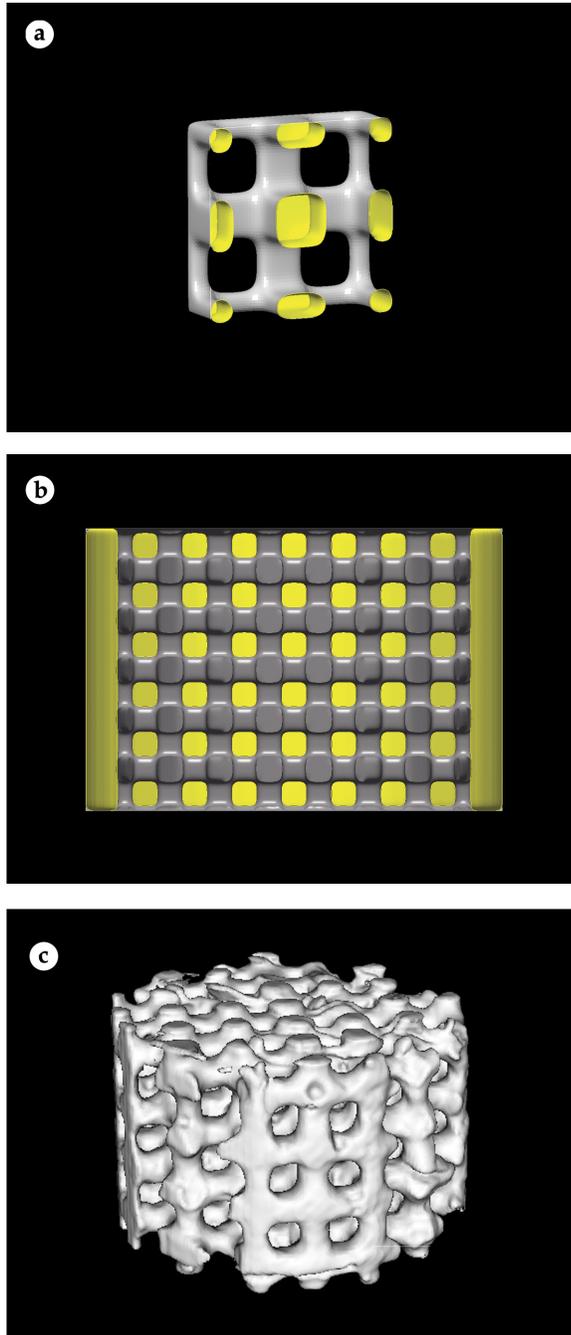


Figure 3.2: Images of SFF design files and fabricated cubic scaffold. a) A four unitcell aggregate showing the pore interconnections [350 x 350 x 350 μm] of the designed cubic architecture. b) Unitcells are used to create the architecture within a cylindrical shaped scaffold with 5 mm diameter and 3 mm height. c) Micro-CT scanned image of the final fabricated scaffold made from indirect SFF techniques.

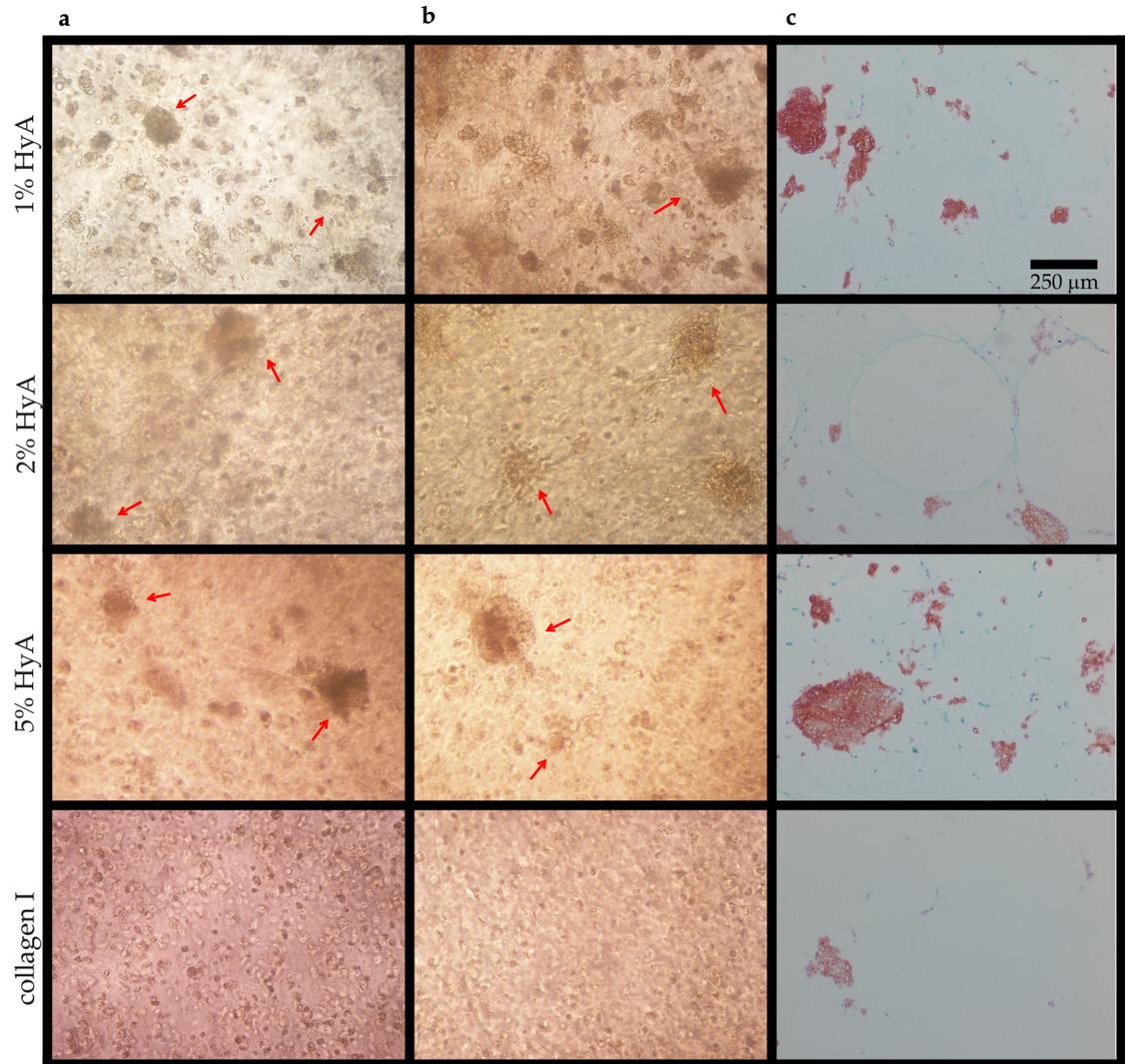


Figure 3.3: BMSC aggregation within 1, 2, 5% HyA and collagen I only hydrogels. a) Phase contrast images of hydrogels after 1 week of *in vitro* culture. The arrows indicate the cellular aggregates that were seen in the HyA composite hydrogels. Larger aggregates were seen in the hydrogels with higher concentrations of HyA, although 10% HyA hydrogels did not have any aggregates (data not shown). Cellular aggregates were not observed in the collagen I only hydrogels. b) Phase contrast images of hydrogels taken after 2 weeks. The number of aggregates did not increase over time, but the size of the aggregates seemed to increase. c) Safranin O stained sections of the 2 week hydrogels show chondrogenic differentiation of the BMSC within the cellular aggregates.

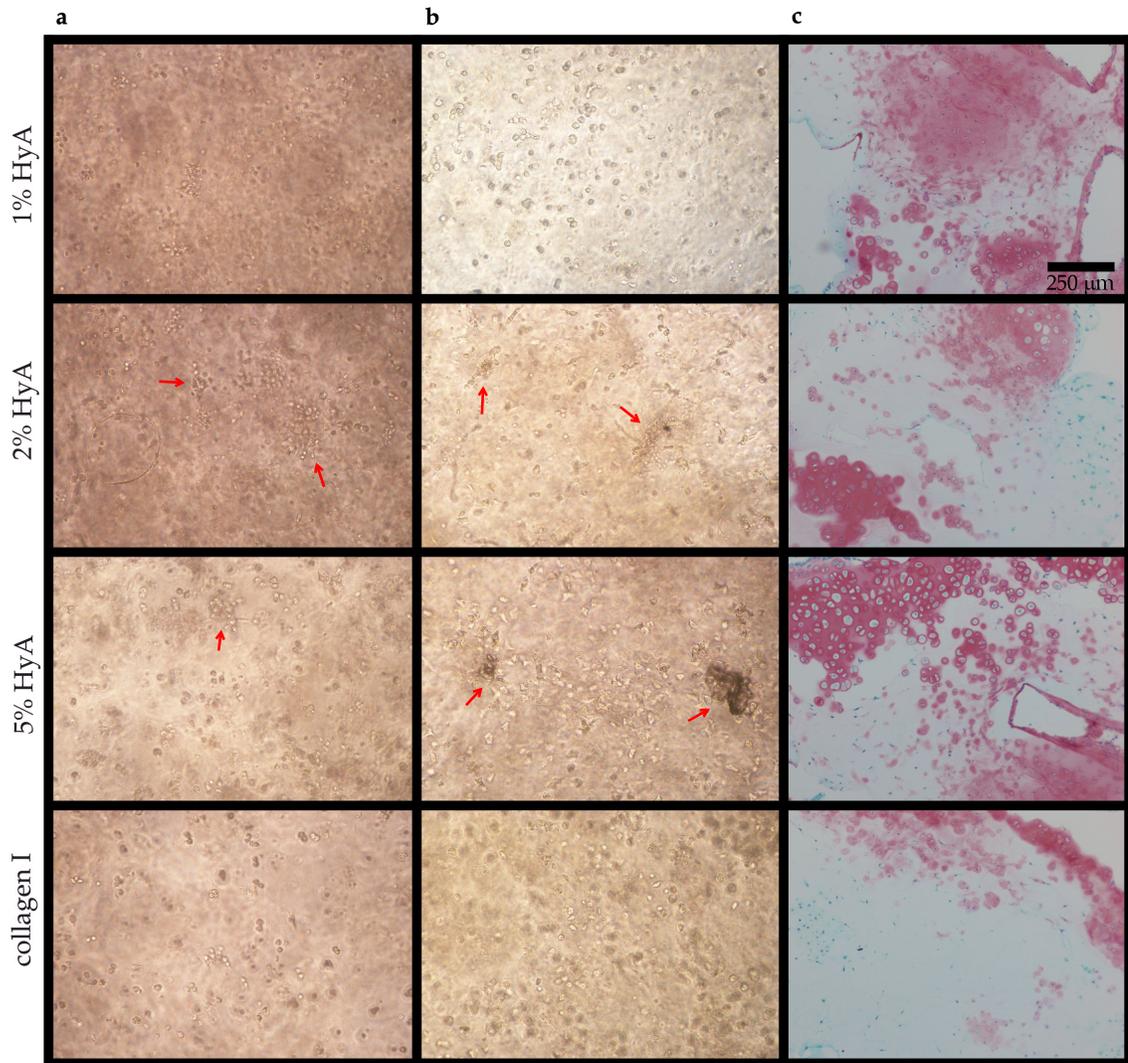


Figure 3.4: Chondrocyte aggregation within 1, 2, 5% HyA and collagen I only hydrogels. a) Phase contrast images of hydrogels taken after 1 week of *in vitro* culture. Cellular aggregates (indicated by arrows) were seen in the 2% and 5% HyA hydrogels only. Aggregates were not seen in the other hydrogel groups. b) Phase contrast images of hydrogels taken after 2 weeks. The aggregates seemed to increase in size over time in the 2% and 5% groups. c) Safranin O stained sections of the 2 week hydrogels show widespread sGAG in the extracellular matrix surrounding the cells in all groups. Larger areas of Fast Green were seen in the collagen only hydrogels, indicating greater dedifferentiation and loss of phenotype in these gels.

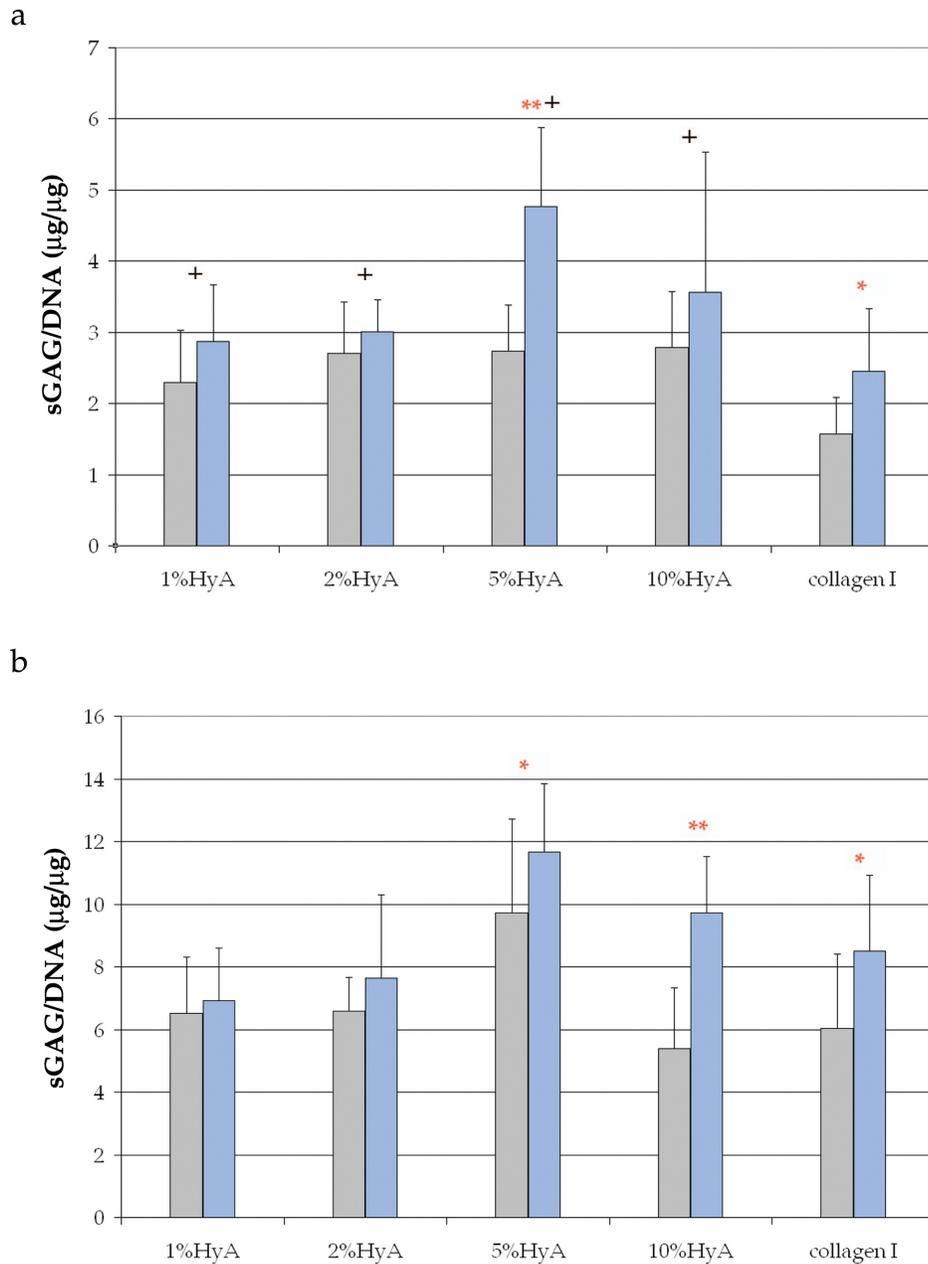


Figure 3.5: DMMB analysis of BMSC seeded hydrogels with varying HyA concentrations. a) One week data showing the sGAG concentration normalized by the DNA content of 1, 2, 5, and 10% HyA/collagen hydrogels and collagen I only hydrogels cultured in basal (gray bars) and defined chondrogenic medium (blue bars). All hydrogel groups with HyA were significantly greater than controls (collagen I only, $p < 0.01$). b) Two week data show an increase (almost 2x) over the 1 week data for all groups. Only the 5% HyA hydrogels were significantly greater than controls ($p < 0.05$). Overall, the highest sGAG ratios were in the 5%HyA group for both timepoints. [* indicates $p < 0.05$ and ** indicates $p < 0.001$ for comparisons between basal and chondrogenic media and + indicates $p < 0.01$ for comparisons between HyA concentration and control groups]

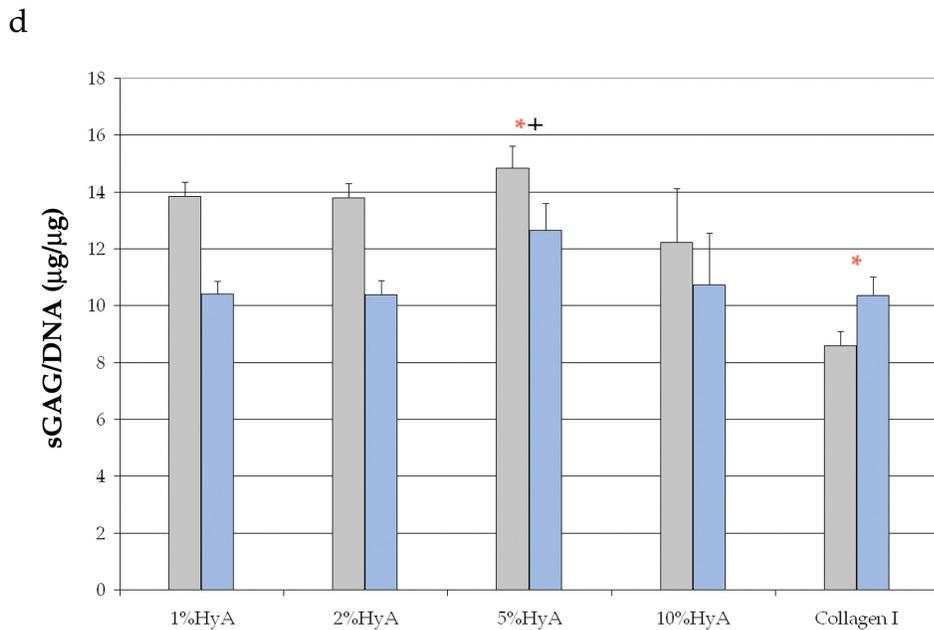
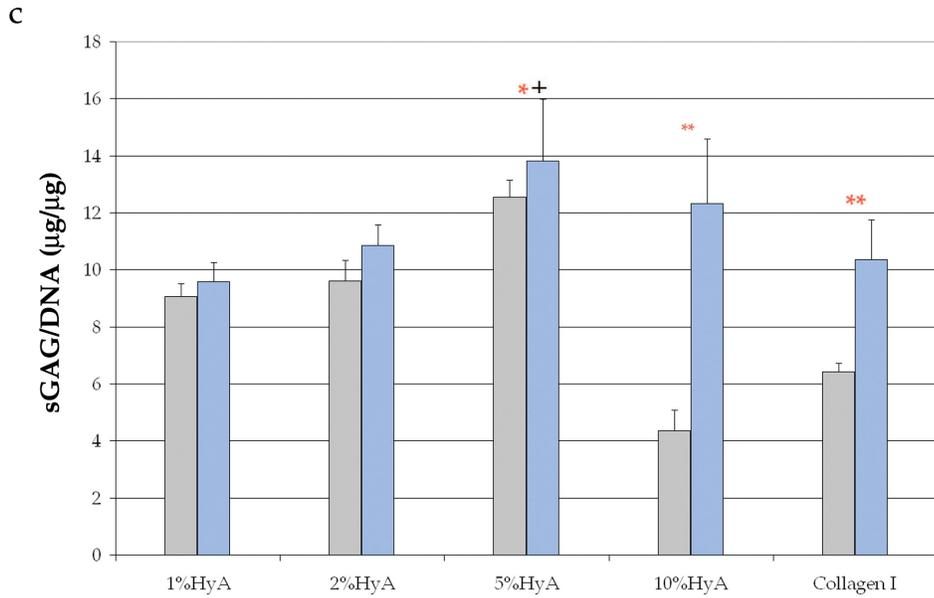


Figure 3.5: (continued) DMMB analysis of chondrocyte seeded hydrogels with varying HyA concentrations. a) One week data showing sGAG concentrations normalized by the DNA content of chondrocytes in 1, 2, 5, and 10% HyA/collagen hydrogels and collagen I only hydrogels cultured in basal (gray bars) and defined chondrogenic medium (blue bars). Only the 5% HyA hydrogels had sGAG ratios that were significantly higher than controls (collagen I only, $p < 0.01$). b) Two week data show an increase in the hydrogels cultured in basal medium, while the hydrogels cultured in chondrogenic medium increased little or decreased. The 5% and 10% HyA hydrogels cultured in basal medium were significantly greater than the control group. [* indicates $p < 0.05$ and ** indicates $p < 0.001$ for comparisons between basal and chondrogenic media and + indicates $p < 0.01$ for comparisons between HyA concentration and control groups]

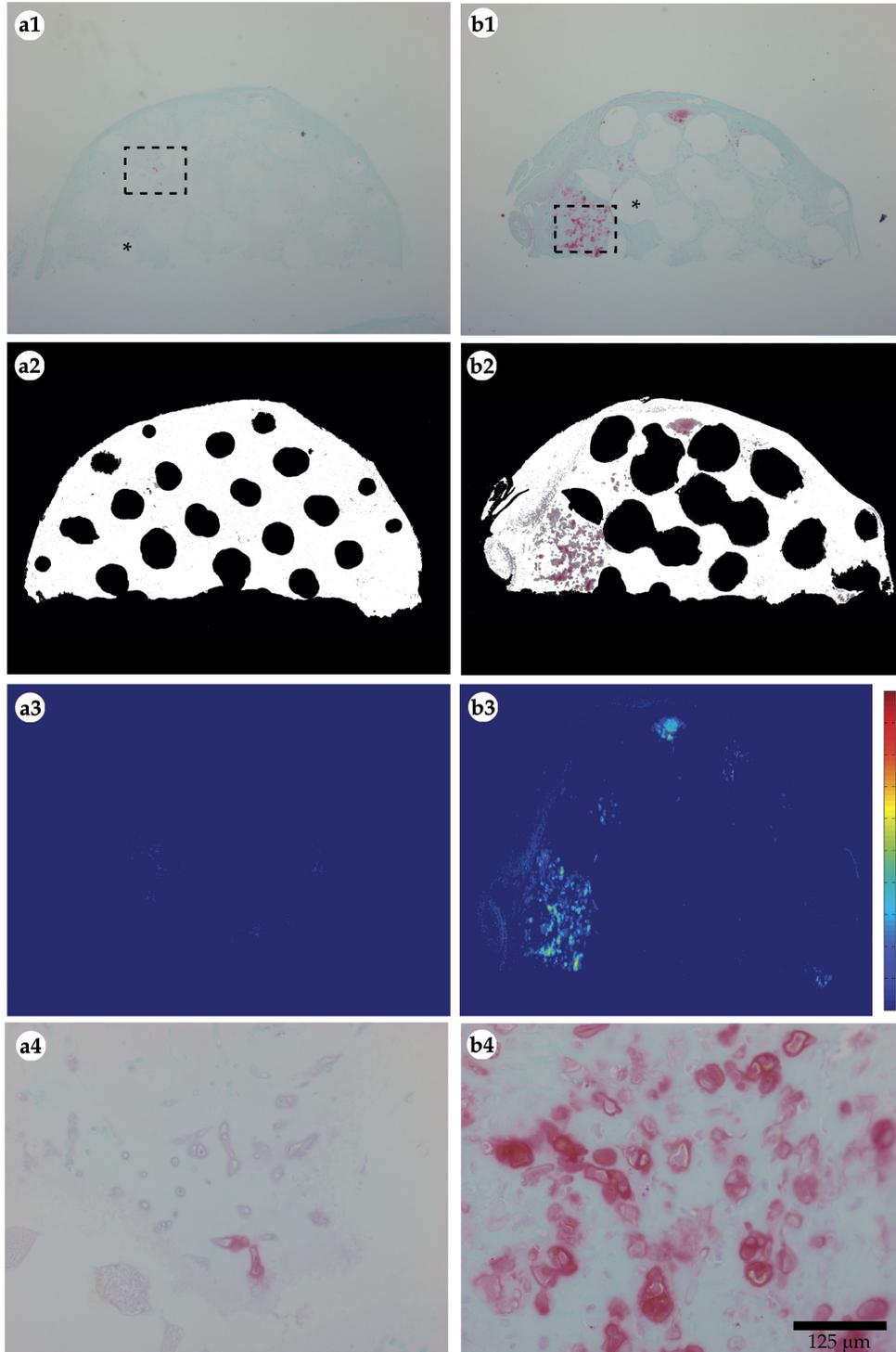


Figure 3.6: Cartilage formation in PCL scaffolds seeded with BMSC in collagen I (a) and 5% HyA/collagen (b) hydrogels and cultured in chondrogenic medium. Sections are stained with Safranin O/ Fast Green. Areas where the polymer was removed (indicate with *). a2,b2) The positive areas of Safranin O shown were extracted using image processing. These areas were quantified to determine the amount of cartilage matrix formation and the stain intensity was correlated to sGAG concentration. The amount of cartilage for these representative specimens is 2% for a2, 20% for b2. a3,b3) The average sGAG concentration calculated for the sections are $2.9 \mu\text{g}/\text{mm}^2$ for a3, $3.7 \mu\text{g}/\text{mm}^2$ for b3. a4,b4) Higher magnifications of the areas within the dashed boxes show round chondrocytic cells surrounded by positively stained extracellular matrix.

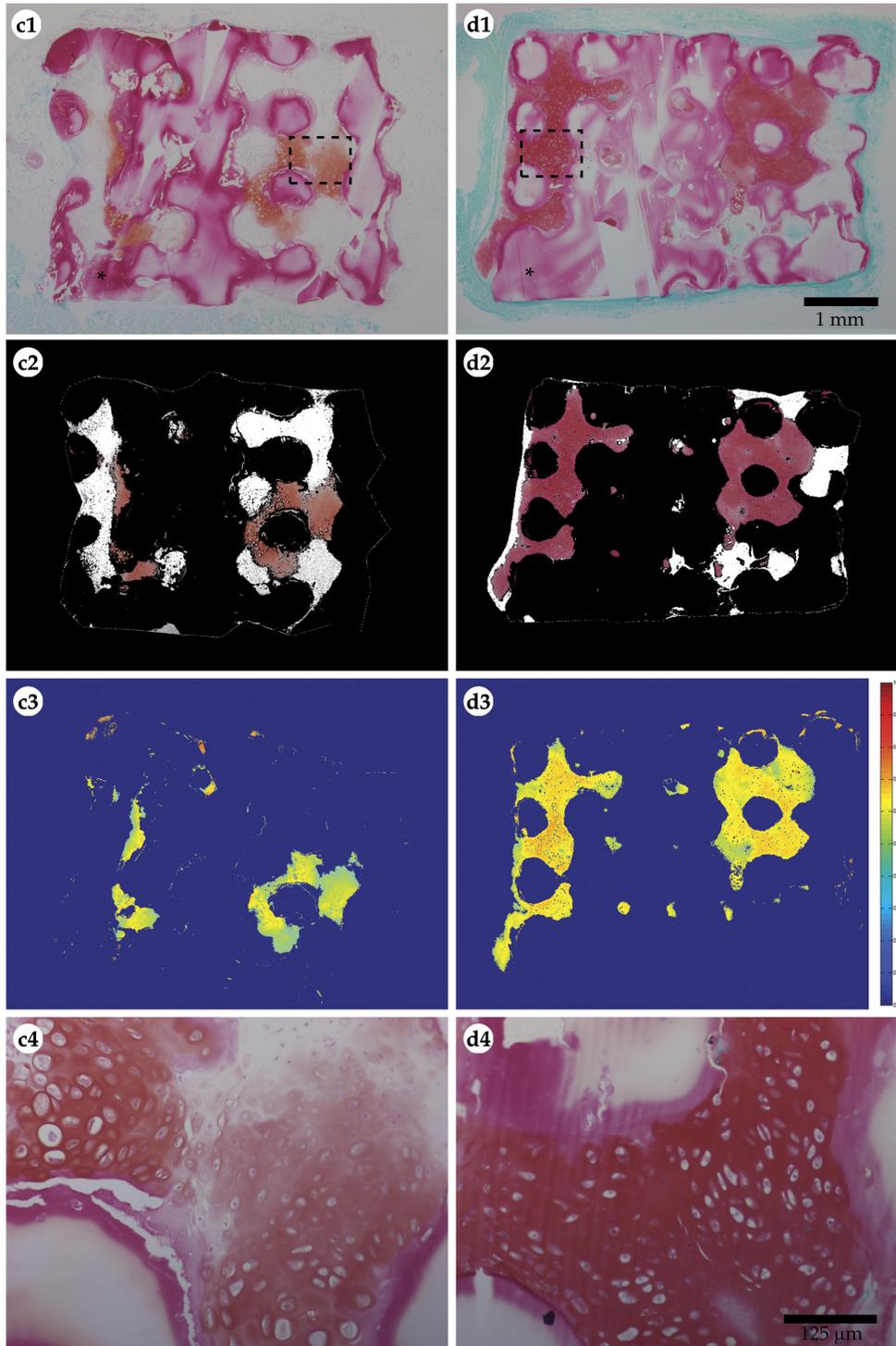


Figure 3.6: (continued) Cartilage formation in PPF scaffolds seeded with chondrocytes using collagen (a) and 5% HyA/collagen (b) hydrogels. Residual PPF scaffolds are visible in the sections (indicated with *). Areas of positive Safranin O staining are extracted (red) as well as the remaining pore areas (white) to determine the percentage of cartilage matrix present in the section, approximately 30% and 60% for c2 and d2, respectively. The stain intensity is correlated to the sGAG concentration in the ECM, 7 and 9 $\mu\text{g}/\text{mm}^2$ for c3 and d3, respectively. Higher magnification images show round chondrocytic cells surrounded by a dense extracellular matrix.

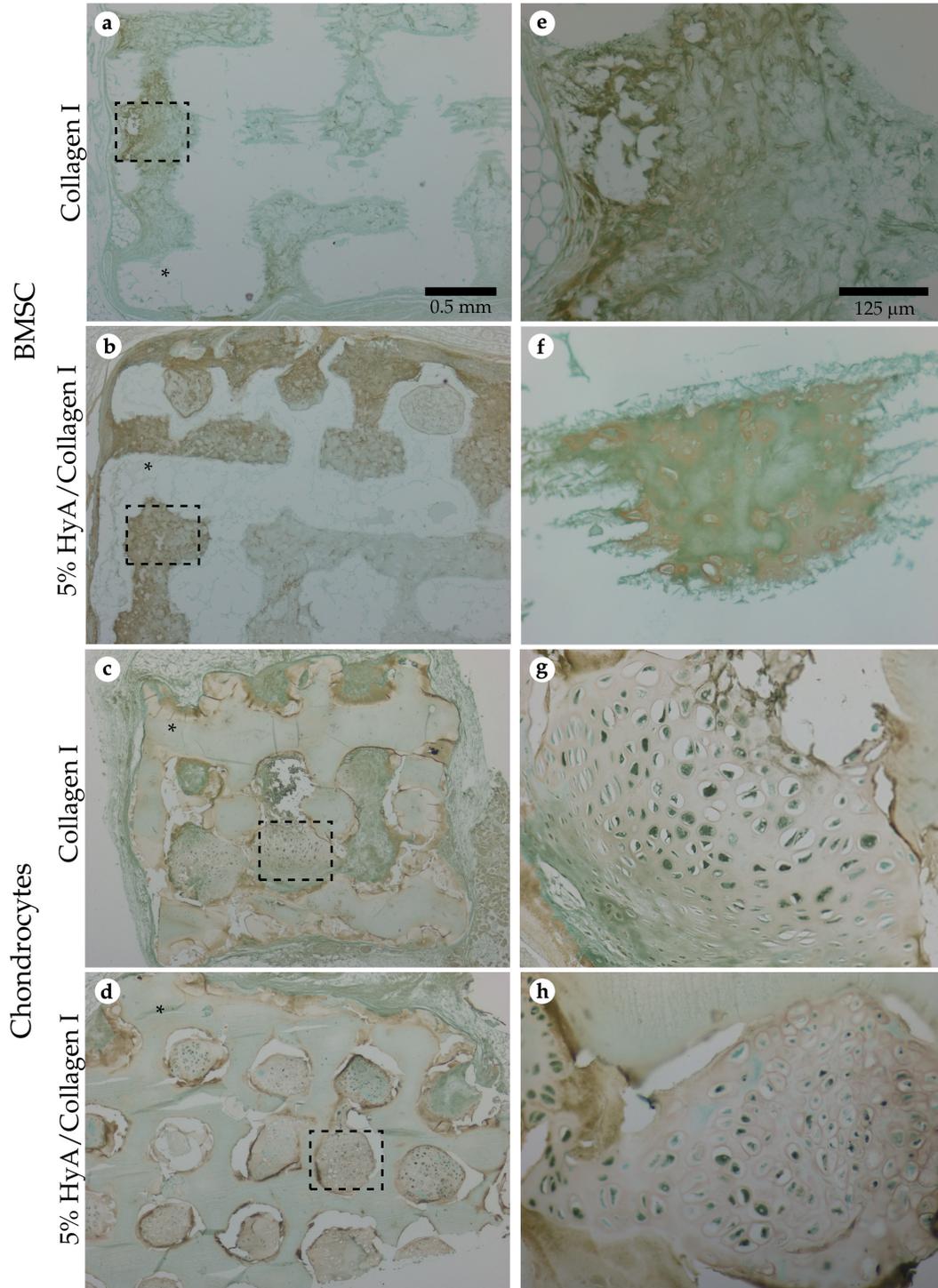


Figure 3.7: Immunohistochemistry detecting collagen II in SFF scaffolds seeded with BMSC and chondrocytes. Residual scaffolds and areas where polymer was removed are indicated with *. Positive staining (brown) is seen in the extracellular matrix surrounding the cells. Collagen II is widespread throughout the pores of the scaffold with chondrocytes and in more isolated areas for the scaffolds with BMSC. More areas of positive staining are seen with the scaffolds seeded using 5% HyA/collagen hydrogels. Higher magnification of areas in dashed boxes showing chondrocytic cells (e - h).

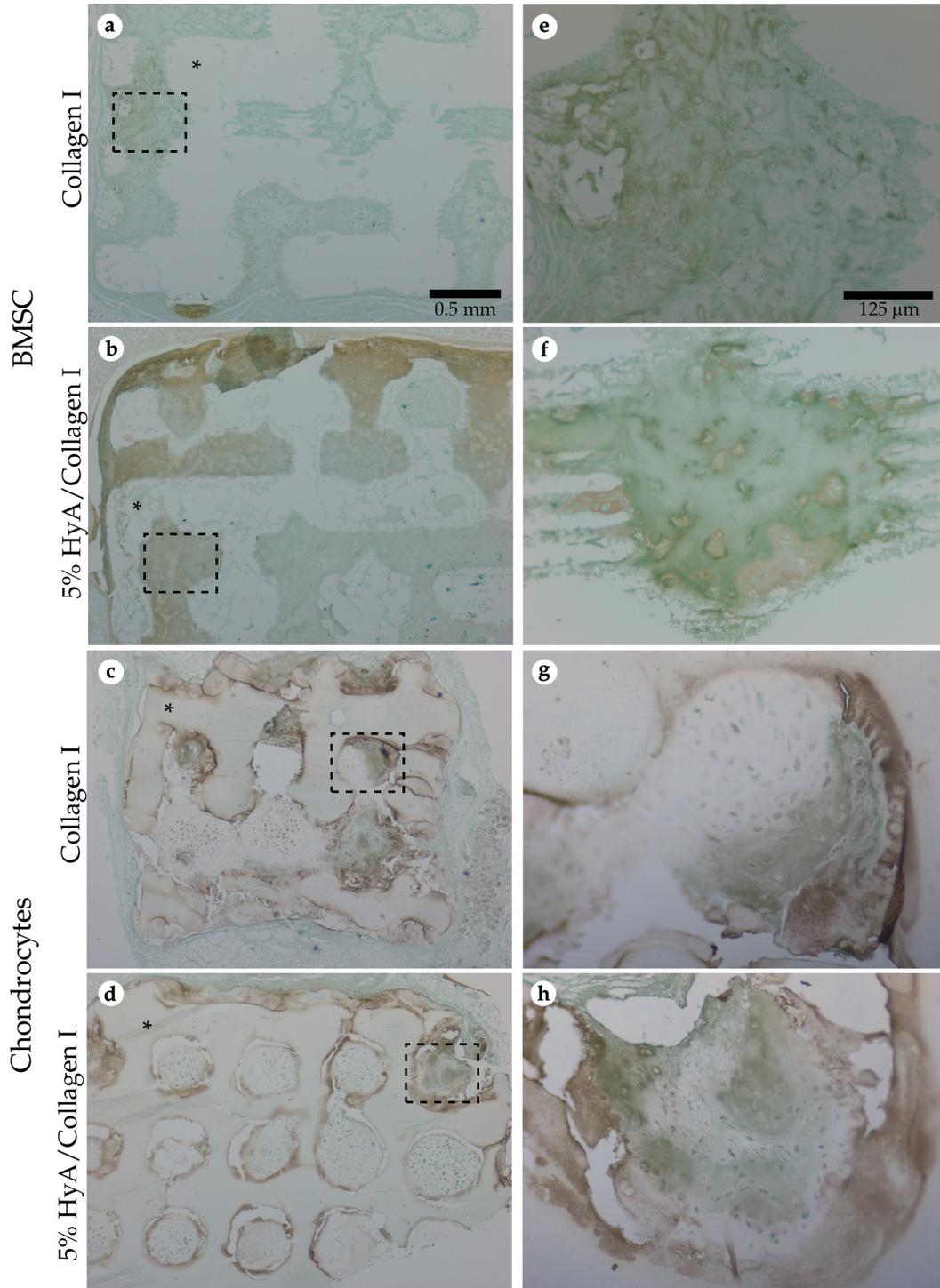


Figure 3.8: Immunohistochemistry detecting collagen I in SFF scaffolds seeded with BMSC and chondrocytes. Residual polymer and areas where polymer was removed are indicated with *. Areas with positive staining (brown) are seen at the scaffold edges and in some outer scaffold pores. Greater areas of positive staining are seen in the scaffolds seeded with collagen I hydrogels for chondrocytes. Higher magnification of areas in dashed boxes are showing areas with collagen I (e - h).

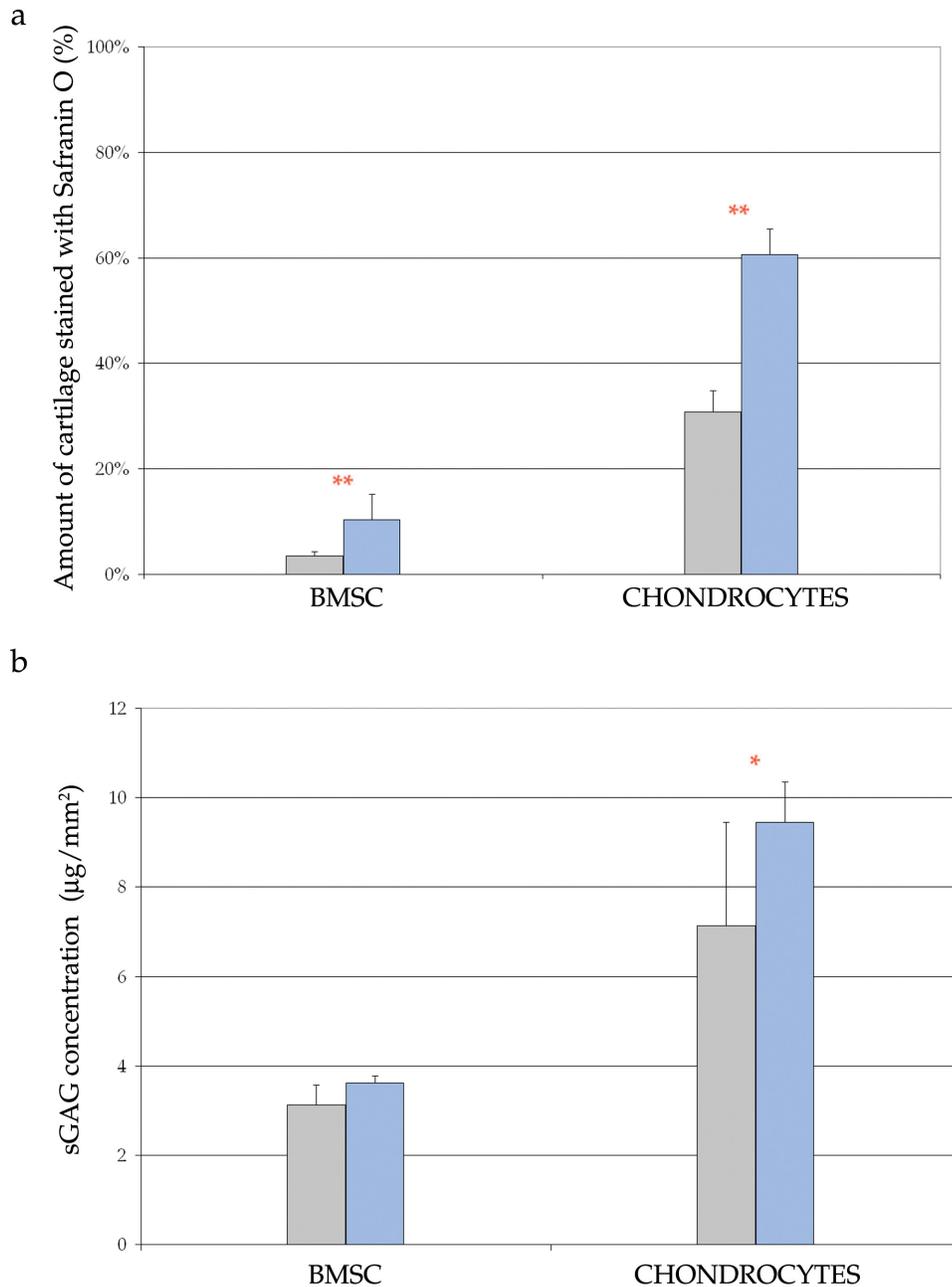


Figure 3.9: Results from Safranin O histomorphometric image analysis of BMSC and chondrocyte seeded SFF scaffolds. Scaffolds were seeded using either collagen I (gray bars) or composite 5% HyA/collagen I (blue bars) hydrogels. a) The amount of positive Safranin O staining is equated to the amount of cartilage matrix present in the sections. The area is normalized by the total pore space available for each section and is expressed as a percentage. Significantly greater areas of cartilage were seen in the scaffolds that were seeded with 5% HyA hydrogels. b) The Safranin O stain intensity is correlated with the sGAG concentration present in the extracellular matrix. [* indicates $p < 0.05$ and ** indicates $p < 0.001$ for comparisons between 5% HyA and collagen hydrogels]

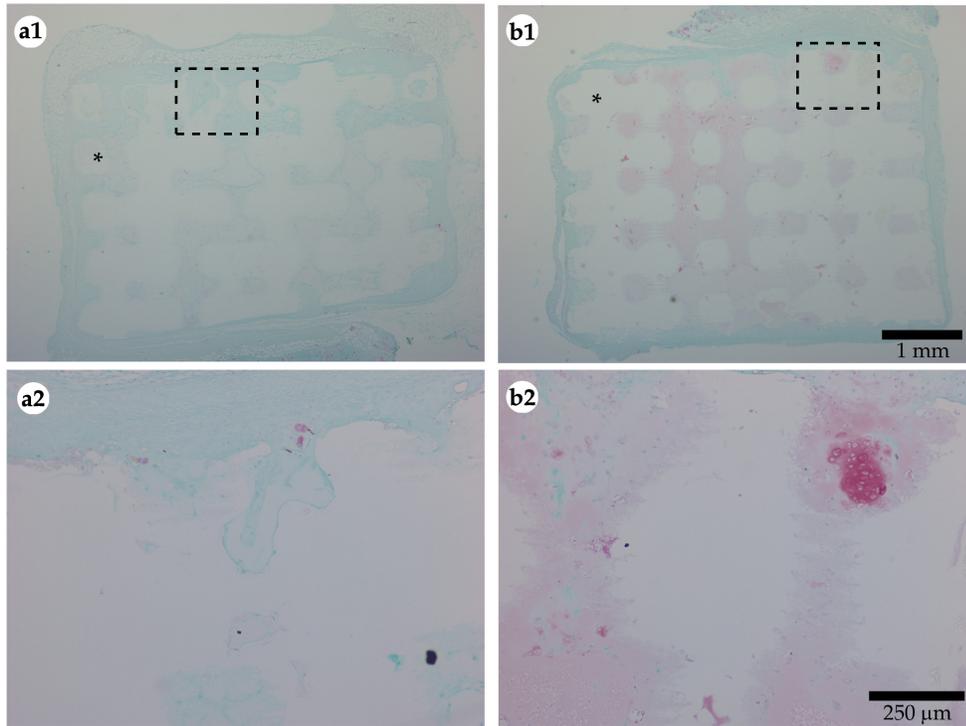


Figure 3.10: Passaged chondrocytes seeded into SFF scaffolds using collagen I and composite 5% HyA/collagen hydrogels. a) Passage 6 chondrocytes in collagen I hydrogels have lost their chondrocytic phenotype after 2 weeks of *in vitro* pre-culture and 4 weeks of *in vivo* implantation. b) Passaged chondrocytes in 5% HyA/collagen hydrogels have not dedifferentiated into fibroblasts and still maintain the ability to produce sGAG, although it is much weaker than primary chondrocytes.

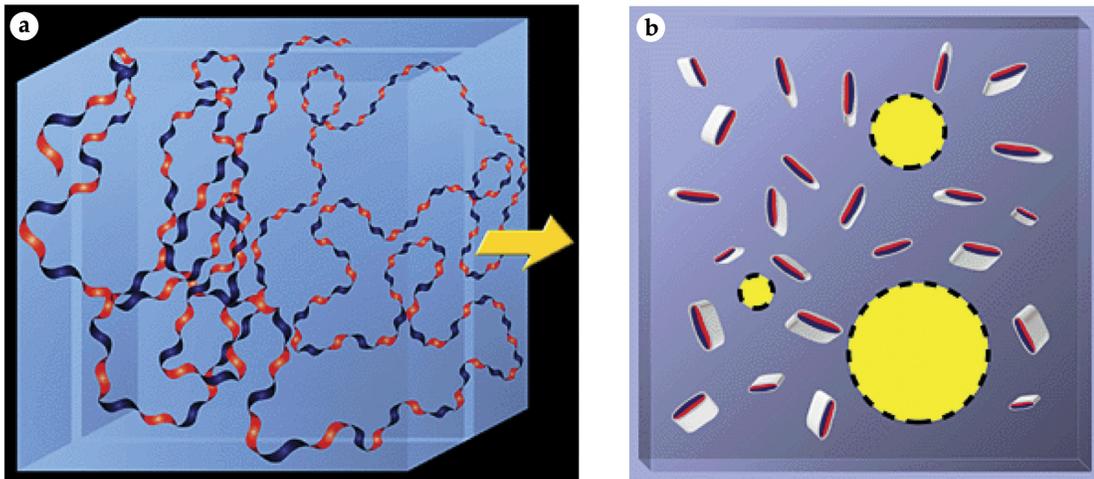


Figure 3.11: Model of hyaluronan molecule in a 3-dimensional matrix (hydrogel). a) The light blue box represents the domain of HyA within a hydrogel. The alternating blue and red strand represents the ribbon structure of HyA with blue (hydrophilic) and red (hydrophobic) faces. b) A vertical slice of the HyA domain shown in (a), illustrating the average pore size and partial exclusion of large molecules due to the presence of hydrophobic patches. The red and blue lines represent portions of the hyaluronan backbone in the representative slice. The gray halo (shading) around the hyaluronan fragments would be the volume of the slice inaccessible to a diffusing molecule. The three yellow circles of varying sizes represent areas available to diffusing molecules. The smallest would have access to most of the volume not occupied by hyaluronan, while the largest would have access to only at the places indicated and would have a harder time moving through the hyaluronan domain. [Hascall and Laurent, 1997]

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