EFFECTS OF CHITOSAN-GELATIN HYDROGELS ON FIBRO-CHONDROGENESIS

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ACRONYMS

3D: three dimension

AFSC: amniotic fluid derived mesenchymal stem cell

AMSC: adipose derived mesenchymal stem cell

BMP: bone morphogenetic protein

BMSC: bone marrow derived mesenchymal stem cell

BSA: bovine serum albumin

CCM: complete chondrogenic medium

CFDA-SE: carboxyfluorescein diacetate, succinimidyl ester

C-G-GP: chitosan-gelatin-β-glycerophosphate

ECM: extracellular matrix

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FACS: fluorescence-activated cell sorting

FBM: fibroblast basal medium

FBS: fetal bovine serum

FGM: fibroblast growth medium

FITC: fluorescein isothiocyanate

GAG: glycoaminoglycan

GP: β-glycerophosphate

G2P: glycerol 2-phosphate

H&E: haematoxylin and Eosin

HA: hyaluronic acid

hESC: human embryonic stem cell

hFF-1: human foreskin fibroblast

hMSC: human mesenchymal stem cell

ICM: incomplete chondrogenic medium

iPS: induced pluripotent stem cell

LMW: low molecular weight

MMP: matrix metalloproteinase

MSCBM: mesenchymal stem cell basal medium

OA: osteoarthritis

OADDL: Oklahoma Animal Disease and Diagnostic Laboratory

OPN: osteopontin

PBS: phosphate buffer saline

PE: phycoerythrin

PEO: poly(ethylene oxide)

PEG: polyethylene glycol

PG: proteoglycans

PVA: poly(vinyl alcohol)

RA: rheumatoid arthritis

RGD: arginine-glycine-aspartate

SEM: scanning electron microscopy

TCP: tissue culture plastic

TGF-β: transforming growth factor beta

CHAPTER I

INTRODUCTION

1.1 Motivation for Regenerative Techniques for Cartilage

There are approximately 600,000 knee replacement surgeries every year in the United States. Some causes for the surgery can be due to but not limited to osteoarthritis or direct injury from athletics. Additionally, osteoarthritis commonly affects the older age groups whereas the sports related injuries are typically found in the age group of adults to young adults (Banzon 2009). Furthermore, osteoarthritis has been found to affect athletes that have suffered a direct cartilage injury that has not been repaired. In 2005, an estimated twenty seven million adults were diagnosed with OA (Center for Disease Control and Prevention 2010). Furthermore, over a ten year period, there was a documented 7,769 sports related knee injuries reported from 6,434 patients; and of those, cartilage lesions consumed 10.63% of the knee injuries (Majewski et al. 2006). Medical options for people suffering from knee injuries include total knee replacement, reconstructive surgery, and rehabilitation that can cause the patients to be in bed rest anywhere from days to weeks depending on the severity of the injury. However, some people suffering from OA or athletic injuries may be unable to have surgery due to the lack of insurance or funds. Knee surgeries can be cost effective depending on a person's

insurance situation and the extensiveness of the surgery. The total cost of medical care for OA was $37.5 Billion in 2009 (Hospital and Health Networks 2011). There are many variables affecting the cost of surgery, some include surgeon, pharmaceutical and facility fees, as well as the cost to obtain and isolate cartilage cells, or chondrocytes. Because chondrocytes have low regenerative capabilities and offer no possibility for natural healing, a replacement strategy has been explored in the form of stem cell differentiation. This technique focuses only on the biological side of cartilage replacement and is limited under clinical conditions. An artificial tissue, serving as a platform, must be both biocompatible and biodegradable for the chondrocytes to proliferate and reconstruct the natural cartilage.

Allografts and biodegradable polymer based structures have been explored as surgical alternatives for cartilage. Extensive studies have been performed to develop a suitable alternative tissue for cartilage as well as deriving chondrocytes from another cell line. The materials used for the tissue development and fabrication are natural and synthetic polymers. These materials are utilized for grafting and injection in physical forms such as porous scaffolds and injectable hydrogels. Since hydrogels, which are cross-linked hydrophilic polymers, contain large amounts of water similar to cartilage; this makes them attractive in cartilage regeneration. In particular, injectable hydrogels are minimally invasive for arthroscopic surgeries and easy to incorporate cells and bioactive agents. An attractive method is using chitosan as the base material to prepare the hydrogels at body temperature using β-glycerophosphate (GP) chemistry. The solution remains in liquid phase at room temperature and gels irreversibly upon increasing body temperature. However, a major weakness of hydrogels is mechanical strength; strategies

to improve the compressive property to comparable levels of cartilage, which range from 35 to 2000 kPa, must be explored (Werkmeister et al. 2010). A weakness of chitosan is its lack of a cell binding domain thus, requiring the addition of polymers that contain this property. The cell binding domain influences cell-cell and cell-matrix interactions in three-dimensional (3D) systems, which are crucial to integrate the extensive signaling pathways, the biophysics, and the biomechanics that regulate the development, homeostasis, and regeneration of tissues.

Allogeneic stem cells capable of differentiating into chondrocytes have been explored in cooperation with 3D matrices. Some efforts have focused on comparing the phenotypic differences between fibroblasts with adult human mesenchymal stem cells (hMSCs) (Blasi et al. 2011). However, there has been no comprehensive study that involves a biopolymer structure that can be used as a minimally invasive alternative for cartilage repair with the inclusion of an autologous cell source that has potentiality to differentiate in to the chondral cell line. Human foreskin fibroblasts (hFF-1) are targeted because they are easily obtained from a small biopsy of autologous skin, able to be programmed into induced pluripotent stem (iPS) cells, and easily populated in tissue culture in serum free medium which minimizes safety concerns related to xenogeneic products.

1.2 Objectives and Hypothesis

The objectives of this study were to investigate a hydrogel system that is easy to utilize in clinical settings to produce cartilage and an autologous cell source such as easily available hFF-1. 3D systems based on mimicking *in vivo* environments provide a

substantial new integrative level of complexity to the cells. An injectable solution system based on the extracellular matrix (ECM) elements present in cartilage was utilized to fabricate gels with properties similar to cartilage at body temperature. Chitosan, which is biodegradable and biocompatible, was chosen as the base polymer for construction of the hydrogel. Chitosan has a net positive charge for immobilizing negatively charged molecules such as glycosaminoglycans and gelatin but contains no cell binding domain. Therefore, gelatin was used for the purposes of cell binding due to its Arg-Gly-Asp (RGD)-like sequence that promotes cell adhesion and migration (Huang, Onyeri et al. 2005). Additionally, gelatin can be combined with chitosan in the absence of a cross-linker (Mao 2003).

1. *The first objective was to evaluate a hydrogel system that is easy to utilize in clinical settings to produce cartilage.* In order to use a hydrogel for cartilage in a clinical setting, we must meet the mechanical characteristics of cartilage. We hypothesized that increasing the concentration of chitosan would increase the mechanical properties. To test the hypotheses, we prepared chitosan-gelatin at concentrations of 2%/0%, 2%/1%, and 2%/2%. First, we analyzed the rheological properties of the hydrogel at a 2%/2% chitosan-gelatin supplemented with glycerol 2-phosphate concentration. Next, we measured the compressive properties of the hydrogels at 37^{o}C using a custom-built two anvil apparatus. These results showed suitable mechanical properties of the hydrogels for cartilage applications.
2. *The second objective was to investigate an autologous cell source such as hFF-1 for chondrocytes and measure viability in a regenerative tissue.* For the purposes of a clinical setting, autologous cell source with minimally invasive harvesting protocol

would significantly enhance the applicability of regenerating cartilage. Hence, human foreskin fibroblasts were cultured in both pellet and hydrogel systems similar to hMSCs and supplemented with a transforming growth factor (TGF-β3) for differentiation. Quantification of differentiation was measured with a Collage Type II ELISA, the expression hyaluronan (CD44) and laminin-binding surface receptors (CD151) via flow cytometry, histology analysis, and the expression of degradation enzymes MMP-2/MM-9 and MMP-13. Additionally, the capabilities of differentiation for an autologous cell source would be similar to that of a stem cell source in both pellet and hydrogel systems. The viability and proliferation of a differentiated autologous cell source was possible in the more concentrated chitosan-gelatin injectable hydrogels.

CHAPTER II

REVIEW OF LITERATURE

2.1 Cartilage Regeneration Techniques

Articular cartilage covers the ends of all bones that form articulating joints in humans and animals. The cartilage acts in the joint as a mechanism for force distribution and as a lubricant in the area of contact between the bones. Without articular cartilage, stress concentration and friction would occur to the degree that the joint would not permit ease of motion. In general, articular cartilage consists of cells called chondrocytes with cell volume averaging only 2 to 3% of the total. Nearly 15% is occupied by an extensive extracellular matrix (ECM) consisting of collagen type II, glycosaminoglycans, proteoglycans, and elastins (Fox et al. 2009). When a person injures cartilage, either by physical activity or deterioration over time, the cartilage is unable to heal spontaneously. This is primarily because articular cartilage has poor regenerative and reparative abilities following injury or degenerative diseases (Toh et al. 2010). Damaged articular cartilage can severely restrict joint function, causing debilitating pain. Thus, small lesions usually result in progressive deterioration and eventual joint diseases including osteoarthritis (OA), requiring joint reconstruction/repair (Hospital and Health Networks 2011).

An alternative strategy mimicking natural cartilage is the key to successfully reducing the cost and care of cartilage issues (Gong et al. 2010, Bobick et al. 2009, Diekman et al. 2010, Tew et al. 2008). Current conventional treatment modalities for damaged meniscal cartilage include the removal and/or surgical repair of the damaged cartilage. Osteochondral allografting and autografting are currently the only options that have demonstrated some potential to restore mature hyaline cartilage. Allografts are transplantable tissues from another person with a different genotype. Autografts are tissues from the patient's body that can be used for transplantation at a different place in their body. Despite developments in surgical procedures, the cartilage tissue replacement with structures consisting of permanent artificial materials has been less than satisfactory, due to many factors such as need for immunosuppressive therapy, and mechanical mismatch of the donor cartilage. In some cases, there is also an increased risk of degenerative arthritis. Recently, tissue engineering approaches have offered new possibilities for the restoration of damaged or lost tissue. Chondrocytes transplantation has been reported in many preclinical and clinical studies.

2.2 Scaffold based Tissue Engineering

Tissue engineering applies the principles of biology and engineering to the development of functional substitutes for damaged tissues (Langer et al. 1993). The concept of tissue engineering is that cells isolated from a patient or donor, propagated *in vitro* in a suitable environment with proper growing conditions, seeded onto a biocompatible and biodegradable platform and once confluent grafted into the patient as a replacement for

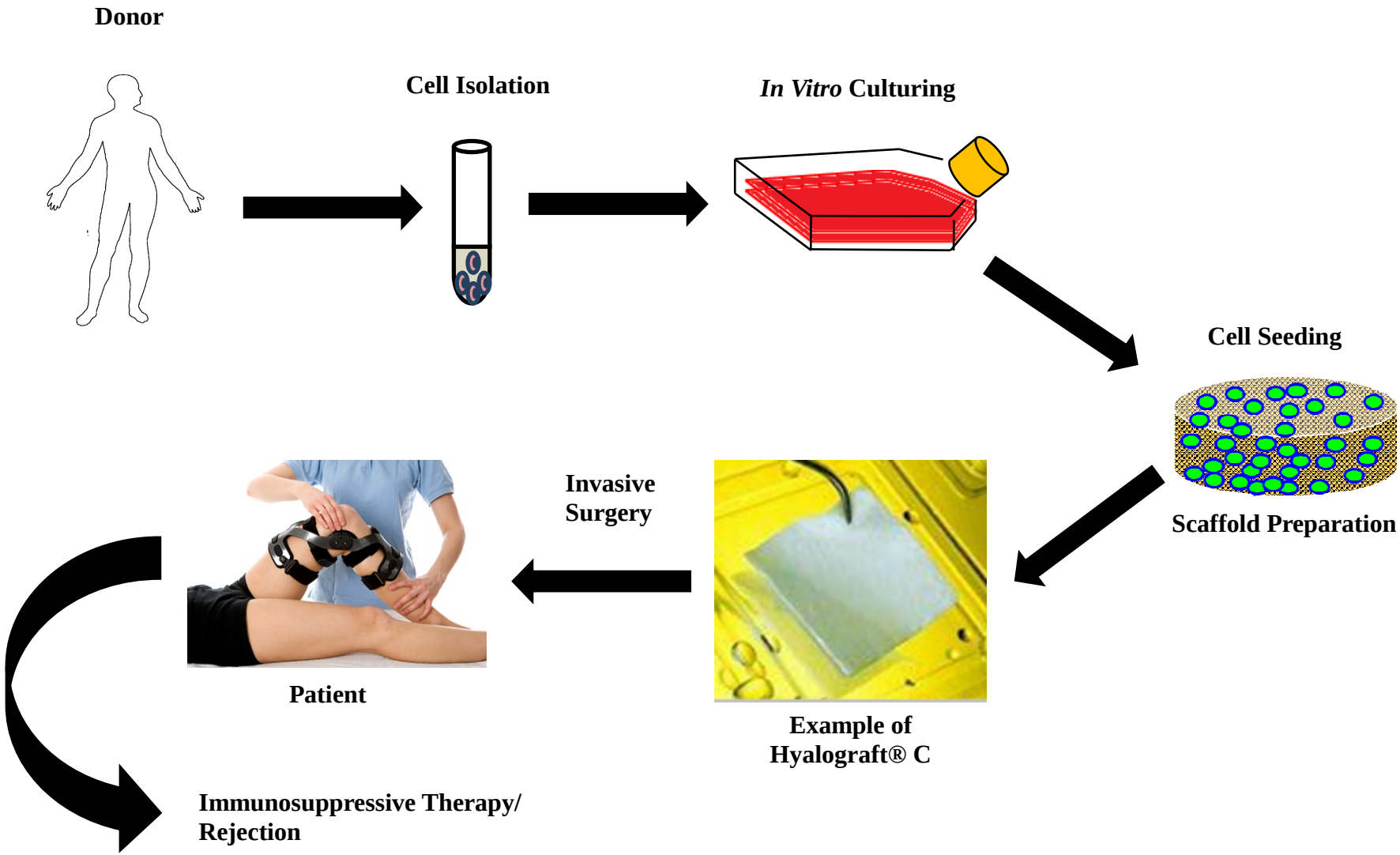


Figure 2.1. Flow chart for traditional tissue engineering techniques used currently. Source: Hyalograft® C Autograft: Anika Therapeutics 2011, Patient: Christopher Ligori and Associates 2011.

the damaged tissue. Figure 2.1 illustrates the steps involved in scaffold based tissue engineering process. The replacement scaffold must be biocompatible, which involves the use of materials that promote functional characteristics and cell attachment but do not provoke unwanted tissue responses, and biodegradable, which implies the ability of the material to degrade into non-toxic materials leaving only living tissue. These characteristics are possessed by materials such as ceramics, metals, natural and synthetic polymers and a combination thereof (Yang et al. 2001). Many in vitro experiments show that cells respond differently in attachment, morphology, migration, and proliferation on three-dimensional scaffolds (Lawrence et al. 2008). Scaffolds provide physical cues to guide cell colonization as well as chemical cues of cell-binding sites to support cell attachment and spreading. Cellular growth and function is influenced by scaffold stiffness of the substrate challenge of tissue engineering cartilage is to provide the essential cells and signals that will establish a cartilage extracellular matrix and recapitulate this molecular organization that forms the basis for the essential mechanical properties of the tissue. Although the polymer based scaffolds have shown promise to regenerate cartilage (Ragetly et al. 2010)**,** implantations of these structures require invasive surgical procedures. Polymer based hydrogels have been explored more recently for use as regenerative cartilage.

2.3 Cartilage Extracellular Matrix and Components

Before exploring the material selection in more depth, a better understanding must be established for the extracellular matrix (ECM) components of cartilage itself. This is to

determine which materials are important for developing a regenerative tissue that would most closely resemble natural cartilage. Further, the function of articular cartilage as a load-bearing tough elastic tissue is based on the unique structure of chondrocytes dispersed in an organized gel-like ECM. The ECM can be considered as the foundation that controls the tissue structure by holding the cells together and regulating the cell phenotype (Stevens et al. 2005). The ECM for cartilage represents the microenvironment for chondrocytes and depends on the interaction with their pericellular matrix to maintain a stable cellular phenotype (Gentili et al. 2009). These components are produced by the resident chondrocytes and can take up a variety of forms in different tissues. Additionally, the ECM stores variety of soluble factor such as cytokines and growth factors and modulates their activation status and turnover (Kresse et al. 2001). The ECM of tissues consists mainly of proteins, glycoaminoglycans (GAGs), glycoproteins and small molecules arranged in a structural support matrix. The ECM components that are specific to cartilage are Collagen Type II, Aggrecan, Hyaluronan, tropoelastins and matrix metalloproteinases (MMPs). More specifically, Collagen Type II makes up approximately 60% dry weight of articular cartilage while the rest of the ECM is comprised of 25-35% dry weight PG and 15-20% dry weight other non-collagenous proteins (Mow et al. 1999).

Collagens are connector fibril proteins and are the principle constituents of cartilage. Together with proteoglycans (PGs) and GAGs filling the extracellular space, collagen type II fibers provide tensile strength and create space for the tissue while allowing diffusion of nutrients, metabolites and growth factors (Kim et al. 1998). The proteoglycans provide the compressive stiffness necessary for normal articulation and

function. Additionally, collagen has been used for drug delivery systems because of its ability to facilitate wound repair (Friess 1998). Hyaline cartilage contains high levels of a cartilage specific proteoglycan, Aggrecan (Roughley et al 1994). As a result of the aforementioned studies, Collagen Type II production was chosen to be the main component to be observed in this study.

Proteoglycans (PGs) are macromolecules of covalently bonded carbohydrate chains GAGs (particularly chondroitin sulfate in cartilage) attached to a core protein and are present in many different forms in ECMs of connective tissues (Iozzo et al 1996). The role PGs in the ECM is to serve as tissue organizers, influence cell growth and maturation of specialized tissue, play a role as biological filters and modulate growth-factor activities, regulate collagen fibrollogenesis and skin tensile strength, affect tumor cell growth and invasion, and influence corneal transparency and neurite out-growth (Iozzo et al. 1998). Aggrecan is the most commonly found PG in hyaline and articular cartilage and is able to interact with hyaluronan to form large PG aggregates (Gentili et al. 2009). Hyaluronan or hyaluronic acid (HA) is a large negatively charged polysaccharide and serves as a lubricant in tissue joints. In adult cartilage, HA binds to aggrecan to maintain high concentrations of aggrecan for tissue compressive resilience (Gentili et al. 2009). The aggregate structure formed represents a major attractor for water molecule that hydrates the cartilage matrix (Jarvelainen et al 1991).

The remainder of components contributes a small portion of the overall composition of the extracellular matrix. The smaller molecules of the ECM consist of tropoelastins, and matrix metalloproteinases (MMPs). Tropoelastins are the base forms for elastins which work as rubber bands for assisting the tissue in returning to its natural state (Iyer et al

2011). MMPs are enzymes that are mainly responsible for the degradation of ECM elements. Loss of GAGs in arthritic patients has been attributed to the increased production of MMP-3 or stromelysin (Fosang et al., 1996). MMP-2 (Gelatinase A), a constitutively produced homeostatic enzyme, degrades gelatin (Makowski et al., 1998). Further, MMP-9 (gelatinase B), which is expressed in acute and chronic inflammations, also degrades gelatin. Degradation of collagen type II involves MMP1, MMP8 and MMP13. Furthermore, MMP-13 has been demonstrated to cleave collagen type II, which is predominant in cartilage, more readily than types I and III (Knauper V et al. 1996). MMP-13 cleaves fibrillar collagens with preference to type II collagen over type I and III collagens, and displays over 40-fold stronger gelatinase activity than MMP-1 and MMP-8. MMPs are expressed at higher levels in patients who have osteoarthritis, rheumatoid arthritis or who have damaged cartilage (Nagase et al. 2003). Thereby the structural integrity and mechanical strength of the tissues is impaired, resulting in irreversible destruction of the joint structures. Together, these ECM components are the building factors of cartilage and are essential for the construction of regenerative cartilages. Now that the biological materials necessary for cartilage regeneration have been discussed, biomaterials used in cartilage regeneration will be described.

2.4 Materials for Hydrogel Fabrication

Hydrogels are the desired scaffolding for cartilage because the gel offers a similar environment for the cartilage cells to grow in compared to the original conditions. Water content within the cartilage generates both mechanical and osmotic pressures which play

a significant role in nutrient nourishment and removal of waste products. These pressures affect fluid flow within the cartilage itself and across the articular cartilage surface/synovial fluid interface. Articular cartilage is an avascular tissue i.e., lacks blood vessels and hence chondrocytic waste products are eliminated when fluid is extruded during compressive loading. Chondrocytes are subsequently nourished when synovial fluid is reinfused into the cartilage during rest. This reinfusion is driven by osmotic pressure differences between the articular cartilage and synovial fluid.

The main advantage for injectable hydrogels is its ability to be liquid at room temperature and will gel at the body temperature. Hydrogels are hydrophilic polymers that are typically crosslinked and consist predominantly of water without dissolution. Injectable hydrogels have been extensively explored more recently because they offer a minimally invasive alternative to arthroscopic surgeries and ease of incorporation of cells and biologically active agents (Burdick et al. 2001, Kuo et al. 2001, Gobin et al. 2001). Hydrogels can be fabricated by a variety of methods and materials including synthetic and natural based polymers.

Synthetic polymers used for hydrogel fabrication come from numerous material sources including but not limited to poly(ethylene oxide) (PEO), polyurethane, polyethylene glycol (PEG), and poly(vinyl alcohol) (PVA). Elliseff et al. have used photopolymerization to form gels in a fast and controllable manner. Notably, PEO and a photopolymerization process have been explored to directly encapsulate chondrocytes while transforming a liquid polymer to a gel. In previous studies, uses of polyurethane hydrogels have had difficulty with cell linkage to the scaffold but further exploration with the incorporation of diisocynates and poly(ε-caprolactone) with methacrylate end

functional groups showed improvement of cellular activity (Werkmeister et al. 2010). Another synthetic polymer that has been used with photopolymerization is PEG where various blends of bioactive derivatives were used to form a matrix substitute (Mann et al. 2001). PVA based hydrogels that have been processed by a freeze/thaw method offers a stable hydrogel at room conditions that is highly elastic. However, many synthetic polymers have demonstrated lack of biodegradability in physiological conditions (Lee et al. 2001), in addition to regulation of cellular interactions. Hence, alternative polymers are explored for cartilage tissue regeneration.

Natural polymers from ECM components are utilized as their role in diverse molecular mechanisms has been extensively studied. A natural polymer such as hyaluronan can be used as a base material for forming hydrogels. Research has been done on hyaluronan based hydrogels and meshes (Toh et al. 2010). The hyaluronic acid (HA) hydrogel was used for differentiating human embryonic stem cell (hESCs) to produce ECM-enriched cartilaginous construct and investigate the hydrogels long-term reparative ability (Toh et al. 2010). Previously, hMSCs encapsulated in alginate hydrogels have been studied to observe the chondrogenic differentiation of hMSCs in a gel system to better define temporal aspects of relevant gene expression and phenotypic changes (Xu et al. 2008). Another polymer often used for hydrogels is collagen. Collagen is commonly used because it is the main component of mammalian ECM tissues such as skin, bone, cartilage, tendon, and ligament. However, collagen hydrogels are limited by their limited range of mechanical properties, potential immunogenicity, and can be economically undesirable (Lee et al. 2001).

2.5 Using Chitosan and Gelatin as Biomaterials in Tissue Engineering

In this study, chitosan was utilized as the base material for preparing the hydrogels at body temperature using β-glycerophosphate (GP) chemistry. This combination is ideal for applications such as drug delivery, cell encapsulation, and injectable tissue engineering because the solution maintains a liquid state at physiological pH and gels when heated to body temperature. Interestingly, chondrocytes revealed capabilities of maintaining cell viability of eighty percent or more over extended time lengths when encapsulated within chitosan-GP in vitro. Additionally, chitosan-GP gels have been used for in vivo purposes and have demonstrated the formation of cartilage after three weeks (Ahmadi et al. 2007). Chitosan was chosen as the base material because it satisfies the criteria such as biocompatibility, and biodegradability (Iyer et al. 2011). Further, it is a polysaccharide mimicking the chemical structure of GAGs. Chitosan is formed through the N-deacetylation of chitin, which is an abundant polysaccharide produced by crustacean shells, and has repeating units of β(1-4) 2-amino-2-deoxy-D-glucose (Nettles et al. 2002). Additionally, chitosan is capable of being processed into multiple forms such as hydrogels without the loss of its functionality but lacks a cell binding domain. Chitosan based hydrogels have been blended with ammonium persulfate and *N.N.N'.N'*-tetremethyl ethelenediamine for reduction of gelation time and enzymatic degradation (Hong et al. 2006). Additionally, gelation and degradation rates as well as mechanical properties have been explored for chitosan injectable hydrogel derivatives using enzymatic crosslinking (Jin et al 2009). The importance of the cell binding domain comes from the necessity of multicellular organisms' requirement of specific mechanisms for intercellular communication and adhesion as well as cell to matrix signaling. The

functional units of cell adhesion are made of three classes of proteins, the adhesion receptors, the ECM and the cytoplasmic plaque/peripheral membrane proteins (Gumbiner 1996). Thus, an additional material that contains a cell binding domain is necessary for the influence of cellular colonization. Gelatin was chosen due to its binding domain and ability to cross link with chitosan without the need of a cross linker. Also, gelatin is a partially denatured derivative of collagen, which is present as a connector for most tissues in the body. The cell binding property of gelatin is contributed by the Arg-Gly-Asp (RGD)-like sequence. Moreover, the linking of chitosan and gelatin results in a structure that can affect spatial distribution of integrin ligands and polycationic chitosan interaction with the anionic cell surface (Huang et al. 2005). Additionally, chitosan and gelatin can be blended together without a cross linker being present. Furthermore, recent studies have shown that the rheological properties of chitosan-gelatin-β-glycerophosphate (C-G-GP) are significantly altered when the concentrations are increased. The gelation temperature and time for gelation for the C-G-GP solutions decrease with increasing concentrations (Cheng et al. 2010). A similar article demonstrated similar characteristics using collagen or starch instead of gelatin (Song et al. 2010, Ngoenkam et al. 2010). The viability of chondrocytes was measure on the starch containing hydrogels and revealed to have an increase in proliferation over a seven day period (Ngoenkam et al. 2010). Little to no mechanical testing for a C-G-GP hydrogel has been done. Alternatively, the compressive strength for the collagen and chitosan supplemented with GP has been tested and has yielded an elastic modulus in the range of 6-22 kPa (Wang et al. 2010). In order to replicate a sustainable regenerative cartilage, the physical characteristics of the

hydrogel needed to be more durable than previous studies to handle the compressive load of the body.

2.6 Sources for Chondrocytes

At first thought, someone may think that chondrocytes from a donor would be the prime choice but this is not true. Monolayer donor chondrocytes gradually lose their phenotype and may not for cartilage. Furthermore, the lack of extensive research on immunogenicity has been done for the donor cells (Fritz et al. 2009). However, extensive research has been done for stem cells and the results have shown that multiple stem cell lines have the capability of producing chondrocytes. The stem cell lineages for sources that are discussed in this study are human embryonic stem cells (hESCs), induced pluripotent (iPS) cells, and human mesenchymal stem cells (hMSCs).

2.6.1 Embryonic Stem Cells:

Embryonic stem cells are derived from blastocysts from *in vitro* fertilized human eggs. Major advantages for hESCs are their ability to self-renew and differentiate into any cell line in the human embryo under proper conditions (Fritz et al. 2009). On the other hand there are many for downfalls to hESCs than benefits. First, there are legal and social issues that arise from their use. Secondly, there is lack of research covering immunogenicity and prevention of teratoma formation *in vivo* as well as spontaneous differentiation upon implantation has occurred for hESCs (Simion,et al. 2010, Fritz et al.2009, Tigil et al. 2009). With the disadvantages outweighing the advantages, another cell source must be examined.

2.6.2 Induced Pluripotent Stem Cells:

An alternative idea is to use induced pluripotent stem cells (iPS) with human foreskin fibroblasts to yield chondrocytes that can be engineered into a scaffold that will act as new cartilage for the knee. Induced pluripotent stem (iPS) cells have the ability to differentiate cells of an abundant source into cells that are either scarce, require a painful procedure to get or are expensive to buy from a company. Adult human cells such as fibroblasts can be reprogrammed to a pluripotent state by introducing several transcription factors into the adult cell genome with iPS cells (Simion et al. 2010). The reprogramming method could be used to derive human iPS cells with properties very similar to embryonic stem cells (ESCs) (Takahashi et al. 2007, Yamanaka 2008). Studies in the mouse model demonstrated that iPS cells exhibit properties typical of ESCs (Takahashi et al. 2007, Okita et al. 2007, Meissner et al. 2007) and could give rise to adult chimeras capable of germline transmission, showing the possibility of redifferentiating mature cells. However, studies have shown that iPS cells have a low efficiency of *in vitro* reprogramming and significant safety considerations to address. For the applicability of the iPS cells in differentiation and clinical applications two major hurdles have to be overcome (Okita et al 2007, Meissner et al. 2007, Takahashi et al. 2006, Blelloch et al. 2007). Retrovirus-mediated transduction followed by drug-dependent selection of iPS cells seriously hinder the applicability of iPS cells in therapeutic use. Although some limitations have been addressed by adapting alternative experimental protocols (Blelloch et al. 2007, Okita et al. 2008, Nakagawa et al. 2008), developing systems without genetic manipulation is necessary. Alternatively, the RNA

in the fibroblasts is rearranged by using methods such as the lentivirus-mediated genetic reprograming (Takahashi et al. 2007).

2.6.3 Mesenchymal Stem Cells:

Human mesenchymal stem cells (hMSCs) derived from different sources have shown capabilities of differentiation into chondrocytes. The three hMSCs sources explored in cartilage regeneration are adipose (AMSCs), amniotic fluid (AFSCs) and bone marrow (BMSCs) derived mesenchymal stem cells (Simion et al. 2010). Adipose tissue is a viable and extensive source for AMSCs because of the cells ability to easily expand *in vitro*. Additionally, protein and mRNA analyses have been done on AMSCs and indications of differentiation into chondrocytes were observed. However, later studies have shown that BMSCs have an increased level of chondrogenic markers as opposed to AMSCs. The discovery of AFSCs was an enhancement to the stem cell study due to their ease of attainability in large quantities and difference in regulations and standards that are set for ESCs. However, similarly to the adipose derived MSCs, the AFSCs lacked in the production amount of cartilaginous matrix when compared to the BMSCs. Overall, the hMSCs have demonstrated viability and potentiality because of their ability to differentiate into multiple lineages, namely the chondrogenic lineage. Furthermore, autologous hMSCs are attractive because of the immunogenicity of the cells after differentiation in to chondrocytes (Fritz.et al. 2009). Risk to the donor during extraction and risk of disease to allogeneic patients during transmission is a disadvantage to the hMSCs. A major issue with hMSCs is their tendency of progeny to evolve into hypertrophic chondrocytes and undergo ossification (Simion et al. 2009).

Table 2.1. Advantages and Disadvantages of Sources for Chondrocytes

Cell Line	Cell Source	Advantages	Disadvantages
Chondrocytes	Autologous (patient) or Allogeneic (donor)	• Immunogenicity (Autologous)	• Loss of phenotype (Allogeneic) • Painful procedure • Lack of cells
Human Embryonic Stem Cells (hESCs)	*In vitro* fertilized eggs	• Potential unlimited cell source • Self-Renewal • Ability to differentiate into any cell line	• Legal and Social Issues • Lack of immunogenicity and prevention of teratoma formation • Spontaneous differentiation
Induced Pluripotent Stem Cells (iPS) cells	Adult human cells	• Ability to differentiate cells from an abundant source • Economic • Less painful procedure for acquiring cells	• Low efficiency of *in vitro* reprogramming • Significant safety considerations to address • Require immune suppressive therapies
Human Mesenchymal Stem Cells (hMSCs)	Adipose, amniotic fluid and bone marrow as well as other sources	• Multiple sources • High viability to differentiate into chondrocytes • Easily to expand *in vitro* • Immunogenicity (Autologous)	• Progeny tendency • Evolution into hypertrophic chondrocytes • Possibility of ossification • Risk to donor during extraction • Risk to allogeneic patient during transmission

Sources: (Simion et al. 2010, Fritz et al.2009, Tigh et al. 2009, Takahashi et al. 2007, Yamanaka 2008, Okita et al. 2007, Meissner et al. 2007).

The Table 2.1 summarizes the cell sources by their advantages and disadvantages. Based on the advantages and disadvantages the stem cell sources have posed, a novel alternative strategy for obtaining chondrocytes has been suggested. In this study, the Human Foreskin Fibroblasts (hFF-1) cell line was used by adopting the same differentiation technique as the bone marrow derived hMSCs. Further discussion of the differentiation technique is discussed in the Materials and Methods section (Ch.3 Section 6). For purposes of being concise, only iPSs and bone marrow derived hMSCs differentiation techniques are explained below.

2.7 Stem Cell Differentiation to Chondrocytes

The chondrogenic potential, easy isolation and vigorous proliferation make hMSC the attractive candidate cell for cartilage tissue engineering. hMSCs have been extensively investigated to regenerate chondrocytes. The chondrogenic potential, easy isolation and vigorous proliferation make hMSC the attractive candidate cell for cartilage tissue engineering. To induce chondrocyte differentiation, various soluble factors and materials making the matrixes on which they are cultivated have been explored. The use of either transforming growth factors (TGF-β) and/or bone morphogenetic proteins (BMPs) is common for differentiation of hMSCs into chondrocytes. Gelatin microspheres containing such as TGF-β3 is shown to promote hMSC differentiation to chondrocytes (Fan et al. 2008). Another process for differentiation into cartilage cells comes by treating the stem cells with bone BMPs with the addition to chondrogenic medium (Gong et al. 2010).

Many have reported multiple comparison analyses using cells harvested from single cartilage biopsy specimens (Grogan et al. 2007), hMSC differentiation into chondrocytes on alginate cultures (Lee et al. 2009). The Sox 9 gene expression is a measurable variable for the stem cells whereas Aggrecan, Collagen Types I and II and osteopontin (OPN) are measured for the chondrocytes (Gong et al. 2010). Cells with greater chondrogenic capacity expressed higher levels of a number of surface molecules, all known to play a key role in mesenchymal condensation (Goldring et al. 2006). These included the hyaluronan receptor CD44, the tetraspanin CD151 and α3 integrin (CD49c). CD151 is expressed at high levels in hMSCs and CD44 is a well-known marker for hMSCs. As the culture continues to grow the appearance of the stem cell diminishes with time; this is due to the stem cell line having successful differentiation into cartilage cells. Cells with higher chondrogenic capacity express lower levels of catabolic mediators and higher levels of surface molecules involved in the early stages of cartilage development, including those involved in establishing cell–cell and cell–matrix interactions (e.g., the hyaluronan receptor CD44 and the tetraspanin CD151). However, the expression level of CD151 was not significantly changed from that of MSCs without TGF-β3 but was reduced reversibly by TGF-β3 treatment during the 3-D alginate culture and that of CD44 decreased reversibly by the 3-D alginate culture alone and irreversibly in the presence of TGF-β3 (Lee et al. 2009).

MSCs cultured in a pellet or on micromass or on a three-dimensional scaffold have been shown to differentiate into chondrocytes more efficiently than individual cells. Cell density is one of the critical requirements for stabilizing the chondrocyte phenotype. In monolayer culture, chondrocytes usually devolve into fibroblastic morphology and

secrete collagen I. The phenotypic instability in monolayer culture makes the pellet culture a useful method to engineer cartilage. When chondrocytes are cultured at a high cell density, the phenotype is kept. Compared to other three-dimensional cultures, such as hMSCs cultured on micromass or a scaffold, the advantages of pellet culture are:

(1) the chondral phenotype is stabilized in pellet, and

(2) the cartilage is engineered without additional materials, which may induce immune-inflammatory reactions.

The hMSCs pellet cultured in chondrogenic differentiation medium shares similarities with native cartilage in cell distribution and matrix composition. Furthermore, spontaneous chondrogenic differentiation of MSCs in a pellet can occur without addition of any other bioactive stimulators. The cell pellet has been used to construct hyaline cartilage, repair chondral defects, and enhance the healing of the bone−tendon junction. Although excellent outcomes are reported, the limitations such as inadequate size and poor process ability are associated with the conventional cell pellet. So there is a potential need to optimize the hMSCs pellet culture for enhancing the chondrogenesis of pellet.

The main variable that is tested for though is Collagen Type II, which is secreted from the cells when they are going through the stages of mitosis. From previous studies, a chitosan-gelatin mixture has been found to support cell proliferation and viability of skin cells. Additionally, the shape of the cells encapsulated in the hydrogels was observed to closely resemble chondrocytes. As for the evaluation of chondrogenic cell viability within hydrogels, Toh et al. used a hyaluronan based hydrogel to measure the amount of Collagen Type II that is secreted from the cells. The group compared the environment

effects of the gel to that of a cellular pellet. Superior chondrogenesis indicated by significant higher levels of s-GAG and Type II collagen was observed in the hydrogel system (Toh et al. 2010). The hydrogel offered a more suitable environment to support cell growth for chondrocytes than the pellet system for both sGAG/DNA as well as Type II collagen/DNA. The limitations that have been associated with regenerating a suitable cartilage replacement useable at clinical setting are the motivation for this study. Therefore in short the variables that are examined in this work is the mechanical strengths of varying chitosan gelatin concentrated hydrogels and size and shape factor, Collagen Type II, histology and flow cytometry analyses for the hFF-1 and hMSC pellet and hydrogel systems.

CHAPTER III

METHODOLOGY

3.1 Sources for Materials

Low molecular weight (LMW) chitosan (200-300 kDa and 75-85% deacetylation), Gelatin Type A (300 Bloom), Glycerol 2-phosphate (G2P), and Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich Co. (St. Louis, MO). Hydrochloric acid (HCl) and ethyl alcohol (EtOH) (200 proof) were obtained from Pharmco. Formalde-Fresh (certified low odor 10% Formalin) was obtained from Fisher Scientific (Pittsburgh, PA). Phosphate buffered saline (PBS), Trypsin/EDTA (0.25% Trysin-0.53 mM EDTA solution), ATCC-formulated Dulbecco's Modified Eagle's Medium (DMEM), and ATCC tested fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC) (Manassas, VA). Clonetics Trypsin-EDTA for human Mesenchymal Stem Cells (hMSC), Fibroblast Basal Medium (FBM) supplemented with Fibroblast Growth Medium (FGM) SingleQuot Kit and Growth Factors, Mesenchymal Stem Cell Basal Medium (MSCBM) supplemented MSC Growth Medium SingleQuots, Chondrogenic Basal Medium supplemented with hMSC Chondrogenic SingleQuots Kit and Transforming Growth Factor Beta 3 (TGF-β3) were attained from Lonza Walkersville,

Inc. (Walkersville, MD). Mouse Anti Human CD151: FITC and Mouse Anti Human CD44: RPE were purchased from MorphoSys US Inc. (Raleigh, NC). Collagen Type II ELISA was acquired from MD Bioproducts Division of MD Biosciences, Inc. (St. Paul, MN). 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester (5(6)-CFDA, SE; CFSE) - mixed isomers was obtained from Invitrogen Corp. (Carlsbad, CA). Matrix metalloproteinase 2 (MMP-2) and matrix metalloproteinase 9 (MMP-9) fluorogenic peptide (DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH) and matrix metalloproteinase 13 (MMP-13) fluorogenic peptide (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 [Cha = L-cyclohexylalanine; Dpa = 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva = L-norvaline] was purchased from CalBiotech (Spring Valley, CA).

3.2 Hydrogel Formulation and Formation

Chitosan-gelatin mixture at a concentration at a desired concentration (0.5%-0.5% w/v or 2%-1% w/v or 2%-2% w/v was autoclaved in DI water. One normal diluted HCl was added to dissolve the chitosan in the solution and allowed to stir overnight. For preparing 0.5%-0.5% chitosan-gelatin solution, one mL of G2P at 0.56 g/mL concentration was added drop wise into 9 mL of the solution in an ice bath to adjust the pH. For higher concentrations, 4 mL of the G2P solution was added drop-wise to nine mL of chitosan-gelatin solution kept in an ice bath in the laminar hood. Solution should be kept in an ice bath while adding the G2P solution and continuous stirring are necessary to avoid precipitation.

To prepare the hydrogel system, 0.5 mL of solution was added to either an hFF-1 or

hMSC pellet in a 15 mL polypropylene culture tube, as described in the following sections. Soon after, the solution and pellet system was supplemented with the medium specific to the cell line; this was done by allowing the medium to flow down the side of the tube and using the fluid properties of the two solutions to not induce mixing. The system was incubated at 37°C and 5% CO_2 in air atmosphere and once the gel had formed after approximately two hours the medium was able to reach the cell pellet through the pores in the hydrogel.

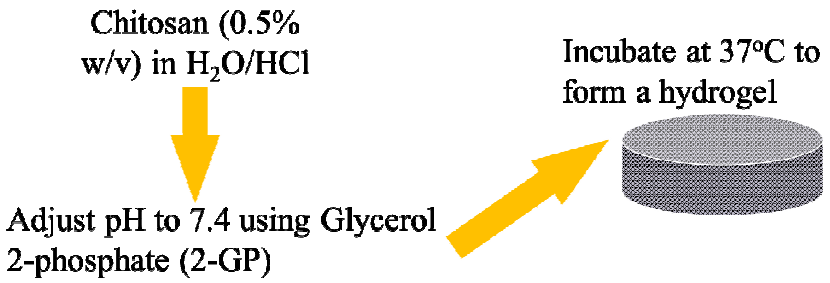


Figure 3.1. Flow chart for chitosan-gelatin hydrogel fabrication. Chitosan-gelatin concentration was 0.5%/0.5% and 2%/1% w/v for cell culture systems and 2%/0%, 2%/1%, and 2%/2% w/v for rheology and mechanical testing.

3.3 Rheology and Compression Testing

Viscous modulus and elastic modulus at various temperatures were evaluated using 1.5 mL solution in a Bohlin CVO controlled stress/strain rheometer (Bohlin Instruments, East Brunswick NJ). Analysis was performed at 1 Hz shear rate with an incremental of 1°C.

Compression testing was done on the hydrogels systems by using an INSTRON 5542 machine (INSTRON, Canton, MA). First, the bottom anvil was converted into a well of size exactly matching the custom-made top anvil using silicon glue. The hydrogels were

formed on the bottom anvil by incubating 5 mL solution at 37^{o}C in an oven. The dimensions of the hydrogel were 25 mm in diameter with a depth of 7mm and circular geometry. The top anvil was also incubated in an oven at 37°C saturated with water. Hydrogel was formed on top of the anvil by creating a well, as shown in Figure 3.2. Hydrogels were compressed at 1 mm/min crosshead speed, similar to previous reports. Using the associated Merlin (INSTRON, Canton, MA) software, data were recorded. The elastic modulus was calculated from the slope of the linear portion (0.1% to 0.3% strain range) of the stress-strain curve.

Figure 3.2. Custom made top and bottom compression testing anvils with hydrogel chamber used on the INSTRON 5542 machine.

3.4 Cell Population Maintenance

Human Foreskin Fibroblasts (hFF-1) cell line was purchased from ATCC (Walkersville, MD) and maintained in ATCC-formulated DMEM supplemented with 15% ATCC tested FBS (ATCC, Walkersville, MD). Cells were incubated at 37°C and 5% CO_2/95% air atmosphere and medium was changed every 2 days. Cells were frequently monitored for shape and confluency. Once confluent, the hFF-1s were detached with Trypsin/EDTA (0.25% Trysin-0.53 mM EDTA solution) (ATCC, Walkersville, MD).

Adult hMSC cell line was purchased from Lonza Walkersville, Inc. (Walkersville, MD) and maintained in MSCBM supplemented with MSCGM supplementation (Lonza Walkersville, Inc., Walkersville, MD). Both cultures were incubated at exhibited normal growth characteristics with respect to size, shape and confluency. Cells were incubated at 37°C and 5% CO_2/95% air atmosphere and medium was changed every 3 to 4 days. Once confluent, the hMSCs were detached with Clonetics Trypsin-EDTA (Lonza Walkersville, Inc., Walkersville, MD).

They were then stained with CFDA-SE, which is an amine-reactive, colorless, non-fluorescent dye that passively diffuses into the cytoplasm of the cells. 250,000 cells were seeded into the controls and hydrogels of each group, as described below.

3.5 Cell Morphology Analysis

Cells cultured on tissue culture plastic and after treatment with trypsin/EDTA were used for initial morphology analysis. Digital micrographs were captured at various random

locations through an EVOS^{TM} AME-i2111 digital inverted microscope (Advanced Microscopy Group, Bothell, WA). These images were quantified for cell shape factor (also called circularity factor and defined as $4\pi \times$ area/perimeter and when the number is closer to 1 the cell shape is closer to that of a circle) and cell area using image analysis software Sigma Scan Pro (SPSS Science, Chicago, IL). For each condition, more than 50 cells were analyzed using many micrographs acquired randomly. Box plot showing the distribution of shape factor and area of cell with 10^{th}, 25^{th}, 50^{th}, 75^{th}, and 90^{th} percentiles and the mean value (thick line within each box) were plotted. Values that were outside 95^{th} and 5^{th} percentiles were treated as outliers.

3.6 Cell Pellet and Suspension Cultures

Cells were tested in three culture conditions in the pellet and suspension cultures and are represented in a concise manner in the figure below.

i) hMSC cell line in complete chondrogenic induction media, serving as the positive control group,

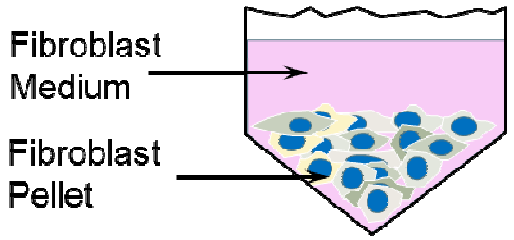
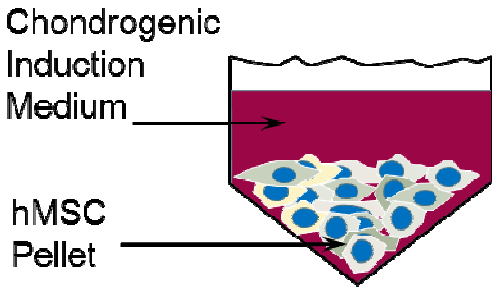
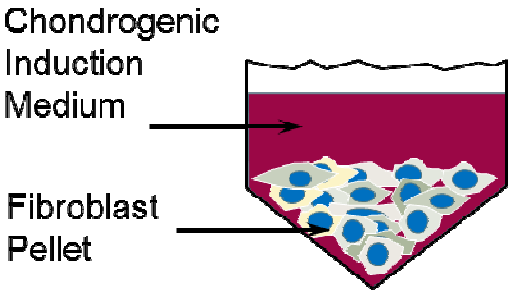
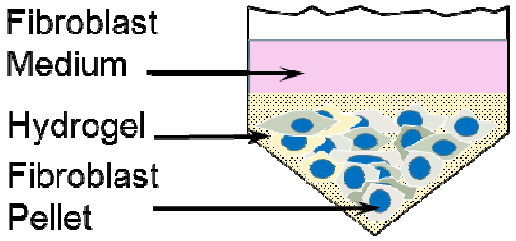
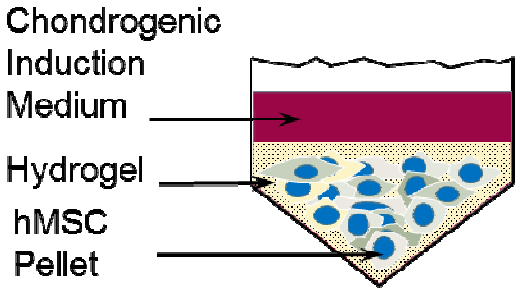
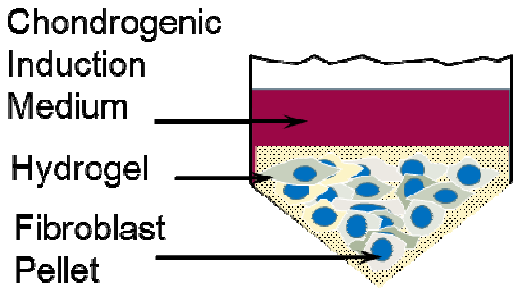
ii) hFF-1 cell line in complete chondrogenic induction media, serving as the experimental test group and

iii) hFF-1 cell line in Fibroblast Basal Medium (FBM) supplemented with Fibroblast Growth Medium (FGM) SingleQuot Kit and Growth Factors, serving as the negative control group.

For the chondrogenesis culture protocol, incomplete and complete chondrogenic induction media were prepared prior to cell culturing. Incomplete medium was

formulated by supplementing the Chondrogenic Basal Medium with hMSC Chondrogenic SingleQuots Kit. The Complete Chondrogenic Induction Medium was prepared by supplementing the incomplete medium with the TGF-β3 solution to a final concentration of 10 ng/mL (Lonza Walkersville, Inc., Walkersville, MD). The TGF-β3 solution was prepared by suspending the lyophilized TGF-β3 in 4mM HCl supplemented with 1 mg/mL bovine serum albumin (BSA) to a concentration of 20 μg/mL. The Complete Chondrogenic Medium (CCM) was prepared fresh before each usage due to the

Table 3.1. Experimental and Control Variables using hFF-1 and hMSC Lineages

Negative Control - Pellet	Positive Control - Pellet	Experimental - Pellet
		
Negative Control - Gel	**Positive Control - Gel**	**Experimental - Gel**
		

short shelf life of 12 hours. Cells were washed with PBS and detached using the respective Trypsin/EDTA solution. Respective cell line medium was used to wash the plate and neutralize the Trypsin/EDTA. The cell suspension was centrifuged for 5 minutes at 270×g for hFF-1s and 600×g for hMSCs, resuspended in medium and counted using a hemacytometer. CFDA-SE was added to the cell suspension at a volume ratio of 1:100 and incubated for 20 minutes. Cells were aliquoted at 250,000 cells per culture and centrifuged at 150×g for 5 minutes in 15-mL centrifuge tubes.

For chondrogenesis cultures, positive control and experimental groups, the medium was removed and the cells were washed and resuspended with 0.5 ml of Incomplete Chondrogenic Medium (ICM). The cells were centrifuged once more at 150×g to form the pellet and the ICM was removed. For the control system, 0.5 ml of Complete Chondrogenic Medium (CCM) was added carefully to not disturb the pellet. For the hydrogel system, 0.5 mL of the chitosan-gelatin/ glycerol 2-phosphate solution was added carefully and 0.5 mL of CCM was added on top of the solution. The caps of the tube were loosened one half turn to allow for gas exchange. The cell pellets were incubated at 37°C and 5% CO_2 in air atmosphere and were not disturbed for 24 hours. Medium was changed twice a week by completely replacing the medium in the tube with freshly prepared CCM. The bottom of the tubes was flicked to release the pellet at the time of the first medium replacement. Culture supernatants were preserved at -80°C for Collagen Type II analysis. The chondrogenic pellets were harvested after 28 days and analyzed via histology.

For the hFF-1 negative control group, the same procedure was followed except washing and incubating in FGM was used to wash and culture cells. Quantities of hydrogel

solution and medium and medium replacement process were similar to the Chondrogenic cultures. Supernatants and harvesting was done for the negative control group as well.

Suspension cultures were prepared using the same conditions and quantities as previously described. After the final centrifugation, the cells were resuspended for the controls as well as the hydrogels. The hydrogels in this case were prepared at a concentration of 2%/1% chitosan-gelatin solution. This was done for the positive and negative control groups and the experimental group. Supernatants of the cultures were preserved for indirect quantification of cell viability within the matrix. The controls were centrifuged at 150×g in order to remove the supernatants and resuspended in fresh medium. The pellets were harvested after 7 days in culture for histological processing and examination.

3.7 Collagen Type II ELISA

Preserved supernatants from the cell pellet culture were tested for the production of Collagen Type II using ELISA kit from MD Bioproducts Division of MD Biosciences, Inc. (St. Paul, MN). The supernatants were thawed to room temperature (22°C - 25°C) and centrifuged before using. The standards for the ELISA were prepared using vendors protocol (300, 150, 75, 37.5, 18.8, 9.4, 4.7 ng/mL). In brief, 700 μL of Assay Diluent was pipetted into the 300 ng/mL standard tube and 500 μL of Assay Diluent into the remaining standard tubes. 300 μL of the 1000 ng/mL standard was added to the 300 ng/mL tube to produce a dilution series. Once the standards were prepared, the microplate was loaded with the standards and the samples at 100 μL/well in duplicate. The plate was sealed and allowed to incubate at room temperature for 2 hours. The Wash

Buffer was prepared by diluting 50 mL of Wash Buffer Concentrate in 450 mL of DI water. The Conjugate was made by diluting 110 μL of Conjugate Concentrate in 11 mL of Conjugate diluent. After 2 hours, the plate was aspirated and washed six times with 200 μL/well with the Wash Buffer. The wells were filled and emptied at each wash and the plate was blotted on paper towels to remove and residual fluid. 100 μL of the Conjugate was pipetted into each well and the plate was covered and allowed to incubate for 2 hours. Due to a 30 min shelf life, the Streptavidin-HRP was prepared by adding 110 μL of Streptavidin-HRP Concentrate to 11 ml of HRP Diluent and kept from light with 5 minutes left in the 2 hour wait time. The wells were aspirated, washed and blotted as previously mentioned and 100 μL of the diluted Streptavidin-HRP was added to each well. The plate was covered and incubated in the dark for 30 minutes. The wells were once more aspirated, washed and blotted and 100 μL of Substrate was pipetted into each of the wells. The plate was covered, incubated and protected from light for 20 minutes. After 20 minutes, the reaction was stopped by adding 100 μL of Stop Solution to each well. The side of plate was tapped gently to ensure mixing and the plate was read for absorbance at 450 nm using a Molecular Devices EMax Precision Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA). Data was collected using the associated Softmax Pro software.

3.8 Flow Cytometry Analysis

Cells were trypsinized, counted, and divided into two groups (~1 x 10^5 cells/group) : i) control group and ii) analysis group. Then cells were centrifuged for 6 minutes at 270×g,

washed in phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA), and centrifuged for 6 minutes at 270×g. Cells were then labeled with monoclonal antibody against FITC conjugated mouse-anti human CD151 (AbDseortec) and RPE conjugated mouse-anti human CD44 (AbDseortec) and incubated for 30 to 60 minutes in the dark at 4°C. Cells were then washed in 5 ml of PBS–1% BSA and centrifuged for 6 minutes at 270×g. Pellets were finally resuspended in 200μL PBS/1% BSA, and the resulting cell suspension was analyzed using a Becton Dickinson FACS Calibur flow.

3.9 Histology Analysis

The pellets from the controls and the hydrogels were harvested after 28 days and fixed with 3.7% formaldehyde for 30 minutes at room temperature. The samples were washed three times with PBS and stored in absolute ethanol overnight at 4°C. The samples were sectioned and stained for Haematoxylin and Eosin (H&E), Masson's trichrome, and Alcian blue stain at 2.5 pH.

For the suspension cultures, the quantities and conditions remained constant. The controls were centrifuged to obtain a pellet culture and the process for harvesting pellet cultures was followed. The hydrogel cultures were left in suspension and fixed with 3.7% formaldehyde for 30 minutes at room temperature. Control and hydrogel cultures were washed thrice with PBS and stored in absolute ethanol overnight at 4°C. These samples were sectioned at 4μm thickness and stained by Oklahoma Animal Disease and Diagnostic Laboratory (OADDL) with the same stains as previously mentioned.

3.10 MMP-2/MMP-9 and MMP-13Analysis

Matrix metalloproteinase activity in the supernatants and fresh media was examined to understand the phenotypic changes in the cells. The activity was measured using fluorogenic substrates (DNP-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH) and (MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2 [Cha = L-cyclohexylalanine; Dpa = 3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Nva = L-norvaline] specific for MMP-2/MMP-9 and MMP-13, respectively. Concisely, 100µL of a 100µM fluorogenic peptide/DMSO solution was added to 100µL of cell supernatant and incubated at 37°C for 20 minutes. Then, the fluorescence was measured at 320nm excitation and 405nm emission Molecular Devices SpectraMax Gemini XS Precision Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA), as described previously (Iyer et al. 2011).

3.11 Statistical Analysis

Statistical analysis was performed by repeating experiments three or more times and collecting samples in triplicate. A one way analysis of variance (ANOVA) with a 99% confidence interval was used for evaluation for significant differences between two groups. Differences were considered statistically significant when $p<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Decreased Thermal Gelation with Increased Hydrogel Concentration

The gelation temperature is the temperature at which the hydrogel begins to form. When rheological tests are performed, the gelation point is determined to be the temperature where the elastic modulus increases significantly and may cross the viscous modulus of the hydrogel. Previous studies have showed that addition of 2-glycerophosphate (2GP) into 0.5% w/v chitosan solution creates a solution at room temperature (or at 4^{o}C) and converts into a semisolid gel at body temperature. However, the elastic modulus of the 0.5% w/v chitosan hydrogel is significantly weaker than that of cartilage, and cannot be utilized in cartilage repair. Further, using chitosan alone is not favorable as there are no cell adhesive sites in the polymer. Hence, addition of gelatin to chitosan was tested for gel formation with equal polymer concentration. Presence of gelatin at 0.5% w/v concentration in chitosan did not alter the hydrogel formation at body temperature and in fact, the gelation temperature showed a reduction (**Figure 4.1 B**). Others have observed similar reduction in gelation temperature of chitosan with the addition of other polymers (Ngoenkam et al. 2010). However, addition of gelatin at similar concentration showed no significant improvement in mechanical properties.

To improve the mechanical properties, polymer concentration was increased to 2% w/v. Rheological analysis indicated (**Figure 4.1 A**) that the gelation is achievable at a lower temperature (15-20^{o}C) with the more concentrated polymer solution. This reduction in gelation temperature is advantages in the clinical settings as it ensures safe formation of hydrogel at body temperature and complete maturation of the interpenetrating polymer network. Further significant improvement in elastic modulus was observed relative to 0.5% w/v chitosan solution. Others have reported that increasing the concentration of chitosan also increases the rate of gelation (Cheng et al. 2010). Analysis of the porous architecture of chitosan based hydrogels with similar concentrations has also been performed using scanning electron microscopy. Chitosan-collagen-G2P hydrogels showed spongy porosity with open pore architecture, suitable for the diffusion of nutrients to the entrapped cells. Hydrogels containg only chitosan revealed loose, irregular and larger pore diameters wheras, the collagen only based hydrogels exhibited an interconnected fibrous network (Song et al. 2010, Wang et al. 2010). This difference can be attributed to the presence of collagen to stabalize the scaffold after the polymerization of chitosan (Song et al. 2010). Thus, the increased concentration of chitosan and gelatin could be utilized in the cartilage regeneration.

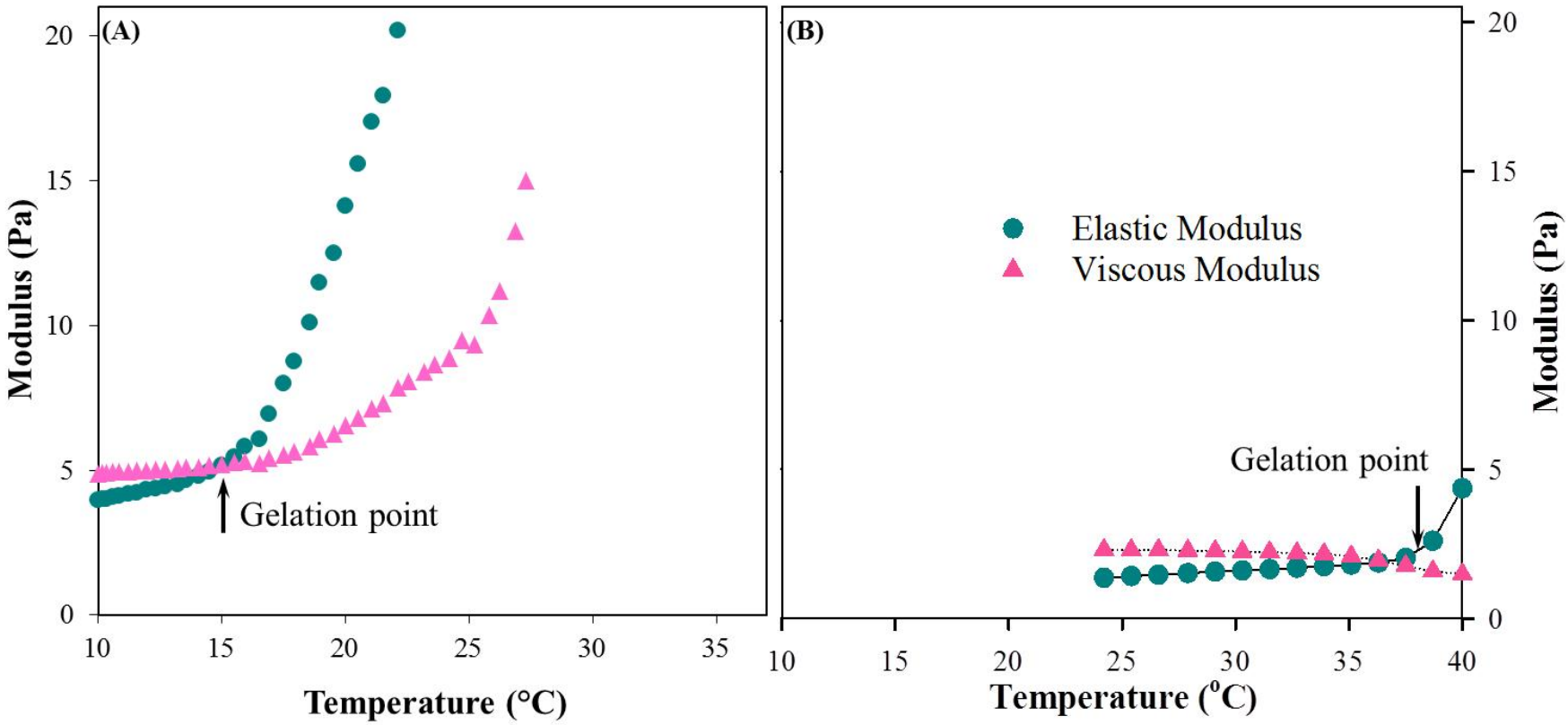


Figure 4.1. Thermal gelation temperature of chitosan-gelatin hydrogels. (A) 2% Chitosan-2% Gelatin. (B) 0.5% Chitosan-0.5% Gelatin.

4.2 Improved Strength and Elasticity of Hydrogels

The elastic modulus for articular cartilage ranges from 0.35 to 2.0 MPa (Werkmeister et al. 2010). Hence the hydrogels with similar mechanical properties are required for clinical utilization in cartilage repair. To understand the improvement in the mechanical characteristics of the hydrogel, compression testing was performed on preformed hydrogels. Since the hydrogels are reversible to liquid form, testing was performed at body temperature. The chitosan-gelatin concentrations tested for improved compressive loading strength were 2%/0%, 2%/1%, and 2%/2%. These results showed (**Figure 4.2 A**) significant improvement in the ultimate stress although strain range decreased with increased polymer concentration.

Alterations in mechanical characteristics were further analyzed by determining the compressive for each concentration assuming Hook's law. Linear region was chosen in the stress-strain curve and the slopes were determined. These results (**Figure 4.2 B**) showed modulus values in the range of 15 to 40 kPa for 2% w/v chitosan with and without gelatin. This is a significant improvement relative to 0.5 % w/v chitosan which was in the range of 0.01 to 0.02 kPa. Addition of 1% w/v gelatin to chitosan increased the compressive modulus moderately and increasing gelatin to 2% w/v did not show significant difference with 1% w/v gelatin. Hence, 2%-1% chitosan-gelatin was chosen for further cell culture analysis. Also, gelation was observed with 2%-2% chitosan-gelatin solutions while forming sterile solutions and could not be used in seeding cells.

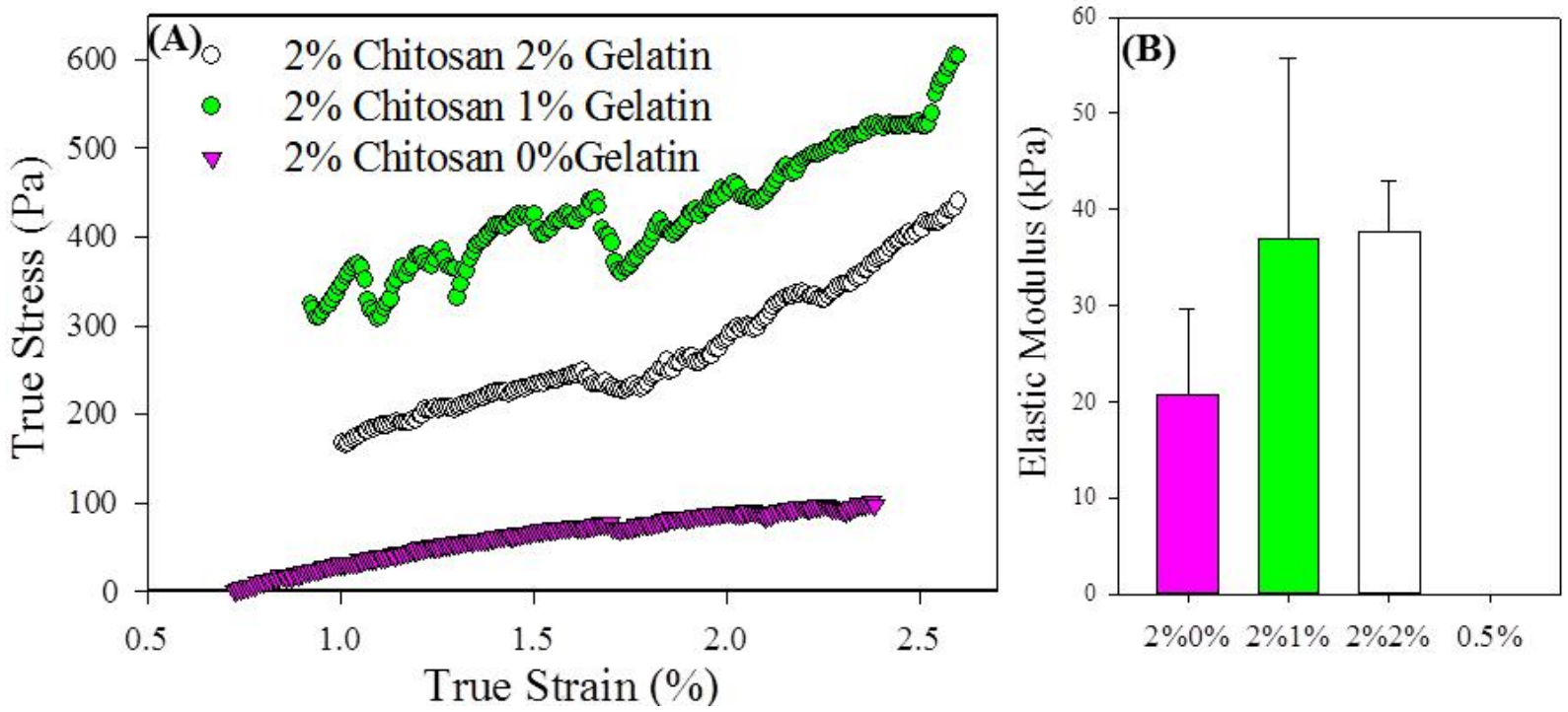


Figure 4.2. Mechanical compression testing of hydrogels. (A) Conventional True Stress - True Strain diagram for 2%/0%, 2%/1%, and 2%/2% w/v chitosan-gelatin hydrogels. (B) Elastic Moduli of the 2%/0%, 2%/1%, and 2%/2% w/v chitosan-gelatin hydrogels.

4.3 Size and Shape Factor Analysis

The objective of the study was to explore hFF-1 as an autologous cell source relative to hMSCs. To understand the difference in the two cell types, their spreading characteristics and shape factors (also called circularity factor) were analyzed when attached to tissue culture plastic and when suspended in a liquid medium. Micrographs were obtained from cell cultures at random time points to assess spreading during adhesion. These results (**Figure 4.3**) showed that hMSCs spread significantly larger than hFF-1s. The shape factor (also called circularity factor) analysis indicated that their shapes were similar when attached to the tissue culture plastic. Both cell types showed significant deviation from the round shape observed in naturally occurring chondrocytes.

Since the round morphology of chondrocytes could be attributed to weak adhesion to a substrate, morphology of cells were assessed when the cells were detached from the surface. Also, hydrogels provide three-dimensional space unlike tissue culture plastic and for better analysis of the outcome of culturing hFF-1s and hMSCs, knowing the cell area in suspension was important. These results showed significant reduction in the cell size when suspended and the shrinkage in hMSCs was much higher than hFF-1s. Further, hFF-1s showed increasing cell size relative to hMSCs. Interestingly, both cell types showed similar shape and approached that of circular cells.

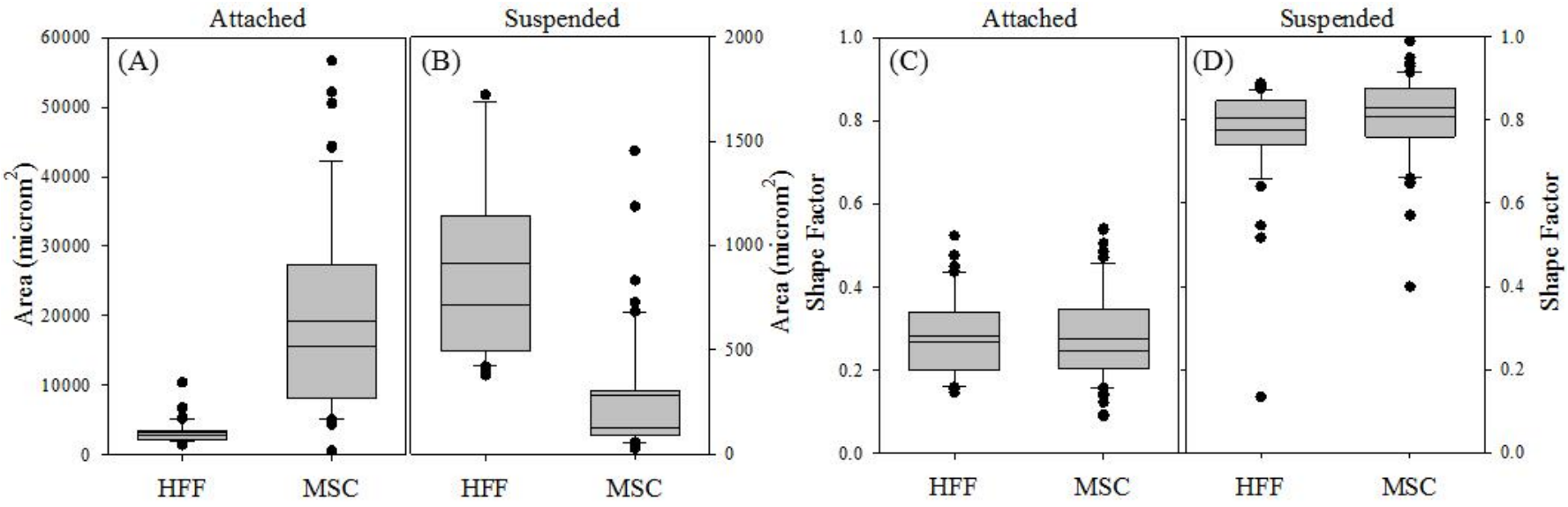


Figure 4.3. Size and shape factor for hFF-1 and hMSC lineages on day zero. (A) Size analysis when attached to the tissue culture plastic (TCP). (B) Size analysis when suspended in serum containing medium (hFF-1) and MSCBM (hMSC). (C) Shape factor analysis when attached to the tissue culture plastic (TCP). (D) Shape factor analysis when suspended in serum containing medium (hFF-1) and MSCBM (hMSC).

4.4 Analysis of Collagen Type II Secretion

Cells were cultured by adapting a system utilized for hMSC differentiation into chondrocytes with and without different concentrations of chitosan-gelatin hydrogels for a week. The hallmark of chondrocyte differentiation is the production of collagen type II instead of collagen type I production by fibroblasts. Hence, to understand whether fibroblasts could be converted to chondrocytes is to assess the production of collagen type II. Hence the phenotypic change leads to an altered biosynthesis of matrix proteins, increasing synthesis of collagen type II instead of collagen type I. The collagen content was measured in the supernatants harvested at different time points up to twenty eight days. The negative control group is the hFF-1s cultured in the pellet form in serum free medium. The positive control group was the hMSCs and experimental group was the hFF-1s in complete chondrogenic differentiation medium, respectively. The collagen secretion from the hMSCs in the pellet system gives a baseline reading for comparison with the negative and experimental groups. Both negative and experimental groups showed similar trend relative to the hMSCs in the pellet system (**Figure 4.4 A**) and were significantly less than hMSCs in all measured time points. This suggested no significant change in phenotypic expression of hFF-1s due to the soluble factors alone, unlike the hMSCs.

Next, the effect of hydrogel system was compared for changes in Collagen type II secretion. hMSCs showed an increase in collagen type II production relative to the system without the hydrogel (**Figure 4.4 B**). These results are consistent with previous studies that have shown collagen type II levels to increase presence of hyaluronan based hydrogels than in the pellet culture systems (Toh et al.2010). Interestingly, the hydrogel

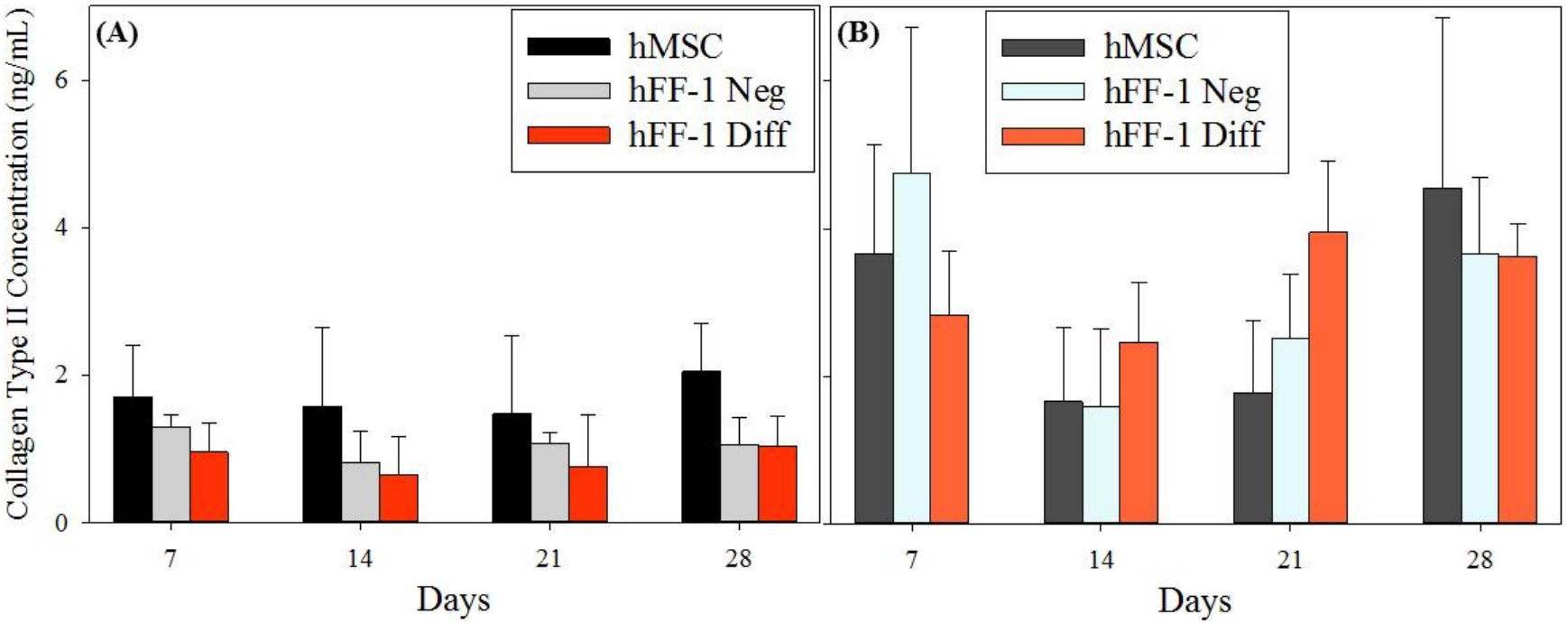


Figure 4.4. Collage Type II secreted into the medium throughout the 28 day culture period. (A) Pellet system for hFF-1control group and hFF-1s and hMSCs in differentiation medium. (B) Hydrogel system for hFF-1 control group and hFF-1s and hMSCs in differentiation medium.

systems containing hFF-1s showed a significant increase in collagen type II, comparable to that hMSCs. The negative control group showed a significant increase on day 7 relative to other cultures, which significantly decreased by day 14 and day 21. On the contrary, the experimental group showed a significant increase at day 21. When comparing the pellet system to the hydrogel system, the amount of collagen secreted into the supernatant was higher for all groups in the hydrogel system.

In the hydrogel system, gelatin was mixed with chitosan and it could be questioned that the observed increase in all hydrogel cultures could be attributed to confounding factors in the ELISA. Hence, further analysis was performed at day 4 and day 7 to understand whether the increase is due to gelatin leaching and non-specific detection by the analysis. For this purpose, collagen content in the fresh medium and the medium exposed to hydrogels without cells were collected after four and seven days. Since hFF-1 negative control was incubated in Fibroblast Basal Medium (FBM), and hFF-1 experimental group and hMSCs were incubated in complete chondrogenic differentiation medium (CCM), both were also analyzed. Further, hFF-1s and hMSCs were propagated in two different media i.e., serum containing media and Mesenchymal Stem Cell Basal Medium (MSCBM). They were also analyzed for comparison.

Figure 4.5 demonstrates a significant quantity of collagen that is contained in the, when compared with the FBM, serum free medium, and the CCM. The same is true for the hydrogel and medium only systems that were cultured up to day seven. Furthermore, the amount of collagen in the medium decreased from the fresh medium to that exposed to hydrogels at both time points. The reduction in collagen type II content suggested that the observed increase in the hydrogel cultures is not due to non-specific leaching and

detection by the assay system. Therefore, the increase in the day seven hydrogel cell cultures in **Figure 4.4 B** is not attributed to leaching of gelatin. The inferences that can be drawn from the ELISA results are that, collagen type II is consistently secreted by each of the groups in both pellet and hydrogel systems, secreted in higher amounts in the hydrogel systems when compared with the pellet system.

Incubation did decrease the collagen type II content in the medium, which can be attributed to the stability of the protein at 37°C and liquid environment as seen in **Figure 4.5**. Also, all pellet cultures showed a reduction relative to the media that was incubated for seven days in hydrogels alone in the absence of cells. Hydrogels with cells exhibited an increase in collagen type II for all cultures during the culture period.

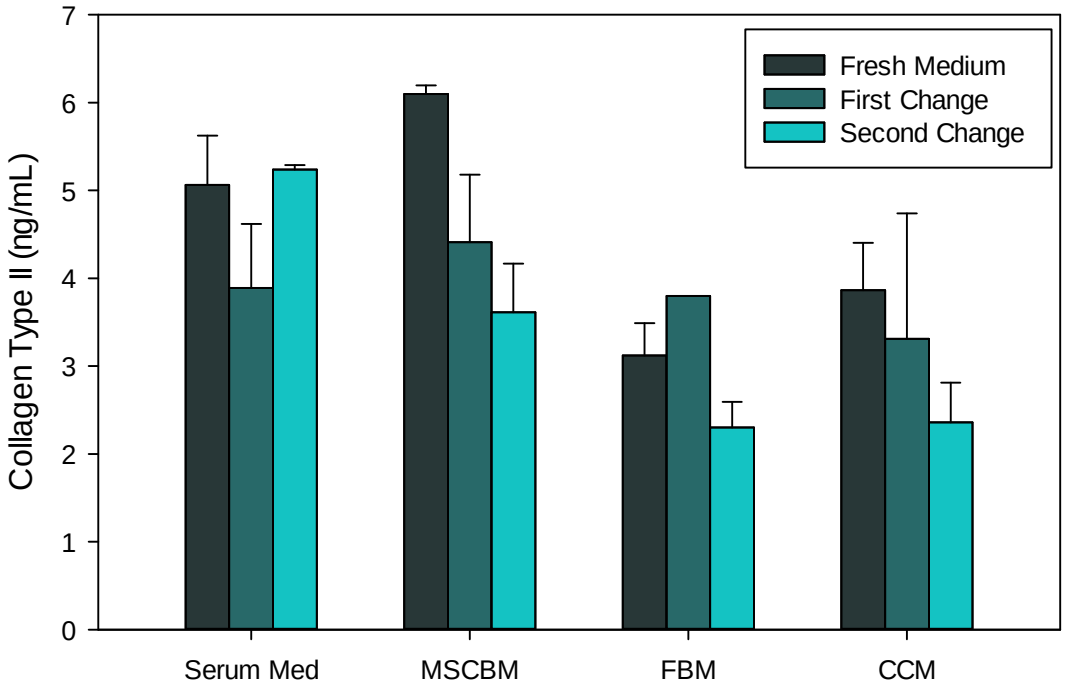


Figure 4.5. Collagen Type II content in serum containing medium, MSCBM, FBM, and CCM for fresh medium (day zero) and medium supernatants of hydrogel systems without cells for first change (day four) and second change (day seven).

4.5 Alterations of CD44 PE and CD151 FITC Expressions on Hydrogel Systems.

Hydrogel systems showed better collagen type II secretion relative to without hydrogels. These changes could be attributed to the cell-matrix interactions, either that present in the matrix or synthesized by the cells. CD151 is a member of tetraspanins, which are membrane proteins that bind to laminin-binding integrins and other proteins to generate functional complexes involved in cell–cell and cell–ECM interactions. The major receptor for hyaluronan, CD44, is highly expressed during MSC condensation and plays an important role in chondrogenesis. These receptor expression changes were measured in all cells after seven day cultures. Similar to literature results, CD151 was expressed at high levels in hMSCs and hFF-1s (Geary et al. 2008). The 0.5% gel had (**Figure 4.7**) little to no change from the unstained control, whereas the 2%/1% chitosan-gelatin hydrogel had an up-regulation for both CD44 PE and CD151 FITC. This is significant because as the chitosan-gelatin concentration increases there is higher reception of hyaluronan and laminan, which suggests differentiation of the HFF-1s. In partial similarity, the hMSC pellet system had an up regulation of CD44 PE but followed the same trend as the unstained control for CD151 FITC. During wound healing, CD151 expression is upregulated within the first week in the epidermis and delayed wound healing is observed in the null CD151 mice (Cowin et al. 2006). The altered expression of CD44 in high-chondrogenic-capacity cells is consistent with a recent report of up-regulation of CD44 on human chondrocytes expanded on type II collagen–coated dishes; these chondrocytes exhibit a superior capacity to generate cartilaginous tissue (Barbero et al. 2006). Decreased CD44 expression of hMSCs reversibly on 3-D alginate culture

alone and irreversibly in the presence of transforming growth factor TGF-β3 has also been reported (Lee et al. 2009). Alterations in CD151 are not related to alterations in

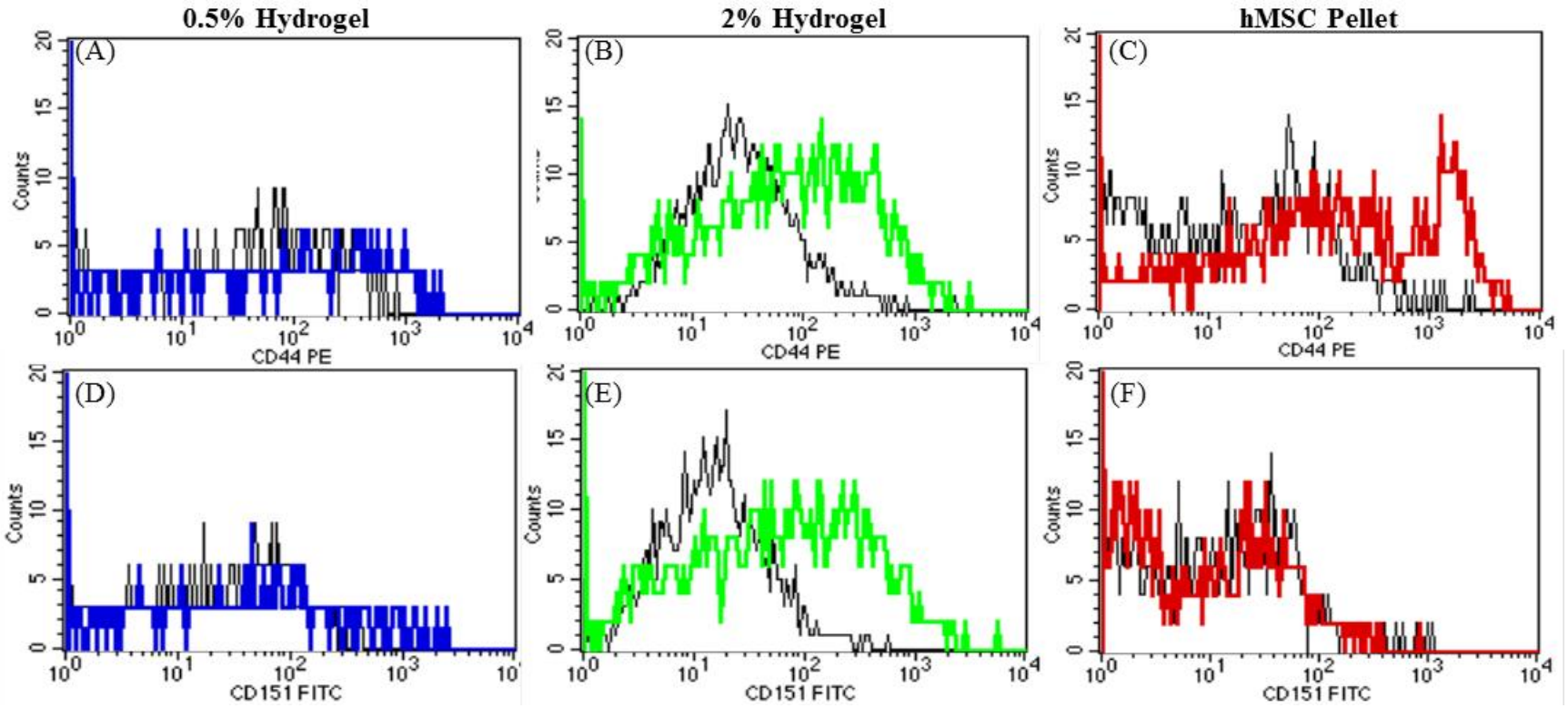


Figure 4.6. Flow cytometric histograms of CD44 PE and CD151 FITC stained hFF-1s and hMSCs in CCM on Day 7. (A) CD44 PE stained hFF-1s cultured in 0.5%/0.5% w/v chitosan-gelatin hydrogel. (B) CD44 PE stained hFF-1s cultured in 2%/2% w/v chitosan-gelatin hydrogel. (C) CD44 PE stained hMSCs cultured in pellet form. (D) CD151 FITC stained hFF-1s cultured in 0.5%/0.5% w/v chitosan-gelatin hydrogel. (E) CD151 FITC stained hFF-1s cultured in 2%/2% w/v chitosan-gelatin hydrogel. (F) CD151 FITC stained hMSCs cultured in pellet form

CD44 in fibroblasts (Liu et al. 2007). Others have reported multiple comparison analyses using cells harvested from single cartilage biopsy specimens (Grogan et al. 2007), MSC differentiation into chondrocytes (Lee et al. 2009, Goldring et al. 2006). Thus further analysis is necessary to confirm chondrocyte differentiation.

4.6 Evaluation of Tissue Morphology and Collagen and GAG Distribution of Tissue

Cellular morphologies and the distribution of collagen and glycosaminglycan were evaluated to understand the quality of the regenerated tissue as well as differentiation of the positive and experimental groups. In the negative control pellet culture without the hydrogel, the cell number and amount of ECM compontents in hFF-1s were much lower and the tissue size was also significantly smaller on the twenty eighth day as seen in **Figure 4.7 A-C**. The tissue size was less than one millimeter for the hFF-1 pellets without hydrogels and 4 mm for the hFF-1 pellet- hydrogels. Higher magnifications of this pellet culture displayed packed cells with minimal ECM. This was confirmed by Trichrome (for collagen) and alcian blue stains (glycosaminoglycans). Thus the increased collagen type II released from these cultures could be attributed to the lack of space for the organization of the matrix element.

Haematoxylin and Eosin (H&E) staining was used to stain the cell nucleus and the matrix in the tissue; cell nucleus and the matrix are stained blue and pink, respectively. In comparison of each of the groups stained with H&E, the positive and experimental groups showed similar distribution of cells and matrix elements (**Figure 4.8 B-F**). However, the tissue harvested from negative control (hFF-1 cultured on hydrogel with

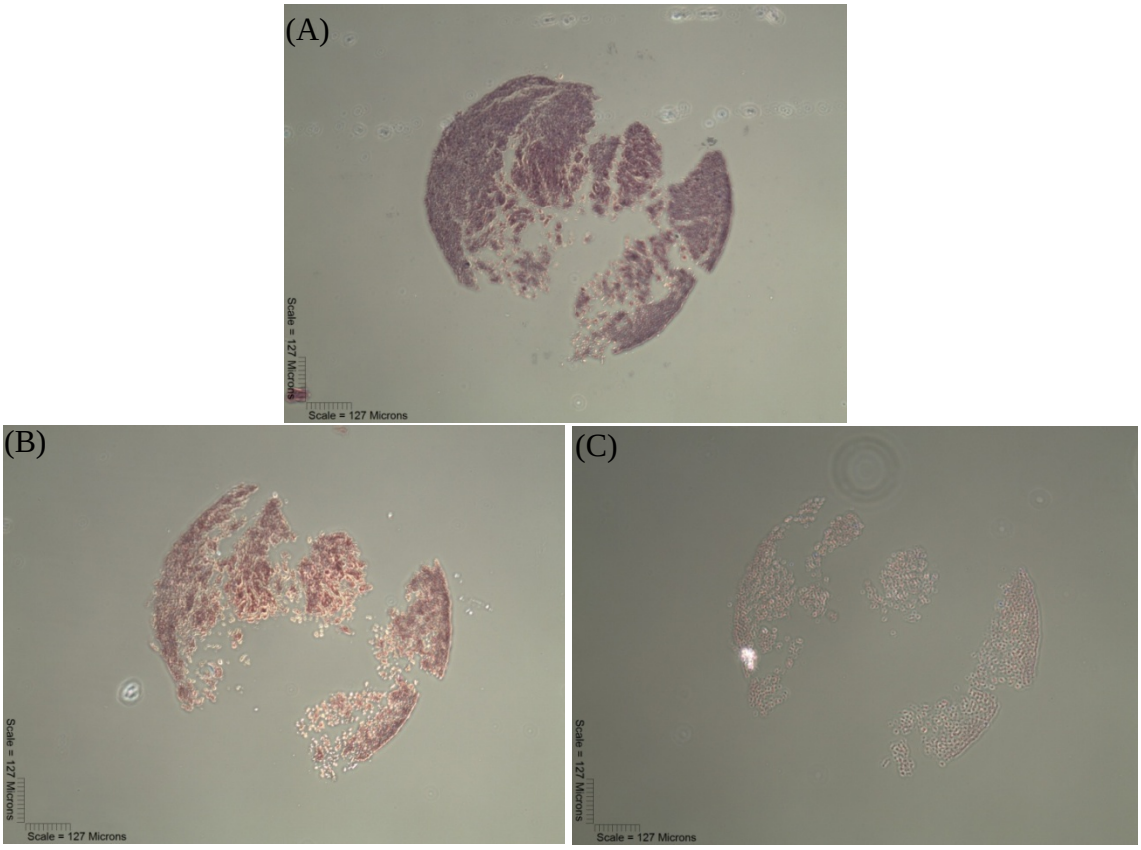


Figure 4.7. hFF-1 Negative Control Pellet culture without hydrogel on day 28. (A) Haematoxylin and Eosin stain. (B) Masson's Trichrome stain. (C) Alcian Blue stain (pH 2.5).

the FBM) was significantly less dense than the other two groups. Further, cells were sparcely dispersed, unlike cell clustures surrounded by matrix elemenets or sparcely dispersed cells in dense matrix components in both hFF-1 and hMSC cultures incubated in CCM. In experimental group of hFF-1 and hMSC cultures histological appearance were similar; both cultures showed round morphology of cells with increased nuclear size relative to the cytoplasm, an indication of chondrocytes. The primary difference was the size of each cell with hFF-1 cultures showing small size relative to hMSCs (**Figure 4.8**

B,C,E,F). This could be attributed to the initial cell sizes. Very few cells were noticed in the negative control group suggesting reduced cell viability (**Figure 4.8 D**).

To understand the various elements within the matrix, tissue sections were stained with Masson's Trichrome stain to evaluate the amount and the distribution of mature collagen surrounding and within the cells. Masson trichrome stains collagen blue, nuclei black and cytoplasm red. The trichrome stain also demonstrated similar collagen distribution from the cells (**Figure 4.9 B-F**). Assembly and maturation of collagen plays a significant role in determining the biomechanics and the quality of the regenerated tissue. Matrix metalloproteinases (MMPs) mediate delicate balance between different matrix elements by a variety of signaling mechanisms (Nelson et al. 2000, Park et al. 2004). Gelatin turnover is mediated either by MMP-2 (Gelatinase A), a constitutively produced homeostatic enzyme, or by MMP-9 (gelatinase B) (Makowski et al. 1998), and upregulated in acute and chronic inflammations. Previously, Pooja et al. have measured the activity of MMP-2/MMP-9 secreted into the growth medium of hFF-1 when cultured in FGM medium to understand whether the increased collagen secretion correlates to altered enzymatic degradation. These results showed a significantly higher MMP-2/MMP-9 activity in all gelatin containing hydrogels at day four than in tissue culture plastic. Thus the increase in on an average, these structures showed two times more enzymatic activity than TCP. This could be attributed to cells recognizing the denatured gelatin in these structures and trying to degrade them.

Apart from collagen, cartilage also contains aggrecan proteoglycan, which contains chondroitin sulfate glycosaminoglycans. In aggrecan, chondroitin sulfate is covalently bound to a core protein, which also has a binding domain for another long chain non-

sulfated GAG hyaluronic acid. These molecules form the complex aggrecan with 100 or more chondroitin sulfate chains bound to a core protein, and help in retaining large number of water molecules. Shock absorption and friction reduction (lubricating action) are intrinsic functions of cartilage and may predominantly be attributable to a decrease in the amount of chondroitin sulfate chains contained by aggrecan. Hence, presence of these molecules was analyzed by alcian blue stain. The products from cellular secrection, acidic mucosubstances, hyaluronan, and sialomucins are stained in dark blue with the background materials being stained in red by Alcian blue with a pH of 2.5. The collagen, cytoplasm and tissue are stained blue, light red, and dark red, respectively. The negative control group did not have any production of GAGs into the matrix as seen in **Figure 4.10 B**. The results displayed similar distribution of matrix elements in the experimental and positive control groups in the 0.5% w/v hydrogel, but were in sparse quantities (**Figure 4.10 B,C**). Whereas, the 2% hydrogel system contianed significantly more concentrated areas of GAGs in the matrix in both experimental and positve control groups (**Figure 4.10 E,F**). However, further analysis is necessary to understand the content of these mucopolysaccharides.

The increase of production in collagen and GAGs in the higher concentrated hydrogels can be attributed to the orientation of the cells with the hydrogels. The 0.5% w/v gels contained encapsulated pellets and in the 2% w/v gels the cells were suspended. Previous reports have shown that viability and proliferation of cells increase with the stiffness or strength of the scaffold (Discher et al. 2005, Yeung et al. 2005). Furthermore, increasing cell differentation generallycorrelates with increasing substrate stiffness but varies from cell to cell (Wells 2008). Also, suspension of the cells addresses the limitation of the

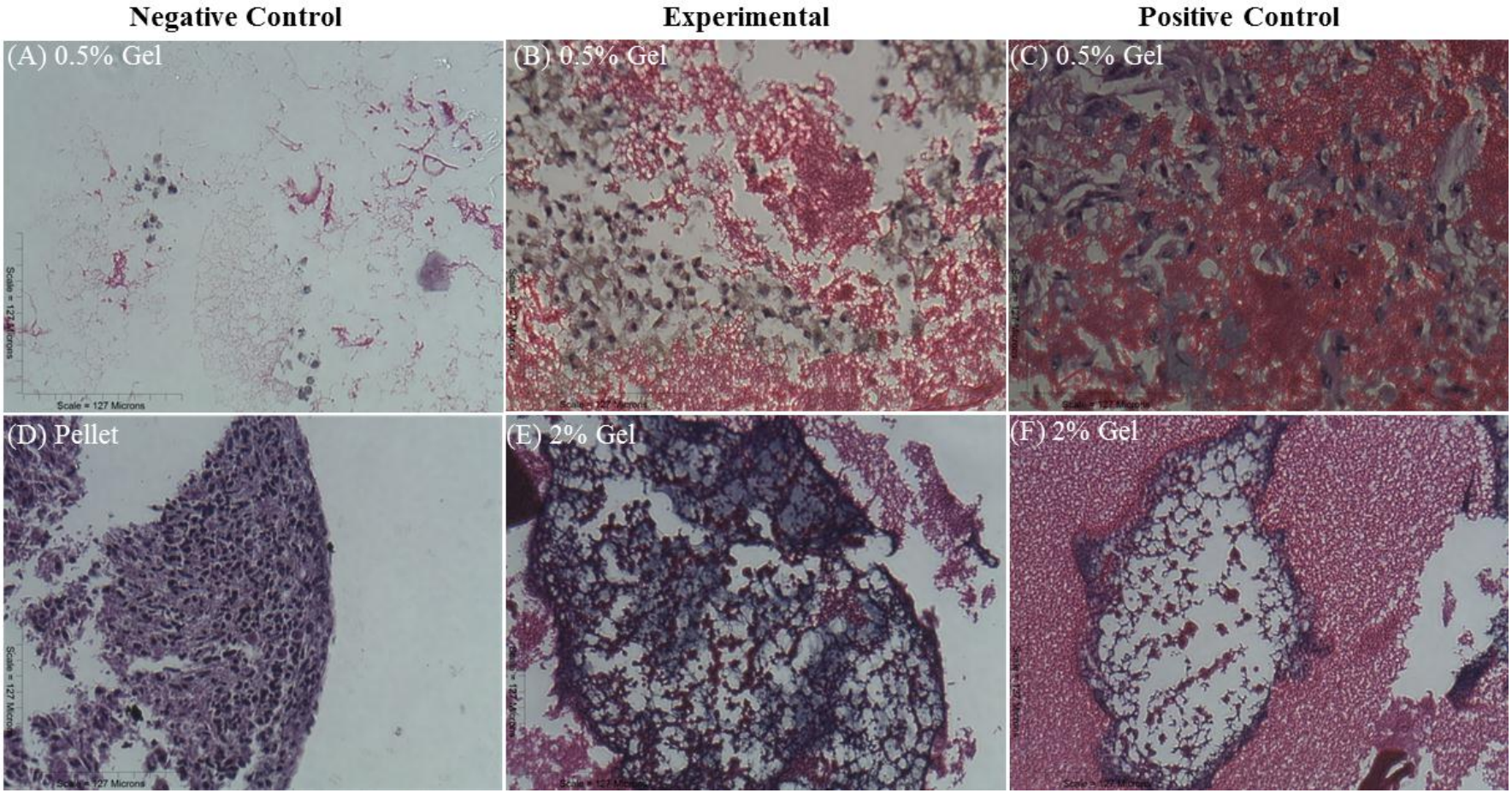


Figure 4.8. Haematoxylin and Eosin stain. (A) hFF-1s in FBM on day 28. (B) hMSCs in CCM on day 28. (C) hFF-1s in CCM on day 28. (D) hFF-1s in FBM on day 28. (E) hMSCs in CCM on day 7. (F) hFF-1s in CCM on day 7.

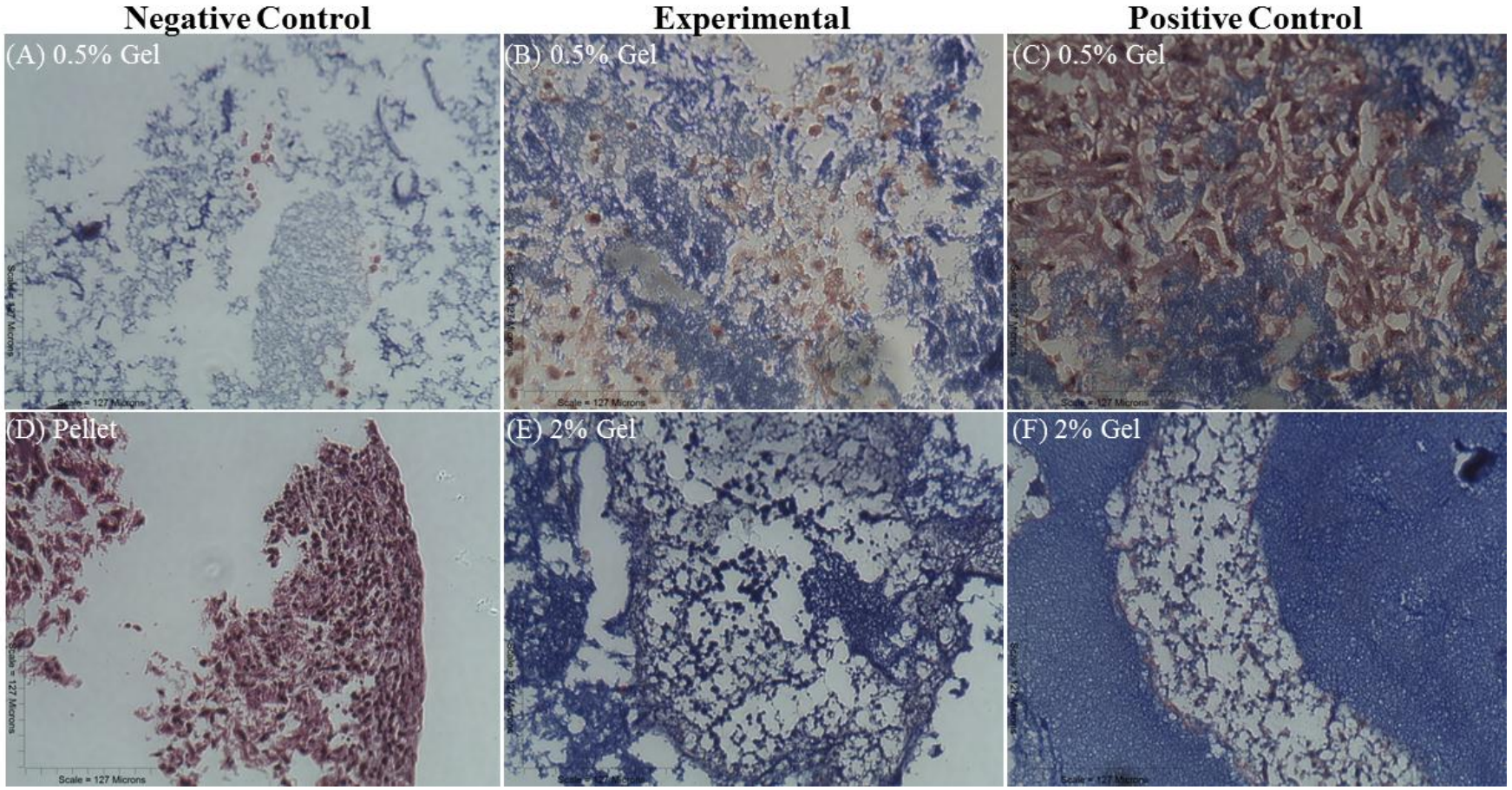


Figure 4.9. Masson's Trichrome stain. (A) hFF-1s in FBM on day 28. (B) hMSCs in CCM on day 28. (C) hFF-1s in CCM on day 28. (D) hFF-1s in FBM on day 28. (E) hMSCs in CCM on day 7. (F) hFF-1s in CCM on day 7.

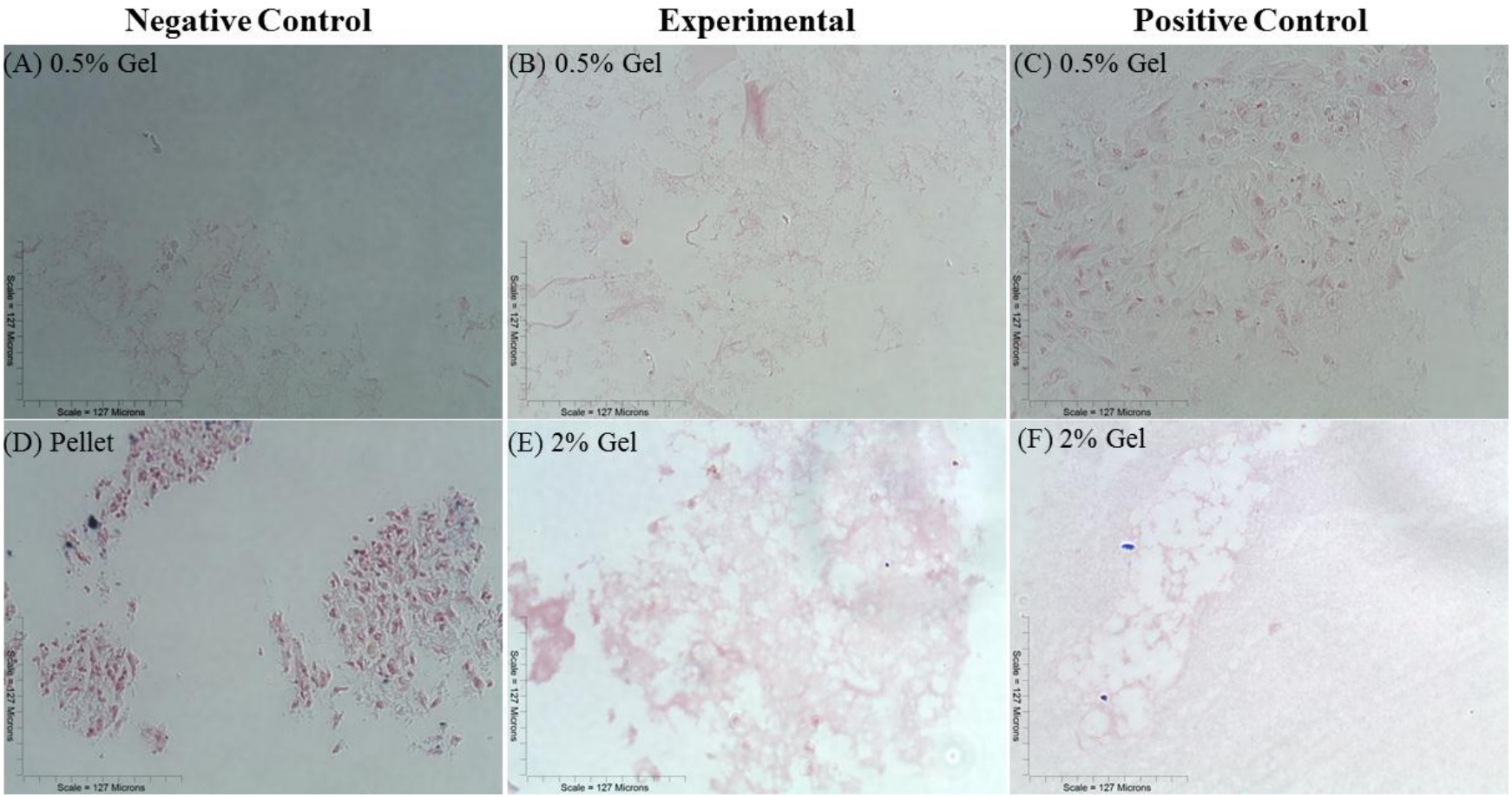


Figure 4.10. Alcian Blue stain (pH 2.5). (A) hFF-1s in FBM on day 28. (B) hMSCs in CCM on day 28. (C) hFF-1s in CCM on day 28. (D) hFF-1s in FBM on day 28. (E) hMSCs in CCM on day 7. (F) hFF-1s in CCM on day 7.

pellet of size inadequecy and poor processing ability. However, the results for the 2% w/v hydrogels require longer culture periods and further analysis of the ECm components and cell viability and proliferation in the hydrogel.

4.7 Matrix Metalloproteinase Activity

MMPs degrade ECM components such as native and denatured collagens, elastin, laminin, and fibronectin (M.S. Agren et al. 1998). Increased activity of these MMPs in cartilage and the synovial tissues has been found in patients suffering from osteoarthritis and rheumatoid arthritis (Nagase et al. 2003). Gelatinases, or MMP-2 and MMP-9, are present for extended periods of time during the wound repair process (M.S. Agren 1994). Additionally, overexpressed collagenase levels have been linked to increased levels of MMP-9 during the involvement in collagen degradation (Iyer et al. 2011). MMP-2 is expressed by MSCs but the activity is inhibited by tissue inhibitors of metalloproteases (Lozito et al, 2011). Additionally, MMP-2 activity in fibroblasts is regulated by the mechanical strength of gels (Tomasek et al, 1997). However, the main enzymes considered to be responsible for collagen and aggrecan degradation in cartilage are MMP-3, MMP-13, and MMP-14. MMP-13 can cleave native fibrillar collagen types I, II, III, and V as well as several other ECM components, including type IV, X, and XIV collagens, large tenascin C, aggrecan, versican and fibronectin. Furthermore, a role of MMP-13, which is produced by chondrocytes, is proposed in the excessive degradation of collagenous ECM in osteoarthritic cartilage (Kevorkian et al. 2004). MMP-13 is expressed by fibroblasts in chronic cutaneous ulcers in vivo but not in normally healing

acute dermal wounds. (Ravanti, L et al., 1999). MMP-13 mRNAs are expressed by human skin fibroblasts cultured in type I collagen gel but not by fibroblasts in monolayer cultures without TGF-β. However, culturing fibroblasts in TGF-β containing medium induces MMP-13 expression.

To understand the implication of incubating the hFF-1 and hMSCs in pellet form on hydrogels, activity of MMP-2/MMP-9 and MMP-13 were measured. First, the activity of the MMP-2/MMP-9 and MMP-13 in the FBM and the CCM for all of the cultures was evaluated using respective fluorogenic substrates. The relative fluorescence units (RFU) per microliter of the supernatant (RFU/μL) were measured for the media supernatants and fresh media. There was a significant difference in the MMP-2/MMP-9 content in the fresh FBM and CCM media (**Figure 4.11 C**). Relative to the fresh medium content, similar MMP-2/MMP-9 expressions were observed in all the negative and positive control samples, whereas the experimental group showed a reduced level (**Figure 4.11 A, and Figure 4.11 A B**).

When MMP-13 activity was measured, there was no difference in the MMP-13 content in the fresh FBM and CCM media (**Figure 4.12 C**). More MMP-13 expression was observed on day seven and day fourteen in the hMSC pellet cultures relative to the fresh media (**Figure 4.12 A**). Similarly, days seven and twenty one of the experimental pellet group displayed an increased expression of MMP-13 content relative to the fresh media (**Figure 4.12 A**). This increase in activity in the pellet cultures may be due to the cells creating intracellular space within the pellet causing degradation of the pellet thus expressing the MMP-13 enzyme. In some cases of differentiation protocols, initial expression of MMP-13 in hMSCs and chondrocytes lead to hypertrophy and bone plate

formation (Bertramet al 2009). Thus, the increased MMP-13 activity could also be due to the hypertrophic conditions. Interestingly, minimal increases of MMP-13 were noticed in the positive and experimental groups cultured with the hydrogels (**Figure 4.12 B)**. This could be attributed to the differentiation of cells to chondrocytes. Alternatively, decreased expressions of MMP-13 could be attributed to the cells attaching to the hydrogels and building the ECM rather than degrading the collagen from the hydrogels.

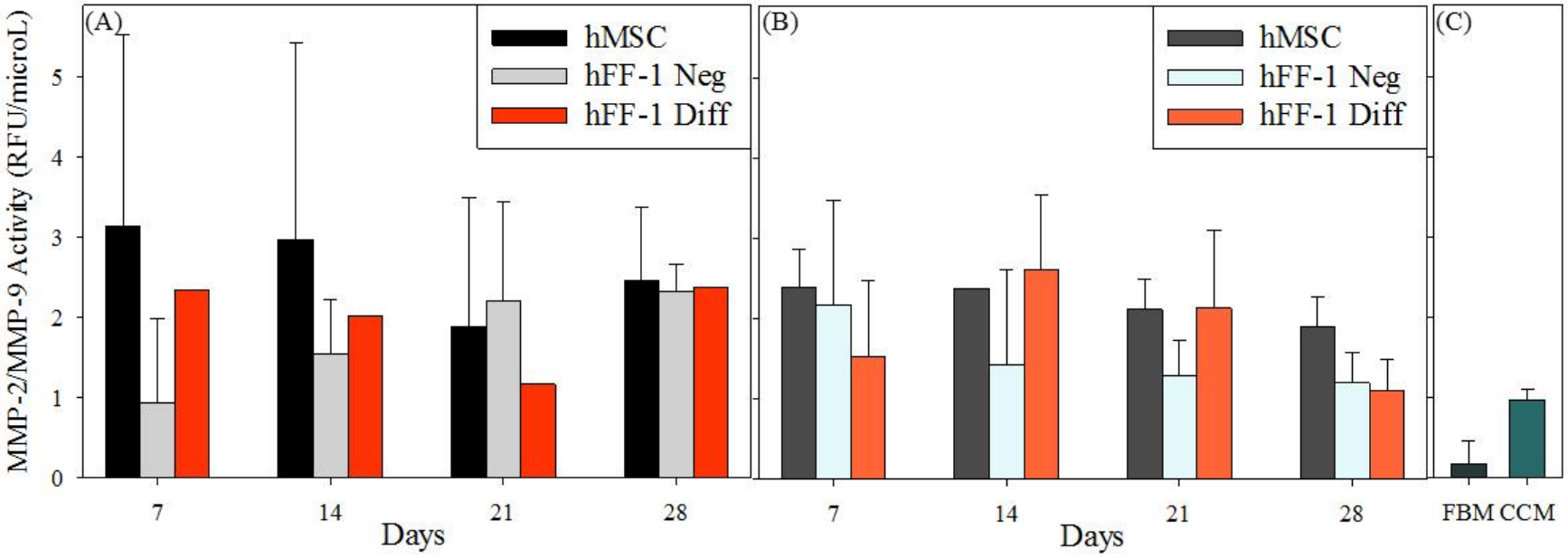


Figure 4.11. MMP-2/MMP-9 secreted into the medium throughout the 28 day culture period. (A) Pellet system for hFF-1control group and hFF-1s and hMSCs in differentiation medium. (B) Hydrogel system for hFF-1 control group and hFF-1s and hMSCs in differentiation medium. (C) Fresh medium used for cultures.

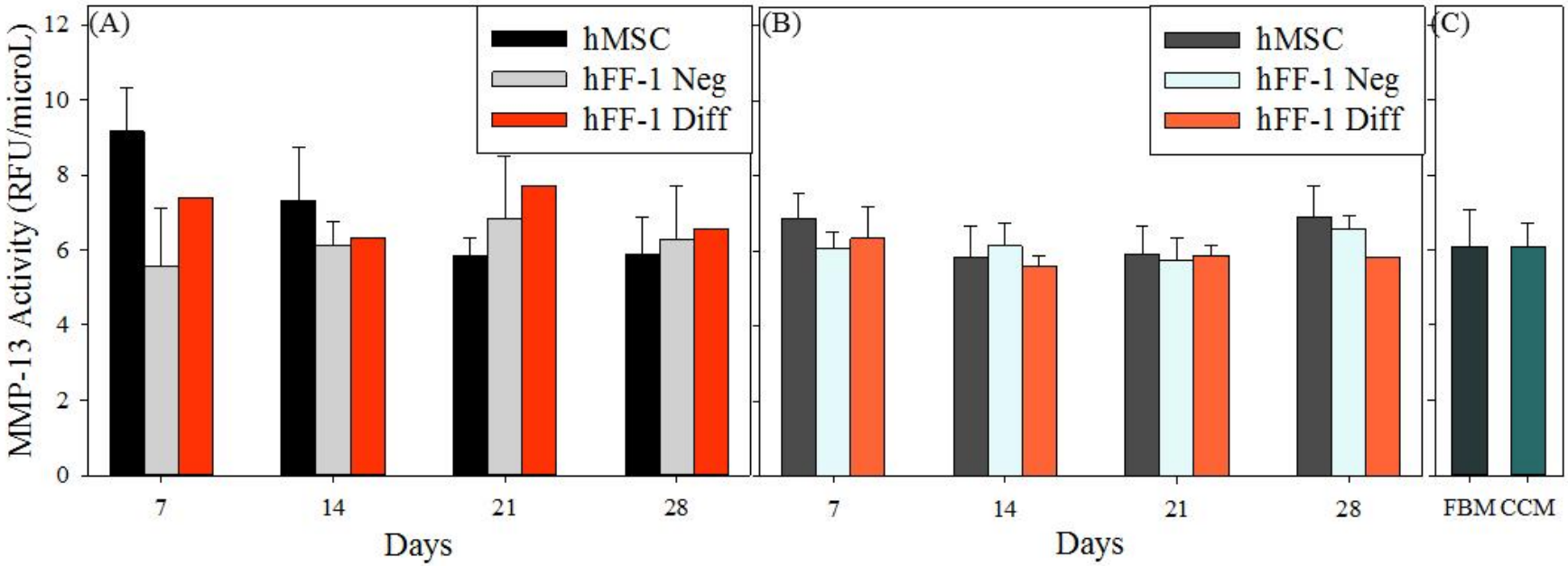


Figure 4.12. MMP-13 secreted into the medium throughout the 28 day culture period. (A) Pellet system for hFF-1control group and hFF-1s and hMSCs in differentiation medium. (B) Hydrogel system for hFF-1 control group and hFF-1s and hMSCs in differentiation medium. (C) Fresh medium used for cultures.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

There were two objectives for this study:

1. The first objective was to evaluate a hydrogel system that is easy to utilize in the clinical settings to produce cartilage. From the thermal gelation studies, the gelation of chitosan occurs with the addition of gelatin and gelation temperature decreases with the increase in chitosan concentration. From the elastic moduli experiments, increasing chitosan and gelatin concentration showed improved elastic modulus.

2. The second objective was to investigate an autologous cell source such as hFF-1s as a source for chondrocytes and measure viability in a regenerative tissue. From the viability, differentiation, and proliferation studies, the hFF-1s were observed to follow similar trends to the positive control hMSCs after twenty eight days of culture in complete chondrogenic medium. This was supported by the cell morphology and collagen distribution studies where the cells and collagen in the ECM produced by each cell line could be observed as embedding in the chitosan-gelatin tissue. Additionally, the cells were noticed to have a round shape which is concurrent with chondrocytes. However, in the initial tissue culture plastic cultures the hFF-1s were examined to be

reduced in size but similar in shape to the hMSCs. Collagen Type II ELISA was performed to evaluate the collagen leaching of the cells into the media supernatant. Both the control and experimental forms of the fibroblasts followed the same progression as the stem cells for in the pellet cultures. The hydrogel cultures showed a significant increase initially in all groups. After the initial jump, the collagen secretion decreased and followed an increasing pattern throughout the rest of the culture period in all groups. Flow cytometry analysis was done on experimental hFF-1 0.5% and 2%/1% w/v hydrogel and hMSC pellet cultures with the detection of antibody surface markers CD44 PE and CD151 FITC. The 0.5% w/v hydrogel culture followed the same trends for both antibodies as the unstained control group. The 2%/1% w/v hydrogel culture displayed an up-regulation in both antibodies. The hMSC pellet culture followed the trend of the CD151 FITC antibody but exhibited an up-regulation in the CD44 PE antibody. Increases in extracellular matrix accumulation were observed in hFF-1 and hMSC cultures containing hydrogel and chondrogenic induction medium. Positive and negative control groups displayed similar results of MMP-2/MMP-9 expression in all samples with a reduction in expression in the experimental group relative to the media. Experimental and positive control pellet cultures showed higher expressions of MMP-13 early in the culture relative to CCM. Hydrogel cultures had minimal increases in all cultures relative to media.

Overall Summary: In summary, a hydrogel system that displays mechanical properties (12-15 kPa) that is more closely useable for clinical settings has been observed, an autologous cell source such as hFF-1s have suggested the possibility of differentiation into chondrocytes as well as viability and proliferation in the explored hydrogel systems.

5.2 Recommendations

1. This study is exploration into the possibility of differentiation of an autologous cell source into a low regenerative cell line and the relationship between the physical and chemical characteristics of the biomaterial structures with respect to the biological responses of the cell lines. Although the mechanical characteristics of the regenerative tissue have been observed, evaluation of the complete makeup of the structure has yet to be experimented. In the future, this can be done by preparing hydrogel systems with the inclusion of hyaluronan, which is another ECM component naturally found in cartilage. This would complete the necessary components in the hydrogel to most closely represent cartilage in its natural state.

2. The size and shape factor analysis is a useful tool for comparing the size and shape of different cell lines. In the case of this study the two cells lines explored are spindle shaped and range in size. The chondrocyte shape on the other hand is round or circular shaped and of a medium size in cells. For this study the initial size and shape analysis was done of each cell line prior to undergoing differentiation. While cells are being differentiated, the cell size and shape are drastically altered to be consistent with the cell line that is desired. Further analysis needs to be done at the end of the differentiation culturing period to determine the transformation of the cell into the chondrocyte cell line. This will be an inclusion to the histochemistry that has been done to complete the analysis of differentiation by physical aspects.

3. The physical structure of cartilage is made up of many extra cellular matrix components, which include Collagen Type II, Aggrecan, Matrix Metalloproteinases (MMPs), and Tropoelastin. Aggrecan is a naturally occurring proteoglycan and Tropoelastin is the base molecule for forming elastin. Both are proteins naturally found in the ECM of articular cartilage. The MMPs associated with cartilage are known as Aggrecanases and are secreted by chondrocytes. There are multiple MMPs found in cartilage and each of them degrades the extracellular matrix proteins such as collagen, Aggrecan, and elastin. In this study Collagen Type II was explored and evaluated in the supernatant of the culture systems. In the future, Collagen Type II, Aggrecan, Matrix Metalloproteinases (MMPs), and Tropoelastin content should be measured in the regenerated tissue. This must be to done to make the hydrogel mimic the structural integrity as well as make up of articular cartilage.

4. Flow cytometry is used for multiple purposes. In this research, it was used for the detection of surface markers CD44 and CD151. Future analysis should include the measurement of more antibodies such as CD168 and CD49a. CD168 is a hyaluronan-mediated motility receptor that binds to hyluronan and alters migratory cell behavior. CD49a is an integrin alpha sub unit involved in cell adhesion and is a receptor for laminin. Each of these antibodies plays an important role in chondrogenesis and is necessary for completion of the work for promotion of clinical studies.

5. Histology is a used for qualitative analysis of in the interaction of cells with the tissue. Haematoxylin and Eosin (H&E) and Masson's Trichrome were used in this study to determine cell morphology and collagen distribution, respectively. For future studies,

Alcian Blue stain at a pH of 1.0 should be explored. This stain is specific for chondrocytes and cartilage extra cellular matrix components.

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VITA

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Scope and Method of Study: The objective of this study was to investigate a hydrogel system that is easy to utilize in the clinical settings to produce cartilage and an autologous cell source for chondrocytes such as easily available human foreskin fibroblasts (hFF-1).

Findings and Conclusions: Evidence of a decrease in gelation point as an increase in concentration was displayed from the rheology data, which was consistent with previous studies. Increase in chitosan and gelatin concentrations significantly increased the elastic modulus to be within the necessary limits of naturally cartilage. Analysis of the cell shape and size factor suggested the possibility of differentiation of fibroblasts into chondrocytes when compared to the differentiation of hMSCs into chondrocytes. From the ELISA analysis, collagen type II secretion into the medium was higher in hydrogels than for the pellet cultures. Additionally, collagen content was observed to be at a maximum on day seven and was determined to be due to components in the medium and not due to gelatin leaching. Flow cytometry profiles demonstrate alternations in CD44 and CD151 expression. Analysis of the histology showed an increase in matrix accumulation in the structures as well as cell to matrix interaction and embedding. Analyses of MMP-2/MMP-9 and MMP-13 showed similarity between fibroblasts and hMSC cultures.

ADVISER'S APPROVAL: Dr. Sundar Madihally