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Synovium-derived stem cell-based meniscal reconstruction on small intestinal submucosa

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**Synovium-derived Stem Cell-based Meniscal Reconstruction on
Small Intestinal Submucosa**

Yunbing Tan

**Thesis Submitted to the
College of Engineering and Mineral Resources
at West Virginia University
in Partial Fulfillment of the Requirements
for the Degree of**

**Master of Science
In
Mechanical Engineering**

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**Morgantown, West Virginia
2009**

**Keywords: Meniscus, Synovium-derived stem cells, Co-culture, Small intestinal
submucosa, Bioreactor, Compressive loading**

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ABSTRACT

Synovium-derived Stem Cell-based Meniscal Reconstruction on Small Intestinal Submucosa

By Yunbing Tan

This study was aimed at investigating the feasibility of co-culturing meniscus cells and synovium-derived stem cells on small intestinal submucosa (SIS) in order to establish an innovative method and further to optimize the culture conditions for engineering *in vitro* meniscus constructs. First, two groups of individual cells and a co-culture group were mixed with fibrin gel and seeded onto SIS discs, then incubated in a serum-free defined medium for one month. Electron and fluorescent microscopy were utilized to evaluate cell attachment and migration. Through histology, biochemical quantification, real time PCR, and compressive modulus, the co-culture group showed greater cell viability and exhibited higher glycosaminoglycan, collagen II, and Sox9 but relatively low collagen I, resulting in the concomitant increase in equilibrium modulus and demonstrating the advantages of co-culturing. Based on these results, we utilized rotating bioreactors and compressive loading for co-culture constructs to improve their function. However, the results were unsatisfactory; we carefully analyzed the reasons and made useful suggestions for future studies.

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LIST OF ABBREVIATIONS

α MEM	alpha modified Eagle's medium
ACL	anterior cruciate ligament
BMP-2	bone-morphogenic protein-2
BMSC	bone marrow stem cell
CD14	cluster of differentiation 14
cDNA	complementary DNA
Col I	collagen type I
Col II	collagen type II
Col X	collagen type X
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
ECGF	endothelial cell growth factor
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FGF	fibroblastic growth factor
GAG	glycosaminoglycan
HGF	hepatocyte growth factor
HMDS	hexamethyldisilazane
IGF-I	insulin-like growth factor-I
IL-1	interleukin-1
MC	meniscal cell
MCL	medial collateral ligament
MFC	meniscal fibrochondrocytes
mRNA	messenger RNA
MSC	mesenchymal stem cell
MWCO	molecular weight cut-off
PBS	phosphate-buffered saline
PCR	polymerase chain reaction

PDGF-AB	platelet-derived growth factor-AB
PGA	poly(glycolic acid)
PL	platelet lysate
PLLA	poly(L-lactide)
PS	penicillin/streptomycin
PTFE	poly(tetrafluoroethylene)
RNA	ribonucleic acid
RT	reverse transcription
SDSC	synovium-derived stem cell
SEM	scanning electron microscopy
SIS	small intestinal submucosa
Sox9	SRY (sex determining region Y)-box 9
TGF- β	transforming growth factor- β

CHAPTER 1 INTRODUCTION

Meniscus is a crescent-shaped fibrocartilaginous structure present at the peripheral aspect of joints. The knee menisci are concave on the top and flat on the bottom, articulating with the tibia. They have several important functions in joint stability, lubrication, nutrition, and shock absorption. As secondary stabilizers, the intact menisci interact with the stabilizing function of ligaments and are most effective when the surrounding ligaments are intact. They serve to disperse the weight of the body and to reduce friction in the knee joint between the lower leg (tibia) and the thigh (femur).

Two common causes of a meniscal tear are traumatic injury and degenerative processes. The former is often seen in athletes and the latter is seen in older patients who have more brittle meniscus. The meniscus tear often occurs along with injuries to the anterior cruciate ligament (ACL) and the medial collateral ligament (MCL). The symptoms of meniscus tear include knee pain, swelling of the knee, tenderness when pressing on the meniscus, popping or clicking within the knee, and limited motion of the knee joint. Injury or loss of meniscus can lead to osteoarthritis and irreversible joint damage¹.

Treatment options for torn menisci vary according to the region of the lesion. The meniscus is vascularized only in the outer third and lesions occurring in the inner two-thirds rarely heal spontaneously. Partial or total removal of the torn meniscus was a standard therapy in the past. However, because of the change in contact pressure and contact area², meniscectomy has been strongly associated with the development of early osteoarthritis³. Experimental studies using meniscus prostheses have yielded unsatisfactory results⁴. Problems with biocompatibility, wear, and inferior material properties of these prostheses have led investigators to pursue a more biological approach through the creation of a tissue-engineered meniscus.

Tissue engineering is the use of a combination of cells, engineering and materials methods, and suitable biochemical and physio-chemical factors to improve or replace biological functions. It is closely associated with applications that repair or replace

portions of or whole tissues, including bone, cartilage, blood vessels, and bladder. In tissue engineering techniques, many fundamental biological factors should be addressed, including cell source, matrix scaffold, bioreactor design, and environmental conditions⁵. Currently there are few tissue-engineering techniques that can regenerate satisfactory meniscus tissues. In order to develop a novel treatment of meniscus tears and defects, we selected a combination of cells, scaffold, medium, growth factors, culturing conditions, and mechanical stimulation based on literature review. We will conduct experiments to test our creative technique.

The objective of this work will be to develop an innovative and effective tissue engineering technique for the treatment of meniscus tears and defects. To test this overall objective, supporting objectives have been identified.

1. Test the biocompatibility of the scaffold with the cells we use, which means whether the cells are viable on the given scaffold.
2. Make sure the scaffold will not generate a negative influence on the proliferation of cells on it.
3. Test and compare the biochemical features of the constructs we obtain with natural meniscus tissues, which is a key point to cure meniscal degeneration or trauma.
4. Test the mechanical properties of the constructs to better validate this technique.
5. Study the effects of rotating bioreactor culture on the meniscal constructs.
6. Study the effects of compressive stimulation on the meniscal constructs.

The overall design of my study can be summarized as shown in Figure 1 on the next page.

Figure 1 Overall design for this study

CHAPTER 2 LITERATURE REVIEW

2.1 *Meniscus Physiology*

2.1.1 Anatomic Structure

The menisci are paired semilunar (C-shaped) fibrocartilage structures that are located between the femoral condyles and the tibial plateau. As shown in Figure 2, they are wedge-shaped with convex outer margin much thicker than the inner margin. The proximal (or femoral) surface is concave, whereas the distal (or tibial) surface is flat to convex⁶.

Figure 2 Human meniscus (shown attached to tibia)⁷

The menisci are approximately 35 mm in diameter⁸ and feature a firm connective tissue attachment to the joint capsule at their outer margin, which usually measures approximately 110 mm in circumference⁹. The anterior and posterior horns of each meniscus are anchored to bone via insertional ligaments. The transverse ligament connects the anterior horns of the medial and lateral menisci and has fibers blending in

with the tibial insertion of the anterior ACL. The medial meniscus is larger than the lateral meniscus in the anteroposterior plane, and both menisci are narrower at the anterior horns and become wider posteriorly. The lateral meniscus is more uniform in width throughout its course (Figure 3). Collagen fibers running along the circumference of the meniscus continue into the anterior and posterior insertional ligaments, which anchor the meniscal horns via interdigitations with subchondral bone through uncalcified and calcified fibrocartilage¹⁰.

Figure 3 Anatomy of the menisci viewed from above¹¹

The most common anatomic variation of the menisci is the discoid meniscus. More common laterally (with an incidence of 1.5% to 16.6%)¹², it appears as a thickened wafer-shaped structure. Medial and bilateral cases of discoid structure have been reported. The Watanabe¹³ classification describes three variations of a discoid lateral meniscus: (1) an incomplete type, featuring a larger than normal meniscus but with normal attachments; (2) a complete type with a full disk covering the entire tibial plateau; and (3) a Wrisberg type, with no meniscal attachments to the posterior tibia or joint capsule but with a normal attachment to the Wrisberg ligament. Currently, discoid menisci are thought to be a congenital phenomenon with some evidence of familial transmission¹⁴.

From an anatomic standpoint, one of the most crucial features of the meniscus is its vasculature. The meniscal blood supply (or lack thereof in the central regions) has profound implications for the healing of meniscal repairs. The lateral, medial, and middle geniculate arteries provide the primary blood supply to the menisci, forming a parameniscal capillary plexus embedded within the capsular and synovial tissues¹⁵. This parameniscal capillary plexus is located along the entire peripheral border of both menisci and features vessels that are oriented circumferentially, with radial branches directed at the joint center terminating in small capillary loops. Prenatally, blood vessels traverse the entire body of the meniscus with the highest density in the peripheral third. Postnatally, a gradual decrease in vascularity of the inner two thirds occurs, along with a decrease in cellularity and an increase in collagen content. The postnatal decrease is thought to be caused by vertical load bearing, which would explain why the non-weight-bearing meniscal horns retain a rich blood supply throughout adult life. In the adult meniscus, capillary loops penetrate no deeper than 10% to 25% of the width of the lateral meniscus and 10% to 30% of the medial meniscus. In addition, the posterolateral region of the lateral meniscus has an avascular area surrounding the popliteal tendon.

The anterior and posterior horns of the menisci are covered by a vascular synovial tissue that is continuous with the synovial sheath of the cruciate ligaments and is found along the periphery of the entire meniscus, extending over a small portion of both the superior and inferior surfaces. These layers do not contribute to the blood supply of the noninjured meniscus, but can play an important role in meniscal healing if stimulated by injury or repair. Rasping or abrading these tissues can stimulate vascular overgrowth onto the meniscus; the synovium contains pluripotential mesenchymal cells that may play a role in meniscal repair.

2.1.2 Biochemical Composition

The composition of the adult meniscus is 70% water; 75% of its dry weight is collagen, 8% to 13% is noncollagenous protein, and 1% is hexosamine¹⁶. The proteoglycan portion of the meniscus, including chondroitin 6-sulfate (40%), chondroitin 4-sulfate (10% to

20%), dermatan sulfate (20% to 30%), and keratin sulfate (15%)¹⁷, accounts for less than 1% of its dry weight and is located primarily in the inner region of the meniscus, with little in the periphery where thick collagen fibers are aligned¹⁸. The compositions of meniscus by region are summarized in Table 1.

Table 1 Compositions of meniscus by region¹⁹

Region	n	Sulfated Glycosaminoglycan (% dry weight)	Water Content	Hydroxyproline (% dry weight)
LA	18	1.80 ± 0.50	75.02 ± 2.14	14.3 ± 3.7
LC	18	1.68 ± 0.56	72.99 ± 2.40	13.2 ± 2.0
LP	18	1.75 ± 0.45	73.39 ± 2.44	15.2 ± 3.1
MA	12	2.20 ± 1.01	72.12 ± 9.73	13.2 ± 3.6
MC	14	2.06 ± 0.68	76.77 ± 2.68	13.9 ± 3.4
MP	18	1.94 ± 0.83	74.88 ± 7.32	13.9 ± 3.6

LA = lateral anterior, LC = lateral central, LP = lateral posterior, MA = medial anterior, MC = medial central, MP = medial posterior.

Collagen types I, II, III, V, and VI have been found within meniscal tissue, which account for 60-70% of the dry weight⁶. Type I collagen is by far the most predominant, accounting for >90% of the collagen within the tissue²⁰. A study done by Cheung²¹ showed that in bovine menisci, the outer two-thirds of the menisci is predominantly type I collagen, whereas the inner one-third is 60% type II collagen and 40% type I collagen. He also found that the concentration of collagen was greater in the outer section, 80.1% dry weight vs. 69.3% dry weight²¹. The meniscus has a unique collagen structure orientation that is related to its function and consists of three different layers (Figure 4). The superficial layer consists of a thin layer of randomly oriented fibers²². The lamellar layer, located just inside the superficial layer, also consists of randomly oriented fibers, with the exception of the peripheral portions at the anterior and posterior sections; here the fibers are oriented radially²². The deep zone consists of circumferentially oriented

fibers with a small amount of radially oriented fibers, also referred to as tie fibers. It has also been shown that the amount of collagen synthesized by the fibrochondrocytes decreases with age²³.

Figure 4 Meniscus collagen structure⁷

Proteoglycans, with large size and anionic charge, endow the menisci with strong shock-absorption properties. This composite acts like a large sponge because of its water retention properties. The proteoglycan and collagen content of the meniscus increases with age until maturity and then remains relatively constant, in marked contrast to articular cartilage, which features a steady decline in proteoglycan content with age²⁴. The ratio of chondroitin 6-sulfate to chondroitin 4-sulfate increases with age while the water content increases significantly because of the accumulation of water-binding proteoglycans in degenerated menisci. Degenerated menisci from knees with osteoarthritis and rheumatoid arthritis contain less collagen, more non-collagenous matrix protein and hexosamine²⁵, and more water; this is attributable to a disrupted collagen network that is unable to resist swelling pressure from the proteoglycans in tissue²⁶. Adams and associates²⁶ also found that meniscal glycosaminoglycans were decreased early on in inflammatory arthritis.

2.1.3 Function and Behavior

Functionally, the meniscus is a shock absorber, helps with load bearing and transmission in the knee joint, improves stability in the knee, and helps with lubrication. Because of all these different functions and the geometry of the tissue, the meniscus is subjected to compressive, tensile, and shear stress (Figure 5).

Figure 5 Free body diagram of the meniscus⁷

One of the first proposed functions of the meniscus was load transmission. Subsequent in vitro studies have confirmed this role and demonstrated that 70% of the load in the lateral compartment and 50% of the load in the medial compartment is transmitted through the menisci². Ahmed and Burke showed that over 50% of compressive load is transmitted through the posterior horns in extension, with 85% transmission at 90° of flexion. The ability of the meniscus to effectively dissipate loads across the tibial plateau results from the fact that the shape of the menisci bridges the incongruence between the relatively spherical femoral condyles and the flat tibial plateau. The meniscus increased the contact area, which allows a decrease in pressure across the central portion of each compartment.

Fukubayashi and Kurosawa²⁷ reported that the femoral-tibial contact area of the knee is 1,150 mm² with menisci and 520 mm² without menisci; accordingly, the peak pressures were recorded across the knee joint at 3 MPa with menisci and 6 MPa without.

Closely related to the load transmission role of menisci is their role in shock absorption. When a sudden load is applied across the knee joint, an intact meniscus can dissipate forces because of its biphasic structure. The solid phase (proteoglycan and collagen) and liquid phase (water) function under compression similar to articular cartilage. Additionally, the unique architecture of the meniscus allows the conversion of vertical forces into tangential and radial forces as the meniscus moves peripherally under load. This conversion is made possible by the circumferential orientation of collagen fibers in the body of the meniscus, with firm attachments at the anterior and posterior horn to tibia. Vertical joint loading causes tension and elongation of these circumferential fibers, effectively converting the vertical load into horizontal “hoop stresses” in the intact meniscus. In an intact meniscus, the compression force at the meniscotibial surface is smaller than that at the menisconfemoral surface²⁸. This dissipation of forces is dependent on the intact circumferential orientation of collagen fibers in the meniscus. In the meniscus, most fibers are circumferentially oriented; thus its greatest tensile strength is circumferential²⁹. This shock-absorbing function is dependent on the strong collagenous attachments of the insertional ligaments to bone. Messner and Gao³⁰ have emphasized the transition from ligament to bone through uncalcified cartilage and calcified cartilage as an effective anchor when stressed by a compressive load. Consistent with studies showing the increased load-bearing function of the lateral meniscus, there is more calcified tissue in the insertional ligaments of the lateral anterior and posterior horns of the meniscus³⁰.

The meniscus also plays an important role in maintaining joint congruity and stability. The menisci serve to create a socket, which improves the articulation between the disparate shapes of the femoral condyles and tibial plateau. When this meniscal socket is intact, axial loading of the knee has a multidirectional stabilizing function, limiting excess motion in all directions. Markolf and associates^{31, 32} have addressed the effect of

meniscectomy on anterior-posterior and rotational knee laxity. With medial meniscectomy alone, anterior-posterior tibial translation at 90° of flexion increased to 1.82 times the baseline; valgus-varus laxity also increased in flexion, to 1.36 times the baseline.

2.2 *Meniscal Injuries*

In order for the meniscus to provide these protective functions, structural continuity must be maintained throughout the entire meniscus from the anterior to posterior insertional ligaments. If a tear of the meniscus or either of the insertional ligaments disrupts this continuity, then the load transmission system breaks down. Hoop stresses cannot be maintained and the result is a functional meniscectomy. Fortunately, a partial-thickness tear may still allow some degree of function depending on the size and direction of the tear. Partial meniscectomy is always preferable to total excision as long as some circumferential fibers remain intact³³. Experimental evidence suggests that the loss of a meniscus results in at least 20% reduction of this shock-absorbing function³⁴.

In the United States, meniscal injuries may be the most common knee injury. The prevalence of acute meniscal tears is 61 cases per 100,000 persons. The overall male-to-female incidence is approximately 2.5:1. The peak incidence of meniscal injury for males is in those aged 31-40 years. For females, the peak incidence is in those aged 11-20 years. In patients older than 65 years, the rate of degenerative meniscal tears is 60%. Surgical procedures of the meniscus are performed on an estimated 850,000 patients each year³⁵. Estimates indicate that at least twice this number of meniscus procedures are performed worldwide.

The two menisci are easily injured by the force of rotating the knee while bearing weight³⁶. A partial or total tear of a meniscus may occur when a person quickly twists or rotates the upper leg while the foot stays still (for example, when dribbling a basketball around an opponent or turning to hit a tennis ball). If the tear is tiny, the meniscus stays connected to the front and back of the knee; if the tear is large, the meniscus may be left

hanging by a thread of cartilage. The seriousness of a tear depends on its location and extent.

Injuries to the meniscus usually consist of tears in the tissue, although separation from the tibial attachments and degeneration of the tissue also occur^{37, 38}. Different styles of tears can occur, such as longitudinal and bucket-handle tears (most common), radial tears, and complex tears. In 1936, King³⁷ was the first to show that tears in the vascularized portion of the meniscus would naturally heal, whereas tears in the avascular portion would not. It has also been shown that longitudinal tears, if they heal, restore native mechanical function, but radial tears, where the collagen structure is disrupted, do not have restored mechanical function after healing.

In the case of older, degenerated menisci that typically split apart or break down under routine, day-to-day or minimal stress conditions, the meniscus itself has not been healthy for some time. A degenerative meniscal defect will almost never bleed, and in fact may often occur gradually, without the patient even being aware of it. When such gradual, degenerative meniscal cleavage or fissuring develops and is discovered by MRI scanning or arthroscopy, while it is still commonly said that the patient “tore” their meniscus, this is actually a mischaracterization. To most people, “tearing” something implies a sudden structural failure in response to a specific, applied force. While the terms “torn” and “tear” are convenient for surgeons to use because they are familiar to patients, it is misleading to use them in relation to a degenerated meniscus that is simply worn, frayed, fissured, fragmented or just plain broken down.

2.3 *Classical Treatments*

Meniscectomy, or removal of the meniscus, is the dominant repair technique attempted for meniscus defects. Due to the poor long-term results of meniscectomy, efforts have also been made to seal tears that occur in meniscus and replacement of the meniscus.

2.3.1 Meniscectomy

The menisci were originally thought to have little importance in knee function and were believed to be functionless remains of leg muscle³⁹. These beliefs led to the practice of removing the meniscus whenever there was a complication. In the 1930s, King³⁷ showed that degenerative effects in the articular cartilage are a side effect of meniscectomy, and widespread attention to this complication occurred after a series of studies in the 1960s and 1970s³⁹. Directly after removal of the meniscus, the mechanical function of the knee joint alters, leading to greater stress applied to the articular cartilage^{40, 41}. Over time, a new tissue is grown by the body to fill the defect, and the cellular distribution within the tissue shows discrepancy⁴². Partial meniscectomy, which is removal of a small portion of the meniscus, usually in the avascular zone, has been shown to cause fewer degenerative changes in the articular cartilage than a full meniscectomy⁴³. A partial meniscectomy has the advantage of leaving the majority of the collagen structure of the meniscus in the knee, allowing the partial meniscus to still absorb some of the shock⁴³. After a partial meniscectomy, a fibrin clot forms in the area of the removed tissue, which then acts as a scaffold for cellular ingrowth, though this does not always occur⁴⁴. McDermott and Amis reviewed the consequences of total and partial meniscectomy and summarized the body of evidence in the literature regarding those factors most relevant to long-term outcome⁴⁵.

2.3.2 Repair of Meniscal Tears

After the realization that meniscectomy causes degeneration of articular cartilage, the healing method of choice started to be repair of the meniscal tear. A variety of different methods have been tried: suture, meniscal arrows, fibrin sealant, laser welding, and abrasion therapy^{46, 47, 48, 49}. Sutures are used to reconnect the torn tissue until it heals together. There are several different suturing methods that have been attempted, the goal being to increase the strength of the wound site^{47, 50}. Meniscal arrows are biodegradable devices that are marketed to replace sutures because they are easier to use⁵¹. Fibrin sealant and laser welding tests have been done to test their effectiveness in sealing tears; in general, they are inferior to suture but can be used in conjunction with suture for better results⁴⁶. Abrasion therapy, which roughens up the tissue surrounding the tear and

removes necrotic tissue, will usually also be used to aid the healing process⁴⁸. Suture, meniscal arrows, fibrin sealant, and laser welding are all techniques that can be used alone to help healing in the vascularized zone.

2.3.3 Meniscal Replacement

Meniscal allograft research has been rather extensive, and the results seem to be promising, though there are no long-term data⁵². The effects of different preservation techniques and attachment methods have been tested. Studies showed that there were few differences between cryopreserved and deep-frozen meniscal tissue in the goat model at 1 year⁵³. The deep-frozen technique kills all of the cells, leaving the allograft to act as a scaffold, whereas the cryopreservation technique leaves 10-30% of the fibrochondrocytes viable⁵⁴. Cryopreserved allografts have been shown to hold a higher water content and a decreased proteoglycan concentration when compared to native tissue⁵⁵. One of the difficulties with full meniscal replacement is attachment to the tibial plate. It has been shown that bone plugs offer the best mechanical results, but these plugs do not simulate the native tissue and long-term results are unknown^{56, 57}. Overall, although meniscal transplantation offers the best results for certain meniscal conditions such as radial tears, complex tears, and degenerative tissue, the current research shows that degeneration of the articular cartilage still occurs and a better alternative needs to be developed.

Different types of meniscal prostheses, including collagen, permanent synthetic scaffolds, and biodegradable scaffolds, have been used in experimental and clinical studies. Several materials have been tried, such as poly(tetrafluoroethylene) (PTFE), Dacron, and polyurethane-coated versions of the previous two materials^{58, 59}. Although the polyurethane-coated PTFE gave the best results, the prosthesis was still mechanically inferior to native meniscal tissue⁵⁸. Tissue engineering of the meniscus seems to be the preferred method for healing the meniscus. van Tienen⁶⁰ highlighted the research on these meniscal substitutes and showed that current research is mainly focused on a biological tissue-engineering approach either with or without additional cell-seeding techniques.

2.4 Treatment via Tissue Engineering

When compared to other musculoskeletal tissues such as bone or articular cartilage, the number is relatively smaller for the studies done in the attempt to tissue engineer meniscus. A large amount of work has been done on a variety of cells and scaffold materials. Knowledge on how fibrochondrocytes react to a variety of growth factors has been discovered through a variety of tests from tissue engineering studies. Some studies have been done on culturing conditions for fibrochondrocytes, but there is still plenty of room for development. Mechanical stimuli were increasingly studied in the area of tissue engineering, especially for musculoskeletal tissues including meniscus. The complex nature of the cells and extracellular matrix make the meniscus a difficult tissue to engineer successfully. Many fundamental biological factors about this technique require attention, including cell source, matrix scaffold, growth factors, culturing conditions, and mechanical stimuli⁵. The overall strategy for meniscal tissue engineering is shown in Figure 6.

2.4.1 Cell Sources

Meniscus cells (MCs) and mesenchymal stem cells (MSCs) have been proposed as potential cell sources contributing to the healing and remodeling response of the meniscus in vascular and avascular regions⁶¹. MCs, especially in the inner third of meniscus, are mostly fibrochondrocytes, where 60% of collagen is type II and five to six times more glycosaminoglycan (GAG) is present compared with the outer third^{21, 62}. However, the proliferative capacity of fully differentiated adult cells is limited and long-term *in vitro* expansion can reduce their functional quality. An MSC is a pluripotent cell that divides many times and its progeny gives rise to skeletal tissues⁶³. Recent studies suggest that MSCs may possess unusual immunologic properties that may permit allo- and xenotransplantation⁶⁴. MSC differentiation requiring more complex cues may be provided by culturing the multipotent cells with more differentiated cells, which guide MSCs toward determined differentiation⁶⁵. Bone marrow stem cells (BMSCs), a popular MSC, have been investigated for their ability to contribute to meniscus reconstruction in

animal models but the results are mixed^{66, 67}. Compared to the other sources of MSCs, synovium-derived stem cells (SDSCs) have the greatest ability for chondrogenesis

Figure 6 Strategy for tissue engineering the knee meniscus⁷

demonstrating the superiority of synovium as a potential source of MSCs for clinical applications in cartilage-like tissue regeneration^{68, 69}. However, to our knowledge, there is no study focusing on using SDSCs as the cell source for meniscus tissue engineering.

Co-culture, a technique with different cells cultured together, has proved to be a powerful tool in tissue engineering, not only to generate tissues and organs consisting of multiple cell types but also to guide and support the tissue formation through cellular interactions with other cell types⁷⁰. In cartilage research, co-culture has been used to study the development of osteoarthritis and the homeostasis of the tissue itself^{71, 72}. There were studies co-culturing expanded articular chondrocytes with bone marrow mesenchymal stem cells, which suggested that co-culture was possibly responsible for the differentiation of the expanded chondrocytes in pellets⁷³. Meniscus also consists of multiple cell types and its structure is complicated⁷⁴.

2.4.2 Scaffold Materials

Scaffolds can be separated into two major categories: natural and synthetic. Many natural scaffolds have been used for tissue engineering of the meniscus: agarose, collagen, small intestinal submucosa (SIS), periosteal tissue, and perichondral tissue. A study done by Walsh and co-workers⁷⁵ using periosteal tissue in a rabbit model showed both hyaline cartilage and bone growing in the repair tissue at the end of the 24-week trial. The results from perichondral tissue were not much better; the 12-month test in sheep showed repair tissue that resembled the meniscus grossly, but the tensile modulus of the repair tissue was much lower than native menisci⁷⁶. Agarose was studied by Aufderheide and Athanasian⁷⁷, and they found that agarose maintained a lower cell number compared to the synthetic scaffold poly(glycolic acid) (PGA). Collagen studies done by Walsh and co-workers⁷⁵ and Mueller and co-workers⁷⁸ yielded encouraging results, but no mechanical tests were performed on the repair tissue. SIS, an acellular extracellular matrix (ECM) and collagen-based xenogenetic biomaterial, has been shown to induce site-specific remodeling of various connective tissues and to promote a reconstructive healing response⁷⁹. Due to minimal immune response⁸⁰ and low risk of disease transmission⁸¹, SIS has gained approval for clinical implant in the United States, Europe, and Australia. Recent studies indicated that SIS has been successfully utilized not only as an unseeded form to promote meniscus regeneration^{82, 83}, but also as a seeded scaffold to regenerate tissue constructs, such as bladder⁸⁴, cartilage⁸⁵, and anterior cruciate ligaments⁸⁶. A

potential clinical problem with the unseeded technique is that unseeded grafts may be limited to a finite size of regenerated tissue; this problem may be overcome when SIS is used in a cell-seeded form.

There is a series of synthetic scaffolds containing carbon fibers that were attempted, but mediocre results halted research into the use of these scaffolds. Veth and co-workers⁸⁷ attempted just plain carbon fibers and carbon fibers embedded in a polyurethane-poly(L-lactide) matrix. The gross results for the short term were promising, however no biochemical or biomechanical studies were performed⁸⁷. They also did a study in which they used PLLA fibers instead of carbon fibers; the results were better, but again no biochemical or biomechanical tests were performed⁸⁸. Another group, Wood and co-workers⁸⁹, also tried using carbon fibers; their embedding matrix was polyester. Their results were mediocre overall, with degeneration of the articular cartilage and little ingrowth of tissue occurring.

Another synthetic material, polyurethane, was studied for the tissue engineering of meniscus. Polyurethane was first used in an attempt to improve healing in a longitudinal tear of a canine's meniscus⁹⁰, and it was used to replace the lateral canine meniscus in a later study⁹¹. They noted better results than what occurred after a meniscectomy, but there was still degeneration of the articular cartilage. Another study consisted of a 50-week canine study where the polyurethane was used to replace a portion of the meniscus⁹². Types I and II collagen were found, however no biomechanical tests were performed and degeneration of the articular cartilage was noted. Unfortunately, the aromatic polyurethane used in the previous studies releases toxic particles during degradation⁹³.

PGA is an alternate synthetic scaffold for meniscal tissue engineering. Aufderheide and Athanasian⁷⁷ found that PGA maintained a higher cell number compared to the agarose scaffold. Another group regenerated whole meniscus using meniscal cells and PGA-derived scaffolds in a rabbit total meniscectomy model⁹⁴. They fabricated a biodegradable scaffold in a meniscal shape from PGA fiber mesh that was mechanically

reinforced by bonding PGA fibers at cross points with 75:25 poly(lactic-to-glycolic acid). However, tissue-engineered meniscus showed some differences in collagen content and aggregate modulus in comparison to native meniscus.

2.4.3 Growth Factors

Numerous growth factors have been used on meniscal fibrochondrocytes to test their effects on the healing of tears or defects, or on protein synthesis under tissue or cell culture conditions. Most experiments have used cell culture approaches, which consist of putting fibrochondrocytes or small tissue explants onto a petri dish or into a flask and then checking the proliferative response of the cells or protein synthesis.

One growth factor that has potential is transforming growth factor- β (TGF- β). Studies by Tanaka and co-workers²³ and Collier and co-workers⁹⁵ showed that TGF- β increases the proteoglycan synthesis of fibrochondrocytes from all different sections of the meniscus in a dose-dependent manner. Studies by Spindler and co-workers⁹⁶ and Bhargava and co-workers⁹⁷ tested the effect of human platelet-derived growth factor-AB (PDGF-AB) on ovine and bovine cells, respectively. PDGF-AB was also shown to increase DNA synthesis by the cells from all three sections. Bhargava and co-workers⁹⁷ also found DNA synthesis increases when hepatocyte growth factor (HGF) or bone-morphogenic protein-2 (BMP-2) was used. BMP-2 and insulin-like growth factor-I (IGF-I) stimulated the migration of fibrochondrocytes from the middle zone by 40-50%. This study also tested the effects of two other growth factors: interleukin-1 (IL-1), which stimulated migration of cells taken from the peripheral third of the tissue, and epidermal growth factor (EGF), which stimulated migration of cells from the inner and outer zones by 40–50%. An earlier study by Webber and co-workers⁹⁸ tested the effect that fibroblastic growth factor (FGF) and human platelet lysate (PL) had on proliferation of fibrochondrocytes; both were found to stimulate growth. Other growth factors, including hyaluronic acid, hyaluronan, and endothelial cell growth factor (ECGF), have been studied in both tear healing and defect repair^{99,100,101,102}.

More recent studies^{103,104} compared different growth factors, like TGF- β 1, PDGF-AB, IGF-I, and bFGF; they showed that TGF- β 1, a member of TGF- β group, is the most promising one in meniscal tissue engineering. Individual and combined effects of three growth factors-TGF- β 1, IGF-I, and FGF-2-were evaluated in synovial cells culture with serum-free chondrogenic medium. The results showed the highest rate of cell growth with the synergistic interaction of TGF- β 1 and IGF-I applied simultaneously compared to any other individual or combined growth factors studied^{105,106}.

2.4.4 Culturing Conditions

Fibrochondrocytes have been cultured in monolayer, in alginate beads, in agarose suspension, and on different scaffolds, some of which have been mentioned before. Collier and co-workers⁹⁵ showed that cells seeded in monolayer produce smaller proteoglycans than cells cultured in alginate beads. Studies by Webber and co-workers¹⁰⁷ have shown that fibrochondrocytes cultured in agarose suspension are capable of expressing their differentiated phenotype. Webber and co-workers^{98, 108, 109} have also done a series of studies that tested the effects of different media on fibrochondrocytes. They tested how a media containing either Dulbecco's modified Eagle's medium (DMEM) or Ham's F-12 media, both supplemented with fetal bovine serum (FBS), affected the cells⁹⁸; the media containing Ham's F-12 seemed to cause faster growth of the fibrochondrocytes. Webber and co-workers^{108, 109} also developed a serum-free medium; it performs better than the serum-free media available commercially, but still does not perform as well as media supplemented with FBS. They also developed an organ culture model, which consists of removing a meniscus from a rabbit model, creating a defect, filling the defect with a scaffold, and placing the meniscus in media. Over time, tissue repair can be monitored. Ibarra and co-workers¹¹⁰ have expanded on this organ culture model; the explant was implanted subcutaneously or put on a dynamic culture system, which consists of an orbital shaker placed inside of CO₂ incubator. Their results showed better healing from the subcutaneous procedure.

In recent years, serum-free medium was broadly used in the studies of meniscal or articular cartilage tissue engineering. Noth *et al.*¹¹¹ treated MSCs with defined serum-free

chondrogenic medium and DMEM with 10% FBS in collagen type I hydrogels; they found that MSC-gels cultured in serum-free medium exhibited higher expression of collagen types II and X. Hoben and Athanasiou¹¹² compared serum-free chondrogenic medium 10% FBS medium; their results showed that serum-free medium might enhance the tensile modulus. Another study by Bilgen and colleagues¹¹³ showed that FBS suppressed TGF- β 1-induced chondrogenesis in synoviocyte pellet cultures while dexamethasone and dynamic stimuli were beneficial. Also, Koay and Athanasiou¹¹⁴ established serum-free, chemically defined conditions to generate fibrocartilage with human embryonic stem cells.

2.4.5 Mechanical Stimuli

Cells can sense changes in their mechanical environment and promote alternations and adaptations in tissue structure and function. Mechanical stimuli regulate such fundamental processes as cell division and differentiation and determine tissue form. Mechanical loading is clearly pivotal to the development, function and repair of all the major elements of the musculoskeletal system, including bone, ligament, tendon, skeletal muscle, intervertebral disc and meniscus. The prevailing clinical opinion is that, in treating musculoskeletal injuries, the repair tissue should first be protected from loading, but then subjected to a carefully-measured regime of loading to promote its remodeling¹¹⁵. Movement is even essential for synovial joints to cavitate during development, and it greatly accelerates the healing of adult tissues.

Although all parts of the musculoskeletal system can adapt to changes in mechanical load, the relationship between structure, function and mechanical demands is perhaps best known in bone. Menisci are subject to a complex pattern of compressive, tensile and shear loads. The distinctive pattern of orientation of collagen fibers in the menisci of the knee joint has been suggested to play a role in enabling the menisci to convert compressive load into tensile load, so that the compression on the tibial plateau is reduced²².

Grodzinsky *et al.*¹¹⁶ reviewed the cartilage tissue remodeling in response to mechanical forces, and focused on the effects of mechanical loading on cartilage and the resulting chondrocyte-mediated biosynthesis, remodeling, degradation, and repair of the tissue. The effects of compression and tissue shear deformation were compared, and approaches to the study of mechanical regulation of gene expression were described. The feasibility of using fibroblast-like synoviocytes was studied to engineer meniscal-like fibrocartilage and to better understand the mechanosensitivity of fibroblast-like synoviocytes by seeding them onto collagen scaffolds exposed to dynamic compressive loads¹¹⁷. However, their applied biomechanical stimulus did not appear to induce fibrochondrogenesis in any treatment group on the scaffolds they used. Considering the fact that fibrocartilaginous tissues experience significant tensile stresses in their normal mechanical environment, Vanderploeg and co-workers¹¹⁸ investigated mechanical tension as a means to modulate matrix synthesis and cytoskeletal organization in bovine articular chondrocytes and meniscal fibrochondrocytes (MFCs). Confocal imaging revealed that all cells initially displayed a rounded morphology, but over time MFCs spontaneously developed a three-dimensional, stellate morphology with numerous projections containing organized cytoskeletal filaments. Both the biochemical and morphological results of their study had important implications for successfully developing cartilage and fibrocartilage tissue replacements and repair strategies. Gene expression levels for extracellular matrix and cytoskeletal proteins in disc cells were quantitatively studied by Chen *et al.*¹¹⁹ in an alginate culture system subjected to static unconfined compression (25% compressive strain) after different time periods (2, 18 and 30 h). Differences in gene expression were observed between annulus fibrosus and nucleus pulposus cells following static compression for the matrix proteins studied. Annulus fibrosus cells responded to mechanical deformation at the 30-h time point, with increasing gene expression for types I and II collagen, aggrecan, biglycan, decorin and lumican. In contrast, nucleus pulposus cells were not responsive to mechanical loading with changes in gene expression for these matrix proteins at any time.

CHAPTER 3 MATERIALS AND METHODS

3.1 *Cell Harvest and Isolation*

Random biopsies of the intimal layer of synovial tissue were obtained aseptically from the knees of two 3-month-old pigs and pooled together for the study. After temporary storage in culture medium at 4°C, the synovial tissue was finely minced and digested at 37°C on an x-y-z shaker (Clay Adams® Nutator; BD Biosciences, Bedford, MA) for 30 min in phosphate-buffered saline (PBS) containing 0.1% trypsin (Roche, Indianapolis, IN) and then for 2 h in a 0.1% solution of collagenase P (Roche) in alpha modified Eagle's medium (α MEM)/10% fetal bovine serum (FBS). The cell suspension was passed through a 70- μ m nylon filter, and the cells were collected from the filtrate by centrifugation. Cells were cultured for 4 days in α MEM with 10% FBS and 1 $\times$ PS (100 U/mL penicillin, 100 μ g/mL streptomycin). Non-adherent cells were removed by a PBS wash on days 2 and 4, and the remaining adherent cells were used for further study.

For negative isolation of SDSCs from primary cultures of adherent synovial cells containing macrophages and fibroblasts¹⁰⁶, cells were detached by trypsinization for 1 min (0.25% trypsin/0.2% EDTA), washed, and suspended in PBS/2% FBS (10^7 cell/mL). The suspension was incubated with 5×10^7 /mL Dynabeads® M-450 CD14 containing a monoclonal antibody specific for macrophages (DynaL Biotech, Oslo, Norway) for 1 h at 4°C on an x-y-z shaker. The conjugated cells and the unbound Dynabeads® were collected using the Dynal Magnetic Particle Concentrator® (DynaL Biotech), and the depleted supernatant with SDSCs was transferred to a second tube. After expansion in flasks, passage 3 SDSCs were used for this study.

For the isolation of MCs, meniscus tissue was minced and digested sequentially in 0.05% hyaluronidase (Sigma, St. Louis, MO) in PBS for 5 min, 0.2% trypsin (Roche) in PBS for 30 min, followed by 0.2% collagenase II (300 U/mg, Worthington, Freehold, NJ) in PBS overnight on an x-y-z shaker (Clay Adams® Nutator). The cell suspension was passed through a 70- μ m nylon filter, and the cells were collected from the filtrate by

centrifugation. MCs were cultured in high-glucose (HG) DMEM with 10% FBS and 1× PS in a 37°C, 5% CO₂ incubator. Passage one MCs were used for this study.

3.2 Labeling of SDSCs

Negatively isolated SDSCs were suspended at a density of 1×10^6 /mL in a serum-free culture medium, and mixed with 5 µL of Vybrant DiI cell labeling solution (1:200, Invitrogen, Carlsbad, CA) by gentle pipetting. After incubating for 20 min at 37°C, the mixture was centrifuged at 1500 rpm for 5 min. The cells were ready for use after being washed twice using pre-warmed medium.

3.3 Preparation of SIS

Intact SIS was prepared according to the method previously described by Badylak *et al.*¹²⁰. Briefly, after harvest of porcine ileum, the mesenteric tissue was removed. The mucosa and lamina propria of the luminal side, as well as the serosal and external muscle layers of the adluminal side, were mechanically removed by manual stripping. The resultant submucosa (approximately 2 mm thick) was irrigated copiously in distilled water and disinfected with 0.1% peracetic acid solution. The SIS was frozen at –20°C overnight in dishes followed by freeze-drying for 5 h. The dry SIS sheet was punched into discs with a diameter of 5 mm and sterilized with ethylene oxide. The SIS discs were stored sterile and dry at room temperature prior to use.

3.4 Preparation and Incubation of Cell-Hybrid Scaffold Constructs

Sterile SIS discs were immersed in 100% ethanol, 70% ethanol, and then PBS (without Ca²⁺ and Mg²⁺). In a centrifuge tube, 75 µL fibrinogen (100 mg/mL in PBS, from human plasma; Sigma), 69 µL PBS with cells [three groups: MCs alone, co-culture of MCs and pre-labeled SDSCs (50:50, SM), or pre-labeled SDSCs alone], 3 µL thrombin (0.1 U/µL, from human plasma; Sigma), and 3 µL CaCl₂ (50 mM) were sequentially added. Then, 10 µL of the cell-gel mixture was pipetted onto an SIS disc in a Petri dish. This procedure resulted in fibrin-SIS composites containing 0.9×10^6 cells per construct. The whole process was completed in 3 min. The dish with constructs was transferred into an incubator at 37°C for 10 min. Medium was added to cover the constructs.

After 1 h, the constructs were transferred into non-tissue-culture-treated 24-well plates, and the medium was replaced with a chemically defined medium (HG-DMEM, 40 $\mu\text{g/mL}$ proline, 100 nmol/L dexamethasone, 0.1 mmol/L ascorbic acid 2-phosphate, 1 $\times$ PS, and 1 $\times$ ITSTM Premix) supplemented with a differentiative growth factor cocktail [10 ng/mL transforming growth factor beta1 (TGF- β 1) and 500 ng/mL insulin-like growth factor I (IGF-I)] for 30 days. For those to be cultured in bioreactors, the constructs were transferred into a rotating bioreactor (RCCS-4; Synthecon, Houston, TX) filled with the same medium as previously mentioned. Over the course of in vitro cultivation, the bioreactor rotation speed was adjusted so that the growing constructs remained freely suspended in the rotating flow. Figure 7 is the picture of bioreactors in the incubator.

Figure 7 Bioreactor culture in 37° C incubator

3.5 *Compressive Loading*

The constructs were transferred into custom-designed 6-well plates after a 3 week culture in rotating bioreactors or static well plates; they were incubated for 3 more weeks with compressive loading. The device was designed and built to apply uniaxial compressive displacements to the samples in the plates. We had 2 samples in each well, and they were placed evenly on the bottom of plate wells. Smooth, impermeable platens were brought

into contact with the samples and to held the medium during incubation. The medium was the same as previously defined chondrogenic medium with 10 ng/mL TGF- β 1 and 500 ng/mL IGF-I. A displacement corresponding to 10% compressive strain was applied every hour for 8 hours, and the loading frequency was 0.5 Hz. Loading of the constructs in compression might give rise to transient stresses and strains that vary non-uniformly throughout the sample; however, transient changes in the constructs were minimal or not existent for a time greater than 25 min^{121, 122}.

Figure 8 shows the experimental equipments for compressive loading, including the well plate under compressive loading during the culture process (left top), the tray of well plates (left middle), inside the plate wells and their covers (left bottom), and the console for both bioreactor rotation and compressive loading (right).

Figure 8 Compressive loading devices

Left Top: Six-well plates under compressive loading during culture
Left Middle: Tray for well plates
Left Bottom: Well structure and corresponding covers
Right: Console for bioreactor rotation and compressive loading

3.6 Scanning Electron Microscopy

Representative constructs (n=2) at day 2 were primarily fixed for 2 h at room temperature in 2.5% glutaraldehyde (Sigma) in distilled water, washed in distilled water 3 times for 20 min each time. Secondary fixation was completed in 2% osmium tetroxide (Sigma) in distilled water for 2 h at room temperature, followed by the same wash procedure after primary fixation. The constructs were then dehydrated consecutively in 25%, 50%, 75%, 95%, and 100% (twice) ethanol for 10 min each, and then in hexamethyldisilazane (HMDS, Sigma) at a ratio of 1:1 with ethanol twice for one hour each time, in HMDS at a ratio of 1:2 with ethanol overnight, and in HMDS 3 times for 4 h each time. The constructs were freeze-dried for 24 h. Each construct was cut into two halves with gold sputter on both sides. The images were recorded by a scanning electron microscope (Hitachi, Model S 2400, Brisbane, CA).

3.7 Histology and Immunohistochemistry

Representative constructs (n = 2) were fixed overnight at 4°C in 4% paraformaldehyde (Sigma) in PBS, dehydrated with ethanol, paraffin-embedded, and sectioned to 5 µm. Consecutive sections were stained with Safranin O/fast green for sulfated GAG and were immunostained with monoclonal antibodies against collagen II (II-II6B3; DSHB, Iowa City, IA) and collagen I (Sigma). Immunohistochemical sections were de-paraffinized, hydrated, treated with 1% hydrogen peroxide to inhibit endogenous peroxidase, and incubated for 30 min with 0.2% testicular hyaluronidase (Sigma) in PBS (pH 5) at 37°C followed by another 30 min with 1.5% normal horse serum and 30 min at 37°C with the primary antibody, then stained using a kit (Vectastain ABC, Burlingame, CA), followed by standardized development in diaminobenzidine (DAB, Invitrogen). The sections were counterstained with hematoxylin.

3.8 Biochemical Analysis

For biochemical analysis, constructs (n = 3) were digested overnight at 60°C with 25 µg/mL papain in PBE buffer (100 mmol/L phosphate, 10 mmol/L EDTA, pH 6.5) containing 10 mmol/L cysteine, by using 400 µL enzyme per sample. To quantify cell density, the amount of DNA in the papain digests was measured using the QuantiT™

PicoGreen[®] dsDNA assay kit (Invitrogen) with a CytoFluor[®] Series 4000 (Applied Biosystems). GAG was measured using dimethylmethylene blue dye and a Spectronic[™] BioMate[™] 3 Spectrophotometer (Thermo Scientific, Milford, MA) with bovine chondroitin sulfate as a standard.

3.9 *TaqMan[®] Real Time PCR*

For studies of gene expression, the total RNA was extracted from constructs (n = 3) using an RNase-free pestle in TRIzol[®] reagent (Invitrogen) and RNeasy[®] Mini Kit (Qiagen, Valencia, CA). Sample mRNAs were quantified and 1 µg RNA was used for reverse transcription (RT) with a High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The primers and probes of porcine chondrogenic genes (collagen I, collagen II, aggrecan, and Sox9) were customized by Applied Biosystems as part of the Custom TaqMan[®] gene expression assays (Table 2). Eukaryotic 18S RNA (Assay ID Hs99999901_s1 ABI) was carried out as the endogenous control gene. Real-time PCR was performed with the iCycler iQ[™] Multi Color RT-PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The cycle parameters were 50°C for 2 min, hot start at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The cycle threshold (Ct) values for 18S RNA and that of samples was measured and calculated by computer software (Perkin-Elmer, Wellesley, MA). Relative transcript levels were calculated as $\chi = 2^{-\Delta\Delta C_t}$, in which $\Delta\Delta C_t = \Delta E - \Delta C$, $\Delta E = C_{t_{exp}} - C_{t_{18S}}$, and $\Delta C = C_{t_{ct1}} - C_{t_{18S}}$.

3.10 *Mechanical Test*

The equilibrium compressive moduli of constructs (n=5) were determined in a uniaxial stress relaxation test. The compressive tests were conducted utilizing a stepper motor-driven miniature compression device manufactured in-house that utilized a 5-mm gage length DVRT (Mirostrain, Burlington, VT) for displacement read out and a 50-gram load cell (Entran, Fairfield, NJ) to monitor the load as in our previous studies¹²³. In brief, discs were equilibrated in PBS containing protease inhibitors then placed in the reservoir on the testing machine. The porous movable platen was advanced until a tare load of 0.00001 Newton was detected. Then a 5% strain step was applied to seat the scaffold

Table 2 TaqMan[®] customized porcine gene primers and probes

Gene	Primer/Probe	Sequence (5'-3')	Genebank Accession	PCR product (bp)
Aggrecan	Forward	GCCACTGTTACCGCCACTT	X60107	58
	Reverse	CACTGGCTCTCTGCATCCA		
	Probe	CTGACCGGGCGACCTG		
Col I α 1	Forward	CCGTGCCCTGCCAGATC	AF201723	78
	Reverse	CAGTTCTTGATTTTCGTCGAGATC		
	Probe	TCGCACAACACATTGC		
Col II α 1	Forward	TCCTGGCCTCGTGGGT	AF201724	65
	Reverse	GGGATCCGGGAGAGCCA		
	Probe	CTCCCCTGGGAAACC		
Col X α 1	Forward	GGCCCGGCAGGTCATC	NM_001005153	80
	Reverse	TGGGATGCCTTTTGGTCCTT		
	Probe	TCAGACCTGGTTCCCC		
Sox9	Forward	TGGCAAGGCTGACCTGAAG	AF029696	96
	Reverse	GCTCAGCTCGCCGATGT		
	Probe	CCCCATCGACTTCCGC		

evenly in the fixture. After this strain was applied the load was allowed to equilibrate, then four consecutive 2% strain steps were applied and each allowed to stress relax. The test specimen was unloaded and left for ten minutes to return to its original stress state. Once again the porous platen was advanced until a 0.00001 Newton preload was seen then a 20% strain step was commanded. From this test the Young's modulus and stiffness was calculated.

The tensile tests were performed in another application specific device that utilized a stepper motor as the prime mover and the above-mentioned load cell. The 5 mm diameter constructs were visualized under a binocular scope and two parallel cuts were made to yield a rectangular shape for tensile testing. The rectangular specimen thickness was measured with a vernier caliper and recorded for later use. The specimen was clamped between the two grips of the tensile tester and the geometry of the specimen was measured with a model 308A video caliper (Colorado Video, Boulder, CO). Small reference points were applied to the specimen away from the gripped ends to minimize

the effects of the non-uniform stress field. The small load imposed on the specimen from gripping was tared out and the video caliper measured the gage length between the two reference points at the start. For the tensile equilibrium modulus test, the hydration was supplied via irrigation with PBS containing protease inhibitors. Five consecutive 2% strain steps were applied and the load was monitored until the rate change of 0.006 MPa over one minute was noted. After this test, the specimen was unloaded, kept hydrated and allowed to rest for ten minutes to recover to its unstressed state. To obtain the Young's modulus and stiffness, a single stretch test was performed. A 25% strain step was commanded and the load verses displacement curve was recorded using a data acquisition card das-16 (Measurement Computing, Middleboro, MA) housed in a Dell laptop with Labtech Notebook software (Labtech , Wilmington, MA).

The four or five consecutive 2% strains and corresponding stresses were plotted, and regression analysis was made and the slope was calculated as the equilibrium modulus. For Young's modulus and stiffness, the one step of 20% or 25% strain and corresponding stress was used, and the ratio of stress to strain was considered as the value of Young's modulus. Combining the geometrical parameters of constructs, including cross-sectional area and thickness or length, we calculated the stiffness using the formula

$$k = \frac{AE}{L}$$

where k is the stiffness, A is the cross-sectional area, E is the Young's modulus, L is the length of construct.

3.11 Statistics

We used a one-way ANOVA F test to compare the differences in biochemical analyses ($n = 3$ for each group), and compressive modulus ($n = 5$ for each group) between the groups. Statistical analysis was performed with SPSS 13.0 statistical software (SPSS Inc., Chicago, IL). Post-hoc power analyses were performed using a significance level of 0.05 and a minimum power equal to 0.80, assuming a two-sided alternate hypothesis. Estimates of standard deviation were obtained from the observed samples.

CHAPTER 4 EXPERIMENTAL RESULTS

4.1 Results from Part I

4.1.1 Microscopic Observation

SEM was used to evaluate the morphology of SIS and adhesion of cells on SIS scaffolds. Figure 9A shows the rough serosal side of SIS while Figure 9C displays the smooth mucosal side of SIS. After saturation with fibrin gel, both sides became more wrinkled (Figure 9B versus 9A, Figure 9D versus 9C). One day after cells were seeded with fibrin gel on SIS scaffolds, the scaffold profile exhibited several layers after freeze-drying (Figure 10A); high magnification showed that the cells were round and evenly distributed (Figure 10B) and some cells were distributed in scaffold wrinkles (Figure 10C) and holes (Figure 10D). Cells of all three groups exist almost everywhere and at a high density on the surface of SIS. Moreover, cells on the surface potentially interact with each other and with the SIS surface according to the higher magnified images, which tells us that SIS, a natural biomaterial, may be a biocompatible material for meniscus tissue engineering.

To trace cell migration and distribution on SIS, SDSCs were pre-labeled with Vybrant DiI (red color) and DAPI was used to counterstain nuclei (blue color) for both SDSCs and MCs. At day 0 after cell seeding, only blue was seen in the MC group (Figure 11a₀) and mixed colors were shown in the SDSC group (Figure 11c₀). In contrast, both the mixed colors and isolated blue were seen in the SM group (Figure 11b₀). The corresponding light microscope images (Figure 11A₀, 11C₀, 11B₀) showed that the cells and fibrin gel mixture attached on the surface of the SIS scaffold. At day 7 (Figure 11a_{7-c}/A_{7-C}₇) and day 30 (not shown), fluorescent images indicated that MCs and SDSCs were still evenly mixed with no detectable separation.

Figure 9 Microscopic images of SIS without cells (A: serosal side without fibrin gel; B: serosal side with fibrin gel; C: mucosal side without fibrin gel; D: mucosal side with fibrin gel)

Figure 10 Microscopic images of SIS with fibrin gel and cells, showing the distribution of cells on SIS scaffold

Figure 11 Fluorescent microscopic images and microscopic images for the constructs at days 0 and 7

4.1.2 Histological and Immunohistochemical Evaluation

The results of histological tests are shown in Figures 12-14. Safranin O staining and immunostaining showed that sulfated GAG (Figure 12A-C versus 12a-c) and collagen II (Figure 13A-C versus 13a-c) accumulated with time (day 30 versus day 7) in all three groups. At day 30, the co-culture of SDSCs and MCs yielded tissue constructs exhibiting more interspersed GAG (Figure 12C), collagen II (Figure 13C), and collagen I (Figure 14C). The SDSC group yielded tissue constructs with more thick and dense expression of GAG (Figure 12B) and collagen II (Figure 13B) and less expression of collagen I (Figure 14B) than the other groups.

Figure 12 Safranin O staining to evaluate GAG product of MC (a, A), SDSC (b, B), and SDSC+MC (c, C) at day 7 (a, b, c) and day 30 (A, B, C)

4.1.3 Quantitative Biochemical Assessment

In order to understand the whole trend of cell number and that of GAG product to cell number ratio during the culture process, we adopt relative DNA amount (the ratio of DNA amount at day 7 or day 30 to that at day 0) and relative GAG amount (the ratio of GAG amount to DNA amount) as assessing indices. Figure 15 shows the changes in relative DNA amount. In the MC group, DNA amount decreased dramatically throughout the period; while in the SDSC group, although DNA amount decreased during the earlier

Figure 13 Immunostaining to evaluate collagen type II product of MC (a, A), SDSC (b, B), and SDSC+MC (c, C) at day 7 (a, b, c) and day 30 (A, B, C)

Figure 14 Immunostaining to evaluate collagen type I product of MC (a, A), SDSC (b, B), and SDSC+MC (c, C) at day 7 (a, b, c) and day 30 (A, B, C)

culture, DNA increased later from day 7 to day 30. Even in the earlier stage, the number of SDSCs decreased more slowly than that of MCs. The co-culture group was in the

middle of two control groups, decreasing all the time but slower than MCs only. In the MC group, the adjusted DNA ratio was $49.8 \pm 4.2\%$ at day 7 versus $31.9 \pm 5.2\%$ at day 30 ($P = 0.0003$); in contrast, in the SM group, the adjusted DNA ratio was $59.2 \pm 15.4\%$ at day 7 versus $53.0 \pm 10.6\%$ at day 30 ($P = 0.5201$), while in the SDSC group, the adjusted DNA ratio was $72.0 \pm 11.6\%$ at day 7 versus $77.8 \pm 16.6\%$ at day 30 ($P = 0.5534$). The data showed that the SDSC group had a stable DNA content at the highest level, indicating that SDSCs were able to maintain cell numbers when co-cultured with MCs.

The comparison of relative GAG amount is shown as Figure 16. With incubation in chondrogenic medium, all groups exhibited an increase in chondrogenic index (GAG/DNA) over time. At days 0 or 7, there was no significant difference in chondrogenic index among the three groups. At day 30, however, the chondrogenic index increased dramatically in the SDSC group compared to that at day 7 ($74.70 \pm 5.33\%$ versus 6.50 ± 0.99); the chondrogenic index in the SDSC group was almost 3.3-fold compared to the SM group and 5.7-fold compared to the MC group. Though the chondrogenic index was the lowest in the MC group, the MCs co-cultured with SDSCs exhibited a significantly increased chondrogenic index (22.74 ± 5.33 versus 13.00 ± 1.39).

Figure 15 DNA ratio adjusted by DNA amount at day 0 (* = $p < 0.05$; ** = $p < 0.01$; and * = $p < 0.001$)**

Figure 16 Ratio of GAG to DNA (* = $p < 0.05$; ** = $p < 0.01$; and * = $p < 0.001$)**

4.1.4 Messenger RNA Analysis

Real time PCR was used to quantify the relative amount of meniscus markers or transcriptional factors in mRNA level. Collagen type I, type II, type X, aggrecan, and Sox9 were measured and the results were shown in Figures 17-20. Each mRNA was compared among different groups at three time points.

For collagen type I, there were distinct trends for different groups (Figure 17). MCs had the highest mRNA level of collagen type I (Col I) for day 0, which decreased continuously to day 7 and then to day 30. For the SDSC group, the content of Col I mRNA increased by day 7 and then began to decreasing to day 30. The co-culture group behaved in a similar manner as the MC group, except that for each time point, the total amount of Col I mRNA was smaller than the latter group.

There is a totally different result for collagen type II (Col II) (Figure 18). Compared to day 30, the SDSC group and co-culture group had a negligible amount of Col II mRNA for day 0 and day 7. Although the change in the MC group is small relative to the SDSC and co-culture groups, there existed an increase-to-decrease trend during the culture process.

Figure 17 Collagen type I gene expression using Taqman® real time PCR test and adjusted by 18S RNA

Figure 18 Collagen type II gene expression using Taqman® real time PCR test and adjusted by 18S RNA

Similar to Col II, aggrecan exhibited a continuously increasing tendency in mRNA level for the SDSC group and co-culture group (Figure 19). However, for the MC group, aggrecan mRNA still increased throughout the 30 days, without the turning point that Col II mRNA had.

Figure 19 Aggrecan gene expression using Taqman® real time PCR test and adjusted by 18S RNA

Sox9 is a transcriptional factor, which can up-regulate the expression of COL2A1 and aggrecan genes. The level of Sox9 mRNA also increased throughout the culture period within each group (Figure 20).

Figure 20 Sox9 gene expression using Taqman® real time PCR test and adjusted by 18S RNA

We also examined Collagen type X (Col X) (not shown), which is a specific marker of hypertrophic chondrocytes, and is induced during osteoarthritis progression. We found that, on the transcription level, the MC group increased gradually, while the SDSC group increased first and then decreased, in contrast to the co-culture group, which decreased first and then increased.

4.1.5 Mechanical Evaluation

In order to evaluate the constructs more comprehensively, we measured the compressive modulus for each construct (n=8) at day 30. As shown in Figure 21, the MC group has the lowest compressive modulus (15.55 ± 5.01 MPa), the SDSC group has the highest (35.18 ± 24.16 MPa), and the co-culture group has an intermedial value and closer to the MC group (22.80 ± 4.30 MPa). We also found that the SDSC group has a much larger standard deviation than the MC group and co-culture group.

Figure 21 Equilibrium modulus of tissue constructs from different groups after a 30-day-incubation

Figure 23 GAG content

4.2.2 Histological Evaluation

Similarly, histological evaluations were made to examine the expression of GAG and collagen types II and I. Unfortunately but consistent with biochemical tests, there was no GAG, collagen type II or I produced for all the 6-week samples (Figure 24 is shown on next page and for collagen type II only). From the microscopic images, we know that most cells were lost after the initial culture in bioreactors, and basically only the SIS structure remained. No GAG or collagen type II was produced during the culture period; some collagen type I can be seen for the control samples at day 0.

4.2.3 Mechanical Measurement

We measured both compressive and tensile strengths to examine the mechanical properties of our tissue engineered constructs, and for each, we calculated equilibrium modulus, Young's modulus, and stiffness (Figures 25 and 26). The tensile strengths are significantly higher than the compressive ones, and equilibrium moduli are higher than corresponding Young's moduli. The relative magnitudes of stiffness are consistent with Young's modulus for both compressive and tensile properties. Among different groups, samples undergoing compressive loading turned out to have higher compressive stiffness,

whereas those cultured in bioreactors during the earlier and/or later stage had higher tensile equilibrium modulus. The detailed numbers are not provided here.

Figure 24 Immunostaining for collagen type II

Figure 25 Compressive modulus or stiffness

Figure 26 Tensile modulus or stiffness

CHAPTER 5 ANALYSIS AND DISCUSSION

The goal of this study was to develop *in vitro* meniscus tissue constructs using a co-culture technique with a tissue-specific stem cell on a clinically approved natural biomaterial. Our data showed that MCs, SDSCs, and co-culture of MCs and SDSCs were able to mix with fibrin gel and evenly adhere on SIS, suggesting good biocompatibility between cells and biomaterial. After the initial drop, the cell number of MCs on SIS continued to decrease with incubation time; this trend was reversed when MCs were combined with SDSCs. The addition of SDSCs could also dramatically increase chondrogenic differentiation of MC-based tissue constructs, resulting in the acquisition of higher equilibrium modulus. Our study indicates that functional menisci can be engineered *in vitro* with SDSCs and MCs co-cultured on SIS-based scaffolds in a serum-free chondrogenic medium.

5.1 *Justification of SIS as Scaffold*

As an acellular ECM^{79, 120}, SIS scaffold is composed of 80% to 90% collagen in the form of oriented fibers, contributing to longitudinal tensile properties, and contains glycosaminoglycans as well as entrapped growth factors, such as basic fibroblast growth factor, vascular endothelial cell growth factor, and TGF- β ^{124, 125, 126}. In this study, our SEM data showed that the seeded cells attached on the surface of SIS well and established a base for their interaction with the scaffold, which potentially allowed the delivery and receiving of signals for cell growth, proliferation and differentiation. Fluorescence images confirmed the biocompatibility of SIS and implied that SDSCs and MCs were compatible with each other. SIS is also a biodegradable biopolymer. SIS has been reported to be entirely resorbed three months after implantation in the bladder of dogs and replaced by deposition of a new host ECM through the body's natural healing process, with successful replacement of the damaged urinary tissue¹²⁷. Many experimental studies have demonstrated the feasibility of SIS in tissue engineering, such as for human bone marrow stem cells to proliferate¹²⁸, for tissue engineering bladder from bone marrow stromal cells¹²⁹, and for anterior cruciate ligament tissue engineering⁸⁶. However, the degradation kinematics and communication between SIS and cultured cells

or host tissues are not yet completely understood, and further research is needed to fully understand the capacity of this multifaceted biological system. What is more, since SIS is a naturally derived material, batch-to-batch variations exist. Despite the shortcomings of SIS, it is still an ideal choice for the treatment of many joint injuries.

5.2 Justification of Pre-treating and Seeding Method

We found that the seeded cells were difficult to immobilize on the smooth and saturated SIS scaffold. However, after freeze-drying and re-saturating with PBS, most PBS in SIS can be easily resorbed by sterile gauze while maintaining SIS in hydrophilic condition. In this study, fibrin gel was applied by mixing with cells, which could be easily absorbed by half-dried SIS. As we know, fibrin is the basic framework on which the repair response of most tissues is initiated. In an *in vitro* culture system, meniscus fibrochondrocytes have been shown to be capable of extricating themselves from their surrounding matrix and migrating into a purified fibrin clot¹⁰⁹. *In vivo* studies have also shown the efficacy of an exogenous fibrin clot to support a repair response in the avascular portion of the meniscus^{44, 130}. Therefore, fibrin can be considered a quintessential model for the matrices on which tissue-engineered menisci are created; it provides a deformable scaffold for even cell distribution, migration and orientation, and the ability to disappear over time¹²³. One of the limiting factors in the use of fibrin, however, is the fragile nature of this scaffold. Therefore, the combined use with other clinically approved natural biopolymers (such as SIS) will complement the weakness of fibrin gel due to its lack of mechanical strength.

Although the SIS preparation method includes processes such as acellularization, sterilization, and lyophilization, this scaffold still retains some active growth factors that can contribute to its biologic activity^{125, 131}. As a xenogenic graft, host immunological response should always be of concern. Allman et al. has reported that porcine SIS elicits an immunological response restricted to the Th2 pathway, which is consistent with a remodeling reaction rather than rejection¹³². When surgically implanted, this biocompatible and biodegradable scaffold provides strength for the reinforcement of damaged or weakened soft tissues, and constitutes an alternative to autograft or allograft.

SIS promotes cell proliferation/differentiation, and induces the synthesis of a new tissue structurally and functionally similar to the original damaged tissue¹³³.

5.3 Justification and Validation of Co-culture Technique

We showed that SIS is suitable for cell attachment and growth; however, different cells may behave differently on the surface of SIS. MCs are a kind of adult cell, and their viability throughout the culture process is not as good as the pluripotent SDSCs. The DNA ratio diagram implies that MCs' death dominate for all 30 days, while although SDSCs die in earlier stage, they survive more and even proliferate to a higher DNA amount later. Due to the high viability and ability to proliferate, SDSCs are a potent cell source for meniscus regeneration.

Like articular cartilage, the inner third of meniscus is under a predominantly compressive load and has poor healing potential. In some studies, the repair (meniscus) tissue was reported to be a cartilage-like tissue^{61, 134, 135}. Also, chondrocytes have been successfully used as a cell source to engineer tissue constructs for the repair of a bucket-handle tear and longitudinal-horizontal lesion (avascular portion) of menisci^{136, 137}. Although meniscus and cartilage are two different tissues, the detailed knowledge already available for articular cartilage metabolism might be very helpful in developing future protocols for engineering avascular meniscus tissue. MSCs from bone marrow appear to have a high propensity for cartilage hypertrophy and bone formation^{138, 139} and therefore may not be ideal chondroprogenitors for the repair of meniscus. SDSCs are a promising stem cell for cartilage tissue engineering because they display greater chondrogenic and less osteogenic potential than MSCs derived from bone marrow or periosteum¹⁴⁰. They were also proven to be superior to other sources of MSCs such as adipose tissue and muscle^{68, 69}. Furthermore, meniscus defect regeneration has been shown to alleviate through the migration, proliferation and differentiation of fibroblasts that originates, presumably, from the adjacent synovium and joint capsule^{83, 134}.

In this study, the co-culture of MCs and SDSCs was evaluated for chondrogenic differentiation capacity in a serum-free chondrogenic medium. Our data suggested that,

during the first seven days of incubation, cell number decreased in each group with the MC group decreasing the most and the SDSC group decreasing the least. This may be due to the change in culture conditions from serum-containing to serum-free medium, in which serum-deprivation would result in cell apoptosis¹⁴¹. In addition, monolayer culture favors cell proliferation while three-dimensional culture benefits cell differentiation. During day 7 and day 30, cell numbers continued to decrease in the MC group; in contrast, cell number started to increase in the SDSC group and although the difference was not statistically significant, the results suggested that SDSCs are more receptive to stimulation by TGF- β 1 in maintaining and increasing cell number of pellets compared to MCs. Cell number in the SM group remained stable. Our data indicate that the co-culture with SDSCs contributes to the maintenance of cell number in MC-based tissue constructs.

The co-culture design is of great importance in this study, and possibly in other meniscal regeneration. As mentioned before, SDSCs play a pivotal role in three aspects: (1) since MCs are limited, adoption of SDSCs can be a cell source for meniscal regeneration because of their high capacity in proliferation and differentiation; (2) the existence of SDSCs may increase the expression of collagen type II and aggrecan, which indicates that tissue engineered constructs under such conditions may be more like the avascular inner zone of meniscus; (3) the action of SDSCs is a potential reason for the down-regulation of collagen type X during the whole culture period, which may contribute to the maintenance of healthy status for meniscus cells, rather than progressing into hypertrophy. On the other hand, MCs are equally important in the co-culture system because they can help direct SDSCs to differentiate into specially aimed tissue, based on the principle of interaction between adult cells and pluripotent cells⁷⁰.

5.4 *Justification of Mechanical Measurements*

The functions of load transmission and shock absorption of meniscus require that the tissue itself should bear strong mechanical properties. Previous study¹⁴² provided detailed values of tensile and compressive moduli for different zones of meniscus, including surface, middle, and deep zones. Effective treatments for meniscal tears or defects must attempt to maintain the mechanical properties of native tissues, and mechanical

measurements are pivotal for judging the outcome of our study. There are many different models and corresponding assumptions to evaluate the mechanical behavior of articular cartilage or meniscus: elastic¹⁴³, viscoelastic¹⁴⁴, biphasic¹⁴⁵ and triphasic¹⁴⁶. Based on the assumption of elasticity, the one-step measurement is sufficient to calculate the Young's modulus and stiffness. Although they are both elastic properties and can be measured in one step, Young's modulus is independent of the geometrical properties, while stiffness is related to the cross-sectional area and the length of constructs from our study. Therefore, we measured strain, stress, cross-sectional area and length of constructs, and calculated both. However, this is rather rough for meniscal tissue, since it is much different from elastic materials. Another popular model is the biphasic one, and in this case, equilibrium modulus is the key parameter, since it is not linear throughout the measurement period. In this study, we applied four or five consecutive strains and allowed sufficient relaxation between every two measurement points. During the relaxation process, there is fluid flow within the constructs; at the equilibrium points after relaxation, there is no fluid flow in the constructs. Equilibrium modulus reflects more genuine situation than Young's modulus for biphasic materials, and it is the basic property we measured in our study. In the second part of this study, to evaluate the constructs from different view points, we measured Young's modulus and stiffness apart from equilibrium modulus.

5.5 Discussion about Biochemical Results from Part I

Histological, immunohistochemical, biochemical, and real time PCR tests gave us consistent results. We can describe overall trends for the changes in density or amount of GAG, Col I and Col II. GAG and Col II accumulated over time for all three groups, while Col I results were a little more complicated. From immunohistochemical tests, the MC group changed little and the SDSC and co-culture groups grew denser from day 7 to day 30. From real time PCR tests, the MC group and co-culture group decreased throughout the culture period, while the SDSC group first increased, then decreased. Since GAG is just one chemical component of aggrecan, the biochemical and histological results can keep certain, but not total, consistence with real time PCR analysis.

The differences between collagen types I and II may be explained by the complex structure of meniscus and the differential capacity of SDSCs. Collagen type I accounts for over 90% of the total collagen content in meniscus, much more than collagen type II²⁰. Due to the gradual death of meniscal cells on SIS, Collagen type I decreased correspondingly in the MC group. While under culture in chondrogenic medium, which is defined purposely to induce cartilage formation, the signals for collagen type II expression may be abundant and therefore increase the content of collagen type II. But for the SDSC group and co-culture group, because of the existence of SDSCs, both collagen types I and II accumulated under this specially defined medium. The expression of aggrecan is similar to collagen type II, increasing along with culture time within 30 days. Previous reports indicated that the peripheral two-thirds of the meniscus solely consists of collagen type I, whereas collagen type II comprises a significant portion of the fibrillar collagen in the inner meniscus²¹. Moreover, aggrecan aggregate content is higher in the inner and middle part than in the peripheral part of the meniscus⁹⁵. Our results indicate that the existence of SDSCs can help increase of the collagen type II and aggrecan content in meniscus, which makes the tissue engineered constructs more like the inner part of natural and host tissue, which is the avascular zone that is difficult to treat.

Concerning the differences between real time PCR analysis and other results, we speculate that it is because they are evaluated from different levels. Real time PCR is at the mRNA level and other testing is at the protein level or equivalent, where change would lag behind those in mRNA. Therefore, in the SDSC group, although the amount collagen type I mRNA decreased from day 7 to day 30, we saw an accumulation of collagen type I from immunohistochemical images due to the increase in collagen type I mRNA during the earlier stage. The same is true in the MC group for collagen type II.

Collagen type X is a specific marker of hypertrophic chondrocytes, and occur exclusively in hypertrophic cartilage of the growth plate, which is an essential feature of cartilage degradation^{147, 148}. There exists no collagen type X in normal meniscus, as reported by Eyre and Wu²⁰ and by McDevitt and Webber⁶. The increase of collagen type

X mRNA for MCs in our study may indicate that under our defined chondrogenic medium, normal meniscus cells may differentiate into hypertrophy and initiate osteoarthritis, in a manner similar to the pathology from hypertrophic chondrocytes. Fortunately, the existence of SDSCs may inhibit the transcription and expression of collagen type X, maintaining the health of the construct, which is another potentially important effect of SDSCs in the co-culture system. However, hypertrophy has not been confirmed, and the underlying mechanism of inhibition needs to be clarified.

The transcriptional factor Sox9 remains at high levels in fully differentiated chondrocytes, in a remarkably similar expression pattern to Col2a1¹⁴⁹. A chondrocyte-specific enhancer in the gene for collagen type II is a direct target for Sox9, and consequently it plays a major role in chondrogenesis^{150, 151}. In our study, the increase of Sox9 mRNA and further Sox9 protein will be beneficial to the expression of collagen type II. This is also consistent with the increasing tendency of collagen type II as stated before. Even for the MCs alone, Sox9 and collagen type II accumulate during the culture period and under our defined medium.

5.6 Discussion about Biomechanical Results from Part I

As for biomechanical measurement, the co-culture group has similar results to the MC group compared to the SDSC group. This is interesting and exciting because the meniscus tissue, which naturally consists of meniscus cells as used in the MC group, is the very goal we want to reach. Furthermore, the compressive modulus of SDSCs alone vary a lot more than MCs and co-culture constructs, which may indicate that the existence of MCs is favorable to keep the compressive modulus in a certain range, rather than fluctuating severely. However, previous studies reported that the compressive aggregate modulus of human menisci is 0.22 MPa¹⁵². This difference may be explained by the different hosts, the differences between *in vivo* tissues and *in vitro* constructs, and the contribution of SIS. Notwithstanding, the result that the compressive modulus of the MC group is lower than SDSCs, a kind of pluripotent cells able to differentiate into chondrocytes under these conditions, is consistent with previous data^{142, 153}.

Another important property of meniscus, tensile strength, was not measured in this pilot study. We did not evaluate this is that in the first part of the study, because we were focused on validating the use of SIS and the biochemical composition of engineered constructs. We know that biochemical composition could determine the functional properties of tissues, so we chose to study the biochemical components first. At the same time, one of the important mechanical features, compressive strength, were tested to confirm the result of biochemical assessment and was compared between groups to obtain useful information for further comprehensive studies.

5.7 *Trouble Shooting for the Biochemical Failure of Part II*

Unfortunately, although we tried several times in the second part of this study in which we used bioreactor incubation and compressive stimulation, we failed to produce any positive data showing the strengths of either bioreactor incubation or compressive stimulation. Instead, we found that the amount of GAG produced within 3 weeks, even within 6 weeks, decreased rapidly relative to the starting point, which is shown in Figure 13. Also, we did Safrinin O staining and immunostaining for collagen types I and II; unfortunately, they did not work as we expected. To better understand these and to avoid the problems in future studies, we analyzed all the factors involved in the second experiment, and made comparisons with the first part to support our analysis.

5.7.1 Cells

The first component we considered was the cells, including MCs and SDSCs. We noticed that the cells used in the two study parts were harvested from different pigs and at different times. The pigs were acquired from the same slaughterhouse, where they were raised routinely. The MC harvest procedures were the same as described in the Methods section; after cell harvest, MCs of passage 1 were stored for use in both experiments. While, SDSCs underwent different treatments: for part I, SDSCs were negatively isolated using Dynabeads[®] M-450 CD14 and a monoclonal antibody specific for macrophages for 1 h at 4°C on an x-y-z shaker; for part II, SDSCs were stored for later use without this treatment. This is one possible reason for the different outcomes between the two experiments. SDSCs without negative isolation contained macrophages and fibroblasts¹²³,

^{154, 155} which may contribute to inflammation and tissue injury as proposed by Glaros *et al.*¹⁵⁶. We suspect that macrophages which were not negatively isolated in the second experiment, probably destroyed the normal metabolism of MCs and/or SDSCs. Thus, even if these cells successfully seeded on the scaffolds, the cells might die quickly or may not excrete matrix proteins as usual. One corresponding suggestion for future research is that SDSCs be stored and used only after negative isolation using Dynabeads[®] M-450 CD14 and a monoclonal antibody specific for macrophages.

5.7.2 Scaffold

Another important element in our study is the scaffold SIS and related treatments. After examination, we found that the scaffolds used in the two experiments were exactly the same, and they were prepared following the identical protocol as described before. Indeed, we observed that the two sides of SIS are different, and even further, the SIS used in Part I possibly had two layers (Figure 29). We did try to split the SIS into two separate layers and to seed cells on each, in order to understand better growth, proliferation, and differentiation on the scaffold. We unfortunately found that each individual layer of SIS was too thin to hold the cell suspension when we seeded the cells as described before. Therefore, we treated the scaffold following exactly the same procedure as in Part I. Moreover, the seeding method with fibrin glue did not change at all, which can further exclude the possibility that the scaffold SIS or related treatments brought about the failure of Part II, which aimed to optimize the culture conditions using bioreactors and compressive loading.

Figure 27 SIS before freeze-drying
Left: before split Right: after split

5.7.3 Medium and Growth Factors

Similarly, we examined the chemical factors of the chondrogenic medium we used during incubation. We used the same high-glucose DMEM together with proline, dexamethasone, ascorbic acid 2-phosphate, PS, and ITSTM Premix, as well as TGF- β 1 and IGF-I. All chemicals were newly purchased and prepared, with the exception of the growth factor TGF- β 1, which was prepared two years ago, stored at -80°C before use, and used in both studies. The concentrations of each chemical agent were kept consistent in our studies. One point to be mentioned is that in Part II, we filtered the medium with the chemicals to avoid contamination; we used a 22- μ m nylon filter. We do not think this additional treatment would remove the chemicals and growth factors before incubation for cell-hybrid scaffold constructs. This supposition can be supported by the relationship between Molecular Weight Cut-Off (MWCO) of filters and the molecular size of the chemicals involved. Tables 3 and 4 specify the molecular weight of chemicals in use and the relationship between MWCO and the diameter of membranes, respectively.

Table 3 Chemicals and corresponding molecular weights

Chemical	Molecular weight	Chemical	Molecular weight
TGF- β 1	25,000	Dexamethasone	392
IGF-I	7649	ITS TM Premix	
Proline	115	Insulin	5808
Penicillin	388	Transferrin	~80,000
Streptomycin	582	Sodium selenite	173
Ascorbic acid 2-phosphate	380		

Table 4 Molecular weight cut-off (MWCO) and membrane diameter

MWCO/Da	Diameter/nm
300,300	140
200,100	55
5,050	30
3,030	22
2,020	18
1,010	15

5.7.4 Hypoxia Incubation

Concerning the culture conditions, we found that one important factor was changed between experiments. In Part I, we cultured the constructs in a hypoxic environment while in Part II, we incubated all bioreactors, static plates, and compression-loaded plates under normal oxygen concentration (20% O₂ versus 5% O₂). This change might have given rise to the completely different expressions of proteins. Adesida *et al.*¹⁵⁷ investigated the potential of human meniscus cells to produce matrix in three-dimensional cell aggregate cultures with a chondrogenic medium at low (5%) and normal (20%) oxygen tension. The expression of type II collagen was greatly increased (400-fold) by culture in 5% oxygen. The gene expression of matrix-associated proteoglycans (biglycan and fibromodulin) was also increased by hypoxia. They concluded that, after expansion in monolayer, MCs could respond to low oxygen tension during aggregate cultures. The same group found that human meniscus cells expressed hypoxia inducible factor-1alpha and increased Sox9 in response to low oxygen tension in cell aggregate culture. They revealed that inner meniscus cells were less responsive to 5% oxygen tension than were outer meniscus cells, and they were both more sensitive than articular chondrocytes from a similar joint. These results suggested that the vasculature and greater oxygen tension in the outer meniscus might help to suppress cartilage-like matrix formation¹⁵⁸.

5.7.5 Rotation and Compression

The most obvious difference in treatment between the two parts is that in Part I, we utilized complete static culture, and in Part II, we used rotating bioreactors and compressive loading, both of which would generate significant physical forces during incubation. Although many studies have been done with rotating bioreactors or compressive loading^{117, 159, 160}, we suspect that the combined use of SIS and fibrin glue in our study might have caused the problems. The structure of SIS is much more compact than other synthetic materials that have been used with fibrin glue in cell culture and tissue engineering with PGA being an example¹⁶¹. The cell-fibrin hybrid cannot migrate into the scaffold fully, thus there is non-homogeneity throughout the disk. This can also be observed and demonstrated in our microscopic images of day 0 samples. For static culture, this may not affect cell growth, proliferation and differentiation, only if the cells are retained within the fibrin glue; under the shear force of the bioreactor rotating, the concentrated fibrin glue may degrade much more quickly than normal, releasing the cells into culture medium, which would be changed every 3 days. Although there are no direct studies that show the degradation kinetics of fibrin glue in a shear environment, we did observe that there was suspended substance in culture medium, especially in that of 1 day after placing the cell-glue-scaffold hybrid in bioreactors. We precluded the reason of contamination for this phenomenon, since we paid close attention when executing the experiments and the medium turned clear after the medium change. Fast degradation of fibrin glue under rotating shear would be an influential factor for the cell loss in our study.

Applying different forces but similarly, the compressive loading would possibly accelerate gel degradation in our study. Presumably, this kind of action would not significantly degrade the gel in medium, since the constructs, including the gel on them, are statically placed in wells. However, Kang *et al.*¹⁶² studied the elasticity of fibrin gels and explained their nonlinear response. They found that the ratio of normal stress to shear stress is one parameter that distinguishes semiflexible and rigid filament models, and is also a factor affecting the elasticity of fibrin glue. Moreover, the non-homogeneous distribution of fibrin gels in SIS would further contribute to quick degradation of the glue, resulting in the loss of cells within the glue. This effect is similar to that of bioreactor

rotation: they both produce physical forces onto the constructs and specifically on the fibrin glue; the non-homogeneous distribution of gels may contribute to gel degradation in both cases.

5.8 Discussion about Biomechanical Results from Part II

Compared to natural meniscal tissues, the compressive or tensile strengths are much lower for the tissue engineered constructs in this study 错误！未定义书签。. This relative weakness might be attributed to the scaffold SIS, the major component of our constructs, which were porous, and the cells, which did not penetrate throughout the scaffold soundly. As shown in the histological images, the inner structure of SIS was rather loose, and possibly contributed to the weak mechanical properties. Meanwhile, common features were found between the natural tissues and our engineered constructs: tensile strengths were significant higher than compressive ones. This is solidly supported by the data from previous studies 错误！未定义书签。, and provides a potential method to better engineer the meniscal constructs *in vitro*, if the problem of cell loss can be resolved.

Young's modulus is a measure of the stiffness of an isotropic elastic material. It is also known as the Young modulus, modulus of elasticity, elastic modulus (though Young's modulus is actually one of several elastic moduli such as the bulk modulus and the shear modulus) or tensile modulus. It is defined as the ratio of the uniaxial stress over the uniaxial strain in the range of stress in which Hooke's Law holds. The stiffness is a measure of the resistance offered by an elastic body to deformation (bending, stretching or compression). Theoretically, the relative magnitude of stiffness is to some degree consistent with that of Young's modulus, and the formula for their relationship is

$$k = \frac{AE}{L}$$

where

k is the stiffness,

A is the cross-sectional area,

E is the Young's modulus,

L is the length of the element.

This relationship was qualitatively confirmed by our data.

Fox *et al.* studied the feasibility of using fibroblast-like synoviocytes to engineer meniscal-like fibrocartilage under dynamic compressive loads¹¹⁷. However, their applied biomechanical stimulus did not appear to induce fibrochondrogenesis in any treatment group on the scaffolds they used. Considering the fact that fibrocartilaginous tissues experience significant tensile stresses in their normal mechanical environment, Vanderploeg and co-workers¹¹⁸ investigated mechanical tension as a means to modulate matrix synthesis and cytoskeletal organization in bovine articular chondrocytes and meniscal fibrochondrocytes (MFCs). Chen *et al.*¹¹⁹ found differences in gene expression between annulus fibrosus and nucleus pulposus cells following static compression for the matrix proteins studied. These conflicting studies needed to be clarified with confirmative data. Our studies showed that compressive loading may increase the compressive stiffness or Young's modulus, and bioreactor rotation is a possible cause for the increase in tensile equilibrium modulus. Lack of cell growth, proliferation, and differentiation for the constructs, there are no proofs to demonstrate that these changes in mechanical properties are due to the biochemical composition of the constructs. On the other hand, the results imply that either compressive loading or bioreactor rotation might change the mechanical properties of the scaffold itself, which were kept in medium.

CHAPTER 6 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In this study, an innovative tissue engineering method was attempted to reconstruct meniscus-like tissues *in vitro*. SIS, which demonstrated good cell attachment and viability, was tested to be a promising scaffold material for *in vitro* tissue engineering. Its porous structure potentially provides plenty of room for cell growth and proliferation, and its natural biocompatibility theoretically allows clinical and *in vivo* trials in future studies. Also, SIS itself may contribute to the mechanical properties of the constructs engineered; apart from the chemical ingredients it contains, it evolves into the constructs.

Co-culture of MCs and SDSCs proved a successful way to tissue engineer meniscal constructs *in vitro*, especially for the inner avascular region. SDSCs potentially act in three main aspects: (1) to help solve the problem of limited cell source for meniscal regeneration because of their high capacity in proliferation and differentiation; (2) to increase the expression of collagen type II and aggrecan, which indicates that tissue engineered constructs under such conditions may be more like the avascular inner zone of meniscus, for which few therapy methods exist; (3) to down-regulate the expression of collagen type X during the whole culture period, which may contribute to the maintenance of health for MCs, rather than progressing into hypertrophy. On the other side, MCs are equally very important in the co-culture system because they can help to direct SDSCs to differentiate into special meniscal tissue.

The second part of this study is a preliminary study for further application of co-culture technique with MCs and SDSCs on SIS. Although it failed to produce meniscal constructs with the expected composition and properties, we analyzed carefully and attempted many explanations, which would be important clues for future studies. After comprehensive scrutiny, there were several possible reasons for the unexpected results. The SDSCs without negative isolation before use could be an important cause, since they contained macrophages that may play a detrimental role in meniscal growth and differentiation. The second component, scaffold SIS together with fibrin gel, distributed

in a non-homogeneous manner throughout the scaffold; this might accelerate the degradation of fibrin gels and the loss of cells as no meniscal matrix was produced. As to culture conditions, normal oxygen concentration rather than hypoxia in this part may also suppress matrix formation, which is the most likely reason for the relative low matrix content for the static culture samples after three weeks. The final and most obvious factors, bioreactor rotation and compressive loading, would be the physical reasons for the cell loss and lack of final matrix in these cell-fibrin-SIS hybrid constructs, compared to the positive results from the first part of this study.

Finally, mechanical tests implied that tensile strengths of developed constructs were higher than compressive ones, and this is consistent with natural meniscus. Stiffness of the constructs was relatively proportional to Young's modulus, and also determined by the geometrical properties of constructs, as we calculated. The differences between groups in compressive stiffness or tensile equilibrium modulus might be caused by physical conditions, such as compressive loading or bioreactor rotation, since no direct biological and chemical data from this study could support the differences in mechanical properties.

6.2 Recommendations

SIS has been suggested as a good biomaterial for meniscal tissue engineering *in vitro*. However, the degradation kinematics of SIS and communication between SIS and cells are not yet fully understood. A good and full understanding of SIS is of great help for the clinic use of SIS, and deserves further research. Similarly, the kinetics of fibrin gel degradation needs further clarification, especially for its use in such cell-gel-scaffold hybrid cultured in a rotating bioreactor or under compressive loading.

Negative isolation may be critical for SDSCs and their role in the co-culture system for *in vitro* meniscal reconstruction. It is suggested that further studies negatively isolate the SDSCs before store and use. At the same time, new seeding method with higher efficiency should be attempted to generate a homogeneous distribution of cells

throughout the whole scaffold. This is very important to meniscal construct engineering for clinic use, especially for the inner “white zone” of menisci.

As for culture conditions, hypoxia is recommended for *in vitro* functional meniscal tissue engineering. Hypoxia is likely to favor the matrix formation of cartilage or meniscus based on literature and our previous studies. Bioreactor culture and compressive loading can be further studied to optimize the culture conditions for meniscal reconstruction.

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APPENDIX PUBLICATIONS

Abstract

- Chen S, Chen DM, He F, **Tan YB**, Pei M. Synovium-derived stem cell: a potential candidate for intervertebral tissue engineering. *2008 E. J. Van Liere Convocation and Research Day*, WVU, April 22-23. (Poster presentation)
- **Tan YB**, Zhang YY, Kish VL, Pei M. Meniscus tissue engineering using synovium-derived stem cells and small intestinal submucosa. *55th Annual Meeting of the Orthopaedic Research Society*. Las Vegas (2009). Trans ORS 34:1207. (Poster presentation)
- He F, **Tan YB**, Chen S, Pei M. Reconstruction of an in vitro microenvironment to rejuvenate synovial stem cells for chondrogenesis. *2009 E. J. Liere Convocation and Research Day*, WVU, April 28-29. (Poster presentation)

Manuscript

- **Tan YB**, Zhang YY, Pei M. Meniscus reconstruction through co-culturing meniscus cells with synovium-derived stem cells on small intestinal submucosa. *Tissue Eng* 2009 (in revision)