IN VITRO SYNOVIAL FIBROCHONDROGENESIS FOR MENISCAL TISSUE ENGINEERING

A Dissertation
Presented To the Faculty of the Graduate School
University of Missouri- Columbia

In Partial Fulfillment
Of the Requirements for the Degree

Doctor of Philosophy

By

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DECEMBER 2008

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IN VITRO SYNOVIAL FIBROCHONDROGENESIS

FOR MENISCAL TISSUE ENGINEERING

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ACKNOWLEDGEMENTS

This work is dedicated to the mentors and collaborators who have contributed to my research training; my doctoral and residency advisor, Dr. Derek Fox, for his guidance and dedication to my development as a surgeon- scientist; the inspiration and excellent example of the members of this doctoral committee, Dr. Jimi Cook, Dr. Carol Reinero, Dr. James Tomlinson, and Dr. Charles Wiedmeyer; the University of Missouri Comparative Orthopaedic Laboratory team, including Dr. Aaron Stoker, Mary Cockrell, and Donna Whitener; and past research mentors, Dr. Brian Beale, Dr. Kurt Schulz, Dr. Mary Christopher, and Dr. Dale DeNardo for fostering the scientific curiosity and early research which led to the pursuance of this degree. I wish to also give profound thanks to my parents, Patricia and Kirk Warnock, and my sister Julie Warnock, for their endless support.

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LIST OF ABBREVIATIONS

ASM: alpha smooth muscle actin

bFGF: basic fibroblast growth factor

BMP: bone morphogenic protein

Col2a1: collagen alpha 1(II)

DMEM: Dulbecco's modified Eagle's media

DNA: deoxyribonucleic Acid

ECM: extracellular matrix

FLS: fibroblast- like synoviocytes

Frzb: Frizzled- motif Associated with Bone Development

GAG: glycosaminoglycans

IGF-1: insulin-like growth factor-1

IHC: immunohistochemistry

IL: interleukin

MMP: matrix metalloproteinase

OA: osteoarthritis

OPLA: open-cell poly-L- lactic acid

PG: proteoglycan

PGA: poly- glycolic acid

PGE-2: prostaglandin E2

PLLA: poly-L- lactic acid

RGS10: Regulator of G- Protein Signaling

rpm: rotations per minute

RNA: ribonucelic acid

RT-PCR: real- time reverse- transcriptase polymerase chain reaction

SOX-9: Sry-type Homeobox Protein-9

TGFβ-1: transforming growth factor beta-1

TNFα: tumor necrosis factor alpha

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ABSTRACT

A series of *in vitro* studies were performed to evaluate the fibrochondrogenic potential of synovial membrane cells, for the long term goal of inducing meniscal fibrocartilage healing and regeneration. The first step in this tissue engineering process was to determine viable synoviocyte culturing conditions, including scaffold type and biomechanical environment. Synovial membrane cells were seeded on OPLA (open cell polly-lactic acid) and PGA (poly-glycolic acid) scaffolds, and cultured in a static environment or in a rotating bioreactor at 51.1 rpm for 12 days. PGA constructs cultured in a rotating bioreactor maintained the highest cellularity and had the highest mean sulfated glycosaminoglycan content, while PGA constructs cultured in static conditions had the lowest cellularity. Dissolution of the PGA scaffolds was also noted. All cell scaffold types and culture conditions produced non-uniform cellular distribution. It was concluded that synoviocyte seeded PGA scaffolds cultured in a rotating bioreactor resulted in the most optimal cell and matrix characteristics seen in this study.

To address the premature PGA scaffold dissolution, PGA scaffolds were coated with 2% and 4% PLLA. Constructs were cultured in the rotating bioreactor and cell viability, distribution, and number were compared on days 14 and 21 of culture. Increased cellularity was noted over time; however, the PLLA coating resulted in severely clumped cellular distribution, and did not prevent rapid dissolution of the PGA scaffolds. Cellular viability ranged from 50% to 90%. It was concluded that PLLA coated PGA scaffolds are not appropriate for synovial tissue culture.

To induce a synovial fibrocartilage phenotypic shift, synovial membrane cells were cultured in a rotating bioreactor, on synthetic polymer scaffolds, under sequential influence of growth factors for 16 and 26 days. The scaffold utilized was a novel synthetic copolymer of 10% poly-L-lactic acid and 90% poly-glycolic acid. One group of cells was exposed to basic fibroblast growth factor (bFGF) only, while another group was exposed to basic fibroblast growth factor (bFGF), followed by transforming growth factor beta- 1(TGFβ-1), and insulin like growth factor- 1(IGF-1). Collagen I gene expression increased over time, and Collagen II and aggrecan gene expression were detected only at the 26th day harvest point. Both growth factor protocols resulted in synovial fibrochondrogenic differentiation as seen by expression of collagen I, II, and aggrecan genes. In particular, the combination of FGF followed by sustained IGF and TGF-β resulted in the highest expression of aggrecan, a constituent of the axial avascular meniscus. The novel polymer blend scaffold showed premature dissolution during the study as well.

The focus of the next study was to produce tangible fibrocartilaginous matrix from synovial membrane cells, by increasing the bioreactor cell seeding density to 9.5 million cells/mL, versus the 400,000 cells/ mL utilized in the previous studies. While this increase in cell culture concentration was critical to producing grossly visible fibrocartilage- like tissue, this tissue synthesis was sporadic, and cell mortality was high. The cellular death likely represented insufficient nutrient delivery to such a high cell count in the rotating bioreactor. Premature scaffold dissolution also occurred and likely contributed to cell death as well.

Autologous osteoarthritic synovium may be a candidate for a cell source for meniscal fibrocartilage tissue engineering. To investigate this fibrochondrogenic potential, normal and osteoarthritic synovial membrane cells were cultured in monolayer with or without the growth factors bFGF, TGFβ-1, IGF-1. In addition, the expression of genes involved in embryonic chondrogenesis, SOX-9, Frzb, and RGS10 were tested, as potential future targets for *in vitro* recapitulation of embryonic chondrogenesis. It was found that i*n vitro* fibrochondrogenesis was

enhanced by growth factor stimulation; while osteoarthritic synoviocytes can undergo a fibrocartilage phenotypic shift, their production of collagenous ECM was less than normal synoviocytes. Expression of SOX-9 and RGS10 were expressed at the highest levels by the osteoarthritic cells, and may be good targets for future optimization of *in vitro* fibrochondrogenesis. Autogenous osteoarthritic synovium may be a viable cell source for meniscal tissue engineering, however, osteoarthritic synoviocytes may require longer culture times or other special treatments compared to normal synovium.

Chapter 1. Introduction

As the human population grows exponentially and the need for cutting edge medical care expands globally, the worldwide demand for replacement tissues and organs has outstripped suitable donor organ and tissue supply. As of Dec 31, 2007, 97,670 people were on the visceral and cardiac organ transplant waiting list in the United States alone, representing an increase in 84% over the past 10 years. Approximately 900,000 musculoskeletal allografts are placed in American patients each year. In humans, organ and tissue donation is dependent on altruistic donation with highly specific donor requirements, limiting the amount of available tissue. High demand has lead to nefarious activities such as 3rd World "organ transplant tourism" and criminal cadaver tissue harvest. Even patients that avoid death or disability when receiving donor organs or tissues face the possibility of acute or chronic tissue rejection, disease transmission, infection, primary disease progression, mechanical organ mismatch, or lifelong immunosupression. Obviously, a medical paradigm shift is necessary to address this problem.

Creating the needed tissues and organs in the laboratory through tissue engineering may provide an alternative to this tissue source problem. Tissue engineering is the science of producing living replacement tissue for the purpose of complete replacement organogenesis, or enhancement of native tissue repair. This multi- disciplinary feat is accomplished through *in vitro* manipulation of cells' differentiation and extracellular matrix formation through chemical and physical stimuli. Growth factors and cytokines are the cellular language of tissue formation in healing processes and embryonic organogenesis, and are commonly used to induce the desired tissue formation in the laboratory. In the native tissue, cells produce, reside within, and communicate through extracellular matrix (ECM); in the laboratory this matrix must be recreated in three dimensions, which can be done with synthetic polymers, gels, and natural substances such as collagen. Biomechanical stimulation is also used to guide cellular differentiation and

ECM proliferation, via cell culture in bioreactors such as spinner flasks, rotating wall bioreactors, and perfusion units.

Of the musculoskeletal organs requiring tissue engineering strategies, the knee meniscus poses particular clinical need. The knee menisci are two c- shaped fibrocartilages anchored to the tibia (medially) and the tibia and femur (laterally), and serve to absorb and distribute weight bearing forces on the tibial plateau, resolve femoral-tibial incongruity, and lubricate the joint; they also have proprioceptive function. These organs are composed of circumferential bands of collagen I bound by radial tie fibers, which convert the compressive forces of weight bearing in to hoop stresses. It has now become an accepted fact that without these structures, painful, debilitating arthritis in the knee will develop. The knee menisci are commonly injured in human beings, either primarily or in conjunction with other ligamentous injury, and can also become degenerative with age. In dogs, the menisci are frequently torn in conjunction with cranial cruciate ligament deficiency. With the advent of arthroscopy, meniscal tearing has also been documented as a naturally occurring cause of hind limb lameness in the equine athlete.

The principal challenge of dealing with meniscal injury is that this organ has a limited ability to heal. The outer 25-30% of the meniscus is vascularized; tears in this region, if no larger than a few millimeters, may be able to heal on their own, and surgically repaired vascular zone tears carry a good clinical prognosis. Depending on species and patient age, the inner 70--75% of the axial meniscus is avascular and does not heal spontaneously. Not only is this poor avascular meniscal repair response correlated with the meniscal vascular supply, but the meniscofibrochondrocytes located in the axial portion of the meniscus have an intrinsic decreased ability to heal and proliferate ECM. The avascular, dense collagenous ultrastructure that is responsible for the healthy meniscus' function is the bane of its adaptation and response in the diseased state. Numerous attempts have been made to induce durable, clinical meniscal healing, such as meniscal darts and suture, meniscal rasping, vascular channels, trephination, fibrin clot, vascularized pedicle flap, and porcine intestinal submucosa implantation. These techniques have

all resulted in variable clinical outcomes, particularly in the treatment of avascular meniscal tears. Meniscal transplant is an option for some severely damaged menisci, however, size and shape mismatch, as well as evidence of late immune reaction are additional complications that preclude the universal use of this procedure. Some rabbit models show successful meniscal healing or regeneration with seeded scaffold implantation, however, to date, a steadfast method to completely restore meniscal form and function in species with naturally occurring meniscal injury has not been reported. Consequently, in both human and veterinary patients, to resolve the pain, popping, and joint locking associated with a torn meniscus, tears in the avascular region of the meniscus are treated by partial menisectomy. Despite surgery's providing temporary relief, patients receiving partial menisectomy will still have progression of painful osteoarthritis.

Using tissue engineering to induce meniscal healing or completely rebuild a meniscal fibrocartilage also has significant procedural hurdles that must be addressed before clinical application. One of the most basic quandaries of tissue engineering is determining the ideal source of progenitor cells. Adult tissue progenitor cells are present in nearly every tissue type in the body, such as bone marrow, fat, blood, and skin; these cells, given specific culture conditions, are able to undergo differentiation into target tissue. Synovium also contains synovial mesenchymal progenitor cells, which can undergo chondrogenesis *in vitro*; clinically, this has been observed as synovial chondromatosis. In relation to the knee meniscus, synovial tissue and cells play a role in meniscal healing and pseudo-regeneration. Synoviocytes also mediate fibrin clot induced fibrocartilage formation in meniscal defects, and contribute to avascular meniscal allograft repopulation. As synovium does have the potential to form hyaline cartilage extracellular matrix constituents and undergo chondrocytic differentiation, synoviocytes may be able to serve as an abundant cell source for engineering meniscal fibrocartilage.

To develop the basic understanding of *in vitro* synovial membrane cell culture for the purpose of meniscal fibrocartilage engineering, this series of studies was performed to determine

basic tissue engineering requirements for synovium, such as culture conditions, viable scaffold types, cultured cell density, and growth factors required to induce fibrochondrogenesis.

Chapter 2. Literature Review

Meniscal Structure

The knee menisci are concave, semilunar-shaped fibrocartilages, wedge-shaped in cross section, and have a thin synovial intimal covering. They are composed primarily of water, type I collagen, type II collagen, and proteoglycans. [1] In the center of the meniscus, collagen I bundles are arranged in a circumferential orientation and bound by perpendicularly oriented radial tie fibers, to accommodate weight bearing forces.[1] Regionally, an individual meniscus consists of a body and a cranial and caudal horn. The cranial and caudal horns of the medial meniscus are attached to the tibial plateau by strong meniscotibial ligaments, which hold the meniscus in place during weight bearing.[2] The body of the medial meniscus also has a close association with the medial collateral ligament and joint capsule. The lateral meniscus is anchored cranially by a meniscotibial attachment, however in dogs, cats, rabbits, and horses, the caudal horn associates with the femur via the meniscofemoral ligament. Seventy percent of humans have a meniscofemoral ligament that is divided into the posterior meniscofemoral ligament of Wrisberg and the anterior meniscofemoral ligament of Humphrey.[3,4] The meniscal horns and attachments are nerve and blood vessel rich, and have important sensory functions.[5]

The major cells of the meniscus are the meniscofibrochondrocytes, which are uniformly distributed and sparse in number.[6] These cells lie in the pericellular matrix in pseudo lacunae on collagen network strands, strand nodes, or within thicker tie- fiber collagen bundles.[6-9] The meniscofibrochondrocytes show 3 phenotypes as round, polygonal, or ovoid shaped cells, and can be seen singly, in pairs, or short rows, similar to cartilage isles.[6-8] Approximately 10-25% of meniscal fibrochondrocytes express α-smooth muscle actin, conferring microcontractile properties to the cells.[10,11] This contractile function is thought to be involved with meniscal reparative responses.[11]

The biochemical composition of the meniscus is 70% water and 30% organic matter, 75% of which is collagen and 8-13% is non collagenous proteins.[12] On a dry weight basis meniscal collagen consists of 85-90% type I collagen, 2-10% type II collagen,[13] 1% type III collagen, (particularly in immature bovids), 1% type VI, and 1-2% collagen type V.[9,12-14] Elastin constitutes less than 0.6% of the meniscal tissue.[15] Glycosaminoglycans constitute 1% of the wet weight and 3% of dry weight of the meniscus,[9,16-18] where the meniscal body has the highest concentration of GAGs, and the periphery, the lowest.[18] The most abundant GAG is chondroitin sulphate, accounting for about 80% of total glycosaminoglycan in the inner zones and 50-56% in the outer zones.[18] This higher level of chondroitin sulfate is consistent the axial meniscus' function to withstand more compressive forces.[19] To this end, bipedal humans tend to have higher glycosaminoglycan (GAG) content, versus smaller quadrupeds such as dogs.[19] The GAGs found in the meniscal periphery tend to be hyaluronate and dermatan sulfate.[18] For small proteoglycans (PG), biglycan makes up 38-53% of the small proteoglycans, and is principally found in the axial region of the meniscus.[17,20] Decorin constitutes 23-32% of the small PG's, and is found in higher proportions in the abaxial meniscus.[20] Perlecan and fibromodulin are found principally in the axial regions of the meniscus.[17,20] Like the other tissues of the synovial joint, menisci also produce lubricin. [21]

The meniscal blood supply originates from the geniculate artery, a branch of the popliteal artery. The menisci can be morphologically divided by zonal vascular content.[22,23] Based on this vascular supply, the red-red zone consists of the abaxial portion of the meniscus, where the blood vessels originating from the joint capsule enter the fibrocartilage. The red zone blood vessels penetrate approximately 10-30% of the outer portion of the medial meniscus and 10-25% in the lateral meniscus.[22,23] This vascular content varies by species and changes with age. At birth nearly all of the meniscus is vascularized in humans, and becomes progressively less vascular, with vessels receding from the center in an abaxial direction.[24,25] The red-white zone is the transitional region where blood vessels of the red zone terminate and the avascular portion of the

meniscus begins. The white-white zone is the avascular, axial meniscal fibrocartilage, devoid of nerves, lymphatics, or blood vessels. This vessel distribution also delineates important functional differences between the zones, with the vascular red-red zone having far better healing capabilities than the white-white zone.

The nervous supply to the meniscus follows the vascular supply. Nerve fibers originate from the joint capsule, run circumferentially, and terminate in the peripheral red zone of the meniscus; only a few fibers branch radially into the meniscus. This abaxial region contains Ruffini, Pacinian, and Golgi Tendon mechanoreceptors, as well nociceptors containing Substance P.[26]

The meniscus can also be divided into four histologic zones, based on collagenous structure, content, and biomechanical function.[27] This zoning has important ramifications for the various functions of the meniscal organ, and allows the meniscus to be anisotropic under tension, shear, and compressive loads. This heterogenous structure also allows conversion of compressive weight bearing loads into tensile hoop stresses. The chondroid zone is located in the avascular, axial portion of the meniscus, and resembles hyaline cartilage, both in gross appearance and histological examination.[18,27] The chondroid zone is composed of 76% collagen,[18] of which 60% is type II and 40% type I collagen.[9,12,28] Type II collagen becomes more widespread throughout the meniscus as ageing ocurrs.[13] Most of the meniscus' sulfated glycosaminoglycans are present in this region at 8% dry weight.[18] In particular, aggrecan is concentrated in this area and shows an organized, spatial network, in contrast to its diffuse distribution in articular cartilage.[13,18] Structurally the chondroid zone tissue is more resistant to compressive forces and more analogous to articular cartilage.[9,12,13,28,29] A round chondroid- type cell is found in this zone, with a cilium, associated centrosome, and adjacently placed golgi apparatus.[6-8] Moving abaxially, the transitional fibrocartilaginous zone contains a mix of type I collagen, plus the type II collagen fibrils from the chondroid zone. The collagenous zone is made principally of the circumferentially oriented bundles of collagen I fibrils, interwoven with collagenous radial tie

fibers which originate from the periphery.[29-32] Some of these collagen fibrils are crimped, as seen in tendons.[31] The resultant force vector where the bundles and tie fibers intersect is parallel to the periphery.[32] In addition, fascicles of collagen type II are found around the radial tie fibers.[9,13,17,33,34] The synovial zone is the outer abaxial rim of the meniscus where the vessels enter the meniscus and the meniscus communicates with the synovium and joint capsule. This region is vascular, can heal, contains predominantly type I collagen, and trace III and V collagen, no type II collagen, and minimal GAGS.[9] By dry weight, this region consists of 93% collagen and 2% glycosaminoglycan.[18] Here the cells are fibroblast-like with extended cytoplasmic processes.[6] In the neonatal condition this zone also contains Type IV collagen, and is strongly associated with blood vessels.[13] Lipid content is also highest in this zone.[18] In a coronal plane, the superficial tibial and femoral faces of the meniscus are covered with a thin meshwork of predominantly type I collagen fibrils, with variable orientation.[9]

The meniscal horns are populated by round chondroid cells and contain collagen I and some collagen II.[14,35] These meniscal attachments have abundant interstitial substance and branched, wavy connective fibers.[36] Large myelinated nerve fibers are present in the meniscal horns, showing a concentrated presence of Pacinian mechanoreceptors.[5] Where the horns attach to the bone, their structure shows distinct zones of uncalcified cartilage, calcified cartilage, and subchondral bone.[37] Collagen X has been identified at the calcified zone of the meniscal horn attachment to bone.[14]

Meniscal Function

Meniscal functions include proprioception,[38,39] joint lubrication,[40] shock absorption,[41] relief of femoral- tibial incongruity,[42] load transmission,[43] and joint stability.[44] It is now well established that intact menisci are crucial for the maintenance of normal knee function. The hoop stress mechanism of the meniscus is an adaptation to the meniscal relief of tibial-femoral incongruity during weight bearing of the incongruous round surface of the femoral condyles on the convex surface of the tibial condyles. The circumferential continuity of the peripheral rim of

the meniscus is integral to meniscal function. Articulation of the apex of the femoral condyles without the menisci creates a focal point of high pressure that is not mechanically conducive to survival of articular chondrocytes.[45] The meniscus allows transfer of the axial compressive load of weight bearing to a tensile force within the circumferentially oriented meniscal collagen fibers.[45-49] The axial load is distributed over the larger surface area provided by the menisci, reducing focal areas of stress on the cartilage.[45] For instance, in humans, menisci appear to transmit approximately 50% of the compressive load through a range of motion of 0 to 90 degrees.[50,51] As the joint compresses, the meniscus undergoes controlled peripheral extrusion and its circumferentially oriented collagen fibers elongate.[45] Large tensile hoop stresses, responsible for restraining the meniscus from radial extrusion, are transmitted through the collagen fibers to the cranial and caudal meniscotibial ligaments.[45-49,52] Formation of these hoop stresses is dependent on intact collagen fibers and meniscal attachments.[45-49] A mechanical analogy of the meniscal hoop stresses is the action performed by barrel stays in an oak wine barrel, against the radial forces of the liquid inside.

Meniscal Injury: Ramifications and Prognosis

Meniscal tears are the most common knee injury in people, with the prevalence of acute meniscal tears at 61 cases per 100,000 persons.[53,54] These meniscal injuries can occur as isolated tears, or can occur with anterior cruciate ligament disruption or other ligamentous instability. The overall male-to-female incidence is approximately 2.5:1, with the peak incidence of meniscal injury for males at 31-40 years of age, versus women at 11-20 years.[53,54] Degenerative tears can be found in as much as 60% of the population over age 65.[55]Arthroscopic menisectomy represents the most common human orthopedic surgery performed annually;[56] surgical procedures of the meniscus are performed on an estimated 850,000 patients each year in the United States,[53,54] and an estimated 1.1 million partial menisectomies are performed worldwide each year, representing $9 billion in medical fees.[57]

In the dog, meniscal tears are extremely frequent sequelae of cranial cruciate ligament (CCL) deficiency disease in the dog, a syndrome so common, it claimed 1.32 billion dollars for surgical and medical care in 2003.[58] Between 50% and 70% of dogs with CCL tears have concurrent damage to the medial meniscus. [56,59-62]

With the advent of veterinary arthroscopy, meniscal injuries have been increasingly identified as a significant cause of lameness and decreased performance in horses.[63-69] Whereas early reports of "mystery stifle lameness" and equine meniscal tears were rare curiosities, the most recent studies included 74 and 80 affected joints, respectively. [63-69]

The functional ramifications for both human and veterinary patients following meniscal injury or disruption is clearly deleterious. Animal models using dogs, cats, sheep, and rabbits show that cutting the meniscal horns or radially transecting the meniscal body prevents development of meniscal circumferential tension, which in turn impairs meniscal load transmission. [45,50,62,70,715072,73,51] Removing part of the meniscus also results in excessive force concentration and articular cartilage damage.[45,62,70,74]Clinical study of traumatic meniscal tears in people revealed that loss of the meniscus also disturbs the distribution of load across the knee joint, [72,73] resulting in degenerative articular cartilage [74,75] and impaired knee function.[76] In humans, meniscal body tears and failed meniscal repairs are associated with significant osteoarthritis of the knee joint, specifically tibiofemoral cartilage defects, decreased tibial and femoral cartilage volume, markedly higher prevalence of radiographic OA in the medial compartment, and greater tibial bone area.[77,78] For instance, one conservative human clinical study showed that 50% of those with a diagnosed meniscus tear have osteoarthritis with associated pain and functional impairment 10 years post diagnosis.[79] Even repaired meniscal tissue does not restore perfect meniscal function nor prevent articular cartilage degeneration.[16,80] In addition, torn meniscal fibrocartilage can move and fold, causing joint capsule pain and locking of the knee. [50,51,81,82] Thus, any derangement of the meniscal collagen fascicles prevents

conversion of weight bearing load to hoop stresses, resulting in excessive cartilage load, cartilage damage, and debilitating OA.

One of the important consequences of meniscal injury is that the meniscus has a limited ability to spontaneously heal. Tears in the red-red zone can spontaneously heal via fibrin clot maturation and remodeling to fibrovascular scar.[23,83,84] For tears >than 8mm, surgical repair is recommended, with tears in the red zone having a 84-100% success rate, using clinical outcomes measures.[85-87] In contrast, tears within the axial, avascular portion of the knee meniscus do not spontaneously heal, and also do not respond well to suturing or other standard surgical fixation.[23,83,84,88-90] This lack of spontaneous avascular meniscal healing is due in part to the lack of vascular supply to this region.[23,83,84] In addition, there is evidence that the meniscofibrochondrocytes within this axial region have an intrinsic inability to heal, [91] and only the peripheral fibrochondrocytes, and not the central cells, can respond to injury by dividing and increasing collagen synthesis.[92] When the meniscus is completely lost, as in surgical total meniscectomy, rabbits[93] and dogs[27,93] can produce a regenerate, peripheral, fibrous mold of connective tissue, however, this pseudomeniscus cannot halt articular cartilage damage and progression of arthritis. Pseudomeniscal regeneration does not occur in humans post complete meniscectomy[94-96] but has been described to occur in conjunction with knee replacement.[97]

Consequently, irreparable tears or tears in the avascular zones of the meniscus are frequently treated with partial meniscectomy, and macerated menisci may require subtotal or complete resection. While removing the torn fibrocartilage improves short term patient comfort and avoids knee locking symptoms, partial and complete menisectomy causes long term progression of painful OA and disability.[94-96,98-107] Knee OA after partial or complete meniscectomy results from the primary joint injury, and the increased contact stress in the cartilage due to the loss of meniscal tissue.[94-96,98-108] Meniscectomy and partial menisectomy are accompanied by the onset of OA because of the high focal stresses imposed on articular cartilage and subchondral bone subsequent to excision of the meniscus.[45,70,94-96,98-108] Tibial femoral contact

area decreases by 75% in human post meniscectomy knees,[109] with a 235%-700% increase in contact stresses after total meniscectomy.[43,50,110] In contrast, partial meniscectomy results in only a 10% decrease in contact area and a 65% increase in contact stress.[109] Clinical studies of partial and complete meniscectomy affirm the importance of loss of meniscal function as a risk factor for subsequent knee OA.[94-96] Not surprisingly, post operative function seems to worsen with increasing area of damaged or resected meniscal tissue[74,111] and degree of functional derangement, such as in radial tears, degenerative meniscal lesions, and extensive partial resections.[98] A significant rate of cartilage loss, at 6.9% per year, is seen in subjects post partial meniscectomy, compared with healthy controls.[112] Regarding clinical functionality, a study of people who received partial meniscectomy showed a post operative 33% sports dropout rate, and an additional 30% shifted from an active to a sedentary lifestyle, with reports of significant physical disability and handicap.[103]

Meniscal tearing also occurs secondarily to anterior/ cranial cruciate ligament rupture/ deficiency in humans and dogs, and is the primary mechanism for meniscal tearing in dogs. In the cranial cruciate deficient human and canine knee, the meniscus becomes a primary stabilizer to cranial translation, and also contributes to varus/valgus stability and internal and external rotational stability.[44,45,113] Consequently, the medial meniscus is vulnerable to caudal shearing injury in the anterior cruciate ligament-deficient knee as it limits limit anterior translation.[45]

In veterinary patients, meniscal injury holds similar clinical sequelae as in the human condition. For the equine athlete, only 47% of horses return to their intended function post partial menisectomy, and a poor prognosis is associated with increasing severity of the meniscal injury, with the presence of concurrent articular cartilage lesions and radiographic abnormalities in the joint.[63] In dogs, not only are meniscal tears common secondary to cranial cruciate ligament deficiency, but an increasing, ageing population of dogs has also received iatrogenic damage to the meniscus in the form of a medial meniscal release procedure, as prescribed by Slocum ***et. al***.[114] during knee stabilization surgery. Meniscal release is detrimental to normal load function,[45]

because it causes significantly decreased articular contact area and significantly increases peak contact stresses.[45-47,49,110,115] This results in cartilage damage, malacia, destruction, progression of osteoarthritis, and advancement of pain and debilitation.[74,110,111,116-121] Performing a meniscal release just caudal to the MCL has the same effect as an entire caudal pole hemimeniscectomy.[45] Thus, these dogs may potentially suffer additional pathologic articular wear with osteoarthritis secondary to this procedure.

To avoid partial or complete menisectomy, numerous techniques have been researched to try to induce avascular meniscal healing. Techniques to successfully encourage avascular meniscal healing by improving vascular access include vascular access channels, trephination, and advancement of vascularized synovial pedicle flap. However, these techniques are limited by insufficient or inconsistent healing responses, premature closure of the vascular access channel, inconsistent neovascularization, intolerable forces on the fragile bridging synovial tissue, biomechanical deformation of access channels, and disruption of meniscal architecture.[88,122-126] Various other means of inducing avascular meniscal repair, including fibrin clot[127] fibrin glue,[128-130] polyurethane glue,[131] meniscal rasping [132-134] have been met with variable results in avascular meniscal tears, often impeded and overpowered by the extent of the injury or its distance from the meniscal periphery.[127-130,132,133,135] Laser sealing of meniscal tears does not work,[136] while laser sealing of fibrin clots does provide some adhesion, but does not return the meniscus to its prior strength.[137] Regenerative medicine strategies have had some success in fostering avascular meniscal regeneration, with the use of using porous polyurethane implants,[138-141]porcine small intestine submucosa implants,[27,142-144]collagen scaffolds,[145,146] collagen sponges,[147] polycaprolactone scaffolds,[148] poly- glycolic acid and lactic-co-glycolic acid scaffolds,[149] estane and polyesterurethane replacements,[150] however, all the these implants failed to completely protect the articular cartilage from damage and hold off the onset of OA. For instances of complete meniscal disruption, meniscal allografting has been a described treatment, but can be complicated by lack of appropriate tissue size and congruency, difficulty achieving tissue

preservation, disease transmission, acute tissue rejection, long term tissue rejection which inhibits cellular repopulation and graft incorporation, incomplete cellular incorporation, structural weakening, and poor function.[151-161] Thus, despite the morbidity and cost of meniscal injury, and the massive effort invested into repairing avascular meniscal defects, complete restoration of the damaged meniscus, both in form and function, has not been accomplished.

Meniscal Tissue Engineering

Tissue engineering is a novel strategy being investigated to address avascular or total meniscal disease. Tissue engineering is the multidiscipline approach to fabricate, reconstruct, or replace living tissue, through manipulation of cultured cells that produce the lost or damaged tissue, materials that serve as delivery vehicles and scaffolds for the cells, bioactive factors and biomechanical stimuli, to facilitate appropriate tissue regeneration or create new organ tissues. Tissue engineering principles focus on: (1) determining a source of healthy cells, which have to be nonimmunogenic, easy to isolate and highly responsive to distinct environmental cues, (2) suitable carriers for the *in vitro* cell differentiation and subsequent transplantation, and (3) a set of defined bioactive molecules driving the process of differentiation and maturation.[162]

One potential source of cells which could be used for meniscal tissue engineering is the cells of the synovial membrane. The synovial membrane is divided into the synovial subintima and the intima, which is 1-4 cell layers thick.[163-171] The intima forms microvilli and larger alar folds, and produces the synovial fluid that is crucial for nutrient delivery to the intra-articular structures, including molecular lubricants such as lubricin and hyaluronan that are essential to normal, low friction joint movement.[172] [173-178] The subintima or lamina propria is a loose areolar connective tissue that contains glycosaminoglycans (chondroitin 4- sulfate and chondroitin -6 sulfate), and fibrils of type I, III, V, and VI collagen, and serves as the supportive stroma for the synovial intima. [176,177,179,180] The subintima also contains nerves, blood vessels, and lymphatics and is attached to the thick joint capsule.[176,177,179] The synovial intima contains several cell types, the Type A synoviocyte, a type of tissue macrophage, which has phagocytic functions, and

mediates inflammation, and Type B cells which are fibroblast- like and perform secretory functions.[176,177,181] Like other adult mesenchymal tissue types of the body, synovium contains tissue progenitor cells (Type C cells) which can undergo terminal differentiation into numerous tissue types, including a chondroid phenotype.[93,182-185] This chondrogenic phenomena is pathologically seen in the clinical disorder synovial chondromatosis,[186] which can occur as primary disease or secondary to joint instability and osteoarthritis.

Synovium first gained attention in relation to meniscal injury when it was found that synovium plays an important role in meniscal healing and regeneration. [187-190] A fibrocartilaginous meniscus-like structure of synovial origin can form in menisectomized stifles, but only if the synovial membrane is left intact. [191] However, menisectomies which include excision of the adjoining synovium result in the complete absence of this fibrovascular replacement tissue.[191] Synoviocytes are thought to play a role in healing meniscal tears [90] and mediate fibrin clot induced fibrocartilage formation in meniscal defects.[127] Synoviocytes have also been documented to repopulate avascular meniscal allografts, undergoing a fibrocartilage phenotypic change. [23,151-155] Synovial membrane has been manipulated as pedicle grafts, grafted through conduits, and free grafted to induce meniscal healing.[132,133,174,187,188,190,192-195] Stifle synovium is an abundant tissue, the synovial membrane regenerates rapidly post surgical synovectomy,[196] and synovium is readily harvested arthroscopically.[197] Cellular adherence on plastic and monolayer cultivation enriches a subpopulation of synovial membrane cells that express cell surface markers of MPC and, of particular importance, these cells can readily undergo *in vitro* hyaline chondrogenesis in response to growth factor stimulation.[182,184,185,198-210 211] Synoviocytes cultured on bioscaffolds can also undergo chondrogenesis and produce hyaline cartilage extracellular matrix constituents *in vitro*.[212] Synovial mesenchymal progenitor cells have superior *in vitro* proliferation capacity and multilineage differentiation potential, as compared with other sources of mesenchymal progenitor cells, such as bone marrow, periosteum, muscle, and adipose tissue,[185,203,210,213] as determined for the purpose of articular cartilage tissue

engineering and regeneration. The number of colony-forming unit fibroblasts (CFU-Fs) identified from synovial lining was reportedly 1 in 12.5 to 80 nucleated cells plated, which was much greater than that of bone marrow, 1 in 104 to 105 cells.[174,214,215] Sakaguchi *et al*. showed that an average of 21,000 cells per milligram of synovial lining collected could be obtained after plating and culture for 14 days; a small sample of synovial lining harvested with a punch biopsy could obtain a high cellular yield.[203] Clearly, synovial progenitor cells can be an excellent cell source for articular cartilage tissue engineering. Because many of the ECM constituents are shared by articular cartilage and meniscal fibrocartilage, these data also suggest that synovium may be able to serve as an abundant cell source for meniscal fibrocartilage tissue engineering, if fibrochondrogenesis can be induced in culture.

Thus, the purpose of the research presented here is to investigate synovium as a potential cell source for meniscal fibrocartilage tissue engineering, to address the challenges of avascular meniscal repair and regeneration. By investigating basic synovial culture conditions, determining suitable scaffold types for synoviocyte culture, and manipulating cell culture density and growth factor exposure to induce fibrocartilage formation *in vitro,* we seek to begin building fundamental knowledge that will one day allow synovial based meniscogenesis in the laboratory.

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Chapter 3. Static and dynamic seeding of equine synoviocytes on OPLA (Open-cell Poly-L-Lactic Acid) and PGA (Polyglycolic acid) tissue scaffolds

Meniscal injuries are a common cause of knee arthritis and disability in humans and dogs, and have been identified as a cause of lameness and decreased performance in horses.[1-3 4-8 6 8] Tears within the axial avascular portion of the meniscus possess a limited ability to heal spontaneously,[9 10] and attempts to successfully encourage avascular meniscal healing [9,11-13, 14 15-18] or regeneration, [16-21 22 23] have been reported with variable outcomes. Complete restoration of the avascular damaged meniscus, both in form and function, has not been accomplished.[15] The majority of meniscal injuries are treated with partial or total menisectomy, which results in articular cartilage damage of the tibial and femoral condyles, and progression of debilitating osteoarthritis.[9,24]

Currently, tissue engineering is being investigated for the treatment of avascular meniscal injury or total meniscal loss. Tissue engineering is the fabrication of living replacement tissue, through manipulation of cultured cells, cell scaffolds, bioactive factors, and biomechanical stimuli, to facilitate appropriate tissue regeneration or create new organ tissues. Synovium may be able to serve as a source for functional fibrocartilage in engineering meniscal tissue, provided the chondrogenic potential of synoviocytes can be optimized. However, ideal culture conditions required to induce or sustain synovial fibrocartilage formation, such as scaffold type and biomechanical stimulation, are not known.

The ideal cell scaffold for use in synoviocyte *in vitro* culture would allow easy cell adhesion, good nutrient delivery/waste change, and support cellular differentiation into the target phenotype with extracellular matrix (ECM) proliferation. Once surgically implanted, the scaffold structure would have to persist for a sufficient time to allow further formation of the vascular, cellular, and matrix components of meniscal tissue, while slowly dissolving and allowing the engineered ECM to take over mechanical function. In addition, the scaffold should not incite a

foreign body reaction, and must prevent secondary joint damage. [25,26] To this end, tissue engineers and polymer chemists are investigating a number of synthetic polymer scaffolds as potential cell carriers. PGA (Poly-glycolic acid) and PLA (Poly-L- Lactic Acid) are biodegradable, biocompatible, poly-esters, that are attractive for tissue engineering because they are readily available, can be easily processed into a variety of structures, and are FDA approved for a number of biomedical applications.[27] PLLA has been successfully used in musculoskeletal tissue engineering for *in vitro* culture of perichondrial cells,[28] adult chondrocytes,[29] human osteoblasts,[30] and human dermal fibroblasts.[31] PGA has been successfully used as a scaffold for meniscal fibrochondrocytes *in vivo*[32] and cultured *in vitro*[33] to form meniscal tissue. PGA has also been used to form articular cartilage *in vitro* with fetal chondrocytes[34] and adult chondrocytes.[35] Based on this prior research, we believe that both PGA and PLA would be viable synthetic scaffolds for the *in vitro* culture of FLS for application in meniscal fibrocartilage tissue engineering.

Cartilage tissue engineering using biodegradable scaffolds is most successful if uniform distribution of cells is achieved.[36-38] Spatial uniformity of cellular distribution has been shown to be optimized through the use of rotating bioreactors.[38] Kim *et al*[39] demonstrated that dynamic versus static seeding of polyglycolic acid (PGA) scaffolds with smooth muscle cells led to significantly greater cell numbers and uniform cell distribution which resulted in increased extracellular matrix production. Furthermore, Aufderheide *et al* have demonstrated higher meniscofibrochondrocyte cellularity of PGA scaffolds when cultured in a rotating bioreactor versus static culture conditions.[33] In addition to the effects of cell seeding, mechanical stimulation of cultured cells has positive effects on cell differentiation, cell viability, and extracellular matrix production through mechanotransductive effects[36-38,40] . With respect to chondrocytes cultured on PGA scaffolds, shear stresses generated from fluid flow in rotating bioreactors have been shown to significantly increase cell concentration as well as synthesis and accumulation of sulfated GAGs, hydroxyproline, and collagen.[36-38] This effect ultimately improves the

compressive properties of the chondrocyte/PGA constructs[36-38] and thus may represent a useful technique for cell-based engineering of functional meniscal tissue.

The purpose of this study was to assess relevant culture conditions for equine FLS to progress toward application in synovium based-fibrocartilage tissue engineering. Our specific objectives were to compare two synthetic scaffold types (PGA vs. OPLA) and two different biomechanical culture conditions (static vs. dynamic) on cellular delivery, cell distribution, and ECM production. We tested the null hypothesis that neither scaffold type nor culture condition would significantly affect the measured outcomes of FLS cellularity, cellular distribution, or ECM production.

Materials and Methods:

Scaffolds

The polyglycolic acid (PGA)[a] product utilized was a 3mm thick non-woven felt, with a fiber diameter of 10μm. The open-cell poly-lactic acid (OPLA) scaffold[b] was a 5mmx3mm, approximately 34mg (dry weight), non compressible, cylindrical sponge. The average sponge pore size was 100-200μm with a hydration capacity of 30μl/ scaffold. PGA and OPLA scaffolds were sterilized in ethylene oxide. Following sterilization, the PGA felt was cut into 5mm diameter discs using a sterile Baker's biopsy punch prior to cell culture to precisely match the dimensions of the OPLA scaffolds.

Tissue Collection and Monolayer Cell Culture

Six 8.0 mm x 8.0 mm biopsies of synovial intima were obtained from the stifle of a horse euthanatized for an unrelated study. This horse's stifle was determined to be free of orthopedic disease based on gross examination of the joint. The tissue was placed in Dulbeccos' Modified Eagle's Media with 10% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B 25ug/mL, 0.002% L-Ascorbate, and 0.01% L-glutamine (supplemented DMEM) in preparation for monolayer culture.

The synovium was sectioned into 2.0mm x 2.0mm pieces using a #10 Bard Parker blade under sterile conditions. The tissue fragments were combined with sterile Type 1A clostridial collagenase solution[c] at a concentration of 7.5mg/mL of RPMI 1640 solution. The mixture was agitated at 37˚C, 5% CO2, 95% humidity for six hours. Cells were recovered through centrifugation, the supernatant decanted and the cellular pellet re-suspended in 5mL of supplemented DMEM. The cell solution was transferred to a 25mL tissue culture flask containing 5mL of supplemented DMEM. The flasks were incubated at 37˚C, 5% CO2, 95% humidity, with sterile medium change performed every 3 days. Synovial cells were monitored for growth using an inverted microscope until observance of 95% cellular confluence per tissue culture flask. Cells were then transferred to 75mL tissue culture flasks. At 95% confluence the cells were subcultured until the 4th cell passage had been reached.

Dynamic Culture

Nine PGA scaffolds (PGA-D group) and 9 OPLA sponges (OPLA- D group) were placed in separate 110mL vessel flasks of the rotating bioreactor system[d] containing 110mL of supplemented DMEM (**Figure 3-1a**). The bioreactor vessels were rotated at 51.1 rpm to allow the scaffolds to free-float and rotate within the culture medium. The scaffolds were presoaked for 24 hours in the bioreactor at 37˚C, 5% CO2, 95% humidity, prior to cell introduction.

FLS were removed from the tissue culture flasks using Accutase[e] and counted. Cells were added to the bioreactor flasks at a concentration of 475,000 cells/ mL. The bioreactor was maintained at 37˚C, 5% CO2, 95% humidity at 51.1 rpm. Fifty percent of the cell culture medium volume was changed using sterile technique every 3 days. Cell counts were performed on discarded media for the first two media changes.

Static Culture

Nine PGA scaffolds (PGA-S group) and 9 OPLA sponges (OPLA-S group) were placed individually in 24 well tissue culture plates, each well containing 2mL of supplemented DMEM (**Figure 3-1b**). The scaffolds were presoaked for 24 hours at 37˚C, 5% CO2, 95% humidity, prior

to cell introduction. Then FLS were transferred from monolayer culture as described above, and pipetted on top of the scaffolds in solution, to reach a total cell concentration of 475,000 cells/ mL. The plates were maintained at 37˚C, 5% CO2, 95% humidity, with 50% cell culture medium changed every 3 days. Cell counts were performed on discarded media for the first 2 media changes.

Histologic Analysis

All scaffolds were harvested on the 12th day of culture. Six scaffolds from each group (PGA-S, PGA-D, OPLA-S, OPLA-D) were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with Masson's Trichrome, Safranin –O, and Hematoxylin and Eosin. Histologic specimens were examined at 10x magnification on a Zeiss Microscope.[f] Images of each section, (three from the scaffold periphery and three from the scaffold center) at 2 o'clock, 6o'clock and 10o'clock positions (**Figure 3-2**) were digitally captured by an Olympus DP-70[g] digital camera and saved as Tiff files. Cell count and distribution were determined by computer image analysis using Fovea 3.0 software.[h] Safranin-O staining, indicating presence of GAG's, and Masson's Trichrome staining, indicating presence of collagen, were subjectively evaluated and recorded.

Biochemical ECM Analysis

Three scaffolds from each group were analyzed for Glycosaminoglycan (GAG) and collagen production. Wet weight of each scaffold was obtained. GAG content of the scaffold was performed using the Dimethylmethylene Blue Sulfated Glycosaminoglycan assay.[41] Samples were lypholized, and a dry weight obtained, and then mixed with 1ml Papain Solution (2mM Dithiothreitol and 300ug/ml Papain). The solution was incubated at 600C in a water bath for 4 hours. Then 5ul of sample was placed into three wells each of a 96-well plate. 245ul of Dimethylmethylene blue (DMMB) reagent, containing 8mg DMMB, 1.5g Glycine, 1.2g NaCl dissolved in 500mL of H2O, was added to each well. The plate was read immediately at 525nm by a Synergy HT – KC-4 Spectrophotometric Plate Reader (BioTec, Winooski Vermont) (38).

Absorbances were compared to a generated standard curve. Absorbances were converted to ug/ug concentrations and total GAG yield in ug using FT4 software (BioTec, Winooski Vermont).

Collagen content of the scaffold was assessed using the hydroxyproline assay, as described by Reddy et al.[42] The papain digested samples used in the above GAG assay were also used in the hydroxyproline assay. 50ul of each papain digested sample was placed, in duplicate, in each well of a 96 well plate. Standards of hydroxyproline were made at concentrations of 0,2,4,8,12,16,20 ug/mL in 50uL of papain digest buffer, containing 20mNaPO4 and 1mmol EDTA.. 50ul of 4N NaOH was added to each well. The samples were autoclaved at 121°C for 20 minutes. 450uL of Chloramine-T reagent, containing 1.27g Chloramine T dissolved in 20mL of 50%n-Propanol and 80mL of acetic citrate buffer, was added to each well. 450uL of Erlich's reagent, containing 15g of p-dimethylaminobenzaldehyde dissolved in 16.8mL p-Propanol and 8.3mL perchloric acid, was added to each well. The samples were incubated in a water bath at 65ºC for 20 minutes. 100ul of sample and standards were transferred to a short- 96 well plate and read immediately at 550nm by a Synergy HT – KC-4 Spectrophotometric Plate Reader (BioTec, Winooski Vermont). Absorbances were converted to ug/ug concentrations and total hyroxyproline yield in ug using FT4 software (BioTec, Winooski Vermont).

Statistical Methods

Mann-Whitney rank sum tests were performed to compare cell counts between each scaffold and culture condition group. To determine significance between periphery and central cell counts within each group, a t-test was performed. For all tests significance was set at $P < 0.05$. All statistical analyses were performed using a statistical software program.[i]

Results

On gross examination, the fibers of the PGA scaffolds and the sponge surface of the OPLA scaffolds were still visible. No live cells were detected in any of the media changes for

either static or dynamically cultured scaffolds, indicating that viable cells rapidly adhered to the scaffolds, bioreactor walls, or tissue culture wells.

PGA scaffolds cultured in a rotating bioreactor had the highest cellularity. Mean total cell count for PGA-D constructs was 1128 cells/ low power field (SD: 575.7) (lpf, field at 10X objective magnification). The mean cell count for PGA-S constructs was 54/lpf (SD: 33.6). The mean cell count for OPLA-D constructs was 375/ lpf (SD: 118.2), which was significantly greater than the mean cell count of OPLA-S (301 cells/ lpf,SD:64.8, P=0.028). Mean cell count for the PGA-D group was significantly greater than all other groups (P<0.001). The PGA-S had significantly lower cell counts compared to all other groups (PGA-D P<0.001; OPLA-D P=0.017; OPLA-S P=0.0217) (**Figures 3-3 and 3-4**).

All groups showed preferential cellular distribution on the periphery of the scaffolds. The mean cell count at the scaffold periphery for the PGA-D group was 1433/ lpf, (SD: 487.3) which was significantly higher than the mean PGA-D central cell count (724/lpf, SD: 314.0, P<0.001) (**Figure 3-5a**). For the PGA-S group the mean peripheral cell count (80/ lpf, SD: 28.3) was significantly higher than the PGA-S central cell count (28cells /lpf, SD: 10.7, P<0.001) (**Figure 3-5b**).

Cells grew in clumps and sheets on the surface pores of the OPLA sponges, as compared to the more sparsely cellular scaffold center. The mean perimeter cell count for the OPLA-D group (476/lpf, SD: 89.8) was significantly higher than the OPLA-D central cell count (295/lpf, SD: 55.3, P<0.001) (**Figure 3-5c**). For the OPLA-S group, the mean number of perimeter cells was 307/ lpf (SD: 52.4) and the central cell count was 287/ lpf (SD: 80.2); this difference was not statistically significant (P<0.464; power of test with α=0.05: 0.05) (**Figure 3-5d**).

Staining for collagen and gycosaminoglycan using Masson's Trichrome and Safranin-O, respectively, was negative for extracellular matrix production in all sections evaluated.

In the PGA-D group, the dimethylmethylene blue assay detected a mean of 22.29 μg of GAG per scaffold, (range 19.34- 28.13μg), with a mean % GAG scaffold content of 0.0345% (μg GAG

per μg scaffold weight). No GAG was detected in OPLA constructs or PGA-S constructs. (**Figure 3-6**)

The hydroxyproline assay did not detect collagen production in any group.

Discussion:

Meniscal injuries are common in humans and dogs and lead to debilitating osteoarthritis. Surgical attempts at inducing meniscal healing have been largely unsuccessful, particularly within the avascular 2/3 of the meniscal tissue. Tissue engineering is being investigated as a novel treatment solution for meniscal deficiency. FLS may be applicable to meniscal engineering because these cells can modulate meniscal reparative responses and undergo chondrogenic differentiation. A necessary step in establishing a cell-scaffold based tissue engineering approach is to determine the optimal cell culturing conditions for tissue development. The current study analyzed the effect of scaffold type and simple biomechanical stimuli on FLS seeding and production of specific ECM constituents. We found that FLS-seeded PGA constructs cultured in a rotating bioreactor had the highest cellularity, with a mean sulfated glycosaminoglycan content of 22.3μg per scaffold. PGA constructs cultured in static conditions had the lowest cellularity. A non-uniform cellular distribution was observed for all scaffold types and culture conditions.

Bioreactors have been reported to optimize cell seeding, uniform cell distribution, and ECM production in cartilage and connective tissue engineering. [33,34,36-39,43-49] The rotating wall bioreactor used in this study provides a dynamic, laminar fluid shear, which perfuses three-dimensionally cultured cells[43]. Medium perfusion encourages cell survival and proliferation by providing efficient transport of nutrients, gases, catabolites, and metabolites and maintaining physiologic media pH.[47,50] Mixing of culture media also promotes cell seeding by creating matched relative velocities of cells and scaffolds, particularly on non-woven PGA scaffolds.[47] In addition, the rotating wall bioreactor limits cellular stress by reducing strong shear forces and cellular impact on the walls of the bioreactor.[43] These conditions may explain why scaffolds

cultured in the rotating bioreactor had greater cellularity than scaffolds cultured in a static environment.

Despite the optimized cellular retention of the dynamically seeded PGA scaffold, we did not see the benefits of more uniform cell distribution previously described for chondrocytes and smooth muscle cells cultured in dynamic conditions.[39,46,51,52] The central portions of PGA-D and OPLA-D scaffolds had lower cell counts than the periphery, as did the statically cultured groups. Therefore, the reported benefit of using a rotating bioreactor for optimizing cell distribution was not realized in this study. Although this distribution difference did not reach significance in the OPLA-S group, the power of that analysis was low and the apparent equal cell distribution represents a potential type II statistical error. In addition, we were not able to provide a comparative examination of FLS dynamic culture techniques against an established gold standard. There are numerous bioreactors types, including rotating wall bioreactors, fixed wall bioreactors such as spinner flasks, wavy wall bioreactors,[48] or flow-through bioreactors such as perfused cartridges,[43] all of which have varying cell seeding efficiencies and distributions depending on the cell type.[43] The gold standard for dynamic and static *in vitro* FLS seeding and scaffold culture has not been determined. The non-uniform cell distribution seen in this study may indicate poor penetration, or may reflect a biological advantage of the perimeter synoviocytes to exposure to nutrient delivery, gas exchange, and mechanotransductive effects in culture.[53]

A higher cell count was seen on the PGA-D scaffolds versus the OPLA-D scaffolds. The non-woven PGA scaffold may favor cellular capture and retention and allow better flow-through of culture media[47] and nutrient delivery, than a sponge type scaffold, in a bioreactor. PGA is a relatively hydrophilic compound, which promotes cellular adhesion, as compared to other scaffold types such as poly-lactic acid.[54-56] This finding of higher PGA cell counts is consistent with other seeding studies done on PGA versus poly-lactic acid scaffolds.[27,33,55]

The OPLA scaffolds had clumped cell distribution in the outermost pores and lower overall cellularity. The OPLA sponge porosity may not allow uniform cell distribution. OPLA sponges are dense compared to PGA non woven mesh; the OPLA sponge has a density of 871mg/cc, as compared to the open-weave PGA scaffold, which has a density of 45-77mg/cc. The 100-200μm pores of the OPLA sponge do not consistently communicate with each other. In addition, this architecture may not allow free media flow through the scaffold, negating the enhanced cell delivery, nutrient delivery, and mechanotransduction provided in the bioreactor.[38,39,46,51,52]

Traditional means for cell quantification have included subjective assessments, manual counting methods and radioisotope DNA quantification methods. Recently, digital image analysis has been validated as a cell counting methodology. [57-60] Based on the reported accuracy of this technique, we applied digital image analysis (Fovea 3.0) for both cell counting and distribution quantification. Using this methodology, cellular density was assessed using a thresholding algorithm[61]. This algorithm allows quantification of cellular nuclei based on their histogram values. Because of the potential of an erroneously low cell count from miscounting clumped cells, regions of clustered cells were hand counted, to confirm the accuracy of the computer analysis.

In dynamic seeding of PGA scaffolds with chondrocytes, seeding efficiency is reported to be greater than 95% within 24 hours.[62] In this study, within the first 72 hours after seeding, no free floating cells could be detected in the bioreactor media, which could represent a high seeding efficiency. It is not known if the cells present on the scaffolds represent cellular retention versus cellular progeny from mitogenesis. This information would be useful to further delineate cell viability and activity in the three dimensional culture environment.

Reported optimal cell-seeding densities for tissue engineering are highly variable depending on cell and tissue type. The musculoskeletal tissue engineering literature documents scaffold cell seeding concentrations of 30,000 fibroblasts /mL,[56] 600,000 chondrocytes/mL,[63] 5 million chondrocytes/ mL,[64] 10 million chondrocytes/mL,[65] and 20 million fibrochondrocytes/ mL.[66]

Because of the paucity of studies investigating FLS as a cell-based approach to tissue engineering, in addition to the general hypocellularity of meniscal tissue, we elected to investigate a single low cell seeding concentration. This may have negatively impacted total matrix production, and thus, our ability to detect production of ECM proteins with the spectrophotometric assays and histologic methods utilized in this study. Other studies have shown the importance of high chondrocyte density for producing ECM.[34,47] In addition, the relatively short [56,63-65] culture time may also account for the lack of detectable ECM components in the static cultured constructs and the dynamically cultured OPLA constructs. Real-time RT-PCR measurement of ECM mRNA may have detected early genomic signaling and potential for ECM production,which was not detected using the biochemical assays in this study.

Synoviocytes typically produce collagen I constituitively,[67,68] however production and deposition of hydroxyproline, a major rate-determining component in the biosynthesis of types I and II collagen, was not detected in this study. Collagen I and II are major ECM consitutents of the meniscus[69] and therefore, collagen production is a major goal of mensical fibrocartilage tissue engineering. Collagen I tends to be present in tissues influenced by tensile forces, and collagen type II in tissues resisting compressive forces.[69-73] Previous studies have shown that high levels of bioreactor shear stress is required for collagen formation by chondrocytes, which is best achieved with a perfusion flow-through bioreactor, rather than a rotating wall bioreactor.[62,74] FLS may require exposure to small tensile forces to achieve gross collagen synthesis in vitro.[75] In the present study the FLS were exposed to the mild shear forces and hydrostatic pressurization in a rotating bioreactor,[72] which may not be the optimal type of force required for synovial collagen I formation. A localization phenomenon of collagen production has also been described in synoviocytes cultured under dynamic compression, whereby cell clusters below the scaffold surface produced collagen.[75] In the present study the majority of cells were located on the periphery of the scaffolds, which may have also accounted for lack of collagen production. A large variety of chondrogenic factors[76-79] that could have optimized chondroid matrix

production,[100] such as hypoxic culture conditions, and certain growth factors, (Transforming Growth Factor-beta, Insulin-like Growth Factor -1 and basic Fibroblast Growth Factor)[76-79,j] were not assessed in this study.

Bioreactor culture in the PGA-D group may have optimally stimulated FLS production of GAG, a constituent of synovial ECM. However, GAG may have been detected merely due to the higher cell content of the PGA-D group. Regardless, production of this ECM constituent is constructive toward meniscal tissue engineering; the axial meniscus contains GAG, which plays a role in resisting compressive load. [73,80] However, the mean GAG content of the PGA-D scaffold of 0.0345% (wet weight basis) was lower than the 0.6-0.8% wet weight in the normal meniscus, and thus represents a sub-optimal response for engineering purposes.[73]

There were several limitations to this study. The synovioctye cultures in this study likely do not represent a pure culture of type B or FLS. Harvested synovial tissue likely contained tissue progenitor cells, subintimal connective tissue, blood vessels, and lymphatics, in addition to Type A and B synoviocytes,[81] The specific identification of Type –B fibroblasts was not performed, but was suspected to be a high percentage based on the selective adherence and gross appearance of these cells in monolayer culture. Specific delineation of the cultured cell type profile could be helpful to determine mechanisms of our results. However, the authors believe that the currently employed methodology of synovial tissue harvest, extraction, and culture is not technically encumbered. Thus, the methods described here possess potential for realistic clinical application in tissue engineering, if cellular production of chondrogenic extracellular matrix can be optimized. An additional limitation of this study was that the cell viability of the constructs was not determined. Non viable synoviocytes usually detach and are subsequently removed in media changes when grown in monolayer; is not know if this also occurs on scaffolds. Techniques such as the Ethidium Homodimer-1/ Calcein AM live/ dead assay would have helped determine which culture conditions were most beneficial for maintaining synoviocyte viability.

We accept the null hypothesis that neither scaffold type nor culture condition affects cellular distribution by FLS. Based on these results, we are unable to validate that the rotating bioreactor or different scaffold types necessarily optimized cellular distribution or enhanced ECM production. However, we reject the null hypothesis for scaffold cell content based on the significant increase in scaffold cellularity observed in PGA versus OPLA cultured in the rotating bioreactor. This combination of PGA and rotating bioreactor does appear to hold promise future meniscal tissue engineering endeavors. Ongoing research is focused on methods to enhance extracellular matrix production, including increased culture duration, increased cell seeding concentrations, and addition of chondrogenic growth factors.

Footnotes

a. PGA Tissue Scaffold, Synthecon, Houston, TX.
b. OPLA tissue scaffold, BD Biosciences, Bedford, MA.
c. Type 1A Clostridial Collagenase, Sigma, St. Louis, MO.
d. Rotating Bioreactor System, Synthecon, Houston, TX.
e. Accutase, Innovative Cell Technologies, San Diego, CA.
f. Zeiss Microscope, Carl Zeiss, Thornwood, NY.
g. Olympus DP-70 Digital Camera, Olympus, Melville, NY.
h. Fovea 3.0, Reindeer Graphics, Asheville, NC.
i. SigmaStat, Jandel Scientific, SanRafael, CA.
j. Pei M, Aaron R, Ciombor D. Modulation of chondrogenesis in synovial fibroblast engineered cartilage by sequential growth factors 51st annual meeting of the Orthopedic Research Society 2005.

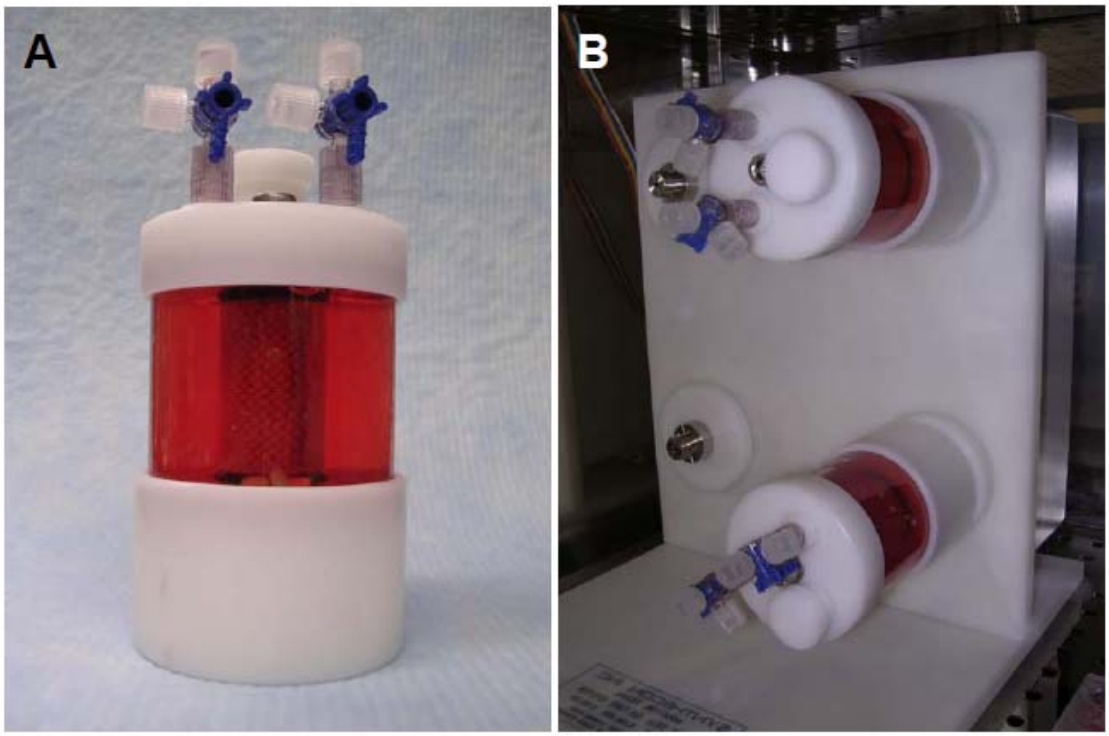


Figure 3-1. Rotating wall bioreactor flask (110mL) containing media and PGA scaffolds (**A**), and flasks loaded on the rotating base apparatus; flasks rotate around their longitudinal axis (**B**).

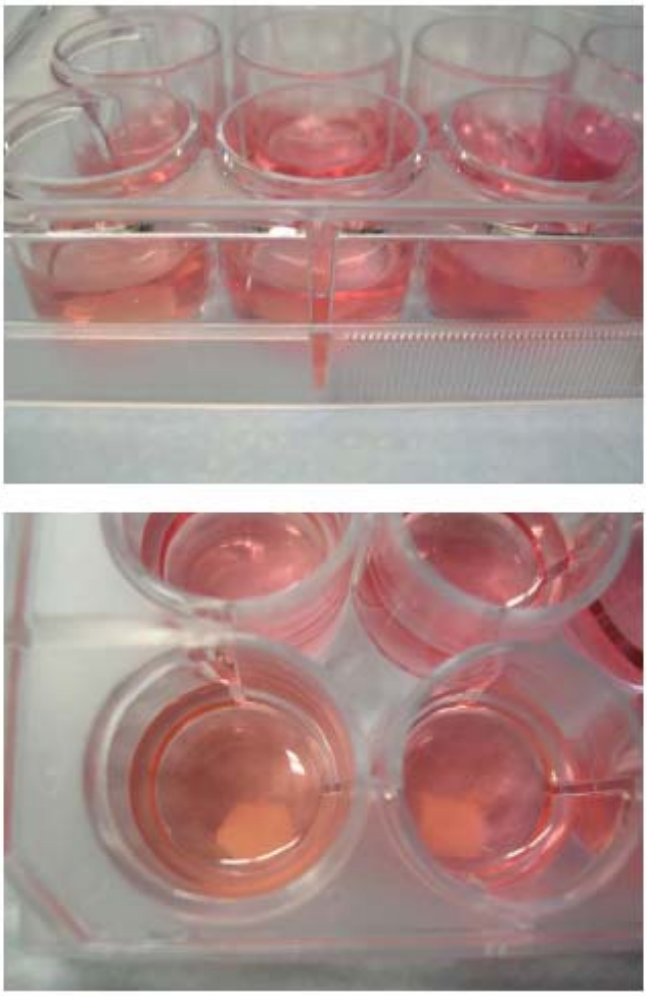


Figure 3-1b. Static culture of synovial membrane cells on PGA scaffolds in a 24 well tissue culture plate, with each well containing 2mL of supplemented DMEM.

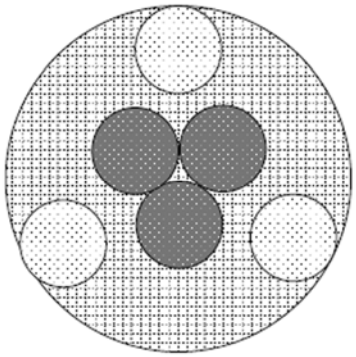


Figure 3-2: Methodology for counting cells at periphery and central regions (dark dotted circles) of the scaffold (cross- hatched circle) using digital image analysis; peripheral cell counts (light dotted circles) were obtained at the 2o'clock, 6o'clock and 19o'clock positions. Circles represent a low power (10X) field of view.

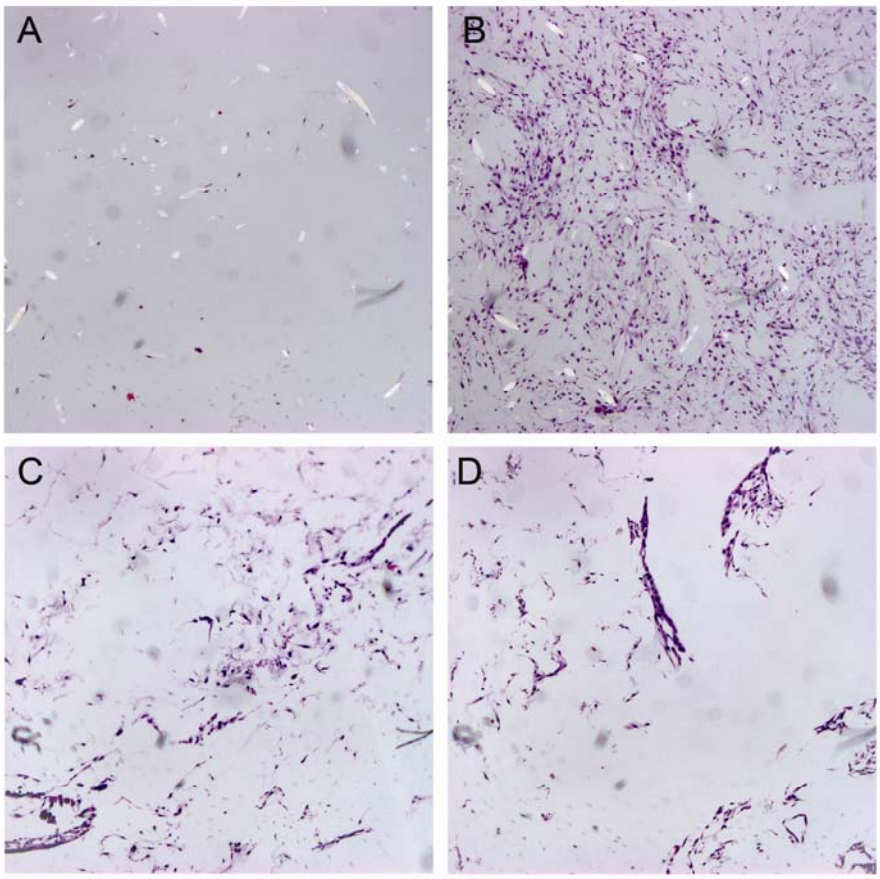


Figure 3-3: Scaffold Micrographs, Hematoxyalin and Eosin staining, 10X objective, 20X total magnification:
A) PGA construct, cultured in a static environment
B) PGA construct, cultured in a dynamic environment (rotating bioreactor).
C) OPLA construct, cultured in a dynamic environment (rotating bioreactor)
D) OPLA construct, cultured in a static environment

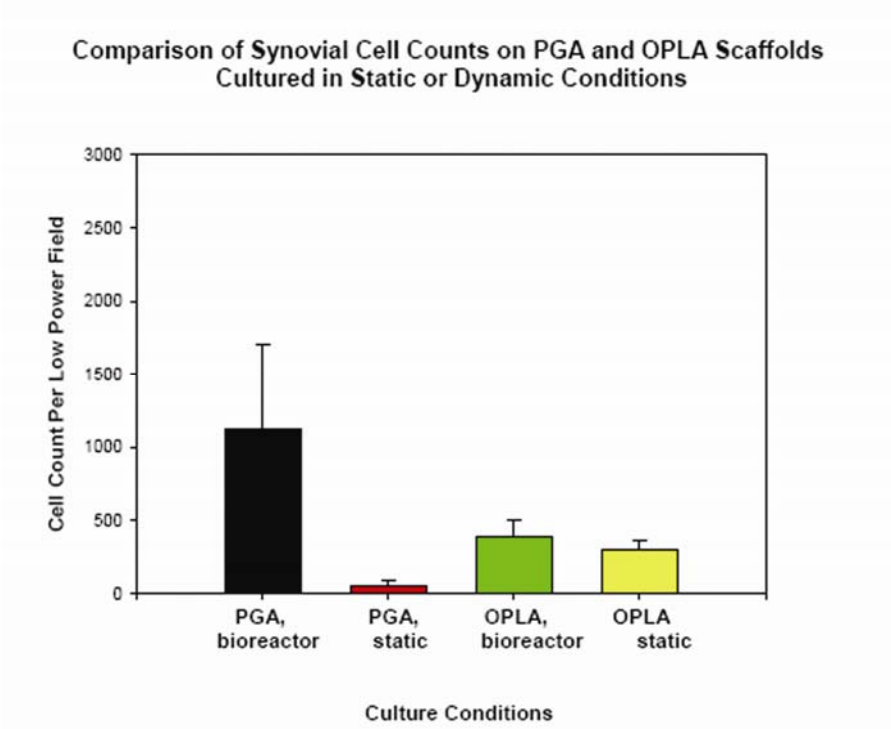


Figure 3-4: Comparison of mean synovial cell counts in PGA and OPLA scaffolds cultured in static and dynamic conditions.

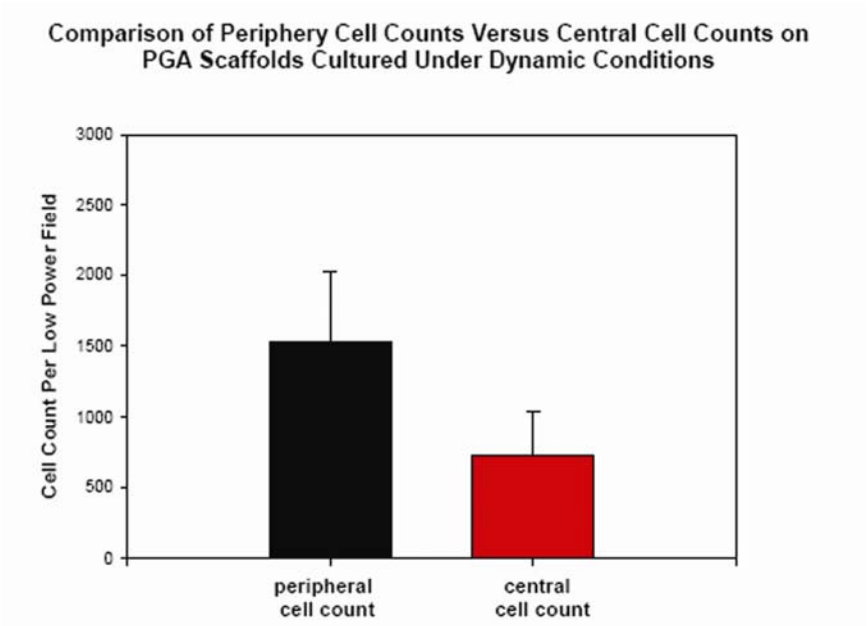
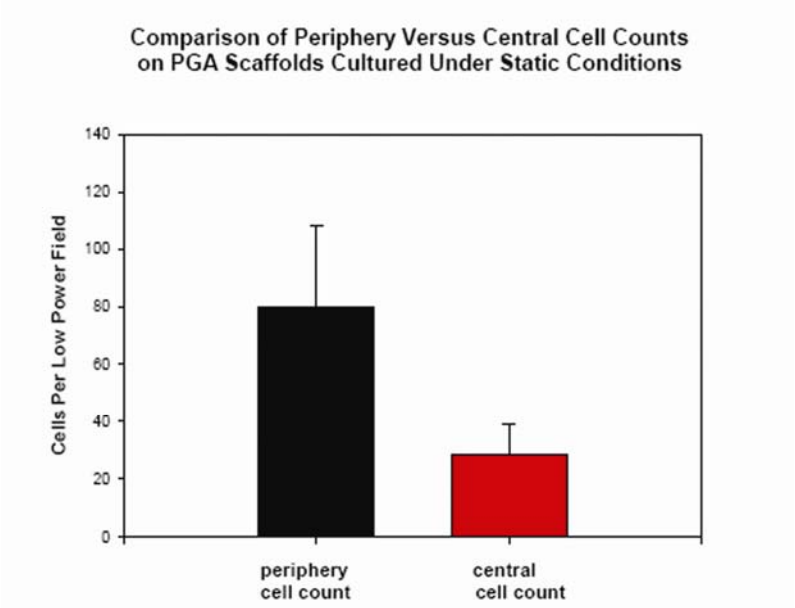
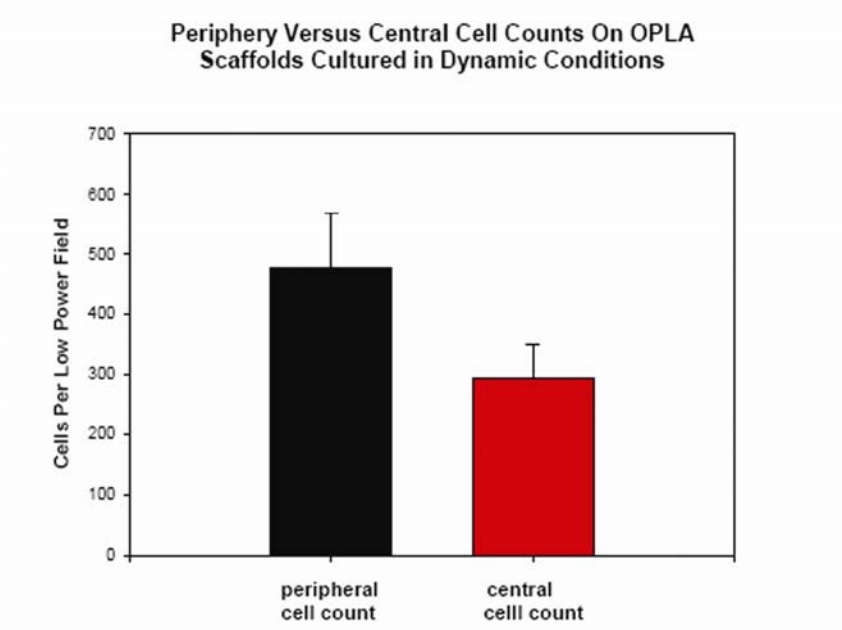


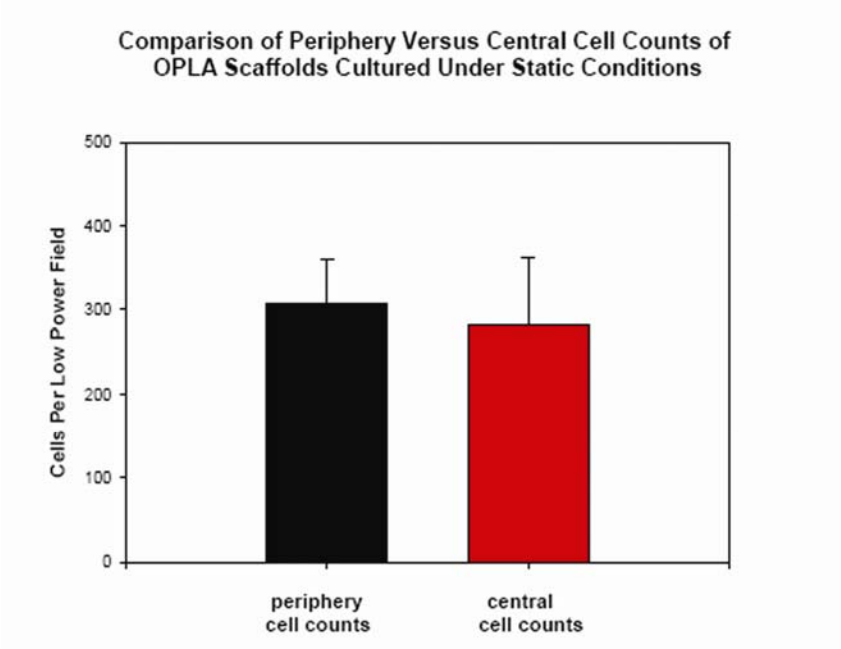
Figure 3-5: Peripheral versus central cell counts in PGA in PGA and OPLA scaffolds cultured in static and dynamic conditions.
a. Comparison of mean peripheral versus central cell count for the PGA-D group.



b. Comparison of mean peripheral versus central cell count for the PGA-S group.



c. Comparison of mean peripheral versus central cell count for the OPLA-D group.



d. Comparison of mean peripheral versus central cell count for the OPLA-S group.

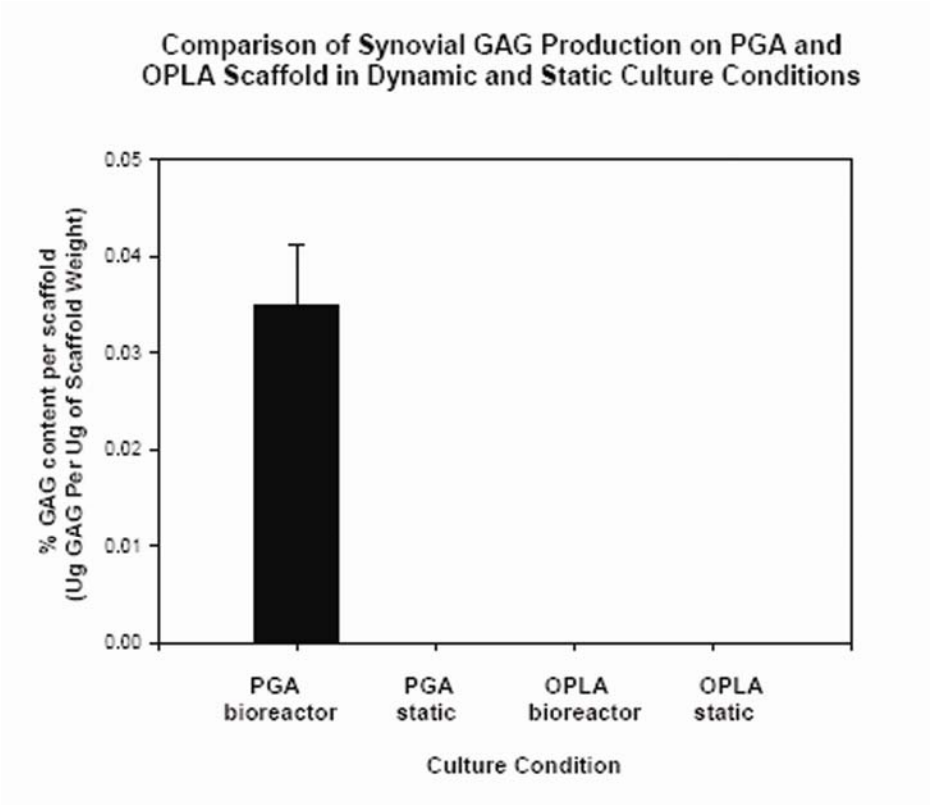


Figure 3-6: Comparison of % scaffold GAG content between PGA-D, PGA-S, OPLA-D, OPLA-S groups (on a wet weight basis).

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Chapter 4: Evaluation of dynamic seeding of equine synoviocytes on 2% and 4% PLLA (Poly -L -lactic acid) coated PGA (Poly -glycolic Acid) tissue scaffolds.

Tissue scaffolds must provide substrate and stability for cellular retention, intercellular communication, and cellular growth to allow seeded cells to proliferate ECM. As scaffolds naturally degrade *in vitro* or *in vivo*, ECM must be produced at a rate to reach or exceed the biomechanical function previously designated by the scaffolds, to maintain tissue integrity. A scaffold must be hydrophilic enough to allow cell adhesion but have a long enough half life to not prematurely dissolve, which would prevent ECM proliferation and cell death. In Chapter 3 we saw that while PGA allows excellent cellularity, it hydrolyzes too quickly (t1/2 =16 days) in proportion to synovial ECM synthesis, for the purpose of longer term culture. While the exact duration of culture required to synthesize a meniscus is not known, the time required is likely longer than this half life of 16 days, based on duration of normal human and canine meniscal development.

PLLA (Poly -L -lactic acid) is FDA approved and commercially fabricated, improving ease of use, and PLLA also has a longer half life of 6 months. In the previous study, we saw that PGA scaffold cellularity was decreased centrally. Coating a PGA scaffold with PLLA could still allow cellular retention centrally, while protecting the outer edges of the scaffold from premature dissolution. PLLA has been used in tissue engineering for *in vitro* culture of perichondrial cells,[1] adult chondrocytes,[2] human osteoblasts,[3] and human dermal fibroblasts.[4] PLLA has also been successfully used for *in vitro* culture of chondrocytes and smooth muscle cells when combined with other hydrophilic substances, such as PGA[5], collagen gel,[6-8] and gelatin.[9] One study comparing *in vitro* and *in vivo* culture of chondrocytes on SIS versus a polyglycolic acid-poly-L-lactic acid matrix mixture (PGA-PLLA) showed that tissues derived from PGA-PLLA scaffolds contained type II collagen, and aggregate and shear modulus values were higher for PGA-PLLA-derived tissues. [5] However, this work included a physiologically irrelevant subcutaneous

implantation model. Further investigation of combination use of PLLA combined with PGA for *in vitro* synoviocyte culture is warranted.

The purpose of this study is to test if PLLA coated PGA scaffolds can provide even cellularity without premature degradation during short term culture times, for use in synovium based meniscal fibrocartilage tissue engineering. We hypothesize that there will be: 1) no difference in construct cellularity, cellular distribution, and cellular viability in 2% PLA coated scaffolds vs. 4% PLA coated scaffolds, 2) no difference in these parameters at 14 days of culture versus 21 days of culture, and 3) 2% vs. 4% PLA coated scaffolds will not show any difference in gross degradation at any time point.

Materials and Methods:

Scaffolds

Polyglycolic acid (PGA)[a] non woven scaffolds were synthesized as a 3mm thick non-woven felt with a fiber diameter of 10μm. Poly-L –Lactic acid (PLLA)[b] was dissolved in methylene chloride as a 2% or 4% solution. The 2% PLLA solution was applied to each scaffold surfaces with an eye dropper, followed by a 2 hour drying period before the next surface was coated with PLLA. The process was repeated for 4% PLLA. The scaffolds were then placed in a vacuum dessicator overnight. Scaffolds were sterilized in ethylene oxide. Following sterilization, the modified scaffolds were cut into fourteen 5mmx 7mmx 3mm squares using sterile scissors and #10 bard parker blades.

Tissue Collection and Monolayer Cell Culture

Synovial intima/ subintima was harvested from the stifles of two horses euthanatized for an unrelated study. The horses were determined to be free of orthopedic disease based on physical examination and gross examination of the joint. The tissue was placed in Dulbeccos' Modified Eagle's Media with 10% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B 25ug/mL, 0.002% L-Ascorbate, and 0.01% L-glutamine (supplemented DMEM) in preparation

for monolayer culture. The synovium was sectioned into 2.0mm x 2.0mm pieces using a #10 Bard Parker blade under sterile conditions. The tissue fragments were combined with sterile Type 1A clostridial collagenase solution[c] at a concentration of 7.5mg/mL of RPMI 1640 solution. The mixture was agitated at 37˚C, 5% CO2, 95% humidity for six hours. Cells were recovered through centrifugation, the supernatant decanted and the cellular pellet re-suspended in 5mL of supplemented DMEM. The cell solution was transferred to a 25mL tissue culture flask containing 5mL of supplemented DMEM. The flasks were incubated at 37˚C, 5% CO2, 95% humidity, with sterile medium change performed every 3 days. Synovial cells were monitored for growth using an inverted microscope until observance of 95% cellular confluence per tissue culture flask. Cells were then transferred to 75mL tissue culture flasks. At 95% confluence the cells were subcultured until the 3rd cell passage had been reached.

Dynamic Culture

Fourteen 2% PPLA coated PGA scaffolds and fourteen 4% PPLA coated PGA scaffolds were placed in separate 110mL vessel flasks of the rotating bioreactor system[d] containing 110mL of supplemented DMEM. The bioreactor vessels were rotated at 51.1 rpm to attempt to achieve free-floating rotation within the culture medium. The scaffolds were presoaked for 24 hours in the bioreactor at 37˚C, 5% CO2, 95% humidity, prior to cell introduction. After this time it was noted that the scaffolds were still not 100% media soaked, and were floating at the apex of the flasks. Using sterile surgical technique, scaffolds were sterily removed from the flasks, pierced centrally, and strung on loops of straight needle 3-0 nylon surgical suture[e] with knots placed adjacent to the scaffolds to prevent bunching on the line. Seven scaffolds were placed per suture. The strings of scaffolds were then placed back in to the bioreactors and presoaked for another 12 hours, at which time complete hydration and submersion were achieved. Scaffolds were then seeded. Synovial membrane cells were removed from the tissue culture flasks using Accutase[f] and counted. Cells were added to the bioreactor flasks at a concentration of 475,000 cells/ mL. The bioreactor was maintained at 37˚C, 5% CO2, 95% humidity at 51.1 rpm. Fifty percent of the

cell culture medium volume was changed using sterile technique every 3 days. Seven scaffolds were harvested on day 10 of culture, and 7 scaffolds were harvested on day 21 of culture (**Figure 4-1**).

Determination of Cell Viability

Cell viability was determined with the use of ethidium homodimer-1 (4ul/ml PBS) and Calcein AM (Acetoxymethylester) (0.4ul/ml PBS) fluorescent stains[g] and the use of Confocal Laser Microscopy. The Confocal Laser Microscope consists of the BioRad Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope[h] equipped with Krypton-Argon and red diode laser. Approximately 1.0 mm sections were made from the scaffold half, with a sliver from what was the scaffold center and a section from the edge. Sections were incubated with the staining agents for 30 minutes at room temperature, placed on a glass microscope slide, moistened with several drops of PBS, 1X, and stained using the fluorescent double labeling technique. The sections were examined under 10x magnification. Images of each section, (three from the scaffold periphery and three from the scaffold center) at 2 o'clock, 6o'clock and 10o'clock positions were digitally captured by an Olympus DP-70[i] digital camera and saved as tiff files. Live and dead cell counts were determined by hand counts.

DNA quantification

One half of each construct was lyophilized and a dry weight obtained. Samples were incubated in 1.0ml Papain Solution (2mM Dithiothreitol and 300ug/ml Papain) at 60ºC in a water bath for 12 hours. The Quant-iT PicoGreen™ double stranded DNA quantification assay[j] was performed. Double stranded DNA extracted from bovine thymus was mixed with TE buffer[k] to create standard DNA concentrations of 1,000, 100, 10, and 1 ng/mL. The standards and 100uL of each papain digested sample (used in the above GAG and hydroxyproline assays) were added to a black 96 well plate. 100uL of 2ug/mL of Pico Green reagent was added to each well and the plate was incubated for 5 minutes. Sample fluorescence was read at 485nm excitation/ 528nm emission by the Synergy HT – KC-4 Spectrophotometric Plate Reader.[l] Absorbances were

converted to ng/mL concentrations and total double stranded DNA yield in ng using FT4 software.[m]

Statistical Methods

Non parametric data was analyzed using Kruskal-Wallis One way analysis of variance on ranks followed by Mann-Whitney Rank Sum tests. Significance was set at $p < 0.05$. Calculations were completed using a statistical software program.[n]

Results

Post PLLA modification, microscopic examination of the scaffolds revealed a heterogeneous conformation: the PLLA could be seen in non uniform globs on the nonwoven fibers of the PGA. Mean scaffold dry weights before soaking and seeding were 1.01mg for 2% PLLA coating and 1.52mg for 4% PLLA coating (P=<0.0001). Scaffolds were noted to be dissolving and more fragile upon manipulation on day 21, particularly on the outer edges as well as around the central suture. Cell content (**Figure 4-2**) as standardized to construct dry weight increased over time: for the 2% group, day 10 cellularity was 102.6 ng dsDNA/mg dry weight, and on day 21 it was 281.79 (P=<0.001). On day 10 for the 4% group, DNA content was 111.01 ng dsDNA/mg dry weight and on day 21 it was 140.2 (P= 0.033). Standardized construct cellular content was higher in the 2% PLLA coating group than the 4% PLLA coating group on day 21 (P=0.037), but not on day 10 (P=0.602). Grossly, both scaffold types were visibly disintegrated at day 21. Mean lyophilized weight on day 10 for 2% PLLA coating was 0.533mg, which decreased to 0.257mg on day 21 (P=0.002). Mean lyophilized weight on day 10 for 4% PLLA coating was 0.481mg, which decreased to 0.381mg on day 21 (P=0.043). As visible under confocal microscopy (**Figure 4-3,a,b,c,d**), cells only adhered to the surface of exposed PGA fibers and had poor penetration to the scaffold centers. Exact viable cell numbers were estimated only because of the marked cellular clumping, but the viability ranged from 50-90% in all groups at all time points. Histologic examination of H+E stained constructs revealed minimal cellular adhesion to the PLLA, in all groups at all times, with cells growing primarily on the exposed

dimples of PGA scaffold, in tightly packed clumps, or adhering to random exposed fibers of PGA (**Figure 4-4**).

Discussion

The method of PLLA coating in this study resulted in uneven distribution of PLLA with exposed areas of PGA fibers. Therefore, cellular adhesion and growth occurred exclusively over the hydrophobic PGA fibers, resulting in clumped cellular distribution and spans of non-cellular regions over the hydrophobic PLLA coating. A clumped cellular configuration is not appropriate for musculoskeletal tissue engineering; cells must be evenly distributed on scaffolds[10] to maximize ECM production and construct compressive properties.[10-14] In addition, cell clumping leaves areas of exposed scaffold which can degrade rapidly for lack of stabilizing ECM formation, resulting in construct failure. In addition central cellularity was poor, possibly because the PLLA coating prevented central cell adhesion and efficient nutrient exchange at that level. PLLA was not able to prevent premature scaffold dissolution in this study. Once the culture media penetrated thought he uneven coated outer surface to the uncoated central PGA fibers, there was no chemical protection to prevent the usual PGA hydrolysis degradation, providing an unstable construct core. Thus, both uneven construct cellularity and uneven PLLA coating exposing PGA fibers to aqueous degradation, may have contributed to scaffold dissolution seen here.

Regardless of 2% or 4% PLLA concentration, the coating of PLLA on the PGA felts was not uniform, and PGA was the principal surface that allowed cell adhesion on the coated scaffolds. Favorable cellular adhesion and growth on PGA is easily explained by the polar surface properties and fibrous configuration of PGA, which helps cell adhesion, allows nutrient transport, and provides a high surface area for cellular adhesion. However, the minimal cell growth over the PLLA is in contrast to data reported in Chapter 3, where cellular adhesion to OPLA sponges in a rotating bioreactor occurred readily. This likely reflects the manufacturing difference in the OPLA sponges, which undergo chemical porosity to assist in cellular retention

and adhesion, versus the slick coating of PLLA with no surface modification, which apparently inhibits cellular adhesion. Although PLLA is widely used in tissue-engineering applications because of its slower degradation characteristics, strength and mechanical properties, it possesses a hydrophobic, inert nature, which affects cell-matrix interactions. [15] This hydrophobic nature was first demonstrated when the scaffolds resisted hydration during the presoaking period, and further demonstrated by the decreasing construct cellularity as PLLA concentration increased from 2% to 4%. Cells adhere to ECM and each other via integrin transmembrane receptors, which bind the cell membrane to Arginine-Glycine-Aspartic acid RGD motifs on the ECM, linking the cell's cytoskeleton to the pericellular environment.[16-28] The integrins must have amino acid motifs or other polar hydrophilic surfaces to allow cellular adhesion and proliferation.[16-28] PLLA can now be modified to take on characteristics of natural ECM, while retaining its desirable strength and longevity. Surface modification and hydrophilization of PLLA can be done with Poly(L-lysine), Arginine-Glycine-Aspartic Acid-Cysteine (RGDC), [15] Arginine-Glycine-Apartic Acid (RGD), [29] Lecithin,[30] Arginine-Glycine-Aspartic Acid-Serine (RGDS), [31] Arginine-Glycine-Glutamate-Serine (RGES), [31] and oxygen plasma treatment with *in situ* graft polymerization of acrylic acid. [32] Biologic surface modification creates an ECM biomimetic scaffold that increases surface area and enhances cellular adhesion. [15,29-32] Future synoviocyte seeding studies will focus on producing biomimetic scaffolds to enhance scaffold cell retention, such as RGD linkage. Thus, utilizing a hydrophilic scaffold (PGA) coated in a hydrophobic compound (PLLA) does not produce a feasible construct for the purpose of meniscal fibrocartilage tissue engineering, and will not be investigated further.

Scaffold cellularity also increased over time in both groups. This likely represents cellular proliferation over time. However, the viability within each group at each time point varied from an acceptable 90% to an unacceptably high mortality rate estimated at 50%. Although these are visual estimates of cellular viability, high cell mortality rates are a concerning blockade to ECM proliferation and live tissue replacement. Cell death may be attributed to

unfavorable growth conditions on the impermeable, hydrophobic PLLA, and scaffold instability and dissolution.

In conclusion, we reject our null hypotheses; there was an increasing trend of cellularity with increasing culture duration, and decreasing construct cellular content with increasing PLLA concentration. PLLA coating did not protect PGA from *in vitro* degradation over time. In addition, the 2% and 4% PLLA coated PGA scaffolds were heterogeneous, leading to uneven cellularity, and therefore are not appropriate scaffolds for *in vitro* synoviocyte culture.

Footnotes

a. PGA Tissue Scaffold, Synthecon, Houston, TX.
b. PLLA, Polysciences Inc.,Warington PA
c. Type 1A Clostridial Collagenase, Sigma, St. Louis, MO
d. Rotating Bioreactor System, Synthecon, Houston, TX.
e. Dermalon, Ethicon, Somerville, New Jersey
f. Accutase, Innovative Cell Technologies, San Diego, CA.
g. Live/Dead Viability/Cytotoxity Kit, [Cat. #1-3224], Molecular Probes Co., Eugene, OR
h. Olympus IX70 microscope, Olympus, Melville, NY
i. Olympus DP-70 Digital Camera, Olympus, Melville, NY.
j. Quant-iT PicoGreen kit, Invitrogen, Fountain Drive, United Kingdom
k. TE buffer, Invitrogen, Fountain Drive, United Kingdom
l. KC-4, BioTec, Winooski Vermont
m. FT-4 software, BioTec, Winooski Vermont
n. SigmaStat, Jandel Scientific, SanRafael, CA.

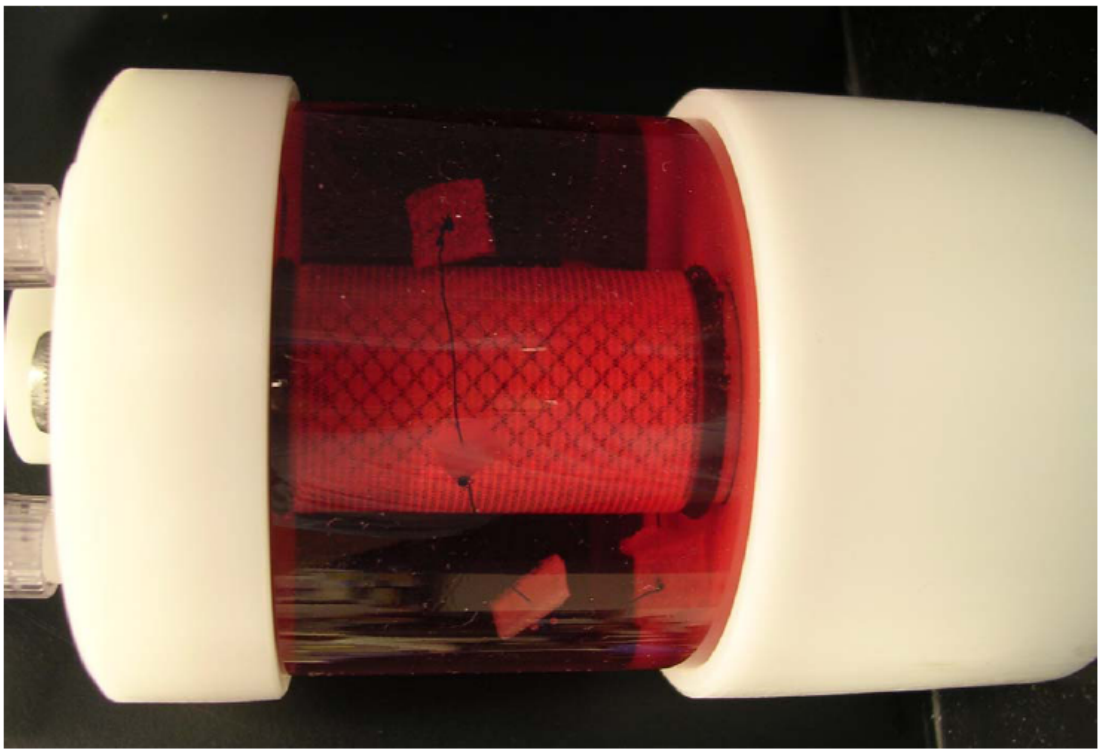

Figure 4-1. Rotating wall bioreactor flask (110mL) apparatus with PLLA coated PGA scaffolds, strung on suture: the 10th day of culture after the first scaffold harvest.

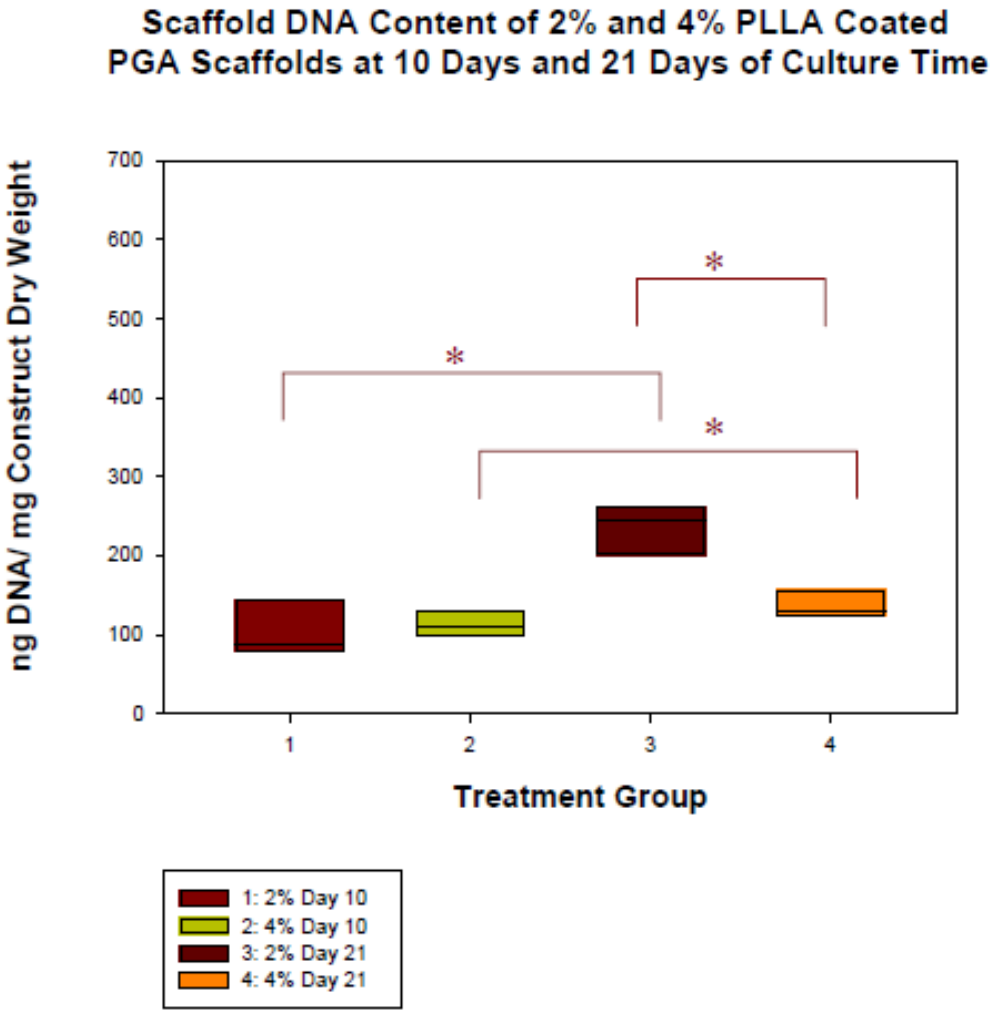

Figure 4-2. Construct cellularity of 2% and 4% PLLA coated PGA scaffolds, on the 10th and 21st day of culture, as determined by the double stranded DNA assay. An asterisk (*) denotes significant differences.

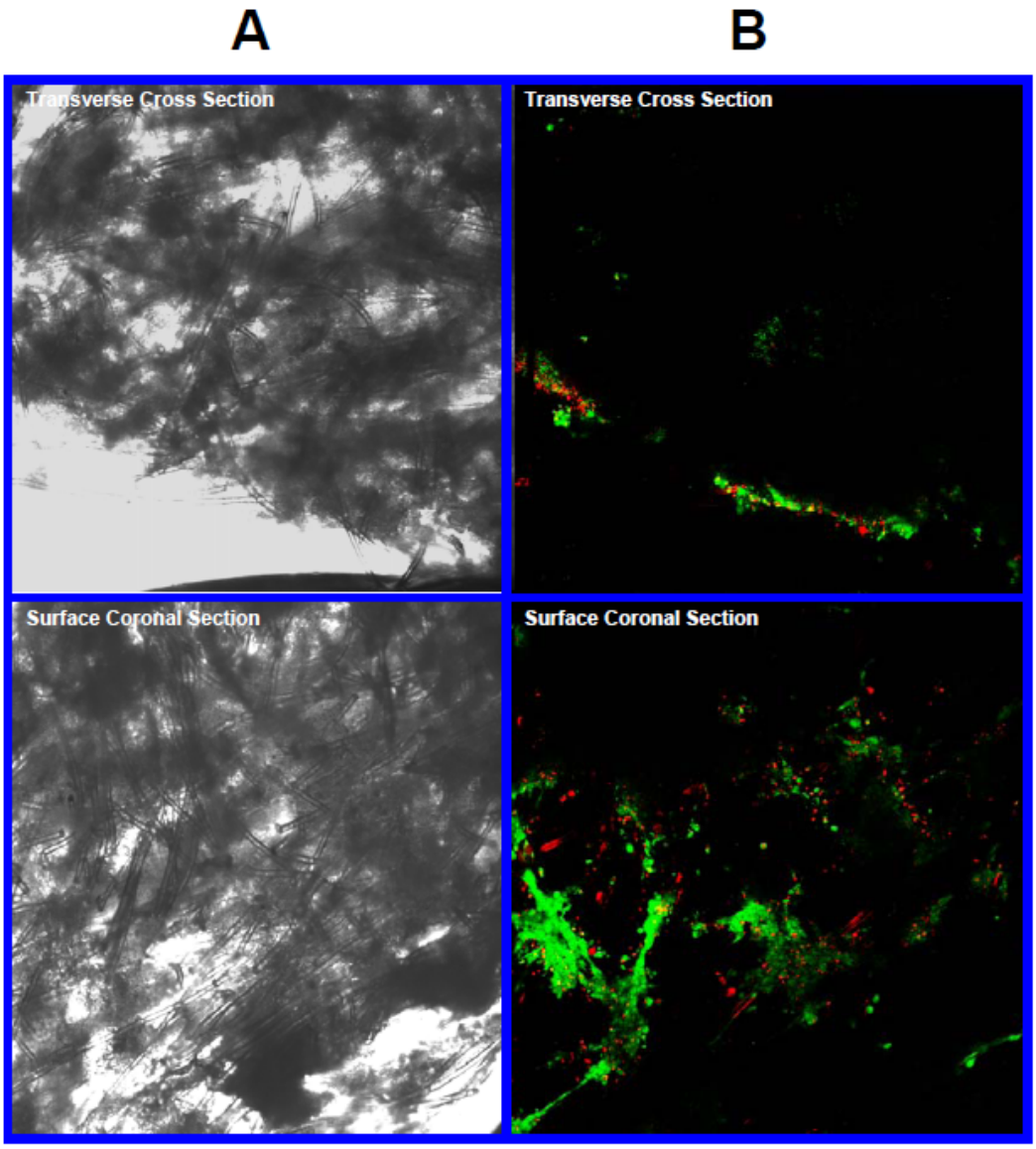

Figure 4-3a. Photomicrographs of 2% PLLA coated PGA constructs harvested on day 10, under standard light (column **A**) and laser (column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 10x objective magnification. Images represent construct transverse cross sections and surface coronal sections. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

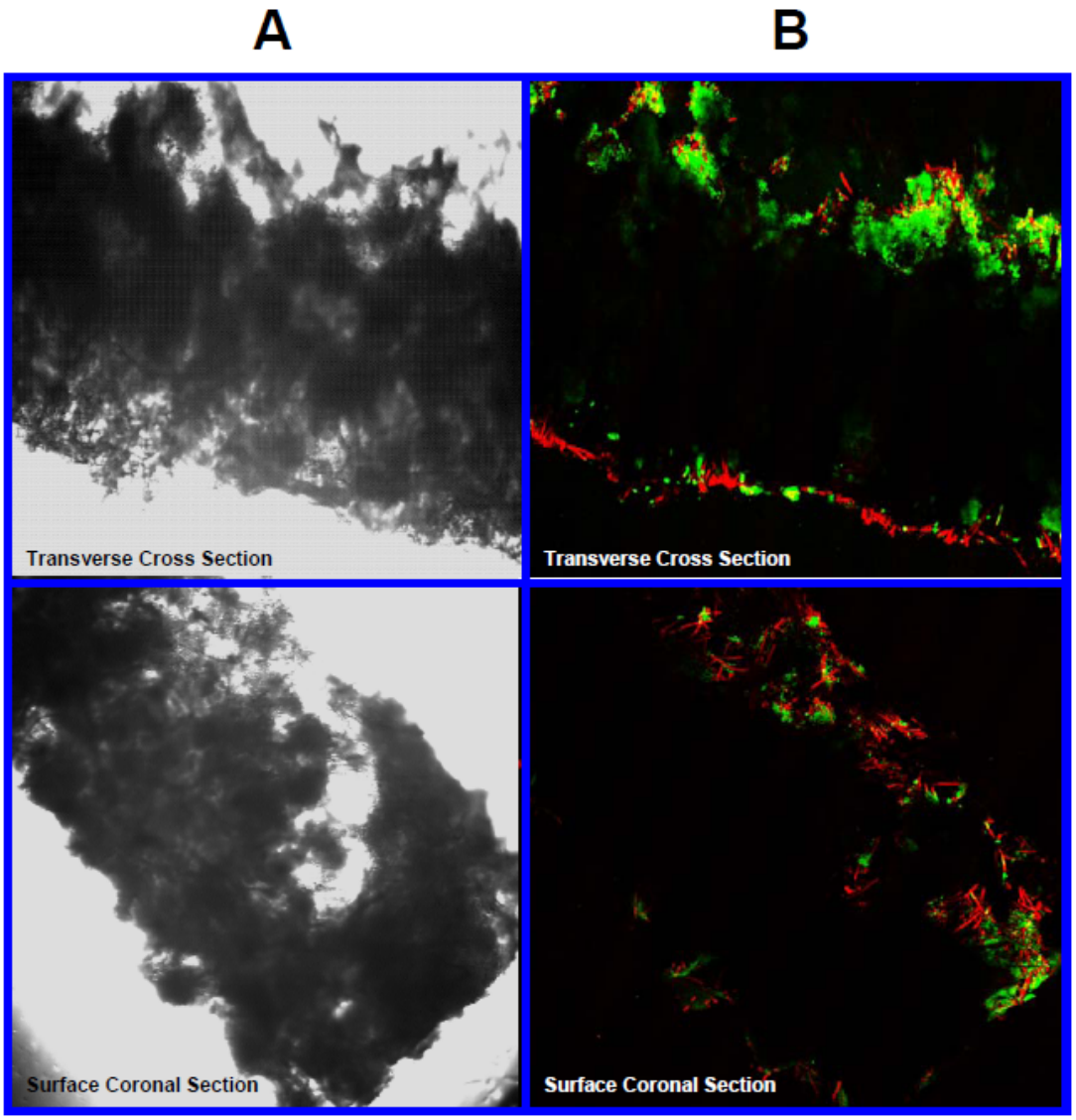

Figure 4-3b. Photomicrographs of 2% PLLA coated PGA constructs harvested on day 21, under standard light (column **A**) and laser (column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 4x objective magnification. Images represent construct transverse cross sections and surface coronal sections. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

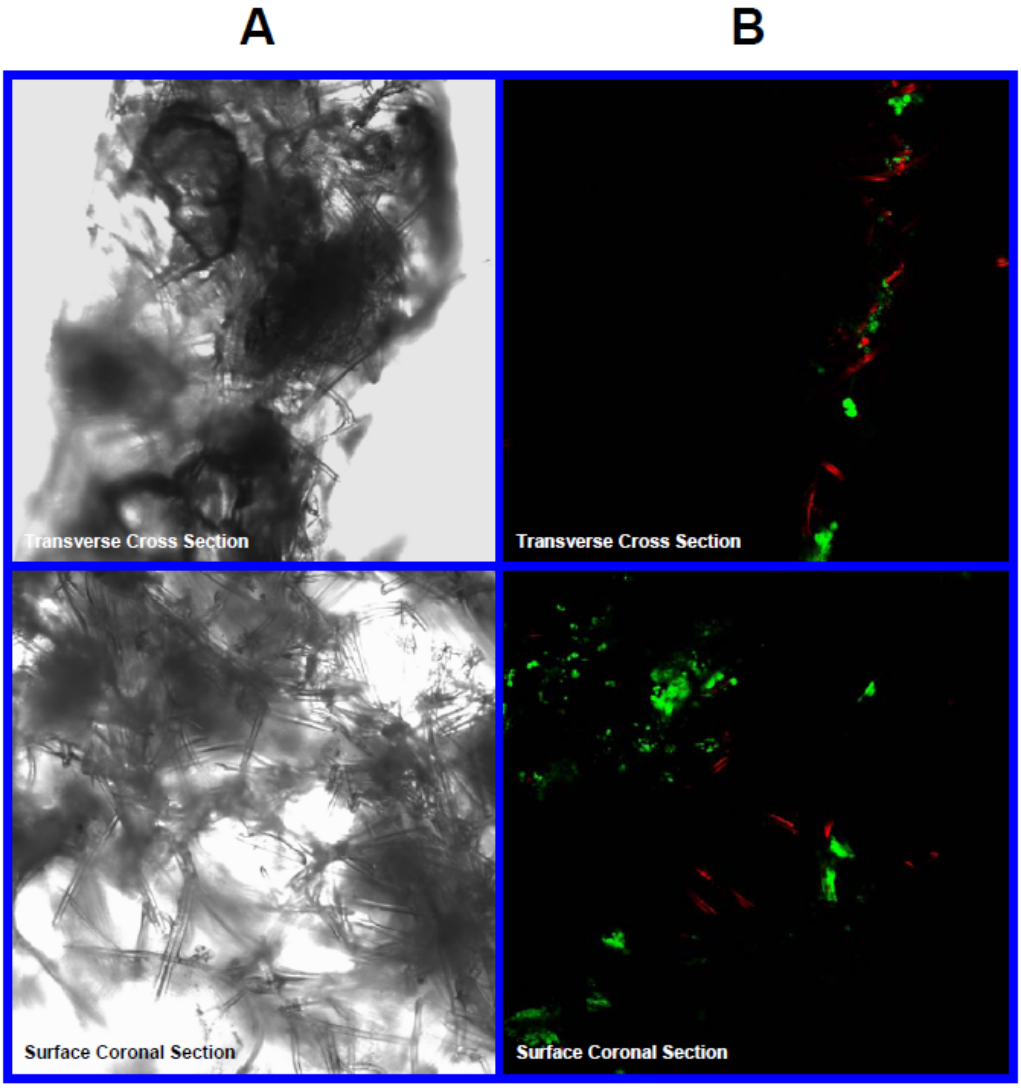

Figure 4-3c. Photomicrographs of 4% PLLA coated PGA constructs harvested on day 10, under standard light (column **A**) and laser (column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 10x objective magnification. Images represent construct transverse cross sections and surface coronal sections. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

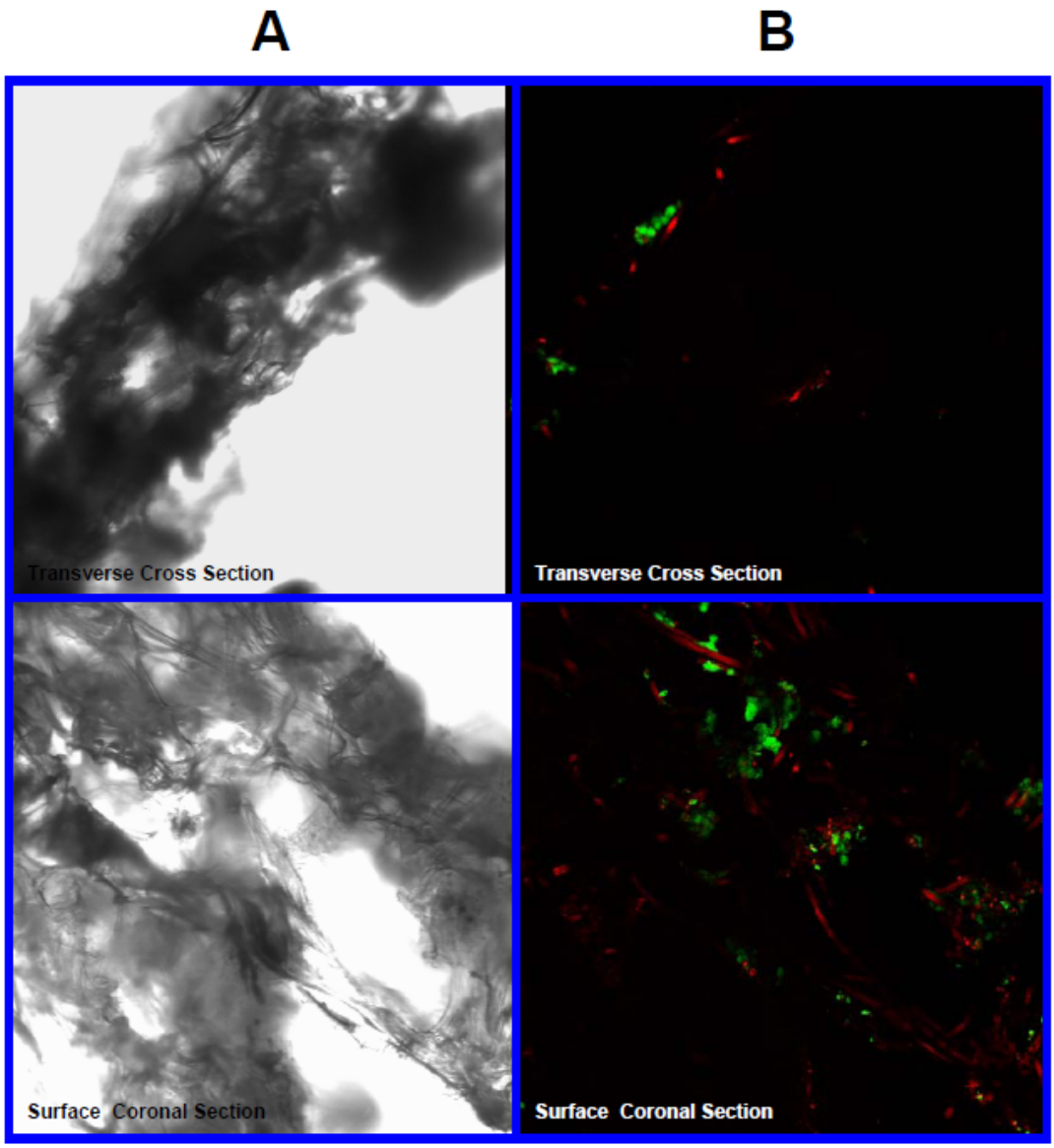

Figure 4-3d. Photomicrographs of 2% PLLA coated PGA constructs harvested on day 21, under standard light (column **A**) and laser (column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 10x objective magnification. Images represent construct transverse cross sections and surface coronal sections. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

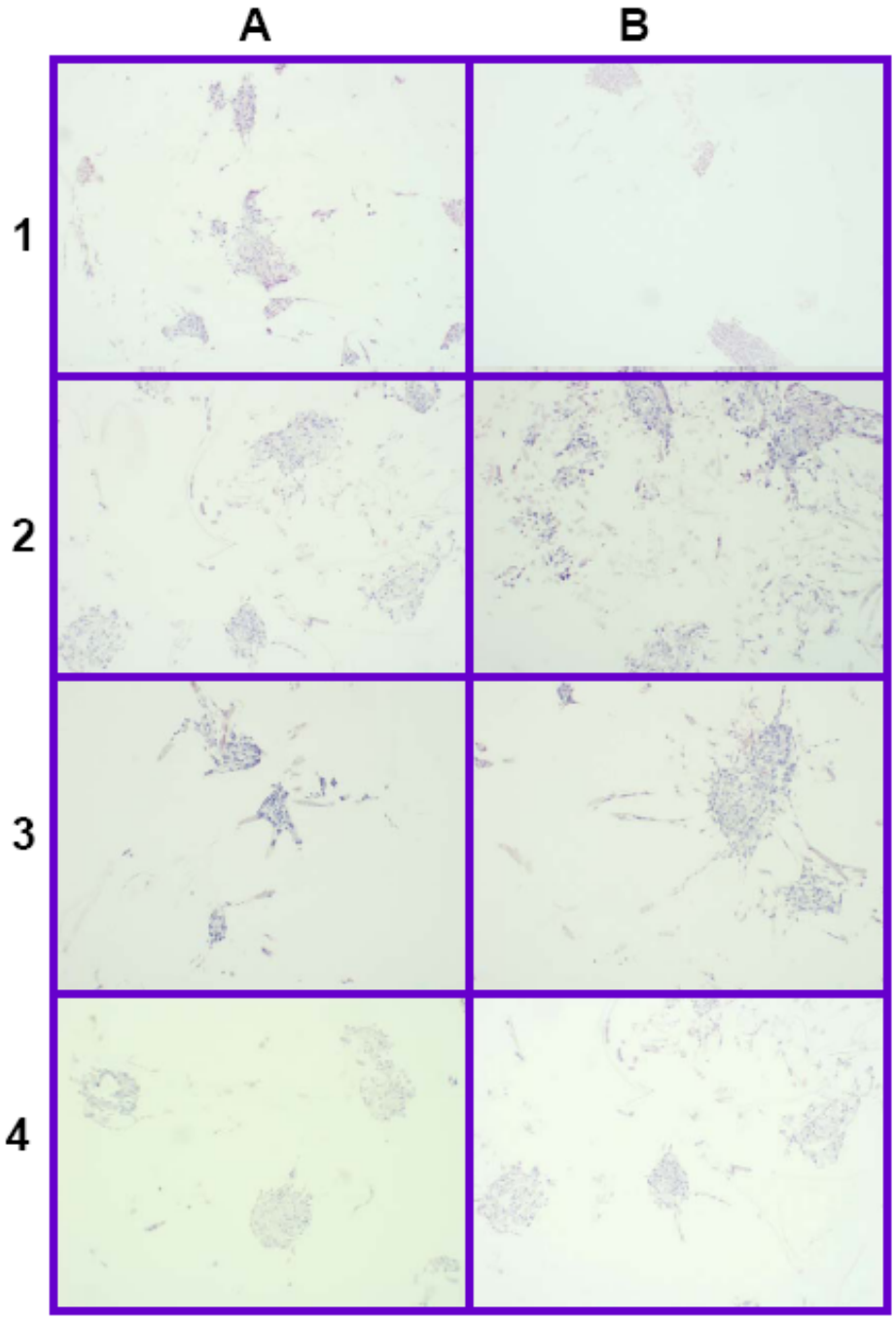

Figure 4-4. Photomicrographs of 2% PLLA coated PGA scaffolds harvested on day 10 (row **1**) and day 21 (row **2**), and 4% PLLA coated PGA scaffolds harvested on day 10 (row **3**) and day 21 (row **4**), H+E staining, 10x objective magnification. Column **A** represents images of the center of the construct and column **B** represents images taken of the scaffold periphery. Note that the cells have grown in dense clusters; PLLA does not stain positive but intermittent PGA fibers can be seen around the cell clusters.

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Chapter 5. Early synovial fibrochondrogenic differentiation by sequential growth factor exposure on a novel 10% poly -(L-lactic acid) and 90% poly-(glycolic acid) polymer blend tissue scaffold

In the past two chapters, we investigated various scaffold types and culture conditions for synovial membrane cells, with the goal of maximizing synovial ECM synthesis, and achieving uniform cellular distribution and viability. We found that synoviocytes cultured in a rotating bioreactor created the most GAG and had the highest cellularity compared to static culture methods. Cartilage engineering with biodegradable synthetic scaffolds is most successful if uniform distribution of cells is achieved,[1-3] and spatial uniformity of cellular distribution has been shown to be optimized through the use of bioreactors.[3] Kim et al[4] demonstrated that dynamic versus static seeding of polyglycolic acid (PGA) scaffolds with smooth muscle cells led to significantly greater cell numbers and uniform cellular distribution which resulted in increased extracellular matrix production. This effect ultimately improves the compressive properties of the chondrocyte/ PGA constructs.[1-3] Rotating wall bioreactors provide a dynamic, laminar fluid shear force on cells, which has positive effects on cell differentiation, cell viability, and extracellular matrix production through the principles of mechanotransduction[1-3,5] Shear stresses are generated from fluid flow in rotating wall bioreactors, which when applied to chondrocytes on PGA scaffolds, significantly increase cell concentrations as well as synthesis and accumulation of sulfated GAGs, hydroxyproline, and collagen.[1-3] Because of this data, the 110mL rotating bioreactor was utilized again in this study for a dynamic culture environment.

PGA (Poly-Glycolic Acid) and PLLA (Poly-L- Lactic Acid) are biodegradable, biocompatible, poly-esters, that are attractive for tissue engineering because they are readily available, can be easily processed into a variety of structures, and are FDA approved for a number of biomedical applications.[6] PLA has been successfully used in musculoskeletal tissue engineering for *in vitro* culture of perichondrial cells,[7] adult chondrocytes,[8] human osteoblasts,[9]

and chondrogenesis of human dermal fibroblasts.[10] PGA has been successfully used as a scaffold for meniscal fibrochondrocytes *in vivo*[11] and cultured *in vitro*[12] to form meniscal- like tissue. PGA has also been used to form articular cartilage *in vitro* with fetal chondrocytes[13] and adult chondrocytes.[14] In previous chapters, use of OPLA sponges or PLLA coated PGA scaffolds resulted in clumped cellularity, poor central cellularity, and lower cell numbers; thus these types of scaffolds were rejected for future use. In contrast, dynamically cultured synoviocytes on non woven PGA constructs had the highest GAG content and tissue cellularity, with central cellular penetration. Based on this and the work of others, we believe that both PGA and PLA would still be viable synthetic scaffolds for *in vitro* culture of synoviocytes, for application in meniscal fibrocartilage tissue engineering. However, while the non woven shaped scaffold proved to be advantageous in a bioreactor, the PGA scaffold began degrading before enough ECM could be synthesized to help maintain tissue contiguity. To address this problem, a polymer blend of PGA fibrils mixed with PLLA fibrils in a non woven felt has been synthesized. This polymer blend scaffold may help provide the cell adherence advantages of PGA with the longer life-span of PLLA to stabilize the scaffold while cells are producing adequate ECM.

Despite investigation of dynamic and static culture conditions and variable scaffold types, no detectable synovial chondrogenic differentiation has occurred in our studies. More sensitive measures such as reverse transcriptase PCR may be required to detect early, subtle chondrogenic gene expression. Application of simple biomechanical stimuli alone has not been able to induce synovial chondrogenic differentiation and chondrogenesis in short term culture.[15] Biochemical stimulation with growth factors may be required for a macroscopic synovial fibrochondrogenic phenotypic shift.

Biologic factors such as growth factors play an important role in synovial based meniscal tissue engineering by inducing synovial chondrogenesis. Three growth factors, transforming growth factor beta (TGF-β), Insulin like growth factor-1 (IGF-1), and basic fibroblast growth

factor (bFGF) have been extensively researched in their normal roles in embryologic skeletogenesis, musculoskeletal tissue synthesis and repair, as well as *in vitro* chondrogenesis.

TGF-β is an important promoter of tissue repair by promoting extracellular matrix formation, including collagen synthesis.[16] Recent research has demonstrated chondrogenesis and formation of type II collagen in synoviocyte aggregates cultured in the presence of TGF-β.[5,17,18] Furthermore, in cultured meniscal fibrochondrocytes, TGF-β increases production of extracellular matrix proteoglycans, aggrecan and biglycan, by 100%,[5,17] as well as type I,II, V, VI collagen.[18] TGFβ-1 has also been shown to induce expression of α-smooth muscle actin in cells derived from the synovium,[16] which plays a major role in wound contraction.[19,16]

Basic FGF is a crucial mediator of cell proliferation and collagen synthesis during healing of musculoskeletal tissues.[20,21] In addition, bFGF has been shown to significantly increase DNA synthesis and extracellular matrix proteins in monolayer cultures of meniscofibrochondrocytes.[16] FGF also induces synovial chondrogenesis *in vitro* [22-24]and increases cell proliferation of human synovial fibroblasts in monolayer culture.[25]

IGF plays a role in the healing attempts of articular cartilage.[26] Treatment of osteochondral defects with IGF-1 increases amounts of proteoglycans and Type II collagen present in the repair tissue.[27,28] IGF-1 improves the repair capabilities of chondrocyte-fibrin grafts,[29] and also consistently stimulates biosynthetic activity of chondrocytes, resulting in accumulation of glycosaminoglycans [30,31] and type II collagen[32] in culture. In another study, chondrocytes were genetically modified to increase expression of IGF-1, then transplanted into equine cartilage defects, resulting in improved early and long-term cartilage healing, with 100-fold increased expression of Collagen type II.[33] IGF-1 significantly increases chondrogenesis of periosteal mesenchymal stem cells *in vitro*, particularly in combination with TGF-β1. [34] Meniscal fibrochondrocytes cultured from the avascular zone of the meniscus are able to regenerate under the influence of IGF-1.[20,21]

Despite the individual biologic attributes of each growth factor, combinations of bFGF, TGFβ-1, and IGF-1 may produce the best fibrocartilaginous tissue *in vitro*. Early research by Pei et al [35] showed that a pulsed growth factor protocol of bFGF, 50ng/mL for 3 days, followed by TGFβ-1, 10ng/mL + IGF- 1, 500ng/mL, maximizes articular-like chondrogenesis of synoviocyte micromass pellets. bFGF, TGFβ-1, and IGF-1 may be applicable to forming fibrocartilage *in vitro* from cultured synovial cells. However, it must be determined if *in vitro* synovial fibrochondrogenesis is possible on scaffolds that can be shaped to the dimensions of a meniscus and surgically implanted. The objective of this study is to assess fibrochondrogenesis of synovial membrane cells, exposed to pulsed growth factors over time, under mechanical stimulation in a rotating bioreactor, on a novel, surgically applicable cell scaffold. We hypothesize that 1) there will be no difference in cell viability, cell number, and scaffold chondrogenesis of synoviocytes exposed to growth factors vs. controls; 2) there will be no difference in cell viability, cell number, and chondrogenesis in cell/scaffold constructs cultured for 26 days vs. 16 days.

Materials and Methods

Experimental Animals

Normal synovium was harvested from three horses humanely euthanatized by an overdose of barbiturate, for reasons unrelated to this study. Animals utilized were free of orthopedic disease, as confirmed on physical examination and gross examination of the stifle joints post mortem.

Tissue Collection

Synovial intima and subintima was collected from the stifle joints using surgical aseptic technique. Fibrous joint capsule was not included in the tissue harvest. Tissues were placed in Dulbeccos' Modified Eagle's Media with 10% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B 25ug/mL, 0.002% L-Ascorbate, 0.01% L-glutamine and transported directly for processing.

Tissue Preparation for Culture

Synovium was sectioned into 2.0mm x 2.0mm pieces using a #10 Bard Parker blade and standard sterile technique in a unilaminar flow hood. The tissue fragments were combined with sterile Type 1A clostridial collagenase solution[a] at a concentration of 7.5mg/mL of RPMI 1640 solution. The mixture was agitated on a plate shaker[b] in an incubator[c] at 37˚C, 5% CO2, 95% humidity for six hours. The digested solution was centrifuged[d] at 1000 rpm for 10 minutes. The supernatant was decanted and the cellular pellet re-suspended in 5mL of Dulbeccos' Modified Eagle's Media with 10% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B 25ug/mL, 0.002% L-Ascorbate, 0.01% L-glutamine (DMEM). The cell solution was transferred to a 25mL tissue culture flask containing 5mL of DMEM. The flasks were incubated at 37˚C, 5% CO2, 95% humidity, with sterile medium change performed every 3 days.

Monolayer Cell Culture

Synoviocytes were monitored for growth using an inverted microscope until observance of 95% cellular confluence per tissue culture flask. Cells were transferred to 75mL tissue culture flasks at a 1:4 dilution using the following procedure: cell medium was decanted from the flask and the cells washed with 5.0 mL of DMEM without fetal bovine serum. To detach cells from the 25 mL tissue culture flask, 1.0 mL of 0.05% Trypsin EDTA was incubated with the cells at 37˚C, 5% CO2, 95% humidity for 10-15 minutes. The cellular suspension was transferred to a 15mL Falcon centrifuge tube and centrifuged[e] at 1.5 RPM, at 15˚C for 5 minutes. The supernatant was decanted and the cell pellet resuspended with 5mL of DMEM; the new suspension was subcultured and transferred into four 75mL tissue culture flasks containing 13mL of DMEM. A cell count per flask was performed by combining 20uL of cell suspension with 20uL of 0.4% Trypan Blue[f] placed on a hemacytometer. At 95% confluence the cells were again subcultured until the 3rd cell passage has been achieved.

Scaffold Culture

A novel tissue scaffold, made of a blend of 10% poly-L-lactic acid and 90%poly-glycolic acid fibers,(PGA/PLLA blend)[g] was used in this study. Scaffolds were manufactured as a non woven felt, which was cut into 10mm x10mm x3mm sections and sterilized with ethylene oxide. Twenty scaffolds were placed in each 110mL rotating bioreactor[h] and presoaked for 12 hours in DMEM without fetal bovine serum, at 37˚C, 5% CO2, 95% humidity. Cells were removed from T-75 tissue culture flasks and counted from the tissue culture flasks using TryplExpress[i] to avoid cell clumping. Scaffolds were seeded with synoviocytes at 400,000 cells/ mL in the bioreactor rotating at 22.5rpm. Three treatment groups, twenty scaffolds per group, were established as follows: Group 1: a control group of serum free media only; Group 2: serum free media containing human recombinant basic Fibroblast Growth Factor (bFGF),[j] 50ng/mL for 3 days, followed by serum free media; and Group 3: serum free media containing human recombinant bFGF, 50ng/mL for 3 days, then maintained in serum free media containing human recombinant Transforming Growth Factor β-1 (TGFβ-1),[k] 10ng/mL, and Insulin-like Growth Factor- 1 (IGF-1),[l] 500ng/mL. Bioreactor cell scaffold culture was maintained at 37˚C, 5%CO2, 95% O2, with 50%of cell culture medium changed, using sterile technique, every 3 days, maintaining the above growth factor concentrations. Ten scaffolds were harvested from each group and quartered for analysis on days 16 and 26.

Scaffold Analysis

Histologic Analysis

Tissue harvested from each flask was cut in thirds and 1/3 fixed in 10% buffered formalin, sectioned, and stained with Hematoxylin and Eosin, Masson's trichrome, Safranin-0, and immunohistochemistry (IHC) for Alpha Smooth Muscle Actin, for histologic analysis. Samples were processed and submitted within 24 hours of tissue collection.

For immunohistochemistry of alpha smooth muscle actin, tissues were cut at 4 microns and placed on plus charged slides, microwaved and left on a 43°C slide warmer overnight to

deparrafinize. The slides were then hydrated and placed in 0.4% pepsin and heated in a 37°C incubator for 20 minutes. The slides were rinsed in tap water and placed in Tris buffer for at least 5 minutes. Subsequent staining was done on the Dakocytomation Autostainer.[m] Slides were treated with 3% H202 for 15 minutes, protein block (DAKO) for 5 minutes. Slides were incubated in primary antibody, Dako M0851[n] at 1:200 concentration for 30 minutes. Secondary and tertiary reagents used included the LINK and LABEL system[o] at 20 minutes each. The chromogen used was Nova Red (Vector) for 10 minutes. Slides were counterstained in Mayer's Hematoxylin[p] for 1 minute, dehydrated and coverslipped. Histologic specimens were examined at 10x magnification on a Zeiss Microscope.[q] Images of each section, (three from the scaffold periphery and three from the scaffold center) at 2 o'clock, 6o'clock and 10o'clock positions (Figure 1) were digitally captured by an Olympus DP-70[r] digital camera and saved as jpeg files.

Determination of Cell Viability

Cell viability was determined with the use of ethidium homodimer-1 (4ul/ml PBS) and Calcein AM (Acetoxymethylester) (0.4ul/ml PBS) fluorescent stains[s] and the use of Confocal Laser Microscopy. The Confocal Laser Microscope consists of the BioRad Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope[t] equipped with Krypton-Argon and red diode laser. Upon construct harvest, approximately 1.0 mm sections were made from each scaffold third, including a sample of the periphery and a sliver from the central portion of the scaffold. The samples were incubated with the staining agents for 30 minutes at room temperature, placed on a glass microscope slide, moistened with several drops of PBS, 1X, and stained using the fluorescent double labeling technique. The sections were examined under 10x magnification. Images of each section were digitally captured by an Olympus DP-70[r] digital camera and saved as tiff files. Live and dead cell counts were determined by hand counts.

DNA quantification

The Quant-iT PicoGreen™ double stranded DNA quantification assay[u] was performed: Double stranded DNA extracted from bovine thymus was mixed with TE buffer[v] to create

standard DNA concentrations of 1,000, 100, 10, and 1 ng/mL. The standards and 100uL of each papain digested sample (used in the above GAG and hydroxyproline assays) were added to a black 96 well plate. 100uL of 2ug/mL of Pico Green reagent was added to each well and the plate was incubated for 5 minutes. Sample fluorescence was read at 485nm excitation/ 528nm emission by the Synergy HT – KC-4 Spectrophotometric Plate Reader.[w] Absorbances were converted to ng/mL concentrations and total double stranded DNA yield in ng using FT4 software.[x]

Determination of Glycosaminoglycan (GAG) Production

The Farndale Dimethyl-methylene Blue Glycosaminoglycan assay was performed. One third of a scaffold was analyzed for GAG production. Samples were lyophilized, and a dry weight obtained, and then mixed with 1ml Papain Solution (2mM Dithiothreitol and 300ug/ml Papain). The solution was incubated at 600C in a water bath for 4 hours. Then 5ul of sample was placed into three wells each of a 96-well plate. 245ul of Dimethylmethylene blue (DMMB) reagent, containing 8mg DMMB, 1.5g Glycine, 1.2g NaCl dissolved in 500mL of H2O, was added to each well. The plate was read immediately at 525nm by a Synergy HT – KC-4 Spectrophotometric Plate Reader.[w] Absorbances were compared to a generated standard curve. Absorbances were converted to ug/ug concentrations and total GAG yield in ug using FT4 software[x].

Determination of Hydroxyproline Production

The Reddy hydroxyproline assay was performed: the papain digested samples used in the above GAG assay were also used in the hydroxyproline assay. 50ul of each papain digested sample was placed, in duplicate, in each well of a 96 well plate. Standards of hydroxyproline were made at concentrations of 0,2,4,8,12,16,20 ug/mL in 50uL of papain digest buffer, containing 20mNaPO4 and 1mmol EDTA.. 50ul of 4N NaOH was added to each well. The samples were autoclaved at 121°C for 20 minutes. 450uL of Chloramine-T reagent, containing 1.27g Chloramine T dissolved in 20mL of 50%n-Propanol and 80mL of acetic citrate buffer, was added to each well. 450uL of Erlich's reagent, containing 15g of p-dimethylaminobenzaldehyde

dissolved in 16.8mL p-Propanol and 8.3mL perchloric acid, was added to each well. The samples were incubated in a water bath at 65ºC for 20 minutes. 100ul of sample and standards were transferred to a short- 96 well plate and read immediately at 550nm by a Synergy HT – KC-4 Spectrophotometric Plate Reader. Absorbances were converted to ug/ug concentrations and total hyroxyproline yield in ug using FT4 software.

Gene Expression

One third of each scaffold was analyzed for extracellular matrix gene expression. Total RNA was extracted and analyzed by reverse transcription polymerase chain reaction (RT-PCR) using primers corresponding to cDNA sequences for type I and II collagens, aggrecan, and the housekeeping gene GAPDH. Scaffold thirds stored in RNALater[y] were transferred to a 0.5 ml screw cap tube filled with 1.0 mm diameter Zirconia Beads and Trizol® reagent[z], and homogenized using a mini-bead beater at 5,000 rpm for 2 minutes. RNA was extracted from the homogenates using the TRIspin method. 5ul of RNA from each specimen was diluted with 95uL of RNAase free water and the absorbances read at 260 and 280nm with a Synergy HT Spectrophotometric Plate Reader. Absorbances were converted to µg/µl using the KC4 software. Equal amounts of sample RNA were converted to cDNA using random hexamer primers and the StrataScript™ RT[aa] enzyme in a GeneAmp PCR System 9700.[bb] PCR primers were designed by Primer.EXE,[cc] and sequences were as follows: Aggrecan, forward: AACTTCTTCGCTGTGAGTGG, Aggrecan reverse: GATCACATTGCCTCGAGCTT; GAPDH forward: CAACCTGGTCCTCAGTGTAG, GAPDH reverse: GAAGCTCACTGGCATGGCCT; Collagen 1 forward: GTCCTTCTGGTCCTCGTGGT, Collagen 1, reverse: ACCATCATCTCCGTTCTTGC; Collagen 2, forward: CCGCAGTGAGCCATGATACG; Collagen 2, reverse: GATGTCTCCAGGTTCTCCTT. Real-Time PCR was performed with the Rotor-Gene RG-3000 using the Quantitect SYBR® green PCR kit[cc] following the manufacturer's guidelines. The PCR profile for all tests consisted of an initial incubation at 94°C for 15 minutes, followed by 55 cycles of 5 seconds at 94°C (melting), 10 seconds at 57°C (annealing), and 20

seconds at 72°C (extension). After the PCR profile a melt curve analysis will be done to ensure specific amplification for each sample. SYBR green fluorescence was monitored during the extension step of the PCR profile, and take off values and amplification efficiencies were then determined using the Rotor-Gene software. Relative expression levels for the target genes studied were expressed as a ratio to the level GAPDH and determined using Q-Gene.

Statistical Methods

An initial power analysis based on data from our pilot study regarding GAG concentration and cell counts indicates that a minimum of 5 samples per group was required to obtain a study power of 0.8, with significance set at $p < 0.05$ (One Way ANOVA Sample Size; minimal detectable difference of means= 20, expected standard deviation of residuals= 3.01). To ensure adequate power and statistical significance for the proposed study, 10 samples per time group per time point were utilized in this study.

Relative gene expression using Q-Gene was expressed as a ratio to GAPDH (Housekeeping gene). Differences in gene expression among groups was determined using the relative expression statistical tool, REST-XL. All statistical analysis of biochemical data, cell viability, and cell counts was performed using computer software programs Statistical Analysis Software[dd] and SigmaStat.[ee] Data from all samples within each group was combined, and medians and means $\pm$ SD were determined for each. For parametric data, comparisons for statistically significant differences were determined using a one-way ANOVA and an all- pairwise multiple comparison procedure (Tukey Test) was performed to determine which groups were significantly different from one another, with significance set at $P<0.05$. Non parametric data was analyzed using a Kruskal-Wallis one way analysis of variance on ranks, followed by Dunn's Method all pairwise multiple comparison procedures, with significance set at $P<0.05$.

Results

Scaffold Cellularity and Viability

At the time of both harvests, cell- scaffold constructs still had the gross appearance of non-woven felts with no visible ECM. By the second harvest period the scaffolds appeared rounded and less robust, and fragmented easily upon manipulation, indicating dissolution. This fragility was worse in the control groups versus the growth factor treated groups. Confocal microscopy and live/ dead assays revealed 98.4%, 94.1%, and 96.5% viability **(Figures 5-1a,b,c)** for Groups 1, 2, and 3 respectively, for the 16th day of culture, with controls having the higher viability than Group 3 (P=0.003), but no difference in viability with Group 2 (P= 0.218) or between Group 2 and Group 3 (P=0.38) There was a drop in viability in Groups 1 and 3, for the 26th day of culture, with 88.4%, 94.4%, and 90.8% viability, with a significant difference in decreased viability for controls (P= 0.010), and Group 3 (P=0.019) but not Group 2 (P=0.948) (Figure 1). At time of second harvest, Group 2 had a higher cell viability than controls (P=0.035) but no difference in viability between controls and Group 3 (P=0.427) and Group 2 and 3 (P=0.104). Based on subjective microscopic examination, the scaffolds showed some single and clumped cellular distribution. Construct cellularity as measured by % dsDNA standardized to construct dry weight was as follows: Group 1: 6.91E-05 %, Group 2: 3.97E-05%,and Groups: 1.19E-05% for the 16th day of culture (P= 0.097) **(Figure 5-2).** Construct cellularity increased for all groups harvested on day 26 (p=0.03) The mean percentage of quantified dsDNA, per scaffold dry weight for group 3 was 0.0153% and 0.0123% for group 2; which were significantly greater than the 0.00075% of group 1 (p<.0001). No significant difference was found between groups 2 and 3 (p=0.202).

Extracellular Matrix Analysis

Mean relative expression of Collagen I mRNA was significantly greater at the 26th day (Group 1: 16.7 copies relative to GAPDH, Group 2: 18.4, Group 3: 19.8) versus the 16th day (Group 1: 12.0 copies relative to GAPDH, Group 2: 10.2, Group 3: 7.0) (p< 0.0001) for all

groups, but was not different between each group for the respective time period as determined by 1 way ANOVA (p= 0.909) **(Figure 5-3a).** Mean relative expression of Collagen II mRNA was significantly greater on day 26 for all groups (p<0.0001) versus day 16 (none detected). Relative collagen II expression on day 26 was significantly greater in groups 2 (0.000326 copies relative to GAPDH) and group 3(0.000181) versus group 1(none detected, p<0.0001, p= 0.0229, respectively) **(Figure 5-3b).** No significant difference was found between groups 2 and 3 (p= 0.065). Mean relative expression of aggrecan mRNA was significantly greater on day 26 versus 16 (none detected) for all groups (p=0.0002). On day 26, relative aggrecan expression was significantly greater in Group 3(1.78E-05 copies relative to GAPDH) versus Group 1 (none detected) and Group 2 (4.57E-06, p< 0.0001 and =0.0019, respectively) **(Figure 5-3c).** No glycosaminoglycan or collagen was detected in the DMMB and Hydroxyproline spectrophotometric assays for any group at any time point. Staining for GAG and collagen were negative at both time points **(Figures 5-4a,b).** Staining for α- smooth muscle actin was positive for all groups at all time points, however there was subjectively more positive immunoreactivity in Groups 2 and 3 on the 26th day of culture, with the most immunoreactivity in Group 3 on day 26 **(Figure 5-5).**

Discussion

In this study, expression of collagen I, II and aggrecan mRNA indicate that early fibrochondrogenic differentiation can occur in equine synoviocytes under the influence of pulsed growth factors. These ECM components are desirable for meniscal tissue engineering because collagen I is the principle matrix and functional constituent of the meniscus; aggrecan and collagen type II is found at the inner zone of the meniscus and in small quantities along radial tie fibers, and are principally involved in resisting compressive forces.[36-40] Synovial membrane contains tissue progenitor cells which can undergo chondrogenic,[41]adipogenic, osteogenic,[42] myogenic,[24] and now, fibrochondrogenic differentiation. Donor age, cryopreservation, and cell passaging, (up to 10-30 passes), do not affect the progenitor cells' chondrogenic ability.[24]

Synovial mesenchymal progenitor cells constitute about 3-10% (5% mean frequency of native population) of human synoviocytes. [42,43] This exact proportion of progenitor cells is unknown in the horse and dog. The actual synovial mesenchymal progenitor cells have a fibroblastic phenotype and are difficult to detect on simple microscopic examination.[24,42] Even under ultrastructural and immunocytochemical assessment, the synovial mesenchymal progenitor cells have features of the type B synoviocytes, including lamellar bodies which are secreted by exocytosis, and surfactant protein A.[23] In an attempt to enhance isolation and culture of these progenitor cells, they have been identified via receptor profiles using flow cytometry and immunohistochemistry. Synovial mesenchymal progenitor cells express adhesion molecules CD 166, hyaluronate receptor CD 44, growth factor and cytokine receptors, integrins, and special markers such as STRO, CD 105,CD9, and CD90.[42-44] These progenitor cells can also exclude Hoechst 33342 dye.[45] However, there is still no one true cellular marker for synovial mesenchymal progenitor cells.[24] In this study, mesenchymal progenitor cells were not isolated via flow cytometry or negative dilution isolation techniques, likely resulting in a mixture of cells, including mesenchymal progenitor cells, type B fibroblasts and subintimal fibroblasts. Despite this, chondrogenic gene expression still occurred. Our long term goal is to produce functional fibrocartilage quickly with the least cost and handling, with production of both type II collagen and type I collagen. Constructing articular cartilage has been the goal of most reported studies on synovial chondrogenesis,[24,42,44-55] not fibrocartilage, with a high collagen I content. Thus, inclusion of collagen I producing subintimal fibroblasts, perivascular fibroblasts, and type B synoviocytes are advantageous for the purpose of meniscal fibrocartilage engineering. Also, these other cells could have an important role in the embryologic "community effect," where multicompetent progenitor cells must be surrounded by similar cells in order to be respond to inductive signals, differentiate, and proliferate ECM.[56,57]

The cells in this study only expressed fibrocartilaginous genes and did not proliferate measurable fibrocartilaginous ECM. The expression of hyaline cartilage specific genes was time

dependent, only appearing at the 26th day harvest; longer culture durations may be required to see ECM formation on porous synthetic scaffolds. Other studies show a lag time in chondrogenic gene expression and time dependent ECM expression.[58-63] One study of synovial chondrogenesis on PGA scaffolds utilized a longer culture duration of 60 days, with successful ECM formation,[52] with histologically visible GAG and Aggrecan and Collagen II gene expression. In another chondrogenesis study, Janjanin *et. al.* cultured mesenchymal stem cells on molded nanofiber scaffolds for 18, 26 and 42 days, in spinner flask bioreactors. His study also found time-dependent increase in cartilage matrix, with constructs harvested on day 42 having higher type 2 collagen and aggrecan expression, and significant time-dependent increase in sulfated glycosaminoglycan and hydroxyproline content.[64] Yet another study of mesenchymal stem cells seeded on silk scaffolds showed that chondrogenic differentiation of human mesenchymal stem cells started after 2 weeks of growth factor exposure, and increased further after 3 weeks' culture time.[65] Studies Based on this data, future studies of synovial chondrogenesis on non woven scaffolds may focus on long term bioreactor culture to allow sufficient ECM accumulation and actual tissue formation.

Duration of culture may not be the only variable affecting synoviocyte ECM production; scaffold cellularity plays an important role in proliferation of collagen and GAG. Obtaining a high cellular density is crucial for stimulating and accumulating cartilaginous ECM formation.[13,66,67,41,56,57 42,68] For example, some multipotential mesenchymal cells (cell line C3H10T1/2) undergo chondrogenic differentiation only when seeded as high-density micromass cultures.[68] In this study, the constructs with the highest cellularity also expressed chondrogenic genes. Scaffold cell density can also be affected by seeding techniques; use of the rotating bioreactor may have inadvertently decreased scaffold cellularity. Pei and Sakimura utilized the convectional perfusion environment of spinner flasks with stationary PGA scaffolds for synoviocyte seeding.[52,69] Spinner flasks with stationary scaffolds create a greater relative velocity between scaffolds and cells, assisting in convective inertial cell-scaffold impacts.[13,52,70,71] Use of

spinner flasks followed by maintenance culture in a rotating bioreactor is now thought to be the best way to seed highly porous, non-woven scaffolds, resulting in essentially 100% yield, versus rotational or static seeding alone.[13,70,71] During seeding in a rotating bioreactor, cells and scaffolds have the same angular velocity, possibly lessening the chance of inertial impacts, no longer making the rotating bioreactor the seeding instrument of choice. Even though cell seeding concentrations in our study were similar to that utilized by Sakimura and workers[52] (400,000 cells/mL versus 240,000/mL) there are several factors that may have reduced our effective cell seeding concentrations. Other studies used fewer scaffolds per bioreactor flask, such as 10 scaffolds per 100mL flask;[52] in our study we used 20 scaffolds per 110mL flasks, which lowers cell# per scaffold and may alter fluid perfusion of scaffolds. Other studies utilizing synoviocyte culture on scaffolds used scaffold dimensions ranging from 5x5x2mm[52,69,72] to 7x7x0.5mm.[41] Our scaffolds were larger at 10x10x2mm, and given an equal total cell concentration, may have effectively lowered cells seeded per scaffold area. In addition, cells could have adhered to our plastic bioreactor wall, reducing the number of cells available for scaffold adhesion. Pre-immersion of PGA/PLLA scaffolds in 100% ethanol, followed by 70% ethanol, then PBS solution can increase wettability and thus cellular adhesion;[69,73] our study presoaked scaffolds in media only. All of these factors could affect final cell # per scaffold, resulting in lower than calculated cell per scaffold seeding concentration, and negatively impacting GAG and collagen production.

Scaffold type may also affect cell retention, synoviocyte phenotypic shift, and fibrocartilaginous ECM production. The majority of studies done on synovial chondrogenesis are done in pellet format, [24,42,44-55] which is not applicable to producing a large, c-shaped fibrocartilage. We chose to culture synovial membrane cells on relatively larger scaffolds, which could potentially be shaped and modified to resemble the form of a knee meniscus. The loose non- woven configuration of the PGA/PLLA scaffolds, while being helpful in nutrient and gas exchange, may prevent accumulation of bioactive ligands used in autocrine and paracrine

signaling, that would favor ECM build up. Scaffold dissolution as seen in our study could also reduce construct retention of early ECM production. The environment that cells live in effects their phenotype, spatial organization, and which ECM matrix components are produced.[74] Use of gel scaffolds, rather than non woven synthetic scaffolds, is another strategy for meniscal tissue engineering. Synoviocyte chondrogenic 3-D culture has been described for alginate[72] and collagen[41] gels, as well as a fibrin gel applied to PGA scaffolds.[69] When synoviocytes are imbedded into these scaffolds immediately they immediately undergo a shape change reminiscent of chondrocytes, rounding into lacunae.[41,69,72] These kinds of scaffolds may provide the advantages of inducing ECM signaling through cytoskeletal change, retaining growth factors, and having mild regional chondrogenic hypoxia.[75] For instance, in chondrocyte culture, agarose constructs contain more glycosaminoglycan, while non woven PGA constructs contain more collagen.[76] Further investigation of compound scaffolds utilizing gels for the axial, avascular portion of the meniscus and non woven felts for the abaxial portion, may ameliorate the conundrum of engineering heterogeneous meniscal tissue. Collagen gels have the added advantage of containing physiologic relevant ECM.[77] In another permutation, scaffolds that elute IGF-I and TGF-beta 1 sequentially may have promise for application in heterogeneous cartilage tissue engineering applications, such as the knee meniscus.[78]

Expression of the aggrecan gene was time and growth factor dependent; a combination of FGF followed by sustained IGF and TGF-β was superior in production of aggrecan mRNA. Expression of the collagen II gene was also time and growth factor dependent, but we could not detect a significant advantage of bFGF treatment versus bFGF followed by sustained IGF and TGF-β for inducing collagen II expression. bFGF's role in embryonic development and its past performance in *in vitro* studies may provide clues to the gene expression profiles seen in this study. FGF's elicit their effects on cells by binding to a tyrosine kinase receptor and a heparin-sulfate proteoglycan, which causes the FGF receptors to dimerize.[79] Activation of the FGF Receptor results in activation of Grb2 (growth factor receptor protein 2) on the plasma

membrane, which recruits SOS to turn on Ras, which activates p38 and ERK 1/2 (extracellular signal-regulated kinase) via the MAPK (mitogen-activated protein kinase) pathway.[79,80] This MAPK pathway plays a key role in a range of cellular responses including proliferation, differentiation and apoptosis.[80] PI 3-kinase and Akt are also activated.[79] bFGF is a chondrogenic growth factor, both *in vitro* and in the embryo,[81,82] by inducing cell division and proliferation of cartilaginous ECM.[83] bFGF acts in the early stages of embryonic development during differentiation of neural crest into skeletal analge and during cartilage differentiation. [84] Under the longer-term influence of bFGF, endochondral and membranous bone are formed instead of cartilage. [84] Early exposure to FGFs strongly increases the activity of the SOX-9 enhancer on the collagen II gene, in chondrocytes and undifferentiated mesenchymal cells. [85] In cell culture, 10 ng/ml bFGF induces cartilage differentiation of mesenchymal stem cells[84] and at 5 ng/ml, bFGF increases the biosynthetic activity and accumulation of GAG in chondrocytes cultured on scaffolds.[31] These doses are much lower than the 50ng/mL we utilized in this study. This *in vivo* embryonic and *in vitro* behavior of a single treatment with bFGF early in the culture period may explain why bFGF alone induced synovial collagen II and aggrecan gene expression in our study. Despite bFGF's chondrogenic properties, combining its use with TGFβ and IGF-1 improves scaffold/chondrocyte constructs, resulting in high fractions of cartilaginous ECM and high compressive moduli.[30] In fact, prolonged culture of chondrocytes with supplemental bFGF for 4 weeks actually creates poorer cartilage tissue formation, resulting in small constructs, low ECM fractions, and low compressive moduli, versus chondrocytes not grown with bFGF.[30] TGFβ and IGF-1 are also chondrogenic on their own *in vitro,*[86] [24,42,49,72] both in monolayer culture and on collagen/GAG scaffolds.[80] However, when given together bFGF, TGFβ-1 and IGF-1 are chondrogenic growth factors and have a synergistic interaction, [87] as seen in our study, with the superior aggrecan gene expression in Group 3 of our study. TGFβ binds with the TGFβ serine/ threonine kinase receptor, resulting in phosphorylation and transport of Smad2/3 proteins into the nucleus. There Smad2/3 associates with Sox-9; the Sox-9 and Smad2/3 complex then bind with

coactivators to transcribe the collagen II gene.[88] TGFβ also acts on p38 through the MAPK pathway.[80] TGF-beta has been touted as the key factor for inducing growth and chondrogenesis of synovial derived mesenchymal progenitor cells. [87] IGF-1 and its tyrosine receptor regulate chondrogenic differentiation in adult mesenchymal stem cells via the PI3K pathway by activation and translocation of the Akt molecule to the nucleus. Akt is recruited to the cell membrane where it is phosphorylated, and subsequently induces the phosphorylation of a number of nuclear and cytosolic proteins, which are involved in the regulation of cell metabolism, growth and survival.[80] When TGF-beta1 and IGF-I are applied simultaneously, chondrogenic differentiation of synovial derived progenitor cells is superior to the sequential application of these two factors.[87] In cultured marrow derived mesenchymal stem cells, treatment with TGF-beta-1combined with IGF-I results in higher type 2 collagen and aggrecan expression, increase in sulfated glycosaminoglycan and hydroxyproline content, as well as increased tissue stiffness.[64] Culture of chondrocytes with the triple sequential combination of TGF-beta1/FGF-2 and then IGF-I yielded large 4-week constructs with high fractions of cartilaginous ECM and high compressive moduli. [30] Interestingly, chondrocytes receiving this growth factor combination do not produce type I collagen. [30] Based on our studies and these other studies, the triple combination of bFGF, TGFβ-1 and IGF-1 is a viable growth factor protocol for further use in *in vitro* synovium based fibrochondrogenesis culture.

There was no difference in relative expression of collagen type I between the treatment and control groups in this study, although Collagen I was more highly expressed than Aggrecan and Collagen II genes. This may indicate that collagen I forms independently of these particular growth factors with the cell types used in this study. It could also indicate contradicting actions of growth factors in Groups 3. IGF-1 has been found to inhibit collagen I synthesis,[89,90] whereas TGFβ-1 enhances expression of type I collagen.[90] Regardless, formation of collagen I is a crucial component in constructing menisci *in vitro*, being the principal constituent of the outer meniscus, with its high tensile properties.[91-94]

While the combined growth factor protocol used in our study favors aggrecan and type II collagen formation in synovial cell pellet culture, [35,69] it also produces the highest rates of synovial progenitor cell growth. [87] Early cultivation for 2 weeks in medium with supplemental TGFβ-1/FGF doubled cellular contents in 2-week constructs compared to unsupplemented controls. [30] bFGF and IGF-1 together induce cell division of fibroblast like cells.[95] The molecular mechanisms of bFGF, TGFβ-1 and IGF-1, with activation of MAPK and Akt, may have increased Group 2+3 construct cellularity through cell division. Interestingly, growth factors also increase cellular binding to ECM,[96-99] which would improve cellular retention, and may also explain the increased cellular content of growth factor treated groups. The growth factors used in this study cause reorganization of the cellular actin cytoskeleton, causing enhanced cell adhesion processes. [100] For example, both TGFβ and IGF-1 increases cultured chondrocytes' surface expression of α3/α5 integrin subunit, and IGF-1 also increases cell surface levels of α1β1 integrin;[96-99] these integrins are crucial to stimulating adhesion of the cells to ECM such as fibronectin and collagen II.[96-98] Some cellular growth factor receptors are even located within some cellular adhesion complexes.[99] Increased construct cellularity is likely useful for fibrocartilage tissue engineering, allowing cellular cross talk to produce ECM[13,66,67,41,56,57 68] that would give engineered meniscal fibrocartilage its form and function.

The cellular viability in this study decreased over time, except in Group 2. It seems that a single dose of bFGF may have been protective against cell death, potentially by stimulating fibronectin formation and other ECM constituents that were not measured in this study.[101] While synovial membrane cells seem capable of prolonged passaging and self renewal, they do not express telomerase and are not immortalized in culture.[24] In explaining cell mortality, it is possible that these synovial membrane cells died of senescence, although this behavior would be very different from what has been reported in synovial cell culture literature. The cells may have also died due to unfavorable growth conditions. The media never showed color changes indicating unfavorable pH shifts, however the degradation products of PGA is glycolic acid,

which enters the tricarboxylic acid cycle and is excreted as water and carbon dioxide. [70] Potentially, both of these metabolic and degradation by-products could cause a regional decrease in pH without a flowing vascular system or perfusion bioreactor, which could affect cell viability. Potentially the scaffold disintegration and increased cellularity seen by the 26th day of culture could have caused an increase in these scaffold degradation/ metabolic by- products. Even though the scaffolds used in this study had 10%PLLA fibers mixed with 90% PGA fibers, the scaffold still behaved as a pure PGA scaffold which has a half-life of 28 days in aqueous solution.[70] The scaffolds were visibly disintegrating and very fragile by day 26, with fragmentation upon manipulation. Loss of this anchoring synthetic "ECM" can cause cellular stress, changing cell phenotype and changing or halting ECM production, because of substrate loss and loss of extracellular matrix- cytoskeleton mechanotransduction.[102-110] For example, fibroblasts that loose their cellular pretensioning through relaxation of collagen scaffolding proliferate cytokines and proteases, switching from a synthetic to an inflammatory phenotype.[104] In chondrocytes and mesenchymal stem cells, integrin receptors' interaction with ECM effects the production of extracellular molecules such as type II collagen and aggrecan. [111,112] Thus scaffold disruption can start a vicious cycle of decreased cellular ECM production, which alters construct stability, allows further scaffold dissolution, and induces more cellular stress. *In vivo* and *in vitro* loss of cellular adhesion to specific components of the ECM can lead to cell death.[113] Life or death depends upon the cell's interaction with the ECM via adhesion receptors such as integrins, which directly regulate apoptosis or survival via the PI 3-kinase and MAPK pathways.[114] Scaffold fragmentation may be the most plausible cause of cellular mortality seen in this study.

The cells in this study produced α-smooth muscle actin (ASM), which was enhanced by growth factor stimulation over time. In human synoviocytes, TGF-β1 induces expression of ASM, while bFGF inhibits it;[16] this may reflect a species difference, where in this study, both growth factors induced ASM in the equine. Although ASM is the major contractile constituent of the intestine, musculoskeletal connective tissue cells including chondrocytes, meniscal

fibrochondrocytes, ligament fibroblasts and osteoblasts, and mesenchymal stem cells can express the gene for the contractile ASM isoform.[19,115,116] In fact, one study of synoviocytes cultured on collagen-GAG scaffolds showed active synovial contraction, pulling the scaffold to 43% of its original diameter after 4 weeks of culture.[19] Expression of ASM and this synoviocyte contractile behavior may explain why, subjectively, the growth factor treated group was less fragile and degraded compared to controls at the end of the study. In synovial membrane harvested from the knee, ASM is associated with blood vessel endothelial cells and vascular origin mesenchymal progenitor cells.[55] ASM is thought to play a potential role in attempted reparative responses in the meniscus, both by the fibrochondrocytes and surrounding synovial membrane.[116] Expression of this ECM constituent may be advantageous to meniscal tissue engineering because of this cellular association with progenitor cells and a potential contractile scaffold stabilizing effect.

Other chondrogenic growth factors and drugs have been successfully used in other studies, including TGFβ-2, TGFβ-3, dexamethasone, bone morphogenetic protein (BMP)-2, BMP-6, and BMP-7, with varying concentrations of additives such as insulin, transferrin, selenium (ITS) and fetal bovine serum.[72,87,117] Hypoxia at 1%, 2% and 5% has also been successfully used to induce chondrogenic differentiation in embryonic stem cells, as well as adult adipose and marrow derived mesenchymal progenitor cells.[118-125] New research using cocultures of meniscofibrochondrocytes and chondrocytes has been able to produce some of the heterogeneous collagenous tissue of the knee meniscus, and may be applicable to synovial based fibrocartilage formation.[90] Perhaps future studies can investigate various combinations of these bioactive factors to induce or enhance fibrocartilaginous ECM production to a greater degree than what was seen in this study.

Based on these data, were reject our null hypotheses: construct cell viability decreased slightly with time, while scaffold cellularity and genomic signaling for ECM formation increased with time and growth factor stimulation. These data suggest that the methodology and techniques used in the present study are appropriate for further *in vitro* research towards development of a

cell-scaffold tissue engineered meniscus. As this study achieved early chondrogenic genomic signaling in synovial membrane cells, but no extracellular matrix formation, future studies will focus on investigating variables that can optimize ECM formation, such as increased seeding numbers, different scaffolds, or altering chondrogenic bioactive factors.

Footnotes

a. Type 1A Clostridial Collagenase, Sigma, St. Louis, MO
b. Titer plate shaker, Lab Line Instruments, Melrose Park, IL
c. Nuaire US Autoflow, Plymouth MN
d. Marathon 3200, Fisher Scientific, Pittsburgh, PA
e. Centrifuge 2702R, Eppendorf, Hamburg, Germany
f. Trypan Blue, Sigma, St. Louis, MO
g. PGA/PLLA blend Biomedical Structures LLC, Warwick RI
h. Rotating Bioreactor System, Synthecon, Houston, TX
i. Innovative Cell Technologies, San Diego CA
j. human recombinant bFGF, BD Biosciences Bedford, MA
k. human recombinant TGF-β, BD Biosciences Bedford, MA
l. human recombinant IGF-1, BD Biosciences Bedford, MA
m. Autostainer, Dakocytomation, Carpenteria, CA
n. Dako MO851, Dakocytomation, Carpenteria, CA
o. Dakocytomation, Carpenteria, CA
p. Newcomer's Supply, Appleton, WI
q. Carl Zeiss, Thornwood, NY
r. Olympus DP-70 Digital Camera, Olympus, Melville, NY
s. Live/Dead Viability/Cytotoxity Kit, [Cat. #1-3224], Molecular Probes Co., Eugene, OR
t. Olympus IX70 microscope, Olympus, Melville, NY
u. Quant-iT PicoGreen kit, Invitrogen, Fountain Drive, United Kingdom
v. TE buffer, Invitrogen, Fountain Drive, United Kingdom
w. KC-4, BioTec, Winooski Vermont
x. FT-4 software, BioTec, Winooski Vermont
y. Ambion, Austin, TX
z. Invitrogen, Carlsbad, CA
aa. Stratagene, La Jolla, CA
bb. Applied Biosystems, Foster City, CA.
cc. The Whitehead institute, MIT Center for Genome Research, MA
dd. Qiagen, Valencia, CA
ee. SAS Institute, Cary, North Carolina,
ff. SigmaStat, Jandel Scientific, San Rafael, CA.

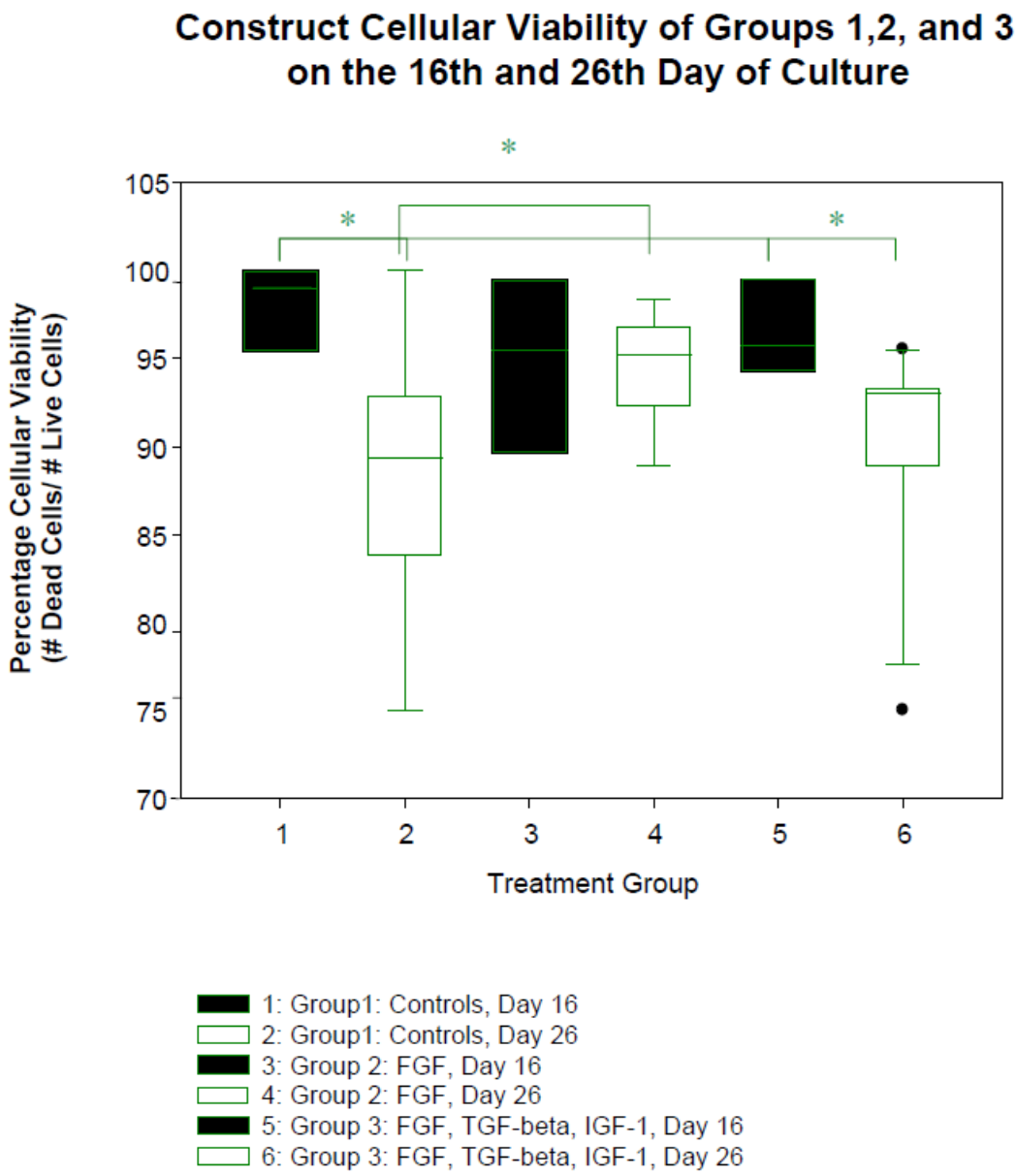


Figure 5-1a. Cellular viability of Groups 1,2 and 3 on the 16th and 26th day of culture, as determined by ethidium homodimer-1 and Calcein AM live/dead assay. An asterisk (*) denotes significant differences.

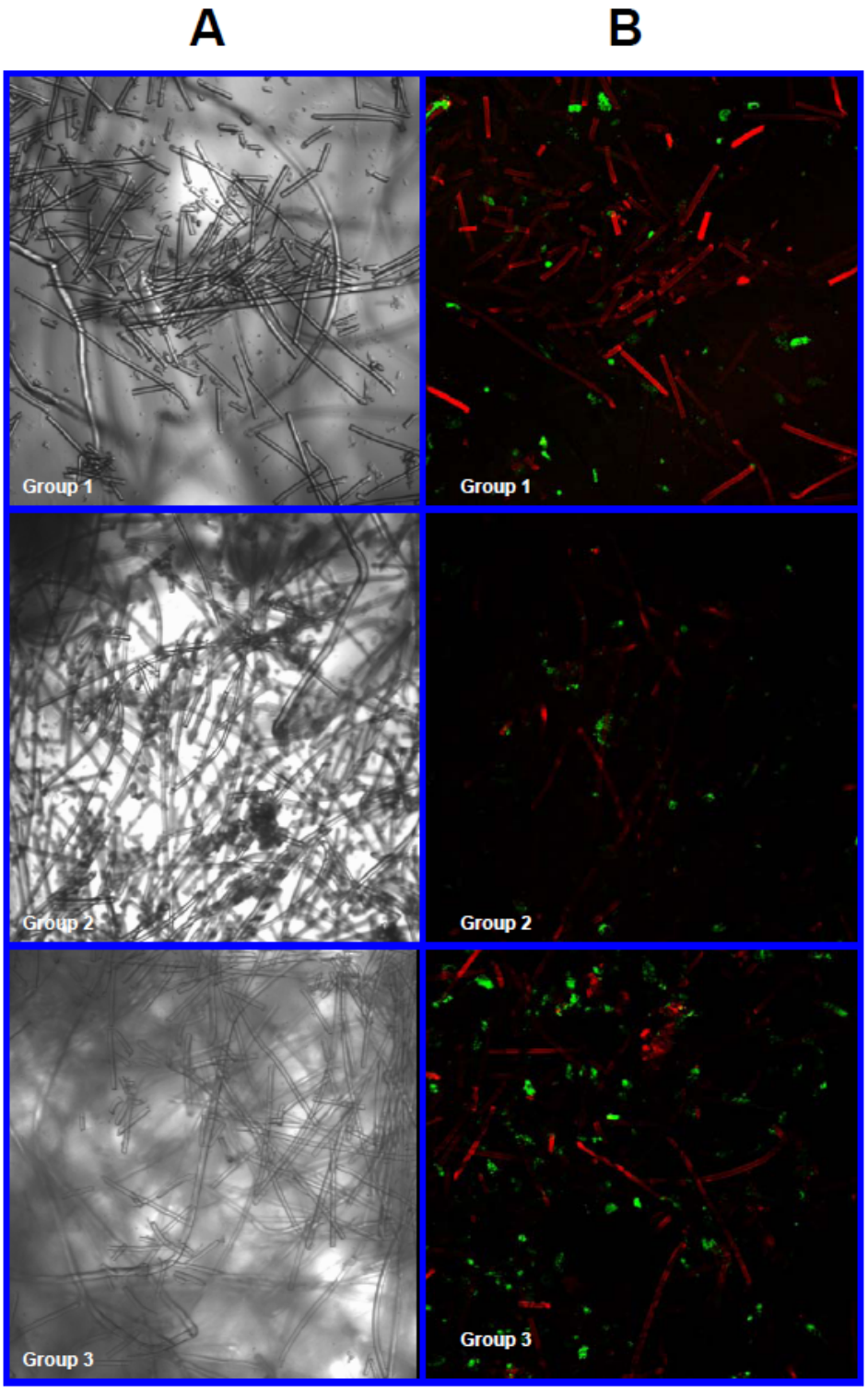


Figure 5-1b. Photomicrographs of constructs harvested on day 16, under standard light (column **A**) and laser (Column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 20x objective magnification. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

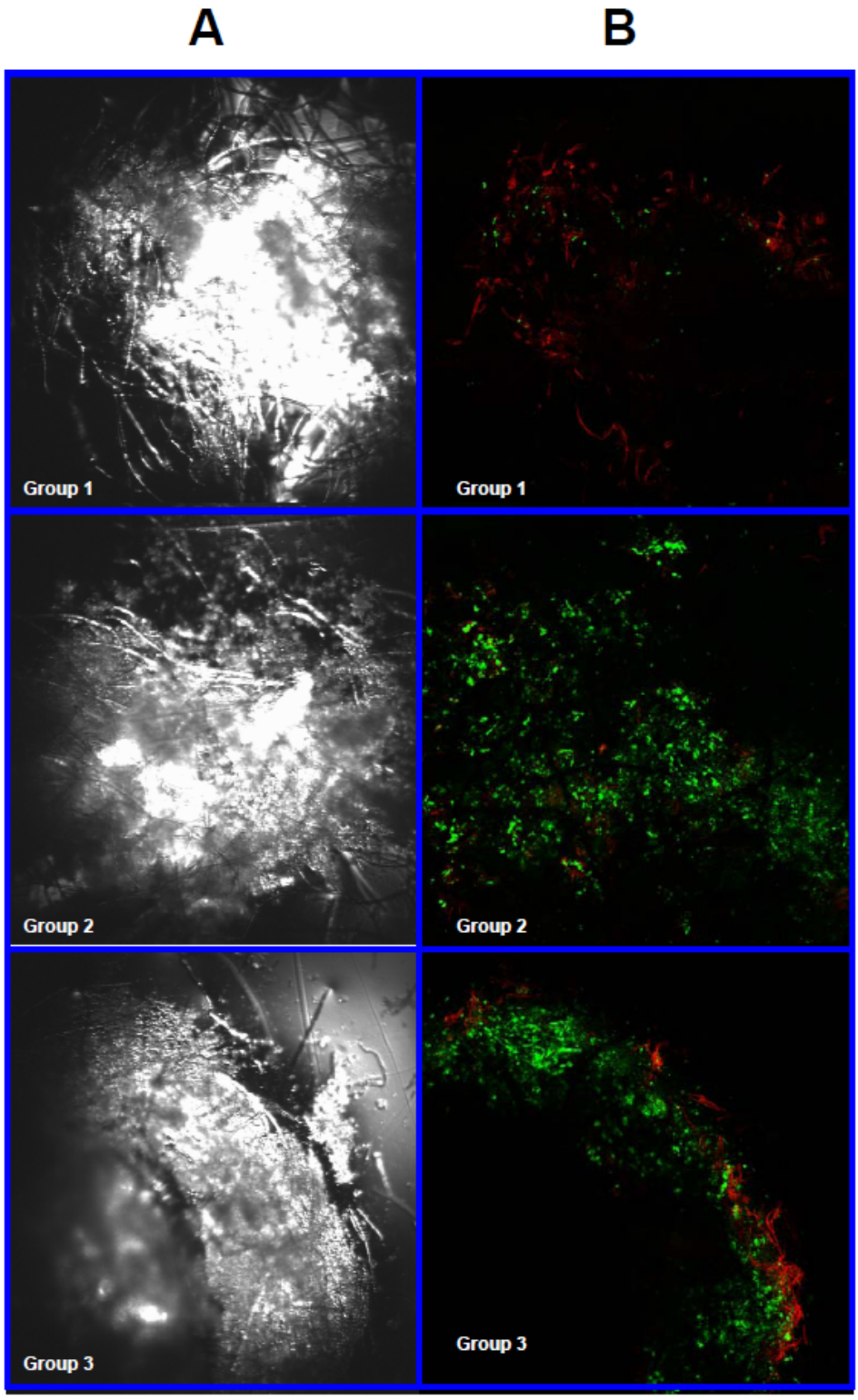


Figure 5-1c. Photomicrographs of constructs harvested on day 26, under standard light (column **A**) and laser (Column **B**), as seen on confocal microscopy using the calcein AM-ethidium homodimer live-dead assay, 10x objective magnification. Green stained cells are alive, red stained cells are dead. Note the spurious red staining of scaffold fibers.

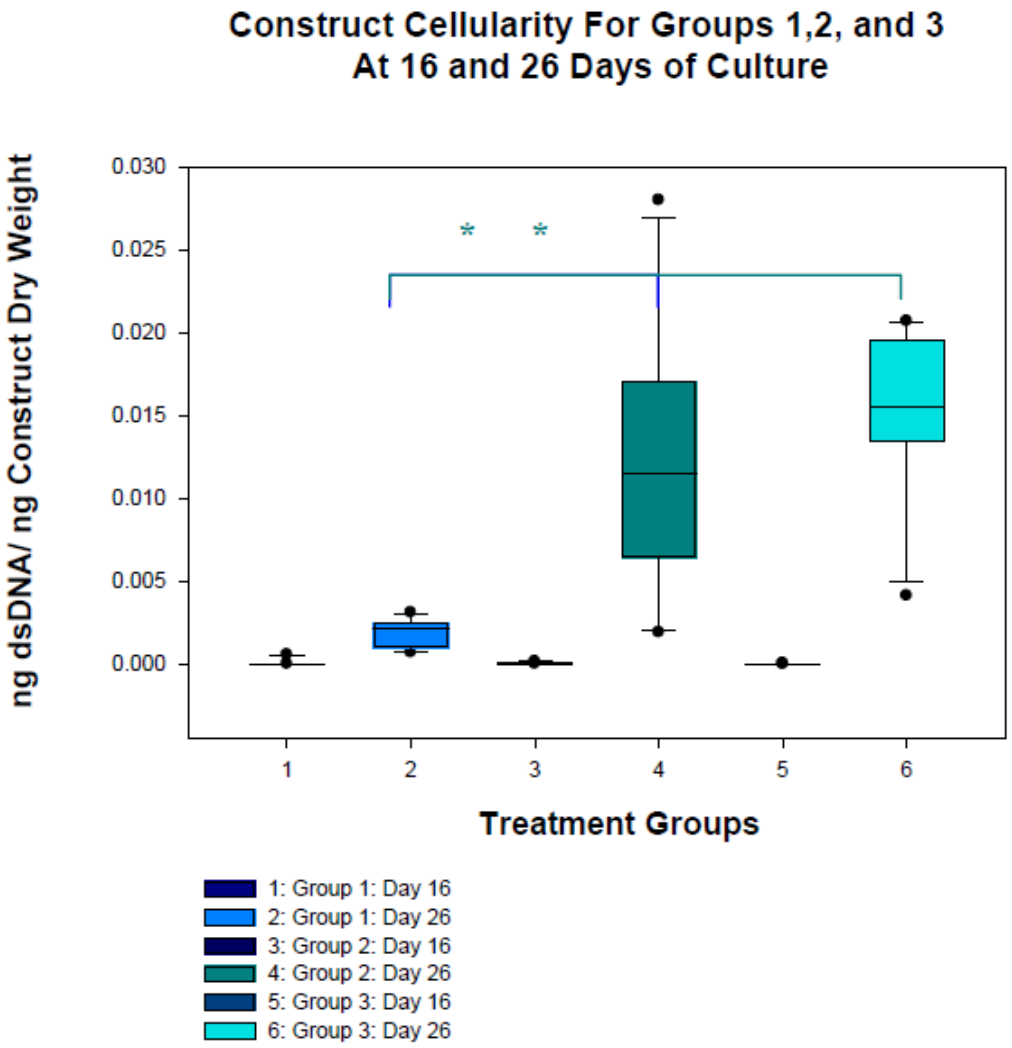
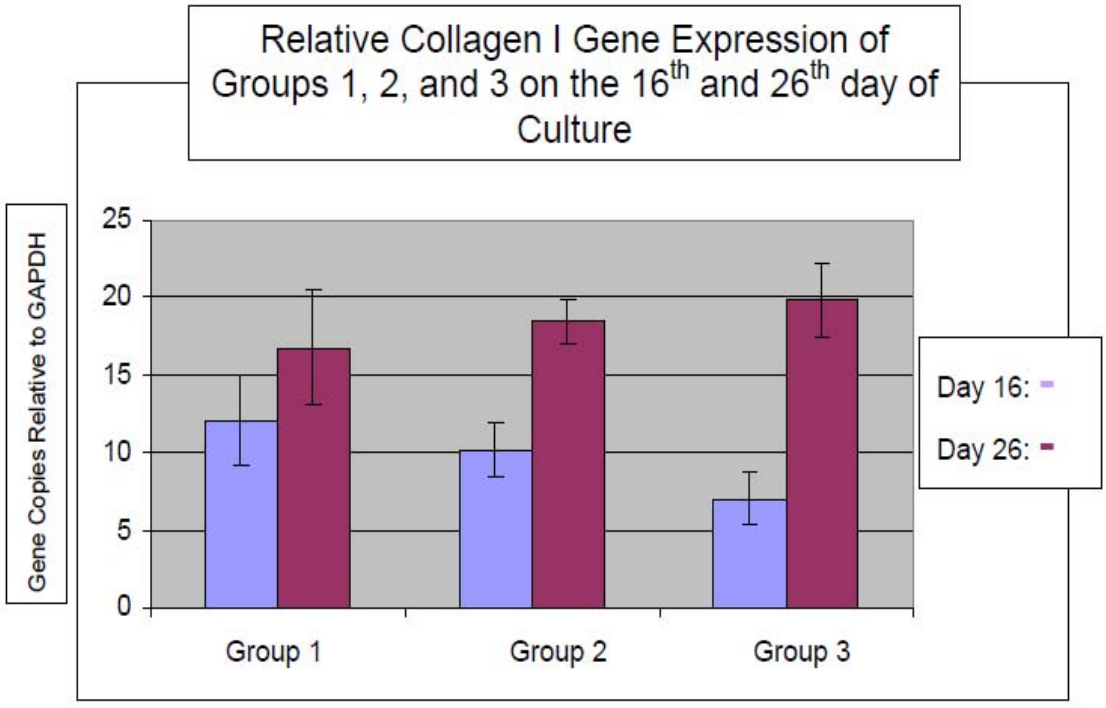


Figure 5-2. Construct cellularity of Groups 1,2, and 3 on the 16th and 26th day of culture, as determined by the double stranded DNA assay. An asterisk (*) denotes significant differences.



Day 16:
Group 2 > Group 1: (p= 0.6980)
Group 3 > Group 1: (p= 0.7152)
Group 2 > Group 3: (p= 0.9737)

Day 26 > 16 all: (p<0.0001)

Day 26:
Group 2 > Group 1: (p= 0.9737)
Group 3 > Group 1: (p= 0.6980)
Group 2 > Group 3: (p= 0.7152)

Figure 5-3a. Relative collagen I gene expression of Groups 1, 2, and 3 on the 16th and 26th day of culture: note that no significant difference existed in collagen type I gene expression between treatment groups on day 16 and 26. However, collagen I gene expression was higher for all groups on the 26th day of culture versus the 16th day.

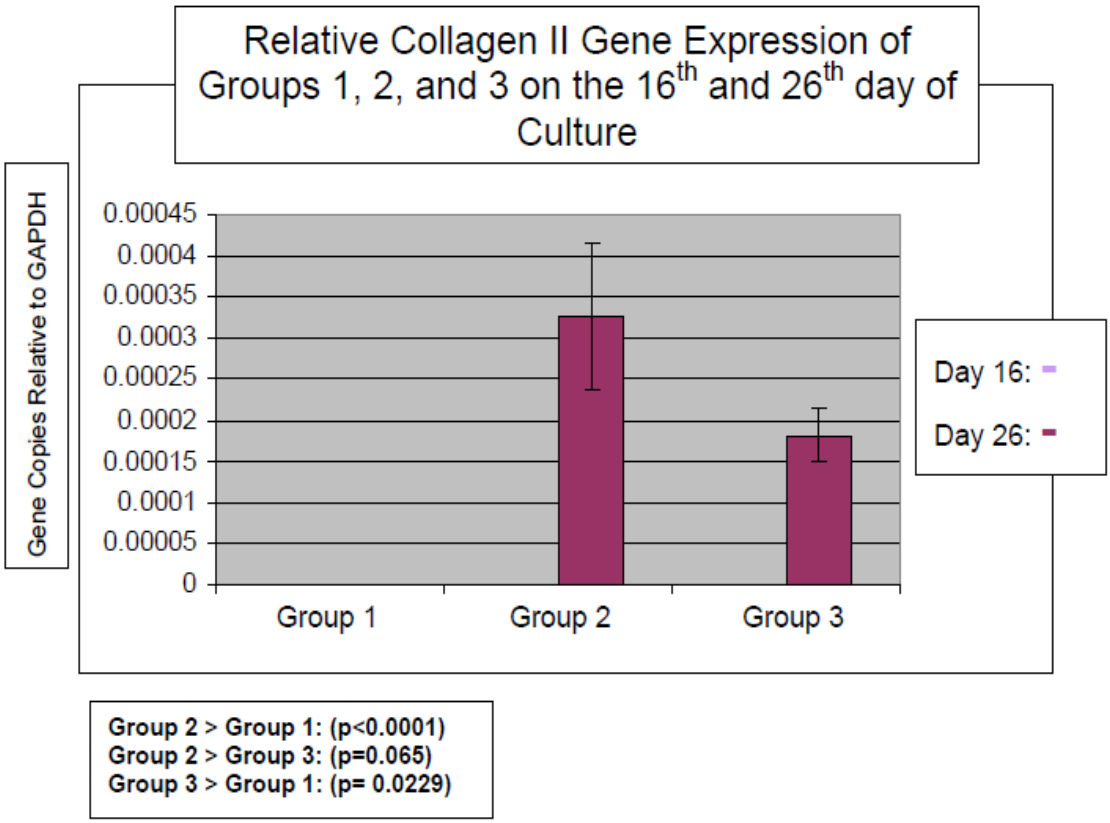


Figure 5-3b. Relative collagen II gene expression of Groups 1, 2, and 3 on the 16th and 26th day of culture: note that collagen II mRNA expression was significantly higher in growth factor treated groups (Groups 2 and 3) versus the control group (Group 1). No expression of the collagen II gene was detected on day 16.

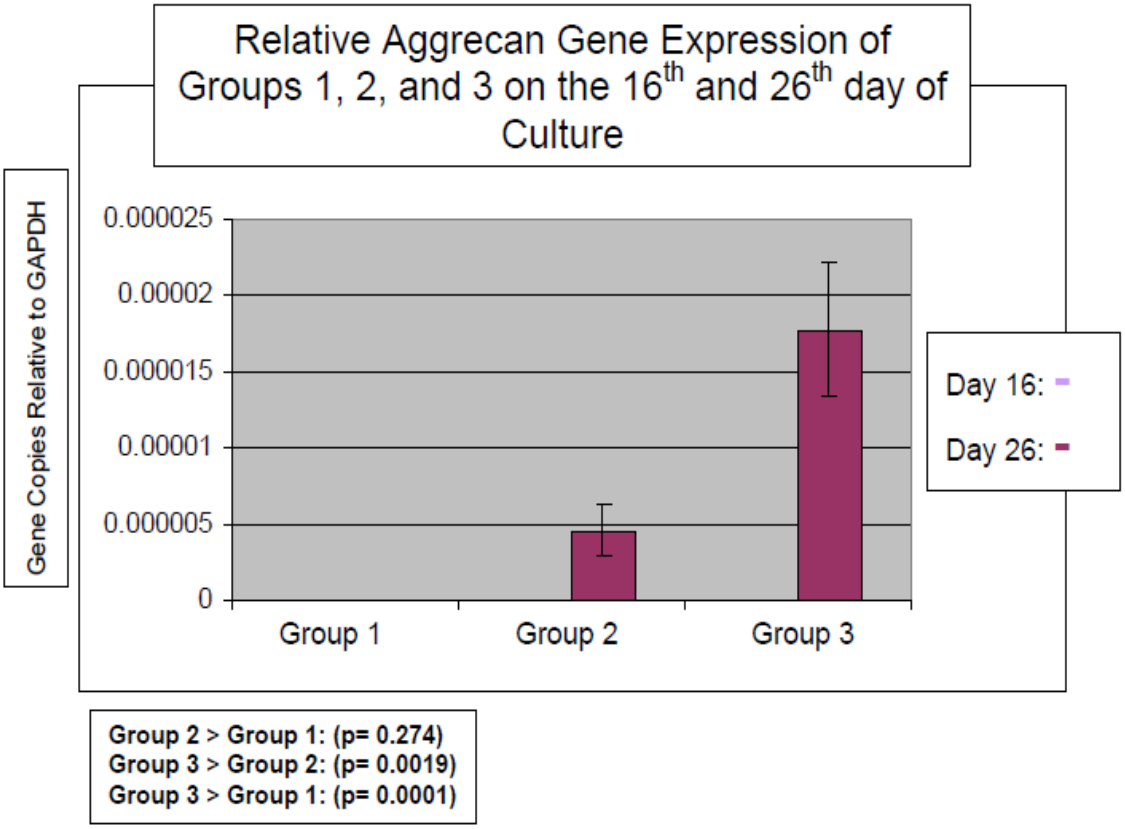


Figure 5-3c. Relative aggrecan gene expression of Groups 1, 2, and 3 on the 16th and 26th day of culture: note that aggrecan mRNA expression was significantly higher in Group 3, treated with sequential multiple growth factors (bFGF, TGF-β and IGF-1), versus Group 2 (treated with bFGF only) or Group 1 (no growth factors). No expression of the aggrecan gene was detected on day 16.

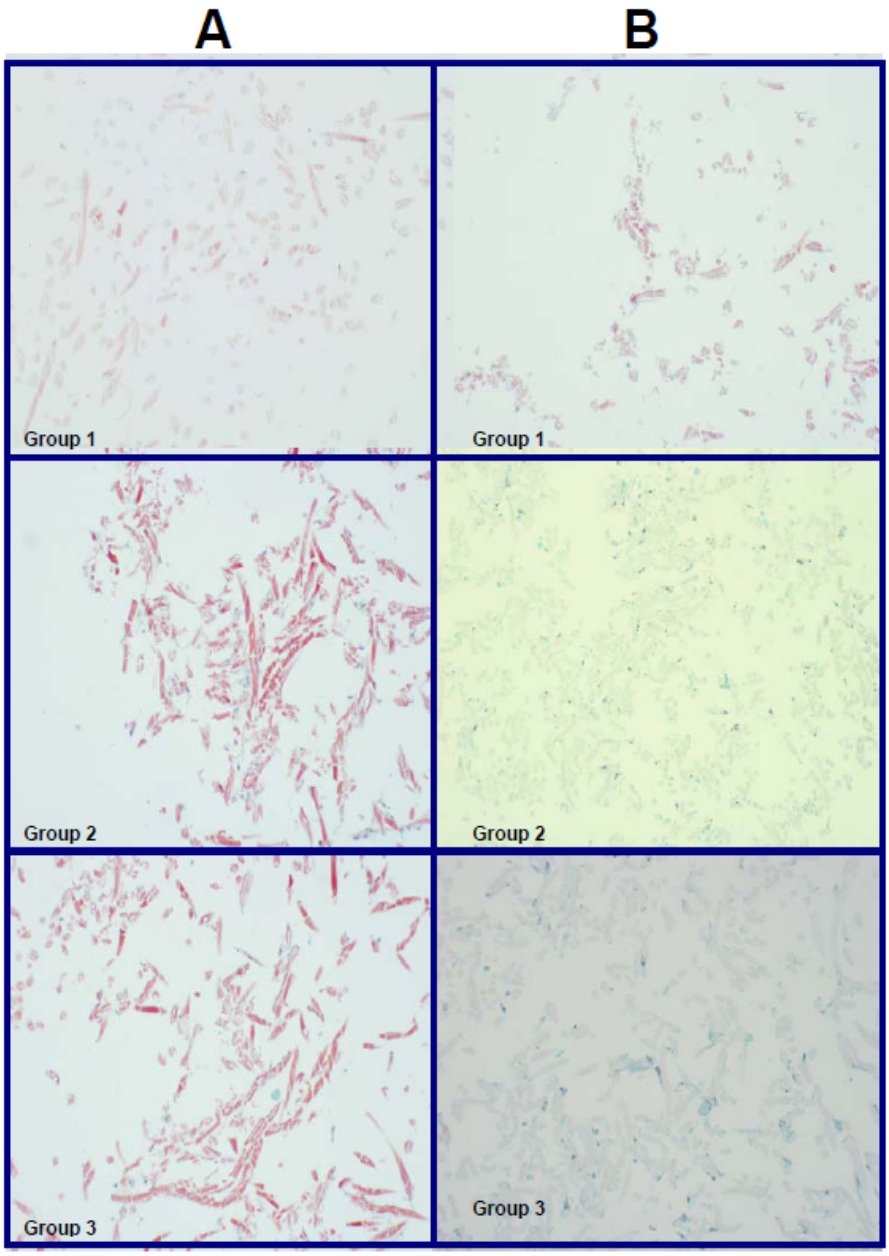


Figure 5-4a. Photomicrographs of constructs harvested on day 16 (column **A**) and day 26 (column **B**), as seen with Safranin- Orange staining, 10x objective magnification. Note lack of glycosaminoglycan staining in all groups at all time points; some scaffold fibers are stained red.

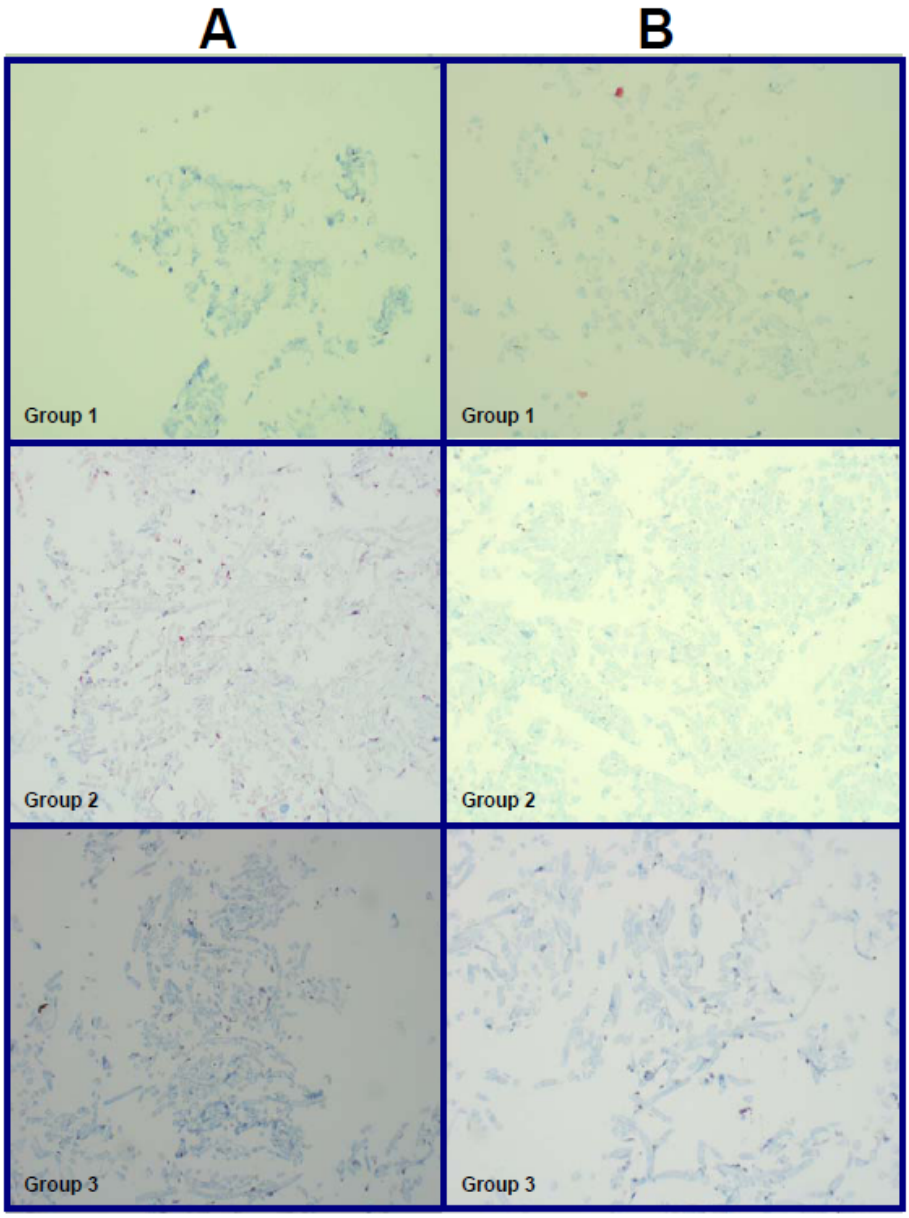


Figure 5-4b. Photomicrographs of constructs harvested on day 16 (column **A**) and day 26 (column **B**), as seen with Masson's Trichrome staining, 10x objective magnification. Note lack of collagen staining in all groups at all time points.

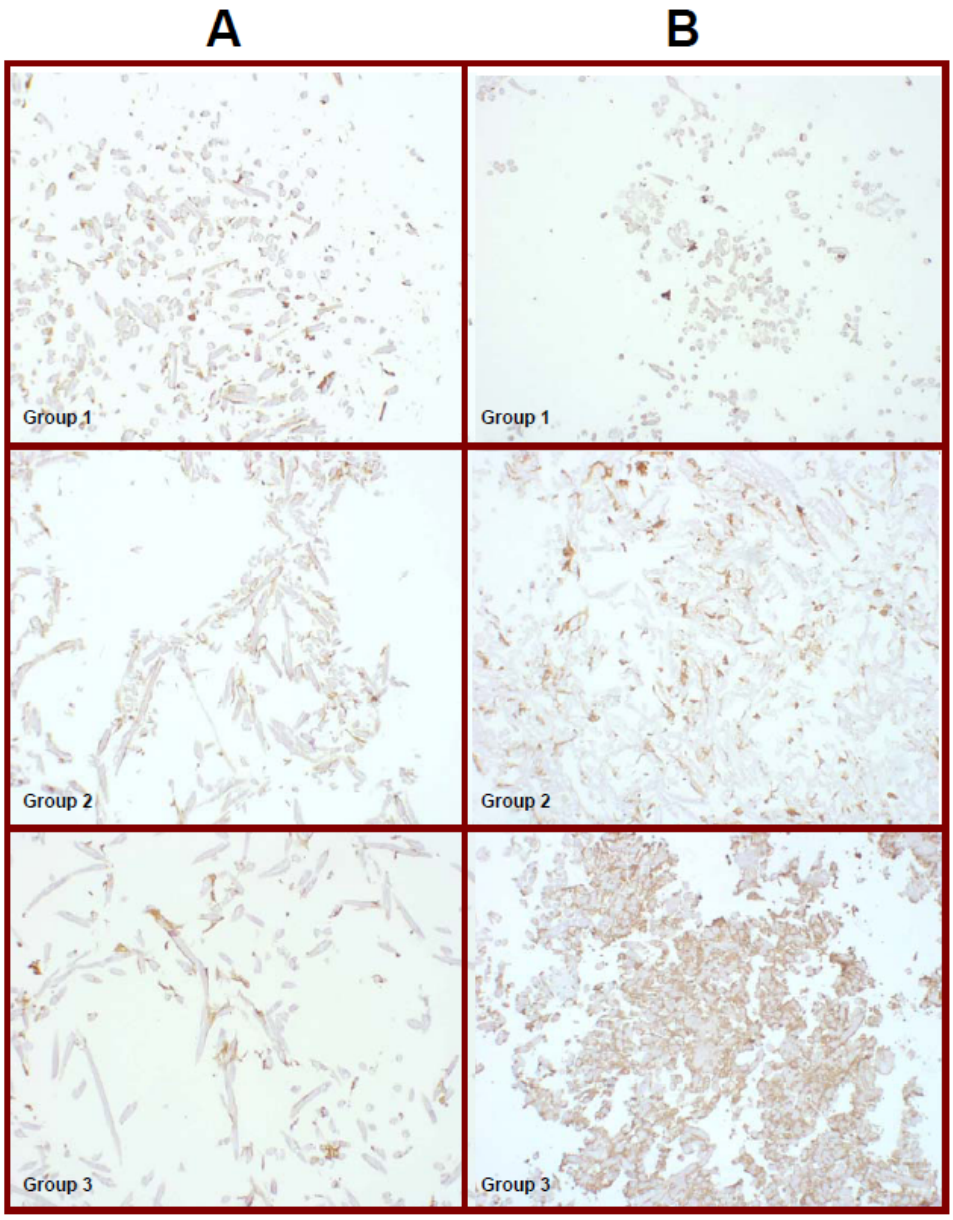


Figure 5-5. Photomicrographs of constructs harvested on day 16 (column **A**) and day 26 (column **B**), as seen with immunohistochemistry for α-smooth muscle actin, 10x objective magnification. Positive immunoreactivity can be seen in all groups at all time points. Note the increased positive immunoreactivity in Groups 2 and 3 (growth factor treated) versus Group 1 (control) on day 26.

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Chapter 6

Comparison of canine synovial fibrochondrogenesis using two cell seeding concentrations on a 10% poly-(L-lactic acid) and 90% poly-(glycolic acid) blend tissue scaffold

Currently, it is unknown how many cells are required to induce and optimize *in vitro* meniscal fibrocartilage tissue formation on a synthetic tissue scaffold. Relatively lower scaffold seeding densities could be used, mimicking the adult meniscus. Conversely, artificially higher cell concentrations may be needed to achieve the paracrine and autocrine cellular cross talk to produce *de novo* ECM *in vitro*. [1-3,4-6 7-9] High cell densities may also be used to mimic the embryonic condition; during the earliest stages of embryonic meniscogenesis, the menisci are highly cellular structures, before significant structural ECM is ever formed.[10] However, a major disadvantage of higher cell culture concentrations includes a greater demand for cell yield and expansion, which increases culture duration and cost.

As the meniscus is a relatively hypocellular tissue, with high ratio of ECM to cell volume,[11-13] we elected to use a low cell seeding concentration in previous studies. However, this may have negatively impacted tissue formation. Cellular seeding density is a major determinant of extracellular matrix (ECM) formation in cell culture. Obtaining a high construct cellular density is crucial for stimulating and accumulating cartilaginous ECM formation.[1-3,4-6 7-9] For example, some multipotential mesenchymal cells (cell line C3H10T1/2) undergo chondrogenic differentiation only when seeded as high-density micromass cultures.[7] Studies on adult mesenchymal stem cell based mandibular condyle tissue engineering, shows improved tissue maturation with 20 million cells/mL seeding density, versus a lower concentration of 5 million cells/ml. [14,15] Chondrogenic differentiation of mesenchymal stem cells in alginate is dependent on initial cell seeding density.[16] However, while high cellular density is helpful for inducing cartilaginous ECM, dense avascular ECM may cause central tissue necrosis, such as what is seen

in pellet culture.[16] A careful balance between avascular tissue perfusion and cellular density must be achieved to reconstruct the meniscus *in vitro*.

Reported dynamic scaffold seeding concentrations of 30,000 fibroblasts /mL,[17] 600,000 chondrocytes/mL,[18] 5 million chondrocytes/ mL,[19] 10 million chondrocytes/mL,[20] and 20 million fibrochondrocytes/ mL[21] have been reported in the literature. The smallest seeding concentration of cells that produced detectable cartilage extracellular matrix on a tissue scaffold was 600,000 cells/mL; however, these cells were juvenile bovine chondrocytes, and therefore ontogenetically primed for cellular replication and ECM formation.[18] Studies of synovial chondrogenesis on PGA scaffolds have achieved cartilage formation on scaffolds using 100million cells/mL.[22] Another study utilized 240,000cell/mL,[23] however over 2 months of time, only GAG was produced, which is a natural synthetic product of synoviocytes,[24] as well as aggrecan and type I collagen genes expression, similar to what was detected in Chapter 5. In gel cultures, concentrations of 4 million synoviocytes/mL[25] and 100 million cells/mL[6] have been utilized. Thus, synoviocytes may require relatively higher cell seeding concentrations in order to created tangible tissue, versus chondrocytes or meniscofibrochondrocytes.

Our prior studies using equine synoviocytes failed to produce constructs with gross tissue formation. While duration of culture, biomechanical stimulation, and growth factors and other environmental stimuli all play a role in synovial chondrogenesis, enough cells must be present on a scaffold to produce ECM and retain the construct shape, taking up biomechanical function as the scaffold naturally degrades. This is particularly relevant with scaffolds made of PGA or PGA polymer blends: cohesive tissue must be formed quickly, as PGA has an aqueous half life of approximately 16 days.[26] The minimum number of cells required for synovial chondrogenesis must be identified, because achieving higher cell numbers requires more extensive initial tissue harvest, and longer and more expensive culture manipulation to achieve higher cell numbers. We propose to seed 90%PGA-10% PLLA scaffolds with two different concentrations of synoviocytes, and induce synovial chondrogenesis using sequential exposure to the growth

factors bFGF, TGFβ-1, and IGF-1. The goal of this study is to compare the prior utilized cell seeding concentration of 400,000 cells/mL to 9.5million cells/mL in an attempt to make fibrocartilage tissue, within the half life of the PLLA-PGA blend scaffold. We hypothesize the null hypothesis, that: 1) there will be no difference in construct cellularity, viability, and fibrocartilage ECM formation between treatment groups seeded with 400,000 cells/ mL vs. 9.5 million cells per mL; and 2) no difference in cell viability, cell number, and chondrogenesis in constructs cultured for 26 days versus 16 days. This study will help to determining a cell concentration necessary to induce production of fibrocartilage extracellular matrix in a rotating bioreactor, for the long term application in meniscal tissue engineering.

Synovial Membrane Cell Culture

Synovium was aseptically harvested from the stifles of one beagle and 2 mixed breed dogs euthanatized for reasons unrelated to the study, and determined to be free of orthopedic disease based on gross examination of the joint. After euthanasia, the joints were aseptically prepared and accessed through a lateral parapatellar incision to the stifle joint and a lateral approach to the shoulder joint via tenotomy of the supraspinatus, infraspinatus, and teres minor tendons. The joint capsule was reflected and the synovial intima/ subintima was sharply dissected from adjacent joint capsule or adipose tissue. The tissue was placed in Dulbeccos' Modified Eagle's Media with 10% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B 25ug/mL, 0.015% (0.15mg/mL)L-Ascorbate, Pyruvate (0.22mg/mL), and 0.01% L-glutamine (supplemented DMEM) in preparation for monolayer culture.

The synovium was sectioned into 2.0mm x 2.0mm pieces using a #10 Bard Parker blade under sterile conditions. The tissue fragments were combined with sterile Type 1A clostridial collagenase solution[a] at a concentration of 7.5mg/mL of RPMI 1640 solution. The mixture was agitated on a titer plate shaker[b] at 37˚C, 5% CO2, 95% humidity for 8 hours in an incubator.[c] The

cellular suspension was transferred to a 15mL Falcon centrifuge tube and centrifuged[d] at 1.5 RPM, at 15˚C for 5 minutes. The supernatant was decanted and the cell pellet resuspended with 5mL of DMEM; the new suspension was subcultured and transferred into 75mL tissue culture flasks containing 13mL of DMEM. The flasks were incubated at 37˚C, 5% CO2, 95% humidity, with sterile medium change performed every 3 days. Synovial cells were monitored for growth using an inverted microscope until observance of 95% cellular confluence per tissue culture flask and then subcultured. At 95% confluence, the flasks were washed with serum free media and the cells enzymatically released from the culture flask using TryplExpress.[e] Cell viability and cell counts were assessed by the Trypan blue assay. A cell count per flask was performed by combining 20uL of cell suspension with 20uL of 0.4% Trypan Blue[f] placed on a hemacytometer. Cells were cultured until 2nd passage to utilize 3rd passage cells in the bioreactor.

Scaffold Culture

Tissue scaffolds from the same batch as used in Chapter 5, made of a blend of 10% poly-L-lactic acid and 90%poly-glycolic acid fibers,[g] were used in this study. Scaffolds were manufactured as a non woven felt, which was cut into 12mm x12mm x3mm sections and sterilized with ethylene oxide. Twenty scaffolds were placed in each 110mL rotating bioreactor flask[h] and presoaked for 6 hours in DMEM without fetal bovine serum, at 37˚C, 5% CO2, 95% humidity **(Figure 6-1).** Cells were released from T-75 tissue culture flasks with TryplExpress[i] to avoid cell clumping and then added to the bioreactor. Two treatment groups, twenty scaffolds per group, were established as follows: **Group 1**: Scaffolds seeded with 9.5million synovial cells/mL, and **Group 2**: Scaffolds were seeded with synoviocytes at 400,000 cells/ mL. All constructs were exposed to the same growth factor treatment: serum free media containing human recombinant basic Fibroblast Growth Factor (bFGF),[j] 50ng/mL for 3 days, then maintained in serum free media containing human recombinant Transforming Growth Factor β-1 (TGFβ-1),[j] 10ng/mL, and Insulin-like Growth Factor- 1 (IGF-1)[k], 500ng/mL. Bioreactor cell scaffold culture was maintained at 37˚C, 5%CO2, 95% O2, at 17.3 rpm, with 50% of cell culture medium

changed, using sterile technique, every 3 days, maintaining the above growth factor concentrations. Ten scaffolds were harvested from each group and quartered for analysis on days 16 and 26. At the first media change cell seeding efficiency was calculated by comparing cell count of free cells in the media with the original cell seeding number.

Histologic Analysis

One quarter of each construct was fixed in 10% buffered formalin, sectioned, and stained with Hematoxylin and Eosin, Masson's trichrome, Toluidine Blue, and immunohistochemistry (IHC) for Collagen I and II.

For immunohistochemistry for collagen type I, tissues were cut at 4 microns and placed on plus charged slides, microwaved and left on a 43°C slide warmer overnight. The slides were then hydrated and placed in 0.4% pepsin and heated in a 37°C incubator for 20 minutes. The slides were rinsed in tap water and placed in Tris buffer for at least 5 minutes. Subsequent staining was done on the Dakocytomation Autostainer.[m] Slides were treated with 3% H202 for 15 minutes, protein block (DAKO) for 5 minutes. Slides were incubated in goat anti-Collagen Type I,[n] at a 1:100 concentration for 30 minutes. Negative slides were treated with goat IgG, 1:1000 in place of the primary antibody. Secondary and tertiary reagents used included the LSAB+ system[o] at 20 minutes each. The chromogen used was Nova Red (Vector) for 10 minutes. Slides were counterstained in Mayer's Hematoxylin[p] for 1 minute, dehydrated and coverslipped. For Collagen Type II, immunohistochemistry, tissues were cut at 4 microns and placed on plus charged slides, microwaved and left on a 43°C slide warmer overnight. They were hydrated and placed in trypsin solution and heated in a 37°C incubator for 60 minutes. Then they were rinsed in tap water and placed in Tris buffer for at least 5 minutes. Subsequent staining was done on the Dakocytomation Autostainer. Slides were treated with 3% H202 for 15 minutes, protein block (DAKO) for 5 minutes. Slides were incubated in rabbit anti-bovine Collagen Type II, AB746P antibody[n] at a 1:100 concentration for 30 minutes. Negative slides were treated with rabbit IgG, 1:1000 in place of the primary antibody. Secondary reagent used was the rabbit

envision+ system[o] at 30 minutes. The chromogen used is Nova Red (Vector) for 10 minutes. Slides were then counterstained in Mayer's Hematoxylin for 1 minute, dehydrated and coverslipped.

Histologic specimens were examined at 10x magnification on a Zeiss Microscope.[q] Images of each section, (three from the scaffold periphery and three from the scaffold center) at 2 o'clock, 6o'clock and 10o'clock positions (Figure 1) were digitally captured by an Olympus DP-70[r] digital camera and saved as Tiff files.

Determination of Cell Viability

Cell viability was determined with the use of ethidium homodimer-1 (4ul/ml PBS) and Calcein AM (Acetoxymethylester) (0.4ul/ml PBS) fluorescent stains[s] and the use of Confocal Laser Microscopy. The Confocal Laser Microscope consists of the BioRad Radiance 2000 confocal system coupled to an Olympus IX70 inverted microscope[y] equipped with Krypton-Argon and red diode laser. Approximately 1.0 mm sections were made from each scaffold quarter and incubated with the staining agents for 30 minutes at room temperature, placed on a glass microscope slide, moistened with several drops of PBS, 1X, and stained using the fluorescent double labeling technique. The sections were examined under 10x magnification. Images of each section were digitally captured by an Olympus DP-70 digital camera and saved as Tiff files. Live and dead cell counts were determined by hand counts.

Results

At the time of seeding, the 110mL bioreactor flasks appeared crowded, with scaffolds touching each other, although the scaffolds were free floating. In Group 1 (9.5 million cell/mL) at the end of the 2nd day of culture, the media changed color from red to yellow, indicating an increased media acidity. Media changes for both groups were performed on the morning of the 3rd day. Cell seeding efficiency was 75% for 9.5million cells/mL vs. 90% for 400,000cells/mL. All cells that were not adhered to the scaffold were dead at the first media change, and cellular

fragments and amorphous debris were noted. A decrease in pH was noted by media color shifting to yellow or orange 12-8 hours before each media change in the first week of culture in Group 1.

At the 16 day harvest time scaffolds were showing gross disintegrating with fragments of scaffold floating in the bioreactor; no intact scaffolds were left for harvest on day 26. On the 16th day of culture, Group 1 (9.5 million cells/mL), 14 scaffolds were recovered, and were severely fragmented and fragile, easily separating with gentle forceps manipulation. Three of the 14 scaffolds had amorphous white tissue arranged irregularly on the scaffold; 11 of the 14 retained a felt-like appearance with no obvious tissue formation. In Group 2 (400,000 cells/mL), 15 disintegrating scaffolds were recovered, and all retained a felt like-appearance. At the 26 day harvest, some scaffold- like debris was harvested from both groups, but its consistency precluded histologic examination; what debris could be collected was used for cellular live dead assay. Confocal microscopy revealed 0% cellular viability in all groups at both time points (**Figure 6-2**).

Histologic analysis

Subjectively, cell numbers were higher in Group 1 versus Group 2 because of the clumps of tissue in three of the 14 recovered scaffolds. In both groups cells were organized singly and in clumps. The 3/14 scaffolds in the 9.5million cells/mL which had formed amorphous white tissue clumps on the scaffolds contained collagen but not GAG on histologic analysis (**Figure 6-3a**). While the tissue was dead at the time of analysis with histologic evidence of autolysis, there was mild positive immunoreactivity for Collagen I and Collagen II immunohistochemistry (**Figure 6-3b**). The remaining 11 scaffolds had sparse cellularity and no microscopic evidence of ECM formation. Sparse cellularity was noted on the Group 2 scaffolds but no ECM formation was seen on Masson's Trichrome or Safranin-Orange staining (**Figure 6-4**). Histologic analysis also confirmed an advanced stage of scaffold dissolution of all constructs examined.

Discussion

In this study, gross fibrous tissue formation was achieved in the high cell seeding group, however, ECM formation was sporadic, occurring only in 3 constructs and in clumped

distribution. Glycosaminoglycan formation was not detected, although our histologic methods are not as sensitive as the dimethyl-methylene blue assay[27,28] used in the previous chapters. This spectrophotometric assay has not been validated for use in autolyzed tissue.[29] This tissue formation required a high synoviocyte seeding concentration, with ECM formation occurring only with clusters of cells. This finding correlates with data from other studies, [1-3,4-6 7,8] showing the importance of achieving high cellular numbers and dense cellular contact to stimulate ECM production.[30] This describes the embryologic "community effect," where multicompetent cells must be surrounded by similar cells in order to be respond to inductive signals, differentiate, and proliferate ECM.[4,5] In addition, the cells must be evenly distributed on scaffolds,[30] which increases ECM production and construct compressive properties.[30-34] However, in this study, cells and tissue formation was highly localized and clumped, possibly due to inefficient fluid dynamics of a crowded bioreactor, indicating that that the biomechanical methods used in this study are inadequate for the above goals.

Increasing cell seeding concentration reduced cell seeding efficiency, i.e. in group I, seeding efficiency was 75%, versus 90% in Group 2. Reasons for this could include a saturation effect once cell counts get as high as 9.5 million cells/mL, or changed fluid dynamics of crowded flasks, which could affect inertial impact seeding of cells on scaffolds and media delivery.

While increasing cell seeding numbers did result in some gross tissue formation, the most striking finding in this study was the profound cellular death and scaffold dissolution seen in both groups. The scaffolds dissolved at a more rapid rate than previously seen. Despite their correct storage with hygroscopic substances in an airtight container, they may have been starting to disintegrate prior to ethylene oxide sterilization. Thus in the future, research with scaffolds will only be done on freshly synthesized batches, and scaffold storage is not recommended. Inability to store large PGA felts will likely increase the cost of using this particular type of scaffold. Scaffolds weakened by dissolution may have applied excessive forces such as shearing, damaging or killing cells as they fragmented at 17.3 rpm. Loss cell attachment to the ECM is well

documented cause cell death, because of loss extracellular matrix- cytoskeleton mechanotransduction.[35-43,37,44,45] Life or death depends upon the cell's interaction with the ECM via adhesion receptors such as integrins, which directly regulate apoptosis or survival via the PI 3-kinase and MAPK pathways.[46] Additionally, because some scaffolds did not form ECM, or formed it in clumps, natural matrix was not distributed evenly enough to prevent the scaffolds from fragmenting. Scaffold fragmentation may be a major cause of cellular mortality seen in this study, because it was seen in both groups and was independent of cell seeding numbers.

Other causes of cell death include degradation products from the chain reaction of cell death and scaffold dissolution. A decrease in pH was noted by media color shifting to yellow 12-8 hours before each media change in the first week of culture in Group 1. Cell death with membrane breakdown results in release of excessive extracellular products such as potassium, phosphorus, calcium, hydrogen ion, and lysosomal enzymes, all of which cause cellular dysfunction and death in neighboring cells.[47-49] As cell fragments and debris were observed at each media change, cell lysis or rupture may have resulted in an *in vitro* tumor lysis- like effect in the bioreactor. Cell death as well as the acidic breakdown products from the rapidly degrading polyglycolic acid [70] may have also contributed to the decrease in pH, exacerbating cell death in Group 2.

Bioreactor limitations may have also contributed to cell death in the 9.5million cells/mL group: the 110mL rotating bioreactor flask likely did not contain enough nutrients for maintaining viability of such a high number of cells. Cellular nutrient exchange within a bioreactor occurs via Michaelis-Menton kinetics,[50] and that critical exchange likely reached the plateau of this curve in this study. The synoviocytes would have starved to death and been unable to excrete metabolic wastes with the insufficient media exchange in the 110mL rotating flask. Future studies may need to focus on more efficient nutrient delivery systems such as perfusion bioreactors or larger volume rotating flasks. Perfusion bioreactors have the advantage of enhancing nutrient delivery while mechanically stimulating cells to influence phenotype and increase matrix production,[50-55]

overcoming the inherent limitations of diffusional transport in conventional culture systems.[52,55,56] Medium perfusion results in better cell viability, due to the convective-diffusive transport of oxygen, compared with oxygen diffusion in other cultivation systems, and efficient removal of harmful cellular metabolic by-products.[30,50,52,55,57] Flow through bioreactors have been successfully used to culture fibroblasts,[58] mesenchymal progenitor cells,[53] chondrocytes,[59,60] osteoblasts,[61] cardiac myocytes,[62] vascular smooth muscle,[62] skeletal myocytes,[63] ovarian cells,[64] neurons,[65] and intestinal smooth muscle cells.[66] The failure of the culture system used here may also indicate the need for a complete change in meniscal tissue engineering strategy, moving to a scaffoldless system and tissue printing.[67-70] Ink jet printers have been used to print various live cell types, including embryonic stem cells, fibroblasts, hepatocytes, ovariocytes, and cardiomyocytes encapsulated in acoustic droplets at rates varying from 1 to 10,000 droplets per second, and with cell viabilities of 89%-98%.[67,70] Bioprinting could be used to fabricate persistent biomimetic patterns[68] such as the radial collagen tie fibers and circumferential fascicles seen in the meniscus. Scaffoldless tissue engineering strategies[71,72]may be the next focus of our research toward synovium based meniscal tissue engineering, which has been reported in synovium based hyaline chondrogenesis.[71,72]

Regardless of scaffold or scaffoldless *in vitro* fibrocartilage synthesis techniques, one of the greatest challenges faced by meniscal tissue engineers is the difficulty of keeping this large, avascular, dense, and relatively low cellularity organ alive *in vitro* and *in vivo*. Methods to deliver media and allow nutrient and waste exchange to the center of dense avascular tissue must be investigated. This may have contributed to cell mortality in the 3 scaffolds that produced tissue; at 12mmx 12mm x3mm our constructs were larger than the average synovial pellet culture, which can also result in diffusional cell mortality.[16] In the human fetus, almost the entire meniscus is vascularized, with the blood supply fueling meniscal growth. [10,73,74] By the second year of life, the vessels start to recede to the outer 1/3 red zone, in time for early ambulation, as the ECM organizes to accept weight bearing.[10,73,74] There have been several attempts to

recapitulate this process in the laboratory using viral transduction of hepatocyte growth factor gene and vascular endothelial growth factor (VEGF) as well as direct application of VEGF. [75-79] Efforts to recreate vascularized meniscus *in vitro* or enhance its healing by increasing vascularity *in vivo* has either not worked, [75-77] or has decreased the compressive strength of the fibrocartilage compared to normal meniscus.[78,79] If an angiogenic strategy were utilized to synthesize and maintain viability of an adult animal sized meniscus, mechanical protection of the joint as well as reversal of the angiogenesis would be requisite. *In vitro* use of artificial dissolvable blood vessels have been recently investigated. Cellulose acetate hollow fiber membranes can be imbedded in collagen gel, and are porous and semi-permeable. [80] These hollow fiber membranes can be connected to micro perfusion bioreactors serve similar functions to a circulatory system; they have been used in 3-D culture of rat bone marrow fibroblastic cells.[80] Channeled elastomer scaffolds have also been used successfully to engineer contractile cardiac tissue *in vitro*.[56] The native juvenile and adult meniscus also contains parenchymal micro-canals, 10-200μm in size, that open onto the surface of the meniscus. The canals play a part in the transport of fluid within the meniscus, and carry nutrients from the synovial fluid or the blood vessels to the avascular sections of the meniscus.[81,82]Synovial membrane also communicates with these holes, furthering the nutrient delivery theory.[83] Presence of anatomic mechanism for avascular nutrient delivery in the meniscus should inspire further investigation of microtube scaffolds that can provide pores or channels for avascular meniscal fibrocartilage construction.

There were several limitations to this study; no quantitative tests, such as gene expression or biochemical assays were performed to assess the levels of specific fibrocartilaginous ECM. However, RNA analysis via real time RT-PCR may have been made inaccurate by cell autolysis and RNA degradation. Additionally, ELISA and spectrophotometric assays have not been validated for autolyzed tissue and may have resulted in misleading data.

In conclusion, at high concentrations, canine synovial membrane cells can produce fibrocollagenous ECM, in 3-D culture, with biomechanical stimulus of a rotating bioreactor. We

accept the null hypothesis, in that there was no difference in cell viability between a high cell seeding concentration of 9.5 million cells/mL (Group 1) and a lower concentration of 400,000 cells/mL (Group2). We reject the null hypothesis in terms of cellularity and ECM formation, with a descriptive increase in cellularity and macroscopically visible ECM with higher cell densities in Group 1. However, because marked premature scaffold dissolution and uniform cell death occurred in this study, stored 10/90 PLLA and PGA blend is not an appropriate scaffold for short duration of *in vitro* synoviocyte culture. In addition, a 110mL rotating microgravity bioreactor is not appropriate for high scaffold and cellular density tissue culture. Future studies will need to focus on alternate meniscal tissue engineering strategies, such as scaffoldless tissue synthesis, perfusion bioreactors, channeled scaffold 3-D culture, or transient meniscal neoangiogenesis.

Footnotes

a. Type 1A Clostridial Collagenase, Sigma, St. Louis, MO
b. Titer plate shaker, Lab Line Instruments, Melrose Park, IL
c. Nuaire US Autoflow, Plymouth MN
d. Marathon 3200, Fisher Scientific, Pittsburgh, PA
e. Innovative Cell Technologies, San Diego CA
f. Trypan Blue, Sigma, St. Louis, MO
g. PGA/PLLA blend Biomedical Structures LLC, Warwick RI
h. Rotating Bioreactor System, Synthecon, Houston, TX
i. Innovative Cell Technologies, San Diego CA
j. human recombinant bFGF, BD Biosciences Bedford, MA
k. human recombinant TGF-β, BD Biosciences Bedford, MA
l. human recombinant IGF-1, BD Biosciences Bedford, MA
m. Autostainer, Dakocytomation, Carpenteria, CA
n. Chemicon, Temecular, CA
o. Dakocytomation, Carpenteria, CA
p. Newcomer's Supply, Appleton, WI
q. Carl Zeiss, Thornwood, NY
r. Olympus DP-70 Digital Camera, Olympus, Melville, NY
s. Live/Dead Viability/Cytotoxity Kit, [Cat. #1-3224], Molecular Probes Co., Eugene, OR
t. Olympus IX70 microscope, Olympus, Melville, NY

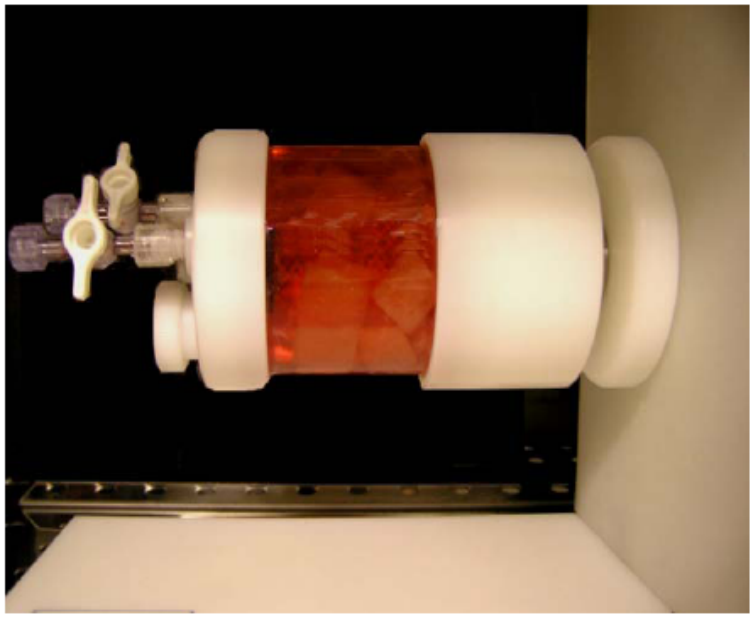

Figure 6-1. Rotating wall bioreactor flask (110mL) apparatus containing 12mm x 12mm x 3mm scaffolds made of a 10% poly-L-lactic acid, 90%poly-glycolic acid blend: this image was taken at the time of presoaking, prior to cell seeding.

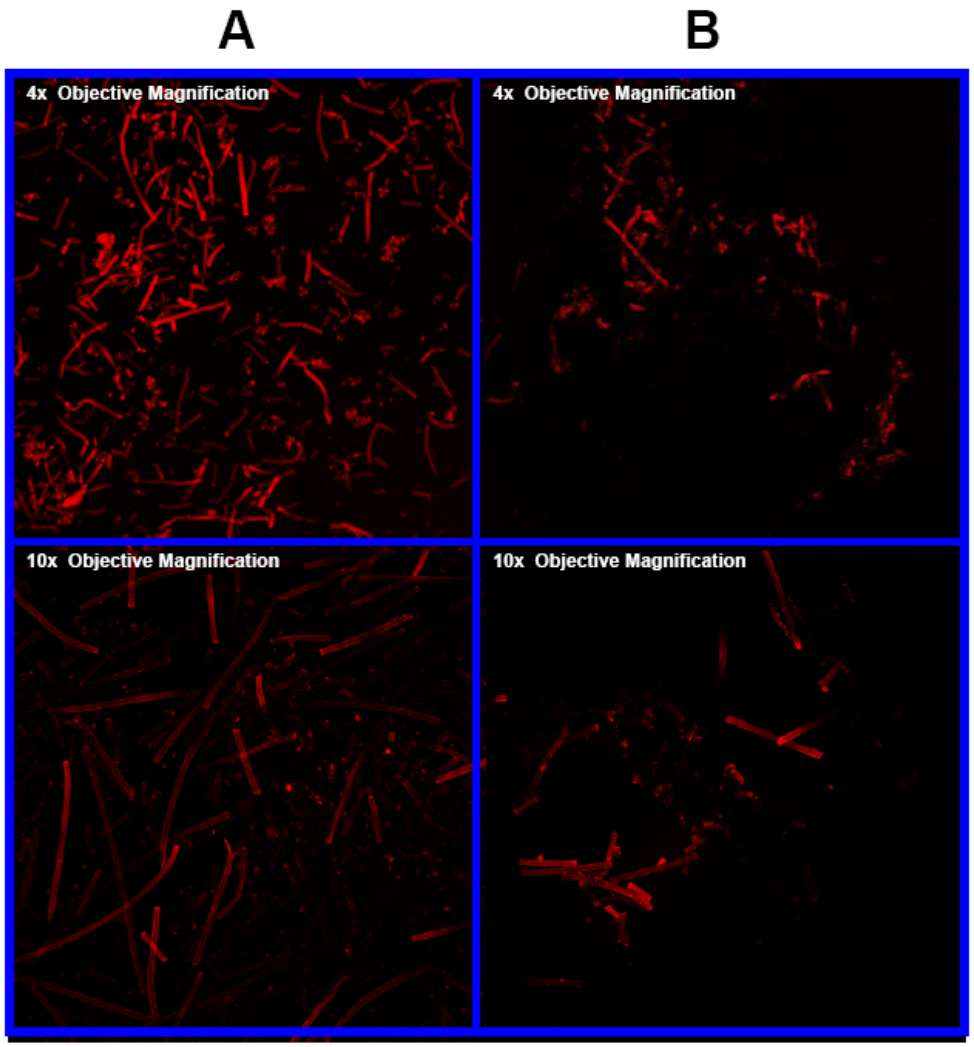

Figure 6-2. Photomicrographs of scaffolds seeded with 9.5million synovial membrane cells/mL, (column **A**) and scaffolds seeded with 400,000 cells/ mL (column **B**), as seen on confocal microscopy, using the calcein AM-ethidium homodimer live-dead assay. The top row represents constructs harvested on day 16, and the bottom row represents constructs harvested on day 26 of culture. Note the complete cellular mortality as noted by red stained cells.

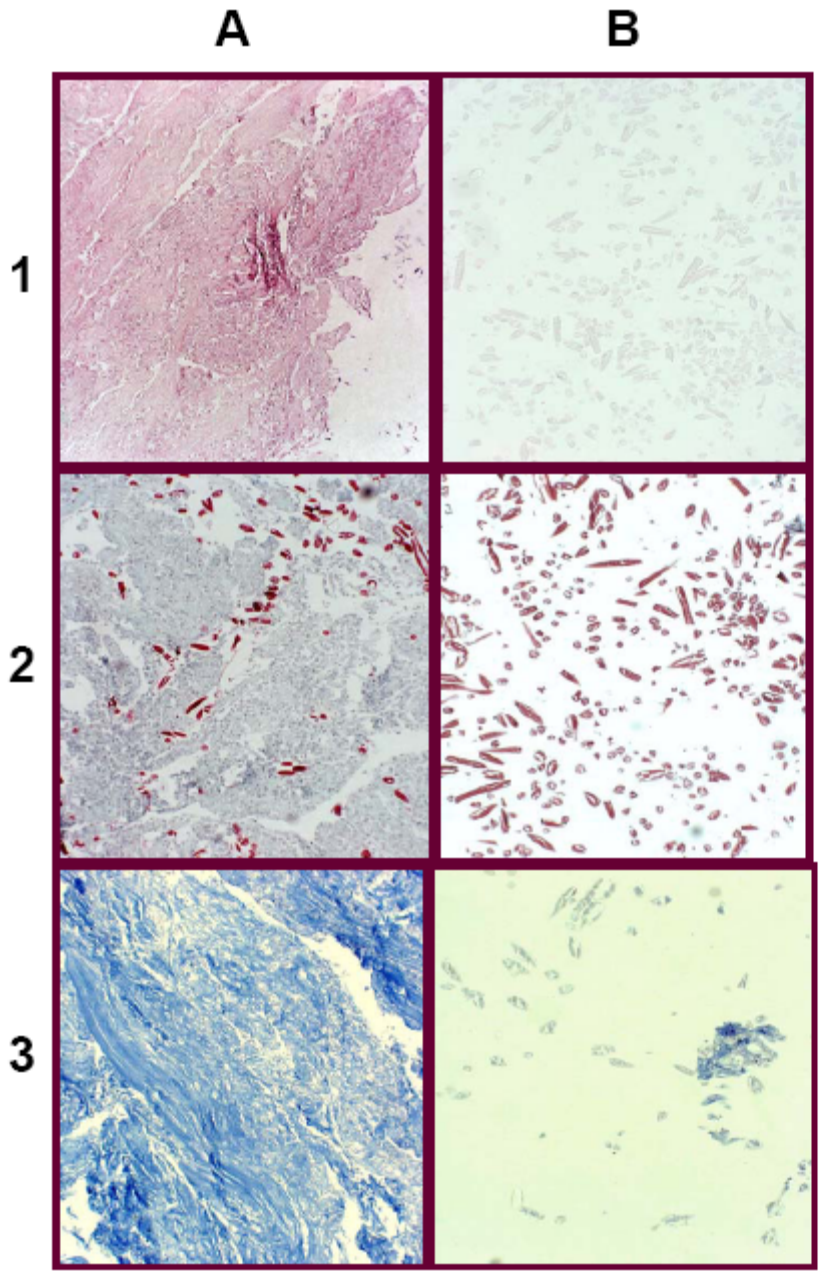

Figure 6-3a. Photomicrographs of Group 1 (9.5million cells/mL) constructs harvested on day 16, 10x objective magnification, representing the biological range of scaffolds that contained ECM, (column **A**) and scaffolds that did not contain ECM (column **B**), as seen with H+E (row **1**), Safranin- Orange (row **2**), and Masson's Trichrome (row **3**) staining. Note the lack of glycosaminoglycan staining and the presence of positive collagen staining.

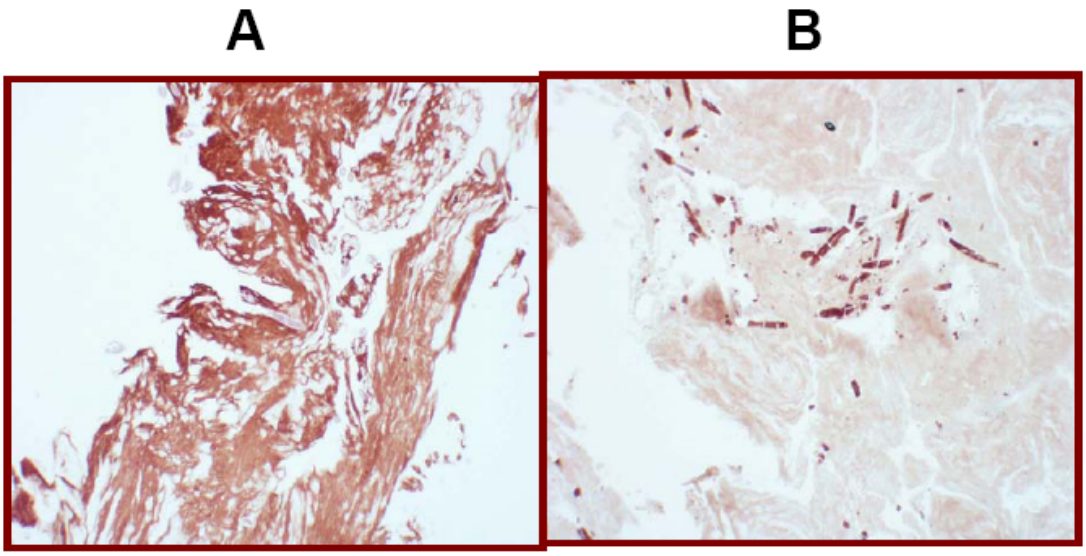

Figure 6-3b. Photomicrographs of constructs in Group 1 (9.5million cells/mL) that contained ECM, harvested on day 16, at 10x objective magnification, immunohistochemistry for collagen I (**A**) and collagen II (**B**): note the strong immunoreactivity to collagen I and mild positive immunoreactivity for collagen II.

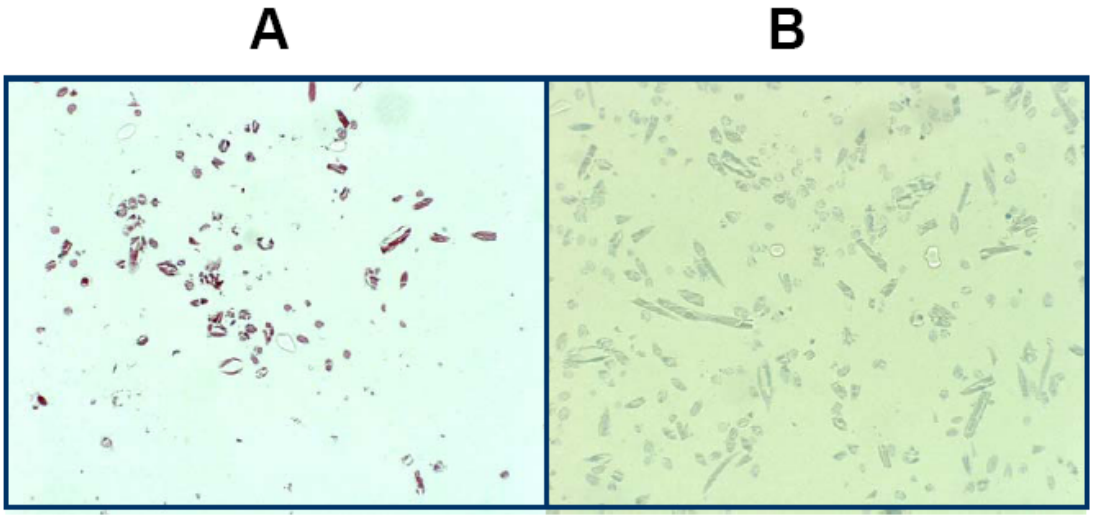

Figure 6-4. Photomicrographs of Group 2 (400,000 cells/mL) constructs harvested on day 16, 10x objective magnification, stained with Safranin-Orange (column **A**) and Masson's Trichrome (column **B**): note the sparse cellularity and lack of glycosaminoglycan and collagen staining.

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Chapter 7. *In vitro* fibrochondrogenesis of normal and osteoarthritic synoviocytes for meniscal fibrocartilage tissue engineering

One of the great challenges in tissue engineering is determining the ideal cell source for *in vitro* regenerate or replacement tissue formation. Because cells cannot be synthesized *de novo*, they must be invasively harvested autologously or taken from deceased organ donors. When determining ideal cell sources for tissue engineering, location of the source tissue, quantity of donor tissue available for harvest, and ability to harvest the cells in a minimally invasive fashion must be considered. As previously described in chapters 5 and 6, as well as in the work of others, synoviocytes can readily undergo *in vitro* chondrogenesis in response to growth factor stimulation.[1-15] Stifle synovium is abundant, the synovial membrane regenerates rapidly post synovectomy,[16] and synovium is readily harvested arthroscopically.[17] Thus, synovium may be an ideal cell source for meniscal tissue engineering. Currently, the majority of these investigations utilize synoviocytes from clinically normal joints, particularly the joints of young, healthy animals. [1-15] In contrast, meniscus deficient joints in veterinary and human patients have clinical osteoarthritis with pathologically affected synovium.[18-42] The use of normal synoviocytes as a cell source for meniscal tissue engineering would require surgical violation of another unaltered joint within the patient's body, or require allogenic donation. Synovium could also be harvested from the diseased osteoarthritic joint. Synovium would be collected from the affected joint at the time of primary diagnosis and debridement of a meniscal tear, with a second surgery required to implant engineered replacement tissue. Advantages for use of autogenous cells for tissue engineering include lack of immunoreactivity[43-45] and decreased risk of infectious disease transmission.[46,47] Thus, use of autologous synovium from affected joints is a rational cell source choice for meniscal fibrocartilage engineering.

Based on synovial propensity for *in vivo* chondrogenesis in osteoarthritic joints, seen in periarticular osteophytes [48-50]and secondary osteochondromatosis,[51-58] osteoarthritic synovium

could be 'primed' for chondrogenic differentiation and possess advantages over normal FLS for fibrocartilage engineering. Twice as many potentially chondrogenic mesenchymal precursor cells have been observed on the tips of synovial villi in patients with OA, as compared to healthy organ donors. [59] The mechanical and biochemical environment of the osteoarthritic joint favors synovial membrane thickening and intimal hyperplasia[60] with an increase in tissue collagen I content. Collagen I is the major structural protein in the meniscus;[61] possibly pathogenic synovial fibroplasia could be applied constructively to *in vitro* fibrochondrogenesis. Microangiogenesis in the arthritic synovial membrane results in blood vessel proliferation,[62-64] and thus by proxy, increased vascular origin progenitor cells[59,62-64] that could also contribute to desirable ECM formation. Conversely, the proliferation of inflammatory mediators and destructive enzymes from the synovial membrane in the diseased osteoarthritic joint[17,63-80] could negatively impact FLS *in vitro* chondrogenic potential.[81] Other cell types in the degenerative joint show decreased ECM synthetic capabilities; for example, osteoarthritic chondrocytes show reduced proliferation and poor ECM formation in response to growth factor treatment (FGF, IGF-I, and TGF-beta) compared to normal young and normal aged chondrocytes in monolayer culture.[82] Mesenchymal stem cells aspirated from bone marrow adjacent to severely arthritic joints showed decreased *in vitro* chondrogenic activity compared to age matched normal marrow.[83] At this time the true *in vitro* chondrogenic potential of canine osteoarthritic synovium is unknown; this crucial variable must be delineated if autologous synovium is to be used for meniscal fibrocartilage tissue engineering.

Tissue engineering can be divided into two philosophical methodologies. Tissue can be synthesized using scaffolds, cells, biomechanical and bioactive factors in a manner created by trial and error science, or tissue can be formed *in vitro*, in a way recapitulating natural organogenesis seen in the embryo. Significant work has been done in elucidating the formation of articular cartilage in the embryo,[84-132] however, little work has been done investigating molecular signaling in embryonic meniscogenesis. Despite this, optimizing in vitro molecular signaling for

articular chondrogenesis could be applied to meniscal tissue engineering. The cellular morphology, collagen and glycosaminoglycan content, and mechanical function of articular cartilage is similar to the axial, avascular portion of the adult meniscus.[61,133-140] Synovial synthesis of hyaline like cartilage can be applied to engineering the axial region of the knee meniscus, which is notorious for its lack of healing and regeneration in all species studied.[141-151] In addition, the mechanism for in vitro fibrochondrogenesis has not been fully elucidated; determining molecular signals in this process may provide targets for optimizing *in vitro* fibrochondrogenesis.

Three candidate genes involved in fetal chondrogenesis were selected for this study. Sry-type Homeobox Protein-9 (SOX-9), is a high-mobility-group (HMG) domain transcription factor that is expressed in chondrocytes and other tissues. In humans, SOX-9 haploinsufficiency results in campomelic dysplasia, a lethal skeletal malformation syndrome, and XY sex reversal. During embryogenesis, SOX-9 is expressed in all primordial cartilages, along with the expression of the collagen alpha 1(II) gene (Col2a1). SOX-9 binds to essential sequences in the Col2a1 and collagen alpha 2(XI) gene (Col11a2) enhancers and can activate these sites in non-chondrocytic cells.[89-92] Regulator of G- Protein Signaling (RGS) is a ubiquitous family of proteins that has recently been shown be involved in regulation of fetal chondrogenesis. RGS10 was found to promote cellular chondrogenic differentiation in mice.[88] Frzb, or Frizzled Motif Associated with Bone Development, first gained the attention of orthopedic surgeons because a variant in this gene produces susceptibility for hip osteoarthritis in women.[152-155] In development, Frzb-1 exerts a strong influence on limb skeletogenesis and is a powerful and direct modulator of chondrocyte maturation, phenotype, and function. Frzb-1 limits embryonic chondrocytes' progression along the maturation pathway. In the epiphysis, Frzb-1 allows epiphyseal chondrocytes to maintain a functional, stable, and immature phenotype, and it organizes the articular joint tissue, supporting joint function through life.[100,119,156-168]

Therefore, the primary objective of this study was to compare the fibrochondrogenic potential of synovial membrane cells from canine normal and OA joints in monolayer culture, by examining fibrocartilage extracellular matrix synthesis in response to chondrogenic growth factors. A second objective was to determine if three genes involved in embryonic chondrogenesis are induced and expressed in adult tissue in response to growth factors. We hypothesized that (1): osteoarthritic synovial membrane cells cultured in monolayer and treated with chondrogenic growth factors would show no difference in fibrocartilage extracellular matrix production and gene expression compared to controls, and (2): osteoarthritic and normal synoviocytes would show no difference in expression of the SOX-9, Frzb, and RGS10 genes known to be involved in fetal chondrogenesis.

Materials and Methods

Synovial Membrane Cell Culture

Synovium was aseptically harvested from the normal stifles of 6 large breed dogs determined to be free of orthopedic disease based on gross examination of the joint. Synovium was also harvested from 5 large breed dogs with osteoarthritis (cranial cruciate ligament rupture and medial meniscal tear: 3 dogs, 4 joints, shoulder osteochondrosis dissecans, 1 dog, 2 joints; chronic shoulder instability, 1 dog, 2 joints). Dogs were euthanatized for reasons unrelated to the study. After euthanasia, the joints were aseptically prepared and accessed through a lateral parapatellar incision to the stifle joint or a lateral approach to the shoulder joint via tenotomy of the supraspinatus, infraspinatus, and teres minor tendons. The joint capsule was reflected and the synovial intima/ subintima was sharply dissected from adjacent joint capsule or adipose tissue. Dogs with osteoarthritis had moderate to severe periarticular osteophytosis, marked synovial hyperplasia, and grade 3-4 Outerbridge cartilage lesions. The tissue was placed in Dulbeccos' Modified Eagle's Media with 20% fetal bovine serum, 0.008% Hepe's buffer, 0.008% non-essential amino acids, 0.002% Penicillin 100I.U./mL Streptomycin 100ug/mL, Amphoterocin B

25ug/mL, 0.015% (0.15mg/mL)L-Ascorbate, Pyruvate (0.22mg/mL), and 0.01% L-glutamine (supplemented DMEM) in preparation for monolayer culture.

The synovium was sectioned into 2.0mm x 2.0mm pieces using a #10 Bard Parker blade under sterile conditions. The tissue fragments were combined with sterile Type 1A clostridial collagenase solution[a] at a concentration of 7.5mg/mL of RPMI 1640 solution. The mixture was agitated at 37˚C, 5% CO2, 95% humidity for six hours. Cells were recovered through centrifugation, the supernatant decanted and the cellular pellet re-suspended in 5mL of supplemented DMEM. The cell solution was transferred to a 75mL tissue culture flask containing 10mL of supplemented DMEM. The flasks were incubated at 37˚C, 5% CO2, 95% humidity, with sterile medium change performed every 3 days. Synovial cells were monitored for growth using an inverted microscope until observance of 95% cellular confluence per tissue culture flask. At 95% confluence 75 mL flasks were seeded with normal or osteoarthritic FLS at a concentration of 350,000 cells/ flask (second passage cells), and randomly divided into four treatment groups: two control groups consisting of normal (**NC**) or osteoarthritic FLS (**OC**), cultured in serum free DMEM, and two treatment groups of normal (**NGF**) or osteoarthritic synoviocytes (**OGF**) cultured in serum free media, exposed to human recombinant growth factors Basic Fibroblast Growth Factor, (bFGF)[b] 50ng/mL for 3 days, followed by Transforming Growth Factor Beta-1, (TGFβ-1)[c] 10ng/mL, and Insulin-like Growth Factor- 1, (IGF-1)[d] 500ng/mL for 13 days. All flasks contained a total of 14mL of control or treatment media. Total culture duration for all groups was 16 days with sterile media changes performed every 3 days.

On the 16th day the flasks were washed with serum free media and the cells enzymatically released from the culture flask using TryplExpress.[e] Cell viability was assessed by the Trypan blue assay.

Histologic Analysis

Tissue harvested from each flask was cut in thirds and 1/3 fixed in 10% buffered formalin, sectioned, and stained with Hematoxylin and Eosin, Masson's trichrome, Toluidine

Blue, and immunohistochemistry (IHC) for Collagen I and II, for histologic analysis. Samples were processed and submitted within 24 hours of tissue collection.

For immunohistochemistry for collagen type I, tissues were cut at 4 microns and placed on plus charged slides, microwaved and left on a 43°C slide warmer overnight. The slides were then hydrated and placed in 0.4% pepsin and heated in a 37°C incubator for 20 minutes. The slides were rinsed in tap water and placed in Tris buffer for at least 5 minutes. Subsequent staining was done on the Dakocytomation Autostainer.[f] Slides were treated with 3% H202 for 15 minutes, protein block (DAKO) for 5 minutes. Slides were incubated in goat anti-Collagen Type I,[g] at a 1:100 concentration for 30 minutes. Negative slides were treated with goat IgG, 1:1000 in place of the primary antibody. Secondary and tertiary reagents used included the LSAB+ system[h] at 20 minutes each. The chromogen used was Nova Red (Vector) for 10 minutes. Slides were counterstained in Mayer's Hematoxylin[i], for 1 minute, dehydrated and coverslipped. For Collagen Type II, immunohistochemistry, tissues were cut at 4 microns and placed on plus charged slides, microwaved and left on a 43°C slide warmer overnight. They were hydrated and placed in trypsin solution and heated in a 37°C incubator for 60 minutes. Then they were rinsed in tap water and placed in Tris buffer for at least 5 minutes. Subsequent staining was done on the Dakocytomation Autostainer. Slides were treated with 3% H202 for 15 minutes, protein block (DAKO) for 5 minutes. Slides were incubated in rabbit anti-bovine Collagen Type II, AB746P[h] antibody at a 1:100 concentration for 30 minutes. Negative slides were treated with rabbit IgG, 1:1000 in place of the primary antibody. Secondary reagent used was the rabbit envision+ system[h] at 30 minutes. The chromogen used is Nova Red (Vector) for 10 minutes. Slides were then counterstained in Mayer's Hematoxylin for 1 minute, dehydrated and coverslipped.

Histologic specimens were examined at 10x magnification on a Zeiss Microscope.[j] Images of each section, (three from the scaffold periphery and three from the scaffold center) at 2 o'clock, 6o'clock and 10o'clock positions (Figure 1) were digitally captured by an Olympus DP-70[k] digital camera and saved as tiff files.

DNA Quantification

One third of the harvested tissue was lyophilized and a dry weight obtained. Samples were incubated in 1.0ml Papain Solution (2mM Dithiothreitol and 300ug/ml Papain) at 60ºC in a water bath for 12 hours.

The Quant-iT PicoGreen™ double stranded DNA quantification assay[l] was used for dsDNA quantification. Double stranded DNA extracted from bovine thymus is mixed with TE buffer[m] to create standard DNA concentrations of 1,000, 100, 10, and 1 ng/mL. The standards and 100uL of each papain digested sample (used in the above assays) were added to a black 96 well plate. 100uL of 2ug/mL of Pico Green reagent was added to each well and the plate is incubated for 5 minutes. Sample fluorescence was read at 485nm excitation/ 528nm emission by the Synergy HT – KC-4 Spectrophotometric Plate Reader.[n] Absorbances were converted to ng/mL concentrations and total double stranded DNA yield in ng using FT4 software.[o]

Biochemical ECM Analysis:

Three scaffolds from each group were analyzed for Glycosaminoglycan (GAG) and collagen production. Wet weight of each scaffold was obtained. GAG content of the scaffold was performed using the Dimethylmethylene Blue Sulfated Glycosaminoglycan assay.[169] Collagen content of the scaffold was assessed using Erlich's hydroxyproline assay, as described by Reddy et al.[170] Median % glycosaminoglycan and hydroxyproline content was standardized to tissue cellularity, (dsDNA content) as a chondrogenic index[171] to identify chondrogenic activity of each tested cell type. and also standardized to tissue dry weight to compare the experimental tissue to normal meniscal ECM content. Hydroxyproline is a approximately 13% of collagen content; hydroxyproline was converted to collagen content using the equation: [ug hydroxyproline x dilution factor x 13=ug collagen].[172,173]

Relative Gene Expression:

One third of the harvested tissue was analyzed via real-time reverse transcriptase PCR to

identify relative expression of collagen I, Collagen II, aggrecan, SOX-9, RGS-10 and Frzb genes as a ratio to GADPH. Primer sequences were designed using Primer EXE.[p] Primer sequences were as follows: GAPDH forward: GTGACTTCAACAGTGACACC, GAPDH reverse: CCTTGGAGGCCATGTAGACC; Collagen I forward: TGCACGAGTCACACTGGAGC, Collagen I reverse: ATGCCGAATTCCTGGTCTGG; Collagen II forward: GGCCTGTCTGCTTCTTGTAA, Collagen II reverse: ATCAGGTCAGGTCAGCCATT; Aggrecan forward: ATCGAAGGGGACTTCCGCTG, Aggrecan reverse: ATCACCACACAGTCCTCTCCG; Sox-9 forward: GAGAGCGAGGAGGACAAGTT, Sox-9 reverse: GCTTGACGTGCGGCTTGTTC; RGS10 forward: GAACCGAGGAAGAGGAAGAA, RGS10 reverse CCTGCATGGTCCTGAGAGTG; Frzb-1 forward: AGCGTGCCAGATTACTGTTG, Frzb-1 reverse GTGGAATCACTGTGGCTAGA. Samples were stored in RNALater[q] at -20ºF. Samples were transferred to a 0.5 ml screw cap tube filled with 1.0 mm diameter Zirconia Beads and Trizol® reagent[r] and homogenized using a mini-bead beater at 5,000 rpm for 2 minutes. RNA was extracted from the homogenates using the TRIspin method. 5ul each of specimen RNA. was diluted with 95uL of RNAase free water and the absorbances read at 260 and 280nm with a Synergy HT Spectrophotometric Plate Reader. Absorbances were converted to µg/µl using the KC4 software. Equal amounts of sample RNA were converted to cDNA using random hexamer primers and the StrataScript™ RT[s] enzyme in a GeneAmp PCR System 9700.[v] Total RNA was extracted via bead beaters and converted to cDNA by reverse transcription polymerase chain reaction (RT-PCR) using primers corresponding to cDNA sequences for type I and II collagens, aggrecan, SOX-9, RGS-10, Frzb and the housekeeping gene GAPDH. Real-Time PCR was performed with the Light Cycler 480[w] using the Quantitect SYBR® green PCR kit[s] following the manufacturers guidelines. The PCR profile for all tests consisted of an initial incubation at 94°C for 15 minutes, followed by 55 cycles of 5 seconds at 94°C (melting), 10 seconds at 57°C (annealing), and 20 seconds at 72°C (extension). After the PCR profile a melt curve analysis was done to ensure specific amplification for each

sample. SYBR green fluorescence was monitored during the extension step of the PCR profile, and take off values and amplification efficiencies were determined using the Light-Cycler software. Relative expression levels for the target genes studied were expressed as a ratio to the level GAPDH and determined using Q-Gene. Differences in gene expression among groups was determined using the relative expression statistical tool, REST-XL.

Statistical Methods

Non parametric data was analyzed using Kruskal-Wallis One way analysis of variance on ranks followed by Mann-Whitney Rank Sum tests. Significance was set at $p < 0.05$. Calculations were completed using a statistical software program.[x]

Results

Gross Observations

Normal and osteoarthritic cells in the growth factor treated groups formed fibrous tissue-like substance, which remained in a cohesive sheet after enzymatic release. (**Figure 7-1a**). Subjectively, the NGF cells achieved this fibrous appearance earlier than the OGF cells. The corners of all growth factor treated samples began to contract slightly by the last 2-3 days of culture, however, OGF cells from a dog with acute anterior cruciate ligament transection formed spontaneous micromasses 6 days after addition of growth factors (**Figure 7-1b**). NC and OC cells were non cohesive and did not produce visible ECM, precluding histologic analysis.

Cellularity and Cell Viability

Tissue cellularity, as measured by DNA content standardized to dry weight, was not different between any group (P=0.19) (**Figure 7-2**). Cellular viability was estimated at 95-99% in all groups, however, an exact measure could not be obtained due to cell clumping and cell retention in the tissue like substance of the NGF and OGF groups.

Histologic Analysis (**Figure 7-3a**)

Histologic analysis of H+E stained sections of the sheets of tissue showed regular layers of eosinophilic fibrous tissue, with rounded cell phenotype in clusters or singly. Some single

cells appeared in lacunae like spaces. The spontaneous micromass specimens showed cellular disintegration, karryolysis and amorphous eosinophilic debris consistent with cell death in the center of the micromass (**Figure 7-3b**). Staining for collagen with Masson's Trichrome was positive in both NGF and OGF groups, but Toluidine Blue staining for GAG was negative.

Extracellular Matrix Analysis

No difference of the relative expression of the collagen I gene was found between groups (**Figure 7-4a**); NC had 6.51e+4 collagen I gene transcripts to copies of GAPDH, OC 1.91e+4, NGF 2.16e+4, OGF 4.57e+4 (P=0.873). Collagen II expression was tested only in the OC, NGF and OGF groups: while OGF (median: 0.54 copies of the collagen II gene transcripts to copies of GAPDH) had the highest relative expression, no significant difference in median relative gene expression was detected between groups (OC: 0.145, NGF: 0.166; P=0.239, P=0.37, P=0.17, P=0.35) (**Figure 7-4b**). Percentage hydroxyproline produced, standardized to DNA content (**Figure 7-4c**), was higher in growth factor treated groups (P< 0.0001) than non-treated groups (0.001%) and higher in NGF (2.791e+3%) versus OGF (801.9%; P=0.03). Percentage collagen content, as measured by the hydroxyproline assay and standardized to tissue dry weight, was highest in NGF (19.5% dry weight) versus OGF (9% dry weight) (P=0.03) and NC (0.06%) and OC(O.08%) (P=0.009, P=0014, P=0.003, P=0.006, **figure 7-4d**). IHC for Collagen I was strongly positive in all NGF and OGF samples (**figure 7-4e**); mild positive staining for collagen II was seen intracellularly and pericellularly in all NGF and OGF samples (**figure 7-4f**).

Relative aggrecan gene expression was significantly higher (P= 0.004) in NGF (median 0.29 copies of aggrecan transcripts per copies of GAPDH) than OC (median 0.014 copies of aggrecan transcripts per copies of GAPDH). The median relative aggrecan expression ratio was 0.57 in OGF, and 0.29 in NGF, however, this difference was not significant (P=1.0). Relative gene expression was not significantly different between OC and OGF (P=0.073), NC (median 0.012 copies of aggrecan transcripts per copies of GAPDH) and NGF, (P=0.223, power 0.121), NC and OGF (P=0.22) (**Figure 7-5a**). Median % glycosaminoglycan standardized to tissue

cellularity (**Figure 7-5b**) was greatest for NGF (1778.0%); both NGF and OGF(1480.6%) were greater than NC(882.3%); (P= 0.016, and 0.05, respectively). GAG content did not differ between NGF, OGF, and OC (753.2%); (P= 0.339, 0.196, and 0.064, respectively), however the post hoc power of these tests was low (0.05, 0.48, 0.036, respectively). On a per dry weight basis, median % glycosaminoglycan content in NC (1.0% of dry tissue weight), OC (0.7%), NGF (0.8%), and OGF (0.9% dry weight), were not significantly different from each other (P=1.0. P=0.52, P=0.30, P=0.87, P= 0.71, P=0.34, **Figure 7-5c**).

Genes of Embryonic Chondrogenesis

OGF (0.540) had the highest expression of SOX-9 versus NC (0.096; P=0.05), OC(0.072; P=0.001) and NGF(0.159; P=0.005); no difference was found between controls (P=0.864) and NC versus NGF (P=0.332), with a power of 0.061 (**Figure 7-6a**). Frzb appeared to be expressed in all groups (median: NC: 0.622, OC 1.5, NGF 0.33, OGF 0.18) with OC having the highest expression, however no significant difference were detected between the groups (P= 0.226) (**Figure 7-6b**). RGS 10 had the highest relative expression in OGF (0.008) versus NGF(0.0005) and RGS 10 was not expressed by controls (**Figure 7-6c**).

Discussion

In vitro synovial fibrochondrogenesis occurred successfully in monolayer culture and was enhanced by growth factor stimulation in this study. Growth factors enhanced both collagen and GAG formation in normal synoviocytes, and collagen only in osteoarthritic cells. Canine normal and osteoarthritic synoviocytes can respond to chondrogenic growth factors and express collagen I, collagen II, and the aggrecan gene, the structural hallmarks of a fibrochondrogenic phenotype shift, in monolayer culture. Expression of these two molecules is advantageous to meniscal fibrocartilage tissue engineering because they are the principal ECM constituents of the axial, avascular portion of the knee meniscus.[139] This chondrogenic shift is also consistent with the findings of DeBari et al,[174] Fiorito et al,[81] Fickert[175] et al and Kurth et al;[176] human synovial

membrane cells harvested from patients with end stage osteoarthritis were able to undergo *in vitro* hyaline chondrogenesis in pellet form[81,174,175] or in alginate gels[176]. In contrast to these studies, the majority of collagen produced by both normal and osteoarthritic synoviocytes in this study was collagen type I, which is the major structural and functional constituent of the knee meniscus.[60,139] Expression of the collagen I gene seemed to be independent of cell pathologic state and growth factor administration in this study. However, actual transcribed collagen I was strongly influenced by growth factor administration in both normal and OA synoviocytes, with only the growth factor treated groups making enough collagen I to form palpable tissue for histologic analysis. Preponderance of collagen I in our tissue may reflect the difference in cell isolation techniques utilized by DeBari et al, Fiorito et al, and Fickert et al; [81,174,175] flow cytometry and dilution limiting techniques were used to specifically isolate CD90+ intimal synovial progenitor cells, whereas in this study, all synovial membrane cell types that adhered to the flask were cultured, and likely included intimal mesenchymal progenitor cells, fibroblast like synoviocytes (type B synoviocytes), and components of the synovial subintima, including vascular fibroblasts and progenitor cells, lymphatic fibroblasts, and subintimal fibroblasts, all of which naturally produce collagen type I.[177] Kurth and workers used the same cell isolation techniques described in our study but did not measure Collagen I production.[176] A difference in collagen production profiles could theoretically be attributed to the different growth factors utilized in each study, although the triple growth factor protocol used in this study has been shown to favor type II collagen formation [7,178] as does the single growth factor protocols (TGF-β1,[81,174] TGF-β3,[175] BMP-2, TGFβ2, or IGF-1[176]) used in the other studies. Pellet and alginate synoviocyte culture may favor an articular cartilage phenotype versus growing sheets of synoviocytes in a monolayer flask. It is unknown if central regional hypoxia exists in pellet culture or alginate gel three dimensional culture, used in the above studies,[81,174,175] which would favor collagen II production, versus a well aerated monolayer of cells in a 75cc tissue culture flask. The cell- matrix interaction in pellets or gels could induce cellular shape change and alter autocrine/paracrine signaling,

resulting in synoviocytes rounding up, assuming a chondrocyte/lacunae phenotype, and producing hyaline cartilage ECM. In addition, the goal of the above studies was to engineer articular cartilage rather than fibrocartilage, so collagen I production was not a major outcomes measure.[81,174,175]

The ECM content of the tissue created in this study was lower than the GAG and collagen content of a normal meniscus, at 2-3% and 60-75%, respectively.[133,139,179] The cells in this study were grown for a short period of time, without scaffolding or biomechanical stimuli to guide ECM formation. Typical reported duration of cell culture resulting in gross cartilaginous tissue formation ranges from 28-32 days,[171,180-184] whereas the culture duration used in this study was 16 days. Biomechanical stimulation, in the form of mechanical stimulation of cultured cells in bioreactors, has positive effects on cell differentiation, cell viability, and extracellular matrix production through mechanotransductive effects.[185-188] With respect to chondrocytes cultured on Poly-Glycolic Acid scaffolds, shear stresses generated from fluid flow in rotating bioreactors have been shown to significantly increase accumulation of sulfated GAGs, hydroxyproline, and collagen.[186-188] This effect ultimately improves the compressive properties of the chondrocyte/PGA constructs.[186-188] Modification of cellular signaling through growth on scaffolding and application of biomechanical stimuli, as well as differential growth factor protocols, may be required to achieve the heterogeneous form and function of a stifle meniscus. Despite falling short of our long term goals to produce meniscal fibrocartilage in its native proportions and quantities, in this study, we were able to construct sheets of tissue like substance from dense monolayer culture of synoviocytes, with the most ECM seen yet of all the studies reported in this dissertation. Recent research corroborates with our success of scaffoldless fibrochondrogenesis. In work by Ando et al, young porcine synoviocytes grown in monolayer culture spontaneously contracted to develop a basic tissue engineered construct, which contained collagen I and III, fibronectin, and vitronectin.[189,190] Further culture of the self forming constructs in chondrogenic media exhibited positive alcian blue staining with elevated expression of

chondrogenic marker genes. When this tissue was implanted into chondral defects, its biomechanical evaluation (6 months later) revealed that repair tissue exhibited mechanical properties similar to those of normal porcine cartilage in static compression and friction tests.[189,190] A dense collection of cells may also be advantageous because of the embryologic "community effect," where multicompetent cells must be surrounded by similar cells in order to be respond to inductive signals, differentiate, and proliferate ECM.[191,192] Indeed, this type of dense cell fibrochondrogenesis mimics human embryonic meniscogenesis, where the fibrocartilages begin as highly cellular organs with minimal ECM, and become less cellular with increasing ECM during development.[193] The findings of our study and the findings of Ando et al may argue for further investigation of scaffoldless fibrocartilage synovial-based tissue engineering.

While canine osteoarthritic synoviocytes can undergo desirable fibrocartilaginous phenotypic shifts for meniscal tissue engineering purposes, their production of fibrocartilage ECM, specifically collagen, was less than that of normal synoviocytes, given identical culture conditions. Fiorito and workers came to a similar conclusion in a study comparing *in vitro* chondrogenesis of pelleted human FLS, harvested from end stage arthritic hips and normal knees, to determine synovial contribution to OA pathogenesis.[81] It was found that the OA FLS had markedly reduced chondrogenic activity compared to normal FLS, as measured by alcian blue pellet staining and Collagen II immunohistochemistry.[81] Relative *in vitro* growth factor effectiveness, including growth factor synthesis, modification, secretion and cellular receptivity could be major contributors to the differential chondrogenesis seen in normal versus OA synovial membrane cell cultures. In humans and mice, normal synoviocytes elute native chondrogenic bFGF and TGFβ-1[194] in monolayer culture but do not externally express TGFβ-1 receptors *in situ*.[195] In contrast, OA FLS also express TGFβ-1 receptors.[191] Since TGFβ-1 is a chondrogenic growth factor naturally found in osteoarthritic joints, is utilized in culture,[196] and has anti-inflammatory properties,[65] it would seem, on a molecular level, that OA FLS should have an

advantage in cartilage ECM synthesis over normal synoviocytes. However, this has not been the case in this study as well as others.[81] Thus, there must be pathologic catabolic processes decreasing or negating the benefit of native synovial anabolic processes. For example, decreased growth factor responsiveness has been documented in OA chondrocytes. Simian chondrocytes show a significant decline in cellular synthetic response to IGF-1 with increasing OA severity, as measured by chondrocyte incorporation of sulfate and proline into GAG and collagen.[197] Cultured human OA chondrocytes from fibrillated cartilage express higher intracellular IGF-1 and plasma membrane-bound IGF Receptor-1.[198] However, they also simultaneously have higher intracellular IL-1α, IL-1β, and plasma membrane-bound IL-1Receptor-I, with the end result of absent coordinated production and accumulation of aggrecan and type II collagen.[198] Perhaps these synthetic- inflammation imbalances are also occurring in OA synoviocytes when they are exposed to growth factors *in vitro*. The signaling cascade resulting from the binding of a growth factor to its receptor and subsequent transcription, translation, and protein activation is innately complex. Each step is interdependent, providing a plethora of pathologic opportunities for decreased growth factor effectiveness. For example, upregulation of pre-receptor TGF binding proteins, or alterations in smad 2,3,4,7 intracellular signaling can alter and interfere with TGFβ transduction, regardless of cell type.[199]

The pathologic hallmarks of osteoarthritic synovium include intimal lining hyperplasia, recruitment and activation of fibroblasts, macrophages, and lymphocytes, (particularly CD4+T cells), perivascular lymphoid aggregates, blood vessel proliferation, and rare neutrophils.[60,65] In synovium, even though twice as many STRO+ mesenchymal precursor cells have been observed on OA synovial villi versus healthy organ donors, [59] the number of STRO+ mesenchymal precursor cells also correlated with increasing lymphocyte inflammatory infiltration.[59] These histologic changes could belie a molecular mechanism for decreased *in vitro* OA synovial chondrogenesis. Fiorito et al found that pelleted human OA FLS express the β1, CD54, CD106, and CD44 receptors, known to regulate recruitment and retention of inflammatory cells.[81] OA

FLS also have increased surface expression of IL-R, [200] the receptor for IL-1β , and TNFR55,[201] the receptor for TNF-α, both of which are the principle pro-inflammatory, destructive cytokines in osteoarthritis.[202] This altered receptor profile could prime these cells for suboptimal chondrogenesis and increased destructive capacity *in vitro*. Thus OA synoviocytes also have receptor priming for inflammatory cellular signaling. OA synoviocytes secrete higher levels of MMP-1, MMP-9, chemokines CXCL12, IL-6, IL-11 and lower TGF-beta and stem cell factor compared to normal FLS.[81] Other work has shown that osteoarthritic synovial fibroblasts proliferate IL-1, TNF-alpha, IL-6, IL-8, IL-18, LIF, PGE-2, cathepsin B, MMP-1, MMP-2 and MMP-3. [65,69,71,72,203-205] Monolayer culture OA synoviocytes do not spontaneously secrete IL-1β and PGE2 until stimulated by the presence of collagen II, which formed in small quantities in our study;[66] this could be counterproductive to *in vitro* cartilaginous ECM formation. All of these synovial origin cytokines, chemokines, and catabolic enzymes are known to directly or indirectly decrease chondrocyte ECM synthetic capabilities and enhance cartilage ECM destruction in arthritis. [65,69,71,72,203-205] Certainly OA chondrocytes have diminished *in vitro* ECM production capabilities as compared to normal chondrocytes.[82] One could hypothesize that *in vitro* autocrine and paracrine action of inflammatory mediators and their receptors in cultured synovial cells[206] could decrease *in vitro* synovial chondrogenic activity or responsiveness to anabolic growth factors. This has been confirmed in the work of Rezzonic et al, who discovered that direct contact with T cell membrane-associated interferon gamma, tumor necrosis factor alpha, and interleukin-1alpha markedly inhibited the synthesis of types I and III collagen by 60-70% in untreated synoviocytes and by 85% in TGFβ stimulated fibroblasts.[207,208] Future studies are required to further characterize *in vitro* OA synovial metalloproteinase expression and its effects on fibrochondrogenesis.

In addition to the type B synoviocytes, the type A round cells of bone marrow origin are present in osteoarthritic synovium and contribute to the inflammatory environment, which may negatively impact *in vitro* synovial chondrogenesis. It is commonly assumed that the type A

synoviocytes, or macrophage like synoviocytes, are eliminated in first pass monolayer culture during the first several media changes, as reported by Krey *et. al*. in 1976.[209] Recent research has shown that the CD68 receptor, one of many cellular macrophage marker, was not detected after 7 days of monolayer culture of synovial membrane cells.[210]However, work by Pei and colleagues[171] showed that synoviocytes derived from conventional monolayer passage techniques, as used in our study, are contaminated with macrophages. Macrophage contaminated synovial cell cultures have decreased *in vitro* chondrogenic potential, with sparse distribution of collagen II and 50% less glycosaminoglycans than uncontaminated cultures, as determined during a 15 day growth period.[171] These macrophages are also increased in numbers and inflammatory activity in osteoarthritis. In osteoarthritis synovial macrophages exhibit an activate phenotype and produce degradative enzymes resulting in the destruction of cartilage.[203,205,211] Osteoarthritic type A synoviocytes secrete IL-1beta, TNF-alpha, IL-8, IL-18, and induce type B synoviocytes to express inflammatory cytokines, chemokines, and destructive enzymes.[65,69,71,72,79,203-205,211] Synovial T cells have also found to be major players in inflammatory upregulation and guided ECM destruction via a specific immune response.[212-215] T cells secrete IL-2, IFNγ, and IL-10 transcripts in the synovial membrane of 50% of patients with OA.[213,215] IL-2 and IFNγ stimulate monocytes to produce IL-1β and TNFα. IFNγ originates form T-helper1 cells and inhibits type II collagen synthesis.[213,215] T cells affect fibroblast and synoviocyte functions directly by contact activation and indirectly, by activation of cytokine production in monocyte/macrophages, which in turn, triggers stromal cell functions. *In vitro* culture of human FLS with membranes of stimulated T cells induces synoviocyte production of PGE2 and MMP-1, and down regulates FLS expression of collagen I and III.[207,208] Since the production of MMPs in Type A synoviocytes is also induced upon contact with stimulated T cells, contact of synovial cells with chronically stimulated T lymphocytes favors matrix catabolism.[207,208] Again, various cell types in addition to the synovial origin cells could contribute to inflammatory signaling that could decrease fibrocartilaginous ECM formation.

Osteoarthritic synovium may have altered growth or ECM proliferation kinetics compared to normal synovium, which could also decrease its *in vitro* chondrogenic potential. Subjectively, in this study, the normal synoviocytes achieved a gross tissue like appearance sooner than the osteoarthritic synoviocytes. At this time a true comparative *in vitro* growth kinetics study of canine normal and osteoarthritic synoviocytes has not been done. If there were marrow derived contaminant cells in our cultures secreting inflammatory cytokines, tumor necrosis factor (TNF) causes cultured mesenchymal cells to undergo cytostasis, with cell cycle arrest in G2.[216] Human osteoarthritic FLS, during the 1st through 4th passage of monolayer culture, consistently undergo chromosomal nondisjunction and endoreduplication, resulting in consistent chromosome aberrations such as trisomy of chromosome 7.[217-219] In 75% of human OA patients, early-passage FLS show numerical chromosomal aberrations involving chromosomes 4, 5, 6, 7, 8, 9, 12, 18, and X .[217] Until the fourth passage, 92% of synovial fibroblasts show increasing rates of chromosome gains or losses.[217] In humans, chromosome 7 carries a number of potentially relevant genes encoding cytokines/growth factors and their receptors, transcription factors, signal-transduction molecules, and molecules involved in matrix formation and cell–extracellular matrix such as collagen I,[220] implying functional consequences for +7 FLS.[217,219] Chromosomal aberrations connote cell division abnormalities and may result in defective growth kinetics and ECM proliferation kinetics. At this time chromosomal aberrations have not been reported in canine synovium grown in monolayer culture, or their effects on cell growth rates and *in vitro* chondrogenesis. Hypothetically, aberrations of canine chromosomes, such as Chromosome 3 containing the cell cycle regulating jak gene, or chromosome 6 containing the collagen α2(1) chain precursor gene could effect cell division rates or cartilaginous ECM formation. [221,222]

The data in this study from the cultured osteoarthritic synoviocytes showed marked variability, making it difficult to tease out statistical significance in comparative collagen II and aggrecan gene expression. Indeed, even though we attempted to equalize the gross pathologic

appearance of the donor OA joint, the sources of arthritic synovium in this study were variable, including naturally occurring cranial cruciate degenerative disease with meniscal tears, Pond Nuki model with meniscal tears, and naturally occurring chronic shoulder instability. These multiple primary diseases could have accounted for data variability. It is interesting to note that other studies also show cartilaginous ECM formation variability in OA synovial membrane cell cultures, despite normalizing joint location, primary disease, and patient age.[176] In our study, the chronicity of the primary OA inciting diseases was variable, with durations of 8 weeks to many years. OA is known to be temporally heterogeneous disease, with different cytokine, growth factor and enzymatic profiles in acute and chronic OA.[60,65,223-225] In the early proliferative phase of OA, synovium displays growth factor-mediated increases in the cellular activity of synovial intimal cells, fibroblasts (resulting in increased collagen fibril production), and endothelial cells (resulting in blood vessel growth).[226] Early OA is also characterized by increased synovial mononuclear cell infiltration with over- expression of inflammatory mediators, compared with late OA.[224,225] Synovial tissue from patients with early OA demonstrates significantly greater CD4+ and CD68+ mononuclear inflammatory cell infiltration, VEGF, NF-kappaB1 and COX-2 expression.[224] Synoviocytes producing TNFα and IL-1β are also significantly more numerous in early OA.[224] It is interesting to note that TNFα, INFγ, and IL-1 increase GAG synthesis in human OA synoviocytes, particularly when these inflammatory cytokines are given in combination.[227] Because of the close cytokine communication that occurs between synoviocytes and chondrocytes, chondrocytic activity could also affect the synthetic or inflammatory state of the synoviocytes. For instance, canine and rabbit chondrocytes in early OA show a marked increase in synthetic activity, including increased synthesis of type II collagen, core protein, link protein fibronectin, decorin, fibromodulin, and biglycan.[228-232] In the later stages of OA, synovial pathology entails intimal hyperplasia, gross fibrosis, mature vasculature and the severest histologic changes.[225,233] Chondrocytes shift to a predominantly catabolic state, with decreased growth factor responsiveness (such as IGF-1) and excessive inflammation and MMP activity.[234-

[237] With this temporal change in synthetic activity, synovial membrane cells' receptor and cytokine profiles also change over time, suggesting differential growth kinetics or ligand responsiveness depending on the stage of disease.[210] Potentially, synovial cells harvested during the early, more anabolic stage of osteoarthritis may have a synthetic advantage over cells harvested from more chronically affected joints, although this remains to be investigated. Temporal variation in synovial chondrogenesis may have accounted for the variability in data from the osteoarthritic synovium groups seen in this study.

Although normal synoviocytes in this study did show increased chondrogenesis versus osteoarthritic cells, osteoarthritic synoviocytes did reveal some spontaneous signaling for cartilage formation; the control OA cells expressed aggrecan and collagen II genes. This may be due to increased mesenchymal progenitor cells [59]and expression of TGFβ-1 and its receptor [191] in OA synovial membrane cells.

The spontaneous contraction of the corners of synovial cell sheets occurred in all growth factor treated groups, and in some samples the release and contracture of the cell sheets was complete and resulted in micromass formation. All of the cells that exhibited micromass formation originated from a Pond-Nuki model hound dog. Micromass formation proved to be problematic for the viability of the center of the tissue, as histologic examination showed evidence of central necrosis. The micromass phenomena may have been mediated by α-smooth muscle actin. Previous studies have shown that FLS express α-smooth muscle actin, particularly of the subintima.[13,59] Spontaneous contraction mediated by this protein has been described by Vickers at al, where synovial cell-mediated contraction of collagen-GAG scaffolds resulted in 43% shortening of the original diameter after 4 weeks.[13] α-smooth muscle actin is also expressed by synovial vascular endothelial progenitor cells,[238] which are associated with synovial mesenchymal progenitor cells in synovial villi.[59] Apparently, IL-4 and TGFβ-1 can induce synovial fibroblasts to a myofibroblast- like phenotype expressing α-smooth muscle actin in monolayer culture.[206,239] Under the influence of IL-4 and TGFβ-1, actin-expressing FLS can

increase from an *in vitro* incidence of 14% to 68%.[206,239] Contraction is inhibited by bFGF,[206,239] at 10ng/mL , which is a lower concentration than what was used in this study. The spontaneous micromass phenomena noted in this study was likely influenced by the growth factor mixture used in the study, and possibly by endogenous secretion of IL-4. IL-4 is secreted principally by T cells, and also occasionally by macrophages,[212] indirectly indicating that bone marrow origin mononuclear cells may have been contaminating the FLS cultures in this study. Contraction could be useful for achieving desired tissue shapes and the formation of scaffoldless tissue constructs.[189,190] In this study the thickness of the resultant thick tissue blobs seen here may have resulted in cell death due to poor nutrient and gas exchange of cells imbedded in dense ECM. In addition, loss of cell—substrate contact during contraction would have changed cytoskeletal mediated signal transduction and may have induced cell apoptosis and halted ECM production in the synoviocytes.[240,241]

There are possible means of enabling or improving *in vitro* chondrogenesis of OA synovial membrane cells. In our study cells were cultured to 2nd passage for a short duration of time. Longer culture durations may allow down regulation of leukocyte adhesion molecules and other inflammatory receptors.[210] Passaging synovial membrane cells beyond the 4th passage could also allow stabilization of potential chromosome abberations[217] that may cause cell division and ECM proliferation difficulties. Cellular adherence on plastic and monolayer cultivation enriches a subpopulation of synovial membrane cells that express cell surface markers of MPC and can undergo chondrogenic differentiation.[175] As shown in the work of Pei et al, negative selection flow cytometry techniques can remove macrophages and other inflammatory cells, from OA synovial cell populations, which would to increase chondrogenic activity.[171] It is unknown if administering NSAIDS to the patient prior to synovial harvest, or providing anti-inflammatory drugs *in vitro* would ameliorate inflammation[227] enough to help OA synovial chondrogenesis. Osteoarthritic cells may simply require more time to elaborate chondrogenic ECM, or may require hypoxia or different growth factor protocols to further stimulate chondrogenesis. Or,

using embryonic signaling pathways of chondrogenesis as a guide, genes involved in the formation of articular cartilage or fibrocartilage could also be identified and specifically upregulated to help engineer fibrocartilage.

SOX-9, a gene involved in embryonic chondrogenesis, was upregulated at the highest levels in the OG group. For the purpose of meniscal tissue engineering, SOX-9 induces Col2a1 and aggrecan formation, as seen in this study, and is of interest as a potential target pathway to meniscal ECM production. Collagen II is a crucial component of the axial portion of the meniscus and is associated with meniscal radial tie fibers;[139] aggrecan is also present in the axial meniscus.[140,242] Embryonic chondrogenesis is a multi-step cellular event: First, undifferentiated mesenchymal cells are committed to a chondrogenic cell lineage, aggregate with each other, and differentiate into chondrocytes. This is followed by unidirectional proliferation, production of matrix proteins, maturation, hypertrophic conversion, and finally replacement by bone. Adequate level of Sry-type Homeobox Protein-9 (SOX-9) is required for mesenchymal condensation, cartilage ECM formation, and physiological inhibition of hypertrophic conversion of proliferating chondrocytes. [84-87,89-92,94-96,99,103-107,109,110,113-116,118,120-123,243-250] When TGFβ, (used in this study), binds with the TGFβ serine/threonine kinase receptor, Smad2/3 proteins are phosphorylated by the activated receptor, and then transported into the nucleus. There Smad2/3 associates with SOX-9; the SOX-9 and Smad2/3 complex then bind with coactivators PGC-1a, TRAP230(which is thyroid mediated), and p300/CBP. This entire group of proteins then binds to the SOX-9 binding site of the enhancer region of the collagen 2a1 gene, resulting in gene transcription.[113] In this way, SOX-9 is essential for formation of hyaline cartilage as the master transcriptional regulator of collagen II and aggrecan gene expression. [90,92,122,249] This cascade also results in Wnt/b-catenin signaling, which inhibits expression of targets such as cyclinD1, a cell cycle regulator, thereby negatively regulating cell proliferation at the hypertrophic cartilage stage and preventing cartilage differentiation into bone.[113] The convoluted steps between binding of TGFβ and transcription of Col21a leave many potential points for molecular regulation and interference,

and more efficient targeting of SOX-9 may help hasten production of collagen II and aggrecan. In this study, expression of SOX-9 was highest in osteoarthritic synoviocytes stimulated with growth factors. This may have been mediated by the TGFβ in our growth factor mix as well as endogenous receptor priming of OA synoviocytes.

Regulator of G- Protein Signaling-10 (RGS-10) is a newly recognized protein involved in embryonic chondrogenesis. G- (GTP binding) proteins are common cellular relay molecules attached to the cytoplasmic face of the plasma membrane, serving to couple trans-membrane receptors to enzymes or ion channels involved in signal transduction cascades.[251] G-protein signaling, one of the most common forms of cellular signaling, begins with activation of a G-protein through stimulation of a transmembrane G-protein coupled receptor (GPCR) by an extracellular ligand.[251-254] The stimulated receptor recruits heterotrimeric G proteins to activate Gα subunits by stimulation of GTP (guanine triphosphate) binding. Effective signaling termination requires rapid GTP hydrolysis and is accelerated by the GTPase activating Regulator of G-protein signaling or RGS proteins.[251-254] RGS proteins catalyze GTP hydrolysis and rapidly terminate signals upon removal of the ligand. [251-254] Cells expressing RGS-10 show increased GAG synthesis and accelerated and increased production of Col2a1 transcripts. [88] RGS-10 induces SOX-9 and its transcription of Collagen II, and proliferation of early chondrocytes.[88] This gene was expressed only in the growth factor treated groups, and most highly expressed in the OA synoviocytes. Thus, RGS-10 must be induced by the growth factors used in this study, and the OA synoviocytes may have intracellular machinery in place and primed to enhance RGS-10 expression.

Frizzled related motif associated with bone development, or Frzb, is a major player in embryonic skeletogenesis. Frzb-1 inhibits binding of Wnt to the trans-membrane receptor Frizzled, preventing activation of the intracellular Disheveled protein, which in turn allows degradation of cytoplasmic β-catenin, maintaining the Groucho/Lef-1/TCP co-repression of gene transcription in the cell nucleus.[100,115,119,152-157,159,160,162-168,255] In the embryo, Frzb-1 keeps

epiphyseal cartilage in a functional, immature, stable phenotype as articular cartilage, in cooperation with members of the fibroblast growth factor family.[158] Formation of articular like cartilage containing Collagen type II and Aggrecan is needed for the axial portion of the meniscus. Formation of type I collagen is easily accomplished *in vitro*, and encouragement of collagen II formation is needed; thus we focused on genes involved in the formation of cartilage types containing collagen II. Expression of Frzb-1 was of particular interest because it is unknown if synoviocytes that undergo *in vitro* chondrogenesis maintain their new phenotype *in vitro* and in the joint, or if persistent addition of expensive growth factors is required. Indeed, synovial origin cartilage is unstable and will not maintain its phenotype in subcutaneous locations,[1] however, there is no chemical or biomechanical impetus for persistence of the fibrocartilaginous phenotype in non physiologic locations. Injection of chondrogenic growth factors into a patient's joint to maintain implanted meniscal tissue would be unacceptable, because of the invasiveness of intra-articular injections, and the causative relationship between excessive TGFβ and synovial fibrosis,[65] pannus formation in Rheumatoid arthritis,[256] and oncogenesis.[257,258] Activity of Frzb-1 or specifically upregulating its expression could be useful in maintaining a chondrocytic phenotype in synoviocytes. Interestingly, all groups in our study expressed Frzb-1; apparently placing synovial membrane cell into the environment of monolayer culture induces expression of Frzb-1.[255] If this gene's function is recapitulated in monolayer cell culture it may insure maintenance of the chondrogenic phenotype when growth factors are withdrawn. TGFβ, which was used in this study is now known to synergize Wnt driven signal pathways in marrow derived stem cells.[259]

There were several weaknesses in this study; the outcomes measures for Collagen I and collagen II were not quantifiable. We could have reduced sample variability by increasing the number of sources of normal and arthritic synovium or normalized the origin and primary disease of the donor joints. Additionally, proliferation of *in vitro* inflammatory cytokines and MMP's

and receptor profiles were not measured to determine a mechanism for reduced OA synovial chondrogenesis.

Thus, we reject the null hypotheses: synovial membrane cells cultured in monolayer, treated with chondrogenic growth factors, show a lessened ability to produce collagenous fibrocartilage extracellular matrix, versus normal synoviocytes, but express the highest levels of SOX-9 and RGS-10 genes. In conclusion, use of autogenous synovial tissue from osteoarthritic joints with growth factor stimulation may provide a viable option for meniscal tissue engineering strategies, because OA synoviocytes can undergo *in vitro* fibrochondrogenesis. However, osteoarthritic synoviocytes may require longer culture times or other special treatments, compared to normal synovium, to produce the same amount of cartilaginous ECM. These data provide justification for continued pursuit of synoviocyte-based tissue engineering strategies for fibrocartilage repair and regeneration.

Footnotes

a. Type 1A Clostridial Collagenase, Sigma, St. Louis, MO
b. human recombinant bFGF, BD Biosciences Bedford, MA
c. human recombinant TGF-β, BD Biosciences Bedford, MA
d. human recombinant IGF-1, BD Biosciences Bedford, MA
e. Innovative Cell Technologies, San Diego CA
f. Autostainer, Dakocytomation, Carpenteria, CA
g. Chemicon, Temecular, CA
h. Dakocytomation, Carpenteria, CA
i. Newcomer's Supply, Appleton, WI
j. Carl Zeiss, Thornwood, NY
k. Olympus DP-70 Digital Camera, Olympus, Melville, NY
l. Quant-iT PicoGreen kit, Invitrogen, Fountain Drive, United Kingdom
m. TE buffer, Invitrogen, Fountain Drive, United Kingdom
n. KC-4, BioTec, Winooski Vermont
o. FT-4 software, BioTec, Winooski Vermont
p. The Whitehead institute, MIT Center for Genome Research, MA
q. Ambion, Austin, TX
r. Invitrogen, Carlsbad, CA
s. Stratagene, La Jolla, CA
t. Applied Biosystems, Foster City, CA.
u. Qiagen, Valencia, CA
v. Applied Biosystems, Foster City, CA
w. Roche, PaloAlto, CA
x. SigmaStat, Jandel Scientific, San Rafael, CA

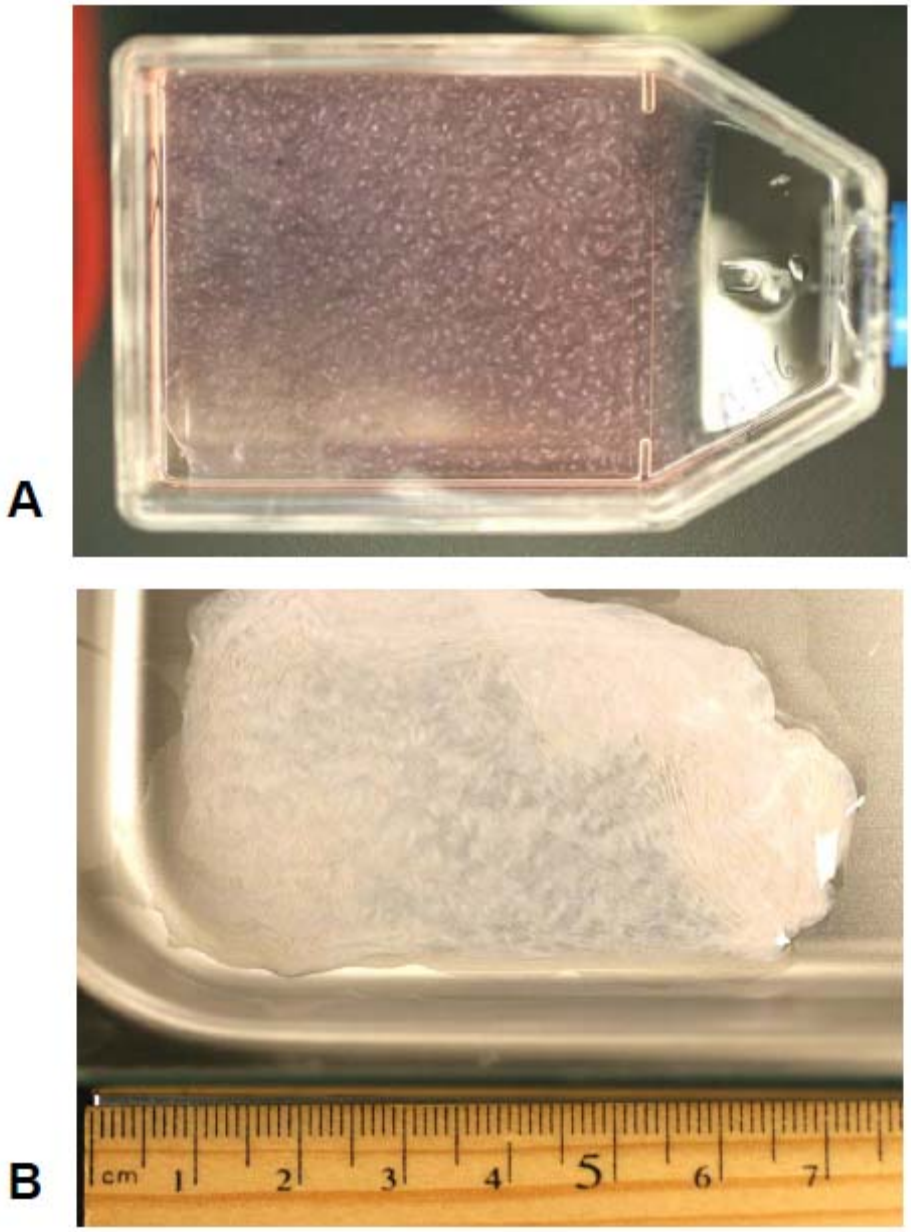

Figure 7-1a. Typical appearance of monolayer tissue in a 75 cc cell culture flask (**A**) and then post enzymatic release (**B**), after 16 days of sequential growth factor exposure. The cell source of this tissue was osteoarthritic synoviocytes harvested from a dog with acute cranial cruciate ligament transection.

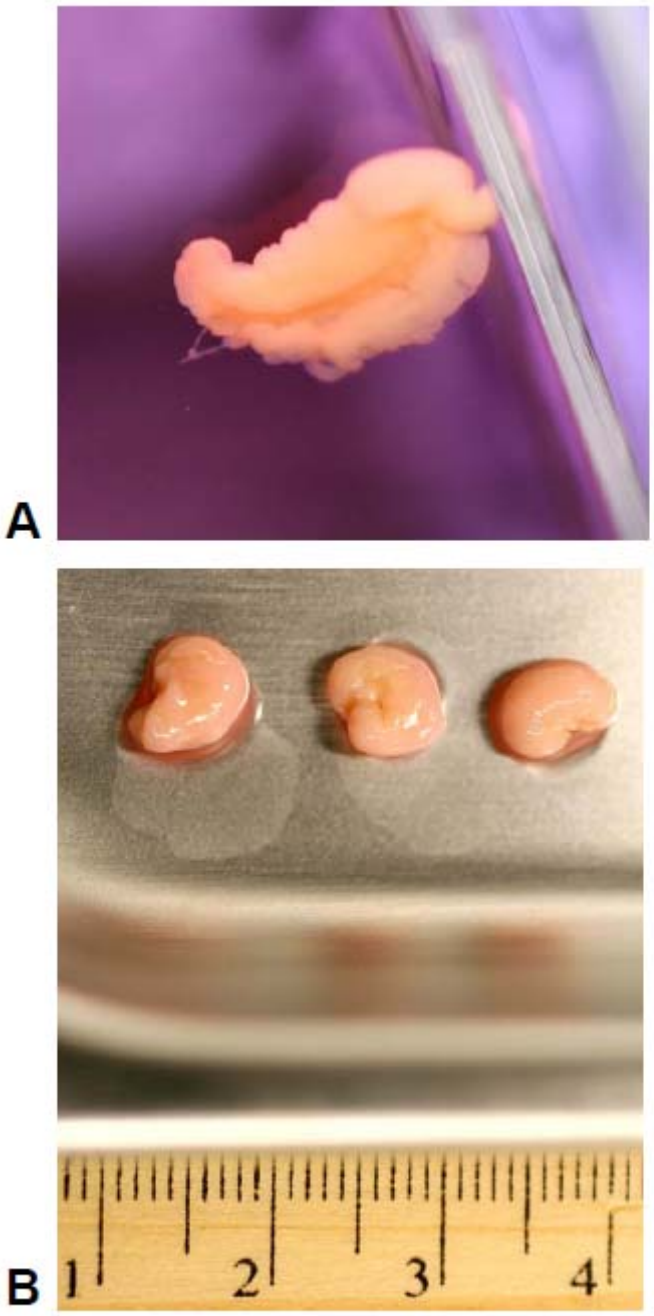

Figure 7-1b. Spontaneous micromass formation in monolayer culture of osteoarthritic synoviocytes from a dog with acute cranial cruciate ligament transection: micromasses are pictured in a 75cc flask (**A**) and at harvest (**B**), after 16 days of sequential growth factor exposure.

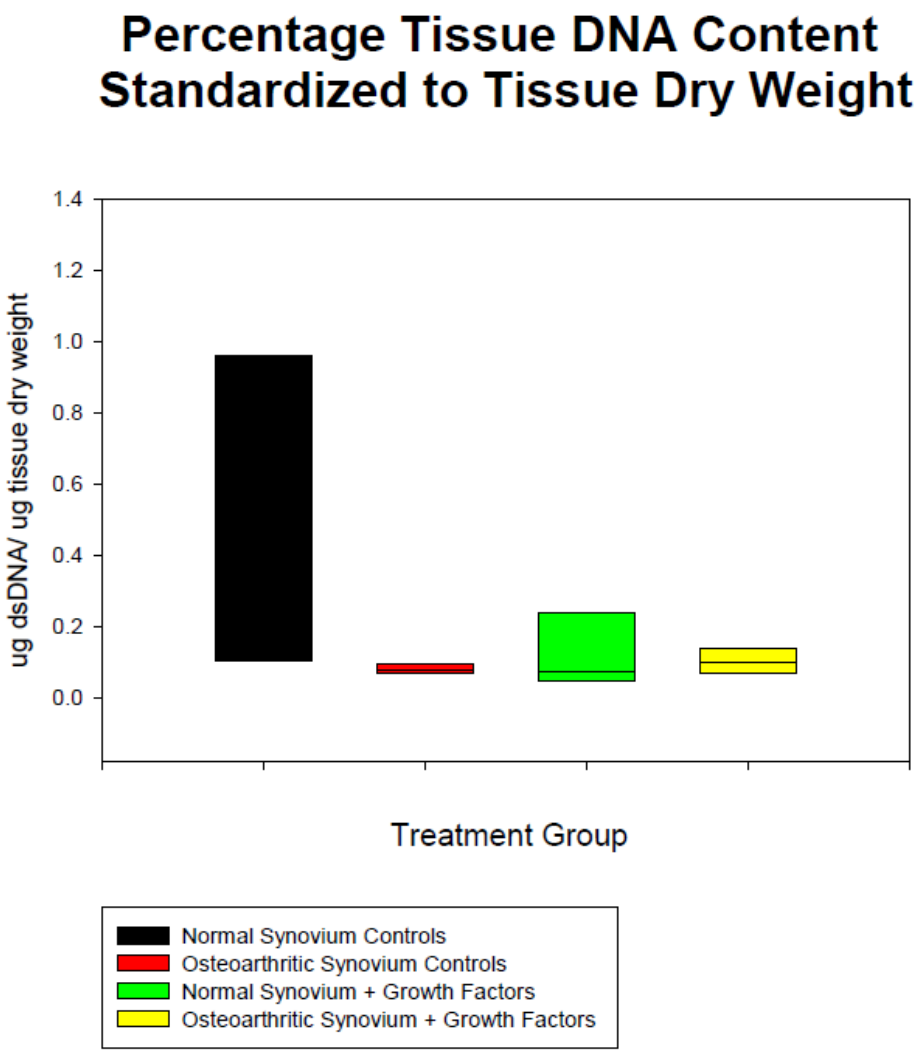

Figure 7-2. Tissue cellularity of NC, OC, NGF, and OGF groups on the 16th day of culture, as determined by the double stranded DNA assay: no significant differences between groups were detected.

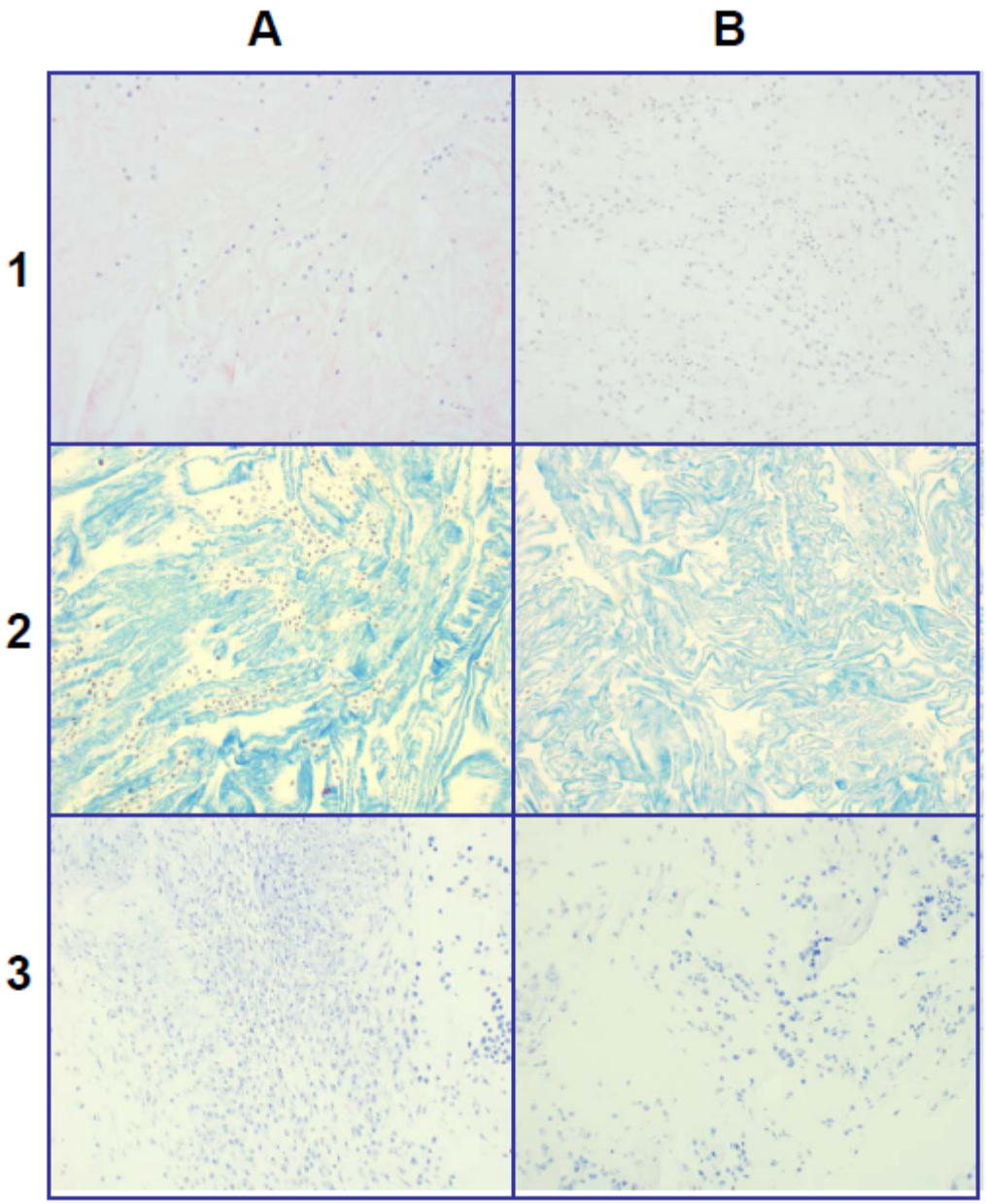

Figure 7-3a. Photomicrographs of NGF (column **A**) and OGF (column **B**) tissues harvested on day 16, 10x objective magnification: Hematoxalin and Eosin stain (row **1**), Masson's Trichrome (row **2**), and Toluidine Blue (row **3**) staining. Note the lack of glycosaminoglycan staining and the presence of positive collagen staining.

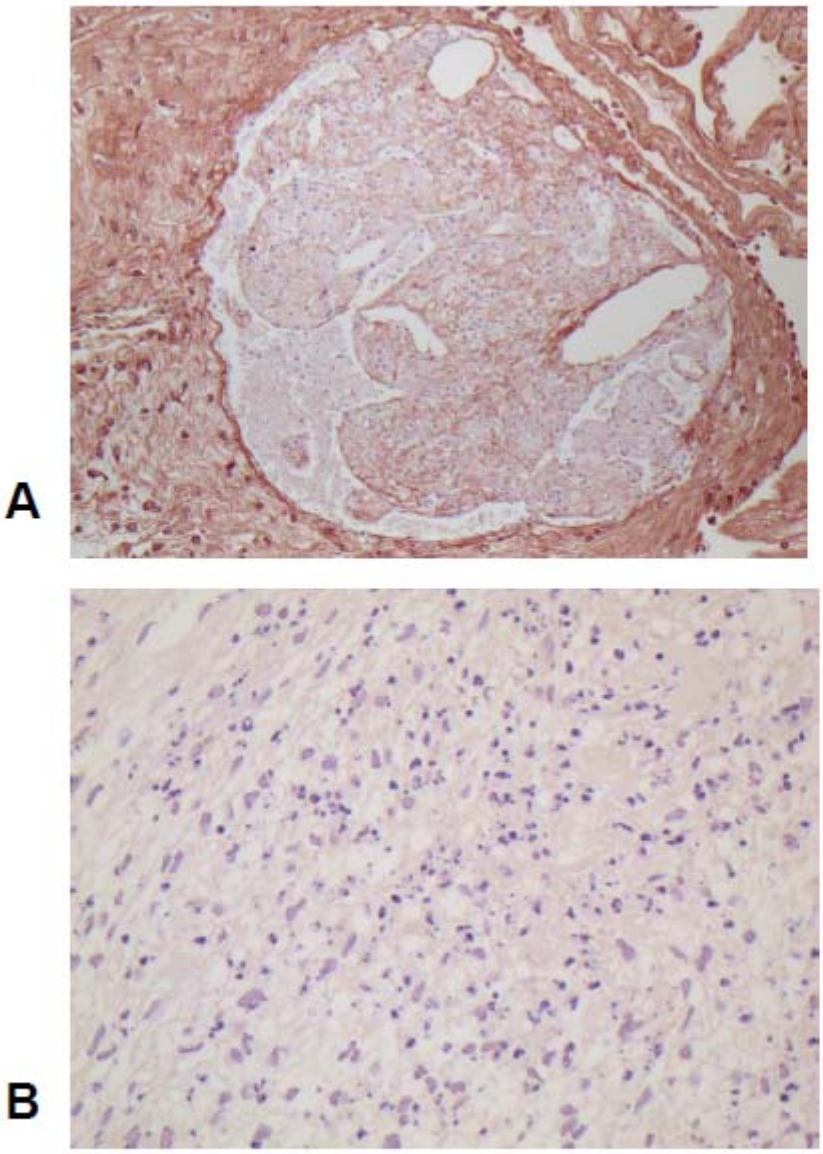

Figure 7-3b. Photomicrograph of tissue in the OGF group that formed spontaneous micromasses, at 10X objective magnification (**A**) and 20x objective magnification (**B**), showing central tissue necrosis (stain: Nova Red, immunohistochemistry of collagen I).

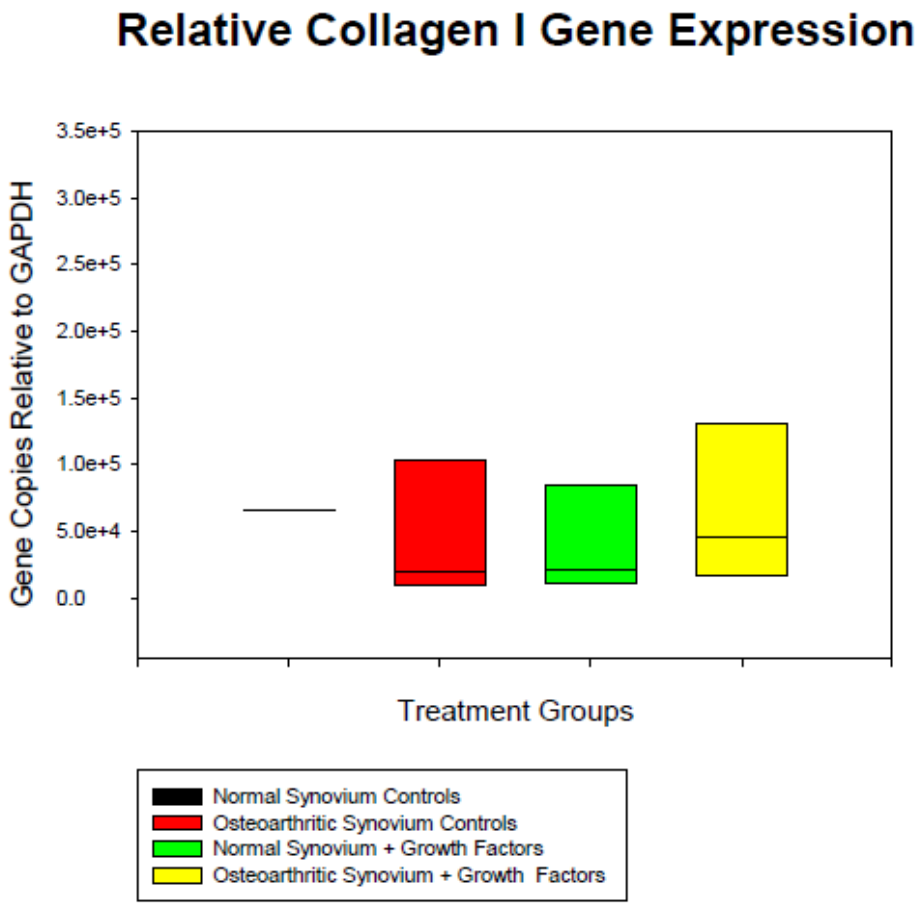

Figure 7-4a. Relative collagen I gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: no significant differences between groups were detected.

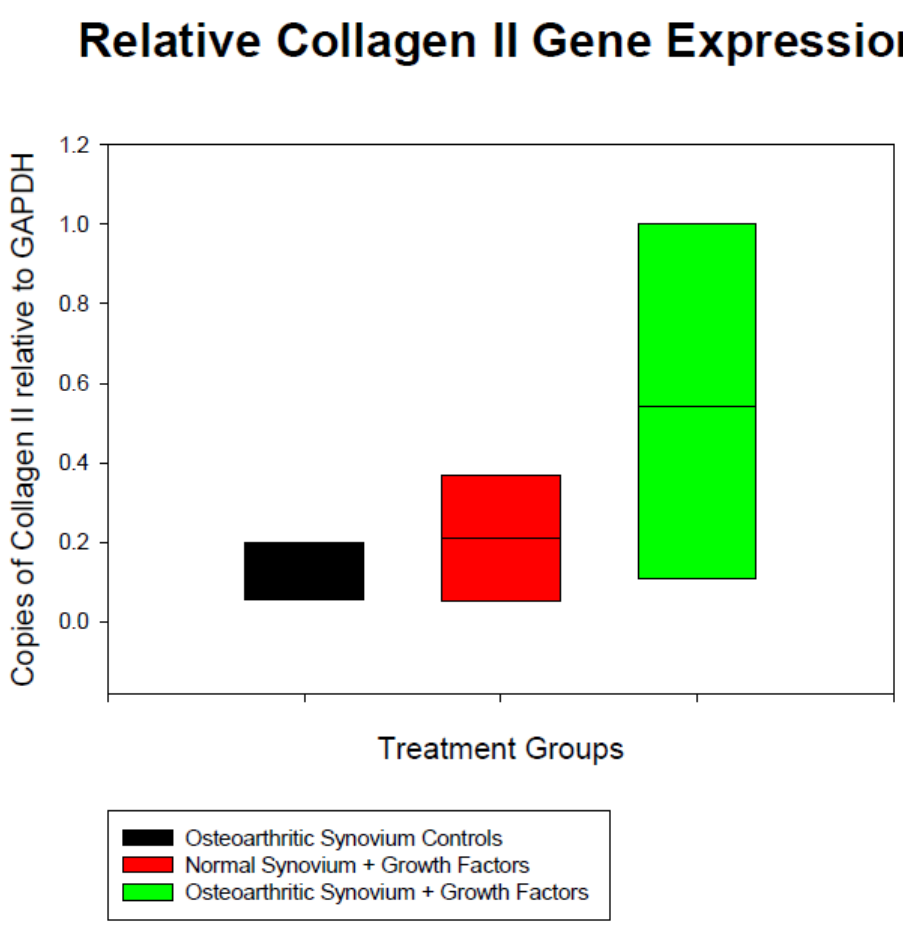

Figure 7-4b. Relative collagen II gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: no significant differences between groups were detected. The NC group was not tested.

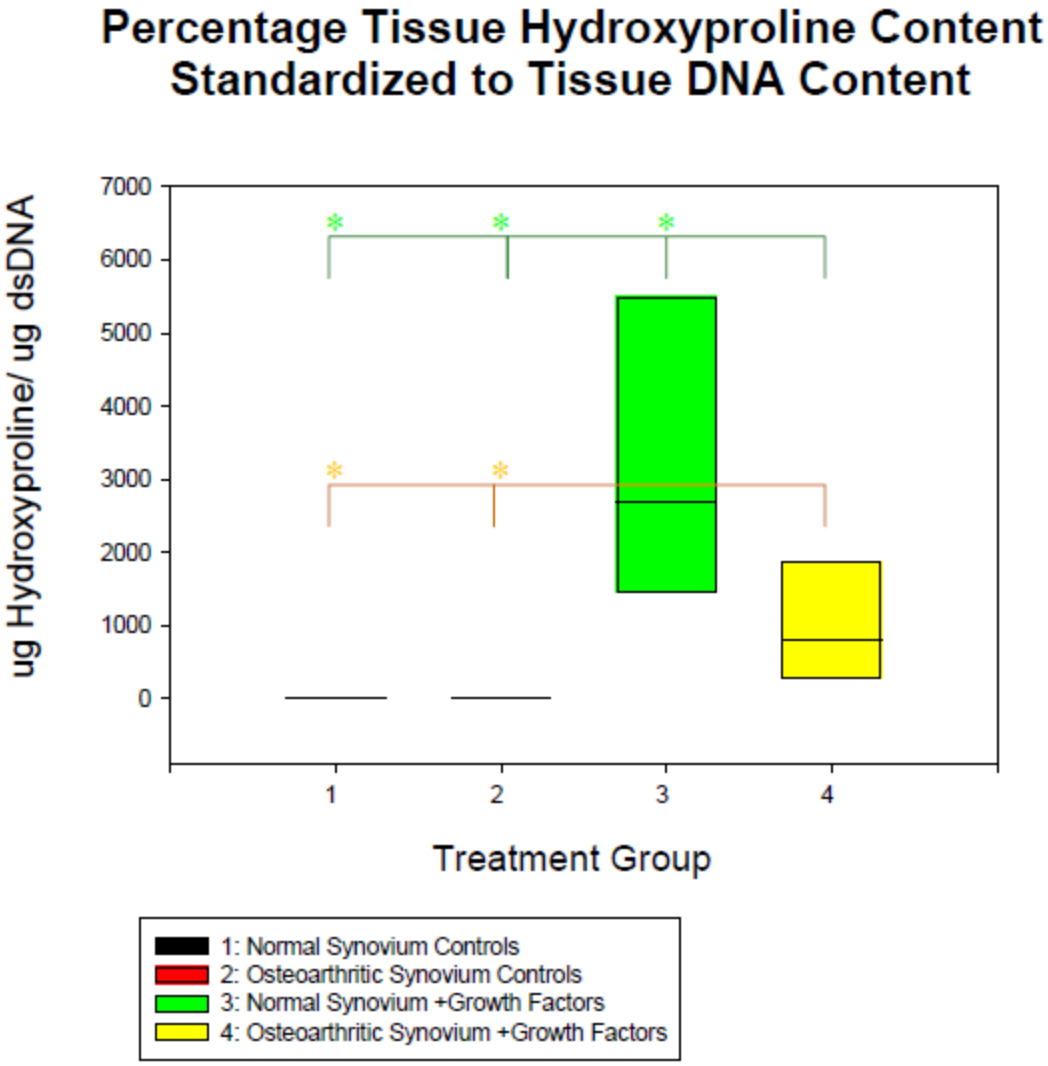

Figure 7-4c. Cellular hydroxyproline production, as determined by tissue content of hydroxyproline per tissue content of double stranded DNA, for the NC, OC, NGF, and OGF groups at day 16 of culture. An (*) denotes significant differences between groups.

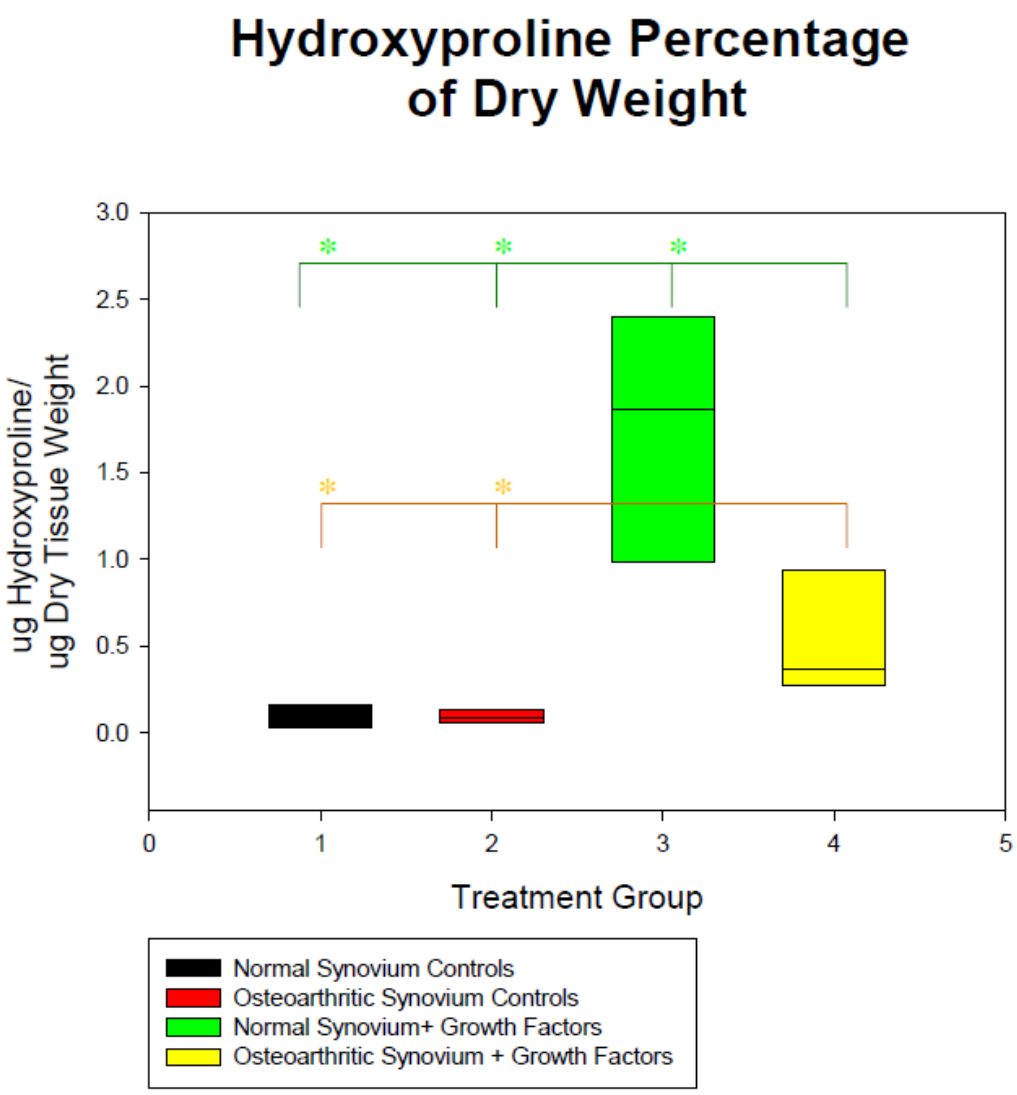

Figure 7-4d. Percentage hydroxyproline content of tissue dry weight for the NC, OC, NGF, and OGF groups at day 16 of culture. An (*) denotes significant differences between groups.

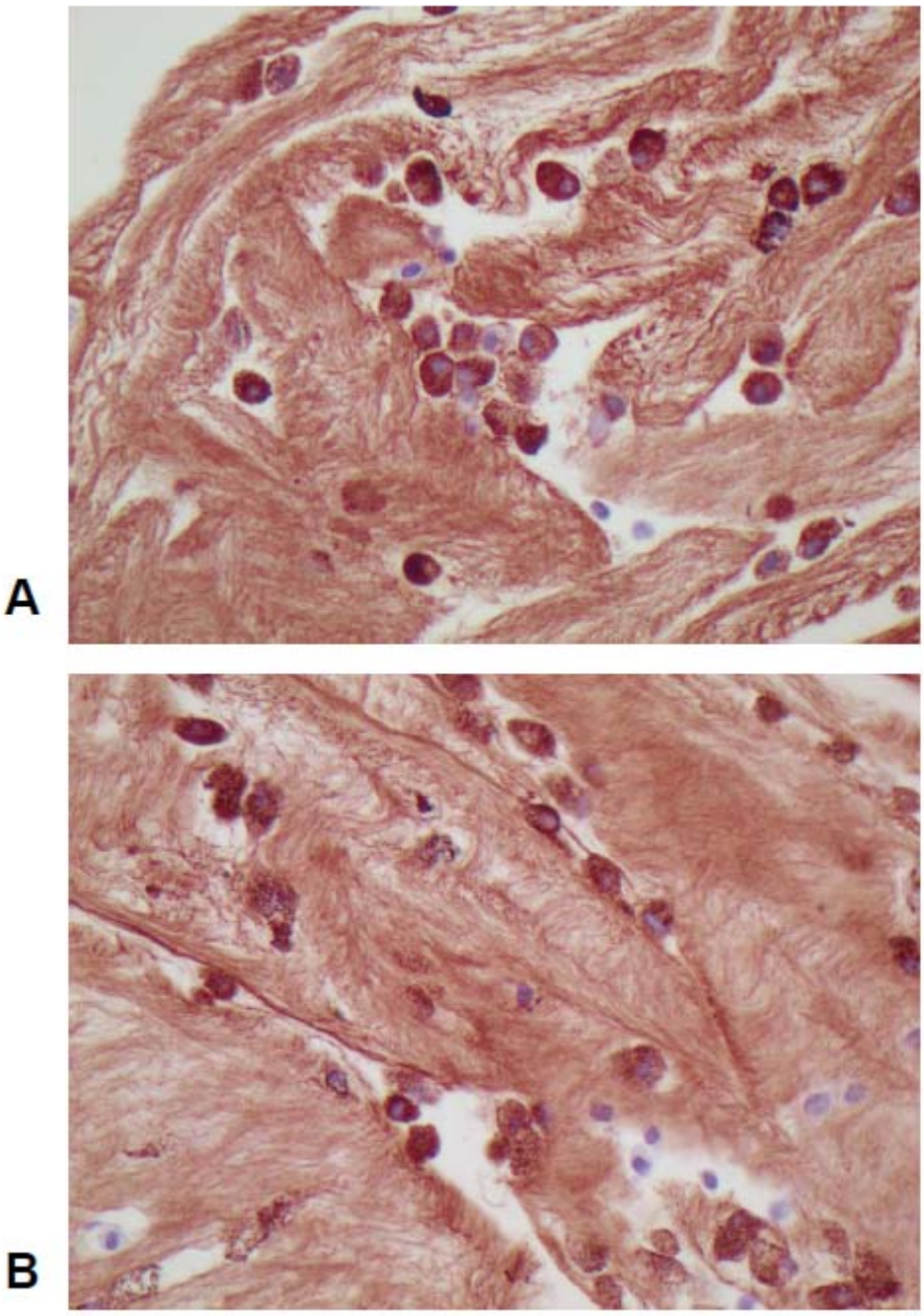

Figure 7-4e. Strong positive immunoreactivity to Collagen I by NGF tissue (**A**) and OGF tissue (**B**), 40X objective magnification: note the rounded cell phenotype and fibrillar ECM.

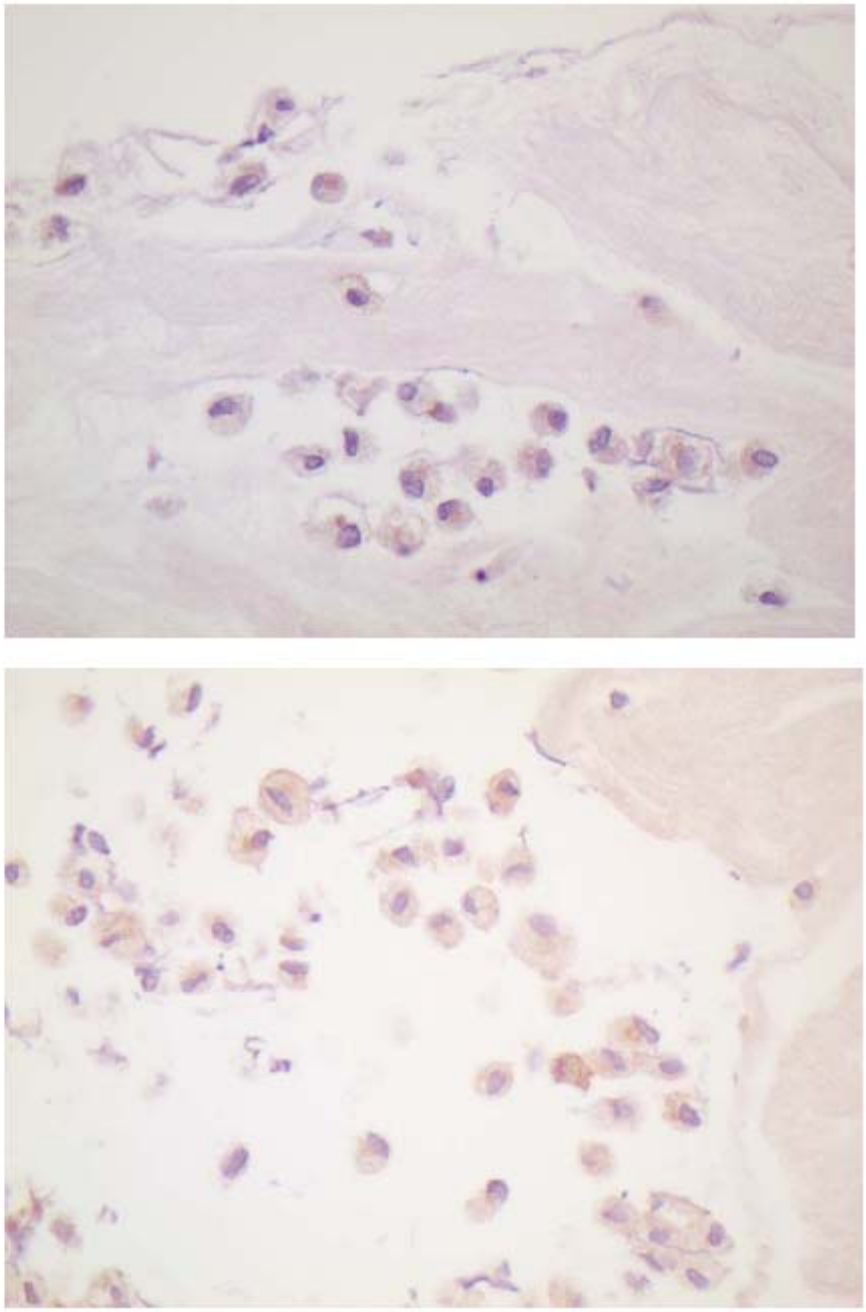

Figure 7-4f. Mild positive intracellular and pericellular immunoreactivity to Collagen II by NGF tissue (**A**) and OGF tissue (**B**), 40X objective magnification: note the rounded cell phenotype.

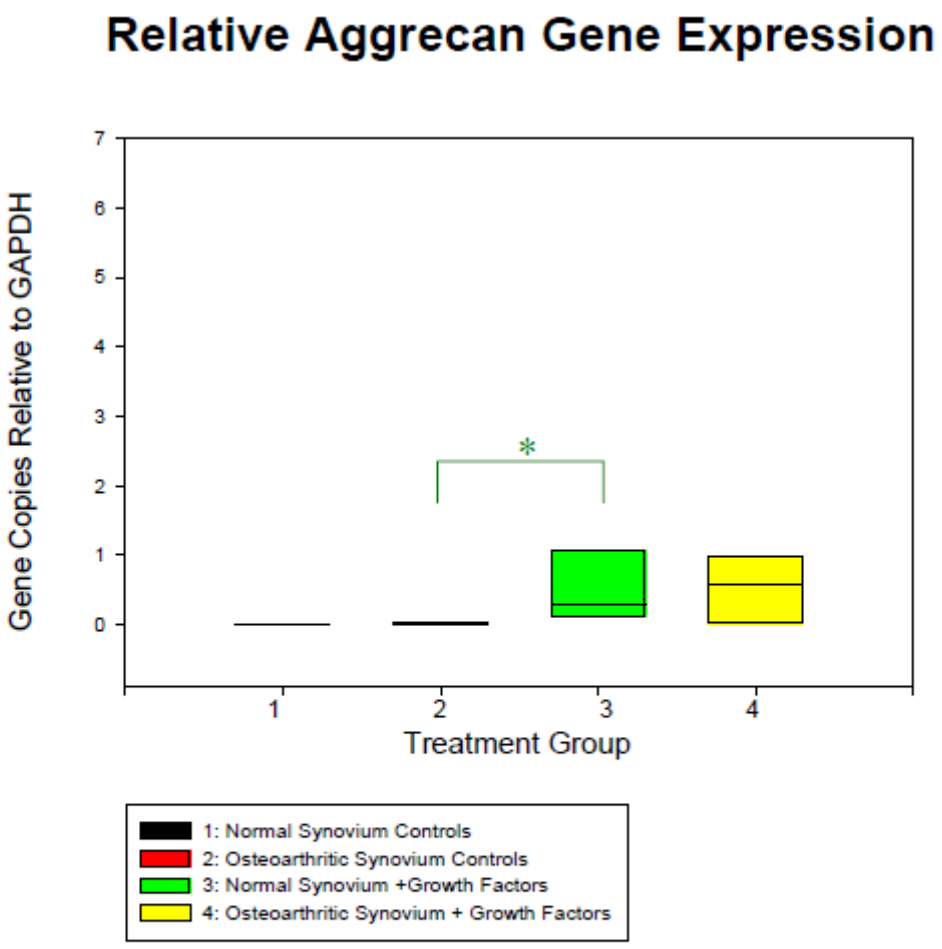

Figure 7-5a. Relative aggrecan gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: a (*) denotes significant differences.

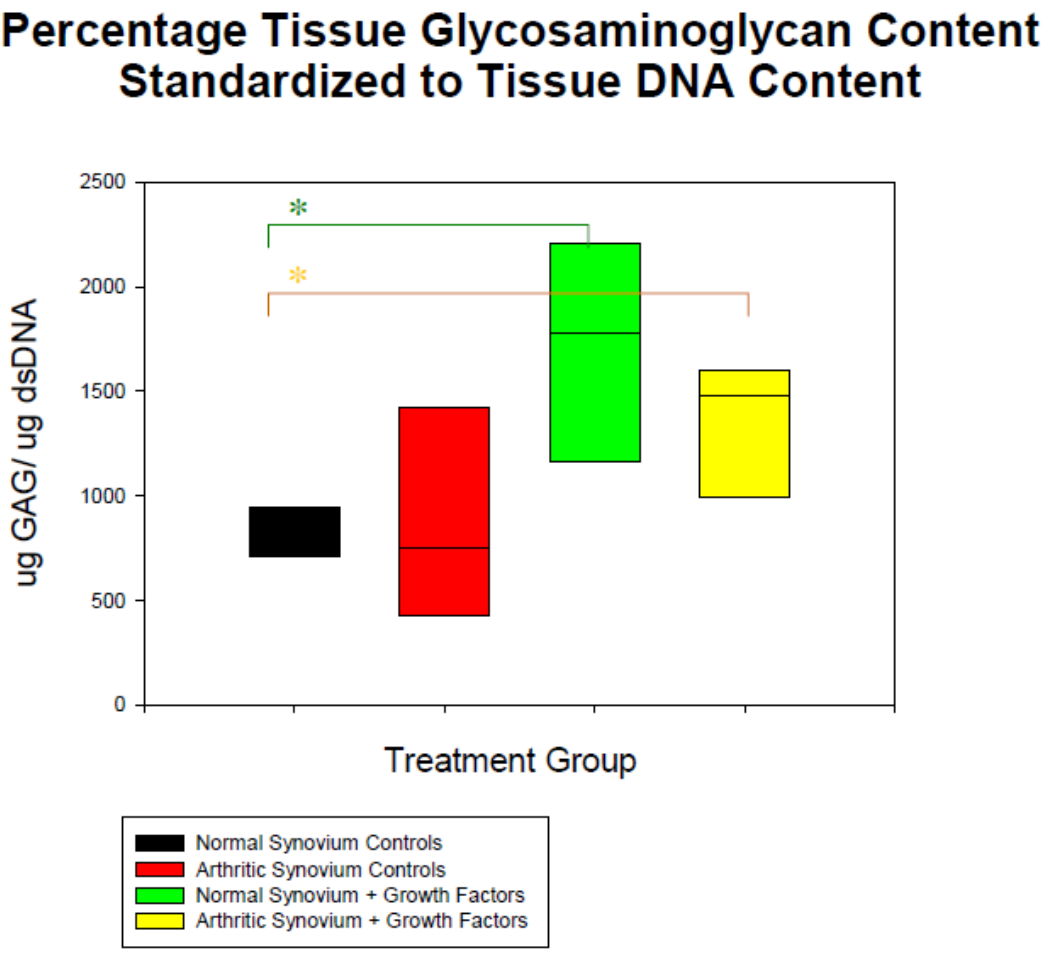

Figure 7-5b. Cellular GAG production, as determined by tissue content of GAG per tissue content of double stranded DNA, for the NC, OC, NGF, and OGF groups at day 16 of culture: (*) denotes significant differences between groups.

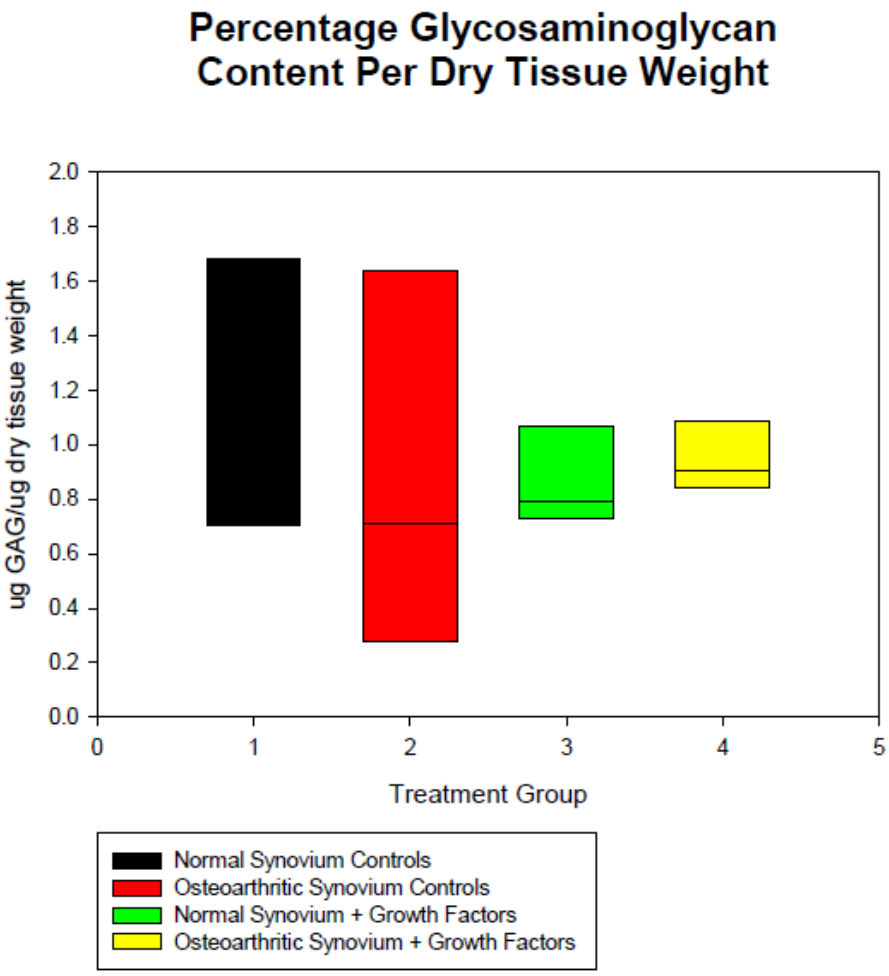

Figure 7-5c. Percentage GAG content of tissue dry weight for the NC, OC, NGF, and OGF groups at day 16 of culture: no significant difference was noted on a per dry weight basis.

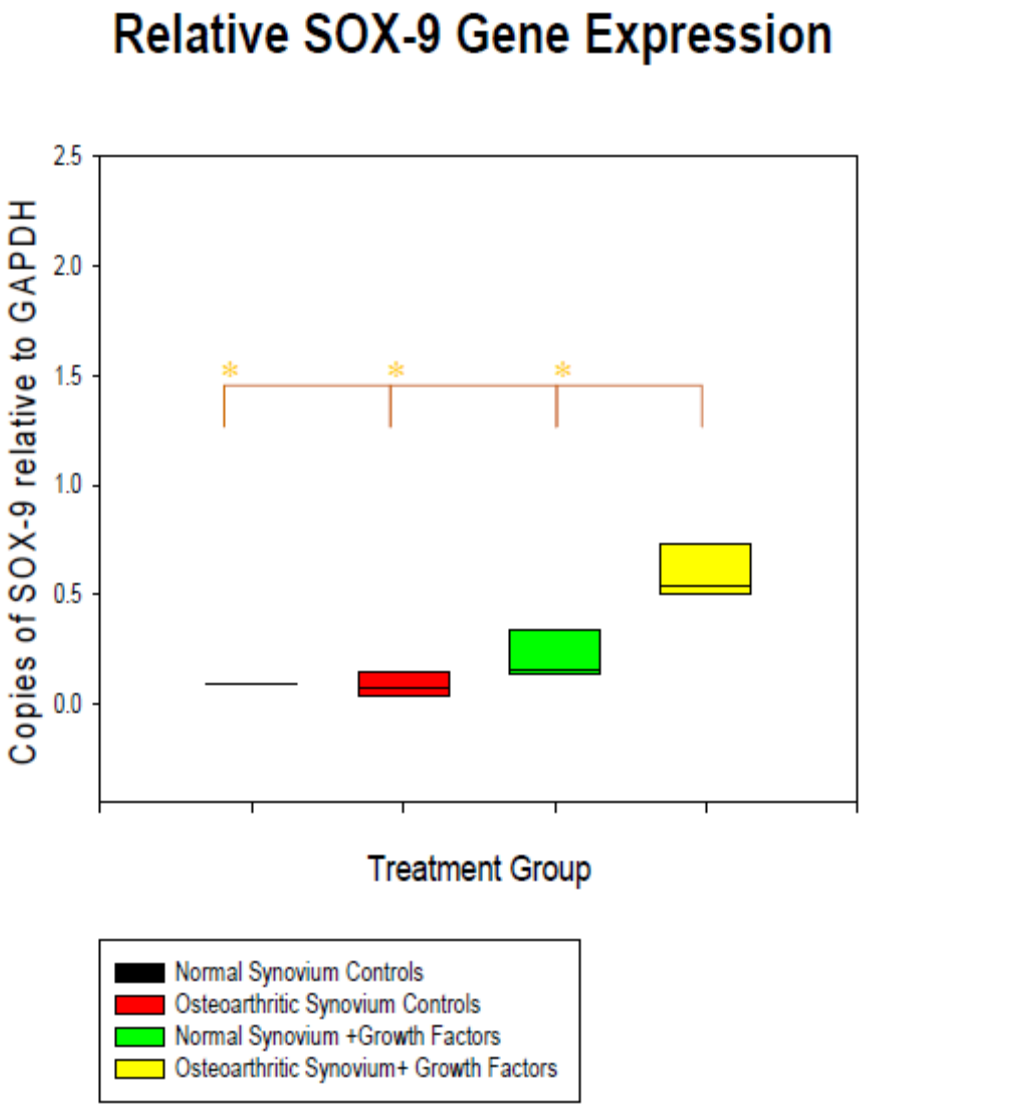

Figure 7-6a. Relative SOX-9 gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: (*) denotes significant differences.

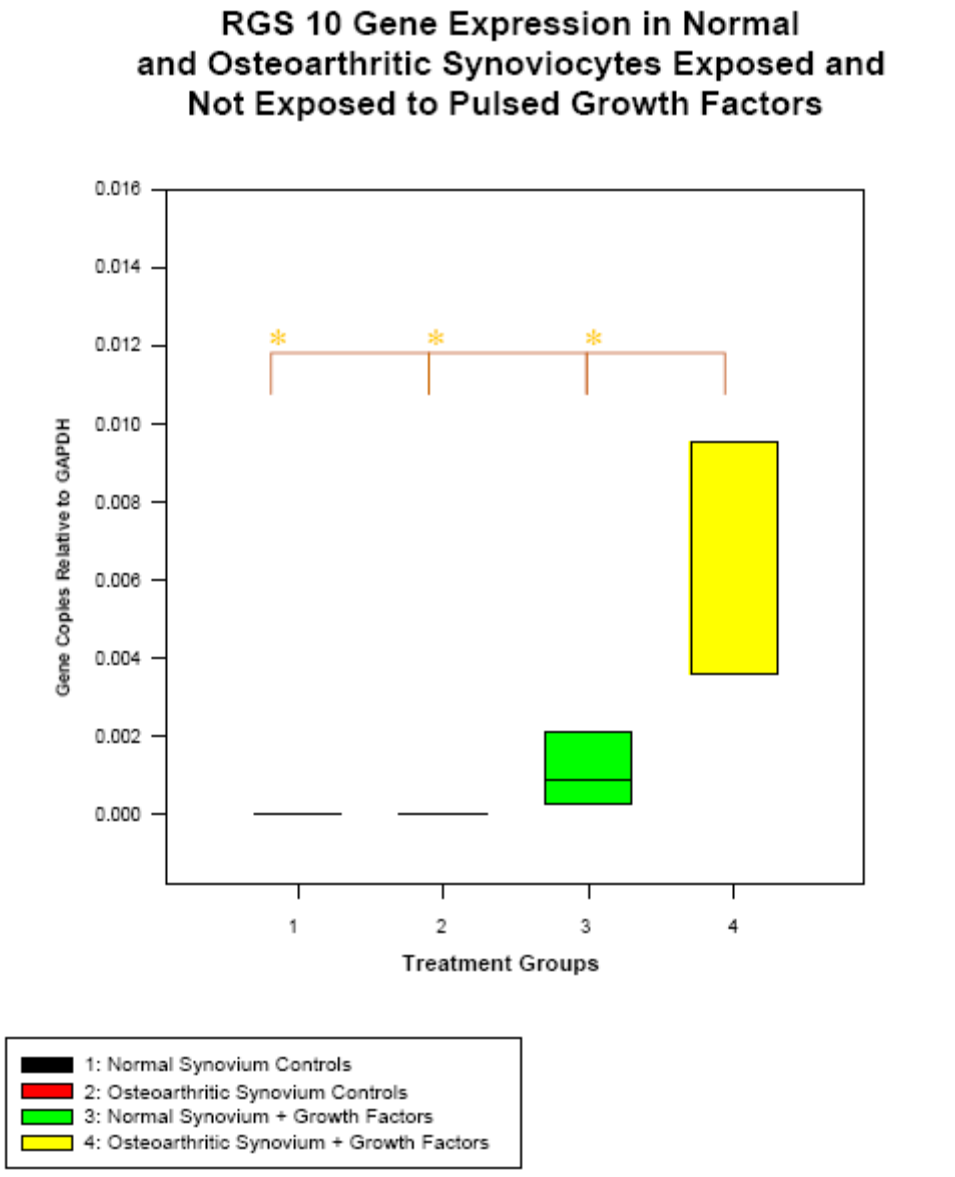

Figure 7-6b. Relative RGS 10 gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: (*) denotes significant differences.

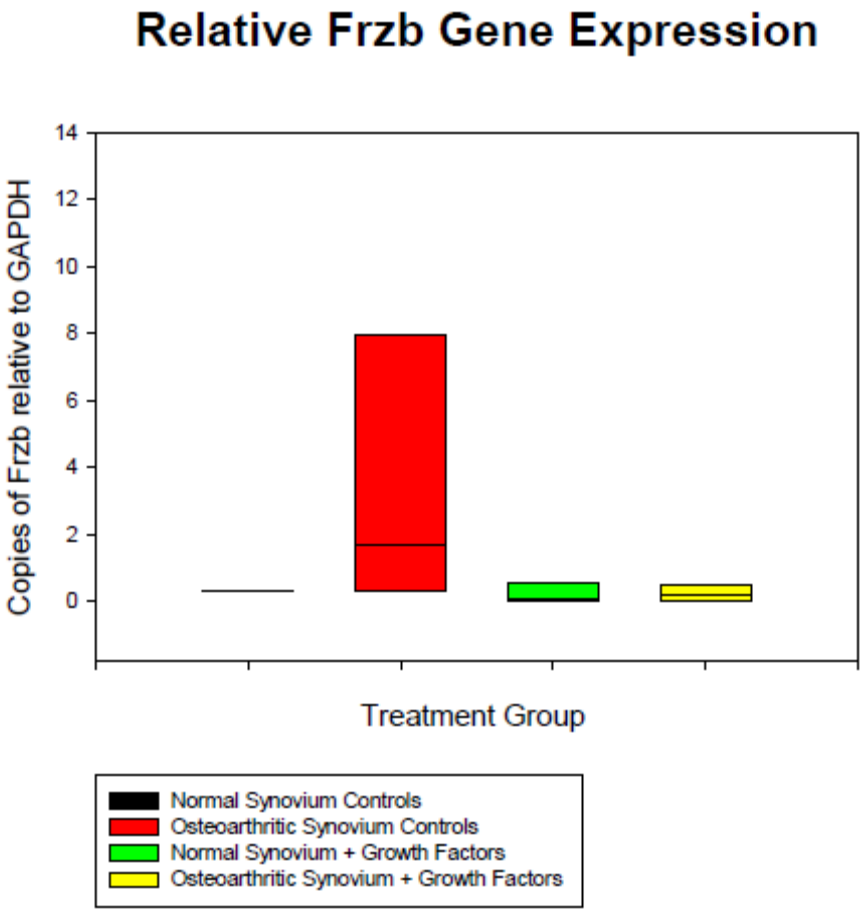

Figure 7-6c. Relative Frzb gene expression in NC, OC, NGF, and OGF groups on the 16th day of culture: no significant differences between groups were detected.

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