Tissue Engineering the Anterior Cruciate Ligament: A Regenerative Medicine Approach in Orthopaedic Surgery

By

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Abstract

Anterior cruciate ligament (ACL) injuries affect over 200,000 Americans yearly, and many occur in young athletes. Current treatment options include tendon autografts and cadaveric allografts. However, these approaches often lead to secondary medical problems, such as donor-site morbidity and immune rejection. Furthermore, in younger patients these grafts fail to grow, leading to additional complications and underlining the need for the development of new approaches that improve the healing and repair of ligaments and tendons. This thesis aims to develop a technique to engineer ACL from autologous mesenchymal stem cells (MSC) and primary ACL fibroblasts using the basic principles of Tissue Engineering. The first part of the thesis characterizes MSCs isolated from tibial bone marrow as an alternative to hip-derived marrow aspirates. The proximity of the tibia to the surgical site of ACL reconstructions makes it a viable source of marrow derived-MSCs for ligament repair, with less stress for the patient and increased flexibility in the operating room. Characterization was performed by fluorescence-activated cell sorting for MSC-surface markers, and assays to differentiate MSCs towards adipogenic, osteogenic and chondrogenic lineages. The second part of the thesis describes the effects of *in vitro* co-cultures of ACL fibroblast and MSC on the expression of ligament-associated markers. The goal was to optimize the cell-cell ratio in order to maximize the positive effects of co-cultures on ligament regeneration. Co-cultures of ACL fibroblasts and MSCs were studied for 14 and 28 days *in vitro*, and the effects assessed with quantitative mRNA expression and immunofluorescence of ligament markers Collagen type I, Collagen type III and Tenascin-C. Finally, based on the enhancing effect observed in co-cultures, the thesis explores a method to regenerate ACL using a three-dimensional polyglyconate scaffold seeded with cell-hydrogel suspensions containing ACL fibroblasts and MSCs. Constructs were analyzed biochemically and by immunofluorescence after 4 weeks in culture with and without mechanical stimulation. Together, our results establish an experimental framework from which a new technique for ACL repair can be developed. The ultimate goal is to foster the design of a one-stage surgical procedure for improved primary ACL augmentation repair that can soon be translated into clinical practice.

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"...And the whole strenuous intellectual work of an industrious research worker would appear, after all, in vain and hopeless, if he were not occasionally through some striking facts to find that he had, at the end of all his criss-cross journeys, at last accomplished at least one step which was conclusively nearer the truth..."

Max Planck
Nobel Lecture
June 2nd, 1920

Table of Contents

List of Figures

List of Tables

Abbreviations

2D	Two-dimensional/two dimensions
3D	Three-dimensional/three dimensions
ACI	Autologous Chondrocyte Implantation
ACL	Anterior Cruciate Ligament
ACLc/ACLcs	Anterior Cruciate Ligament Cells/ACL fibroblasts/ACL cells
ADMSC(s)	Adipose-derived Mesenchymal Stem Cell(s)
A-M	1:1 co-culture of ACLcs and MSCs/ 50%ACLcs:50% MSCs co-culture
APC	Allophycocyanin
BM	Bone Marrow
BMSC(s)	Bone Marrow-derived Mesenchymal Stem Cell(s)
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CO_2	Carbon Dioxide
CT	Threshold Cycle
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetate
FBS	Fetal Bovine Serum
FDA	US Food and Drug Administration
FITC	Fluorescein Isothiocyanate
HBSS - -	Hank's Buffered Salt Solution, Calcium and Magnesium free
HRT	Healing Response Technique
IACUC	Institutional Animal Care and Use Committee

IOC	International Olympic Committee
IRB	Institutional Review Board
ISCT	International Society for Cellular Therapy
MCL	Medial Collateral Ligament
mRNA	Messenger RNA
MSC(s)	Mesenchymal Stem Cell(s)
OCT	Optimal Cutting Temperature
OR	Operating Room
PBMSC(s)	Peripheral Blood-derived Mesenchymal Stem Cell(s)
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin-chlorophyll proteins-cyanine 5.5
PEGDA	Polyethylene Glycol Diacrylate
PGA	Polyglycolic Acid
PGA:TMC	Polyglyconate; Polyglycolic Acid:Trimethylene Carbonate
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Poly-L-lactide
PRF	Platelet-Rich Fibrin
PRP	Platelet-Rich Plasma
qPCR/qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
TMC	Trimethylene Carbonate
TTO	Tibial Tubercle Osteotomy
UV	Ultraviolet
WADA	World Anti-Doping Agency
w/v	Weight/Volume

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Chapter 1.

The Anterior Cruciate Ligament and Our Approach for Potential Primary ACL Augmentation Repair

1.1. Anatomy and Physiology

Ligaments and tendons are dense connective tissues that have the function to provide stability and support connections within the musculoskeletal system. Ligaments provide connections between bones, while tendons connect muscles to bones. The anterior cruciate ligament (ACL) is an extrasynovial structure located inside the knee joint (1). Within the knee joint, the ligament at the proximal end is attached to the posteromedial fossa of the lateral femoral condyle, while distally it attaches to the tibial plateau at the anterior intercondylar fossa (1) (Figure 1.1).

Structurally, the ACL is comprised of two bundles, the anteromedial and the posterolateral bundles (2). These bundles are composed of fascicles made of collagen fibers, glycosaminoglycans and cells (2). Approximately 94% of a fascicle is made out of collagen, with a 9:1 composition ratio of collagen type I to collagen type III, and minimal content of collagen types II, IV and VI (2-4); the remaining 6% includes various proteoglycans, glycosaminoglycans and ligament fibroblasts (2).

The middle geniculate artery, medial inferior geniculate artery and lateral inferior geniculate artery provide a limited blood supply to the ACL (1,5,6). Some scientists have hypothesized that part of the reason the ACL has a limited ability to self-heal is the scarcity of its blood supply (7). The ACL is a well innervated structure, with most of the nerves provided by the posterior articular branches of the tibial nerve (1). An important feature of ACL innervation is its heavy supply of mechanoreceptors for proprioception, including Ruffini corpuscles, Golgi tendon organs, Pacinian corpuscles and various nociceptors (1). These features suggest that the ACL is a highly sensitive structure with an important role in detecting and relaying joint position (1,8). Furthermore, some researchers suggest ACL nerves may trigger muscle reflexes that can protect and stabilize the knee during motion (9). These neural properties of ACL, along with possible improvement in graft vascularization, suggest that future therapies should explore sparing ACL remnants during reconstruction and/or repair (9-11).

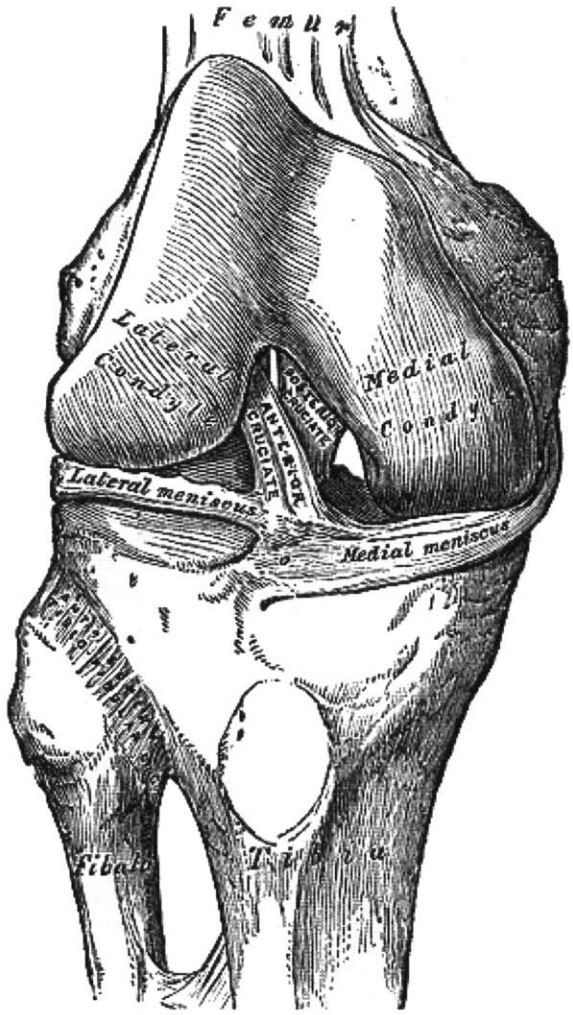

Figure 1.1. Knee joint anatomy. Image from Gray's Anatomy, 1918.

The primary function of a ligament is the transmission of tensional forces between bones to maintain uniform joint motion and avoid abnormal joint displacements (12). Physiologically, the ACL functions to prevent excessive anterior translation of the tibia and maintains rotational joint stability (13). The ACL is also important in preventing medial displacement of the tibia (14,15). More specifically, each bundle of the ACL plays a unique biomechanical role depending on joint position, with the anteromedial bundle carrying greater loads during knee flexion, and the posterolateral bundle playing a more prominent role during knee extension (9,16).

1.2. Injuries and Healing

Injury to the ACL represents one of the most common athletic injuries and the most common ligament injury involving the knee joint (17,18). The literature estimates that close to 200,000 ACL injuries occur yearly in the United States (9). Approximately a third of ACL injuries are caused by contact, where a planted lower leg receives an externally applied torque leading to rupture (19). Non-contact ACL injuries account for over two-thirds of all injuries; in these cases a sudden leg deceleration causes a tear when there is simultaneous quadriceps contraction and full knee extension (19), or when the deceleration leads to an excessive valgus load of the knee joint (20). In the United States, more than 100,000 ACL reconstructions are done each year (18,21,22). In fact, the literature reports that the incidence of ACL injuries is close to 1 in 3500 knee injuries, with the majority of patients electing surgical reconstruction for repair (23,24).

As studied by Woo and colleagues in medial collateral ligament (MCL) samples, it is generally agreed that following injury, ligament healing follows the same three overlapping stages of other soft-tissue healing responses: [1] the hemorrhagic/inflammatory phase, where bleeding occurs at the site of ligament rupture forming a clot that over a period of around 2 weeks transforms from granulation tissue into an immature collagen matrix, thanks in part to the migration and activity of inflammatory cells; [2] the proliferative/reparative phase is characterized by the migration of fibroblasts to the site and the initial deposition of a disorganized extra-cellular matrix; and [3] the remodeling/maturation phase, where the collagen and extra-cellular matrix undergoes reorganization and realignment towards the load-bearing axis of the tissue (7,12,22).

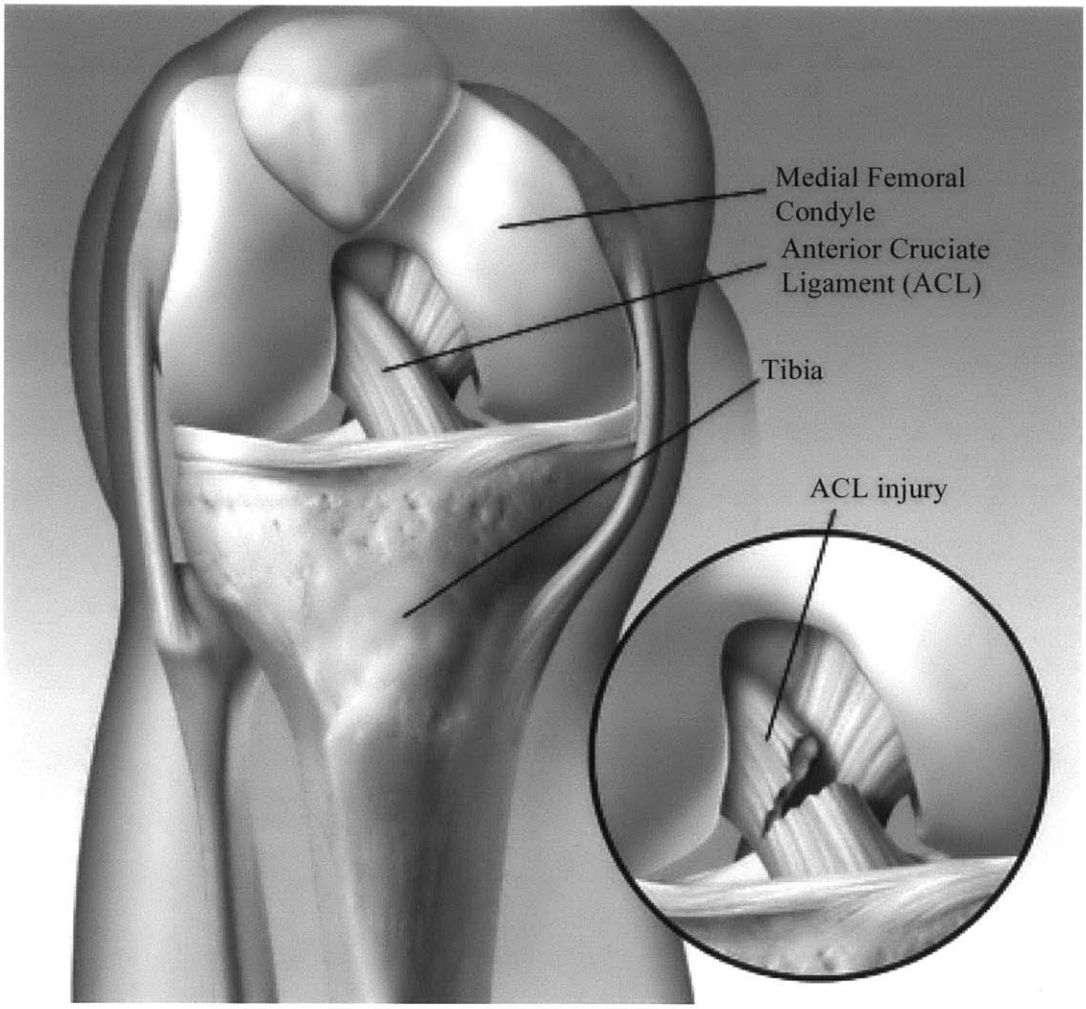

Figure 1.2. Right ACL tear. Image from MayoClinic.com article "ACL Injury", with permission. Courtesy of © Mayo Foundation for Medical Education and Research. All rights reserved.

It is important to note that in contrast to most ligaments and tendons in the human body, the ACL is characterized by an inability to successfully self-heal after injury. Because of the ACL's limited blood supply, some investigators have postulated that its observed poor healing capacity stems in part from an inability to supply the injury site with sufficient resources for healing (7). Moreover, subpar vascular responses of the injured tissue, and observed increased ACL fibroblast difficulty in initiating cell migration and triggering angiogenesis may also play a role (7,25,26). Additionally, recent studies have also suggested that in anterior cruciate ligament healing, there is a lack of formation of a bridging matrix that connects the torn ends of the ACL as seen in other ligaments, such as the MCL (11). Unlike other self-healing ligaments, the ACL

does not form this temporary "bloody clot" or "fibrin scaffolding", likely due to increased plasmin and urokinase plasminogen activator activity (11,27), and as a result healing cannot be effectively initiated, regenerative cells cannot be recruited to the site and the proliferative/reparative phase cannot proceed adequately (11,27,28).

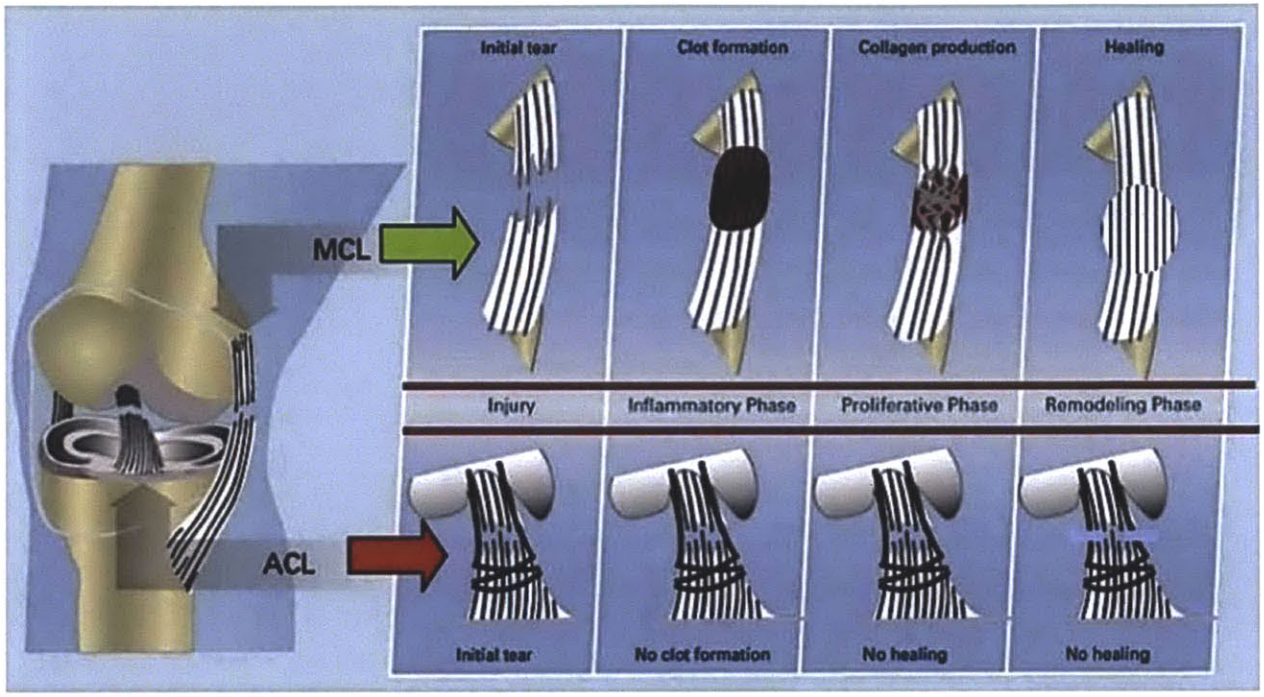

Figure 1.3. Murray 2009 theory on impaired ACL healing. Image previously published in reference (11). © Elsevier. Reprinted with permission.

1.3. Current Treatments

After ACL rupture, knee instability occurs, especially in young and active patients. Currently, surgical reconstruction is the gold standard for ACL repair, especially for patients that wish to continue with athletic and active lifestyles (9,23,29-31). To date, there are only a few materials available for the repair of ACL injuries requiring surgical intervention. Biological grafts available include autografts and allografts. Autografts are taken from the own patient to replace tissue at another site. For ACL reconstruction, these autografts are typically harvested from the patellar tendon, hamstring tendon, or quadriceps tendon, with an overall reconstruction success rate upwards of 70% (9,29-31) (Figure 1.2). Though the choice of autograft is largely

dependent on surgeon preference, patellar and hamstrings tendons are the most commonly used grafts, with failure rates of 7 and 16%, respectively (9,29). In addition to the risk of graft failure, this technique is limited by the amount of tissue that can be obtained from the donor site, and has the potential to cause donor site morbidity, including functional muscle weakness, chronic articular pain, patellar rupture, and increased long-term risk of osteoarthritis (17,29,31-34). However, it is important to note that, even though some types of grafts may accelerate the development of osteoarthritis, evidence suggests that the risk of developing osteoarthritis post-ACL injury is high regardless of the type of treatment (35,36); and that surgical intervention does not decrease the long-term incidence of osteoarthritis compared to conservatively-treated injuries (35).

In addition to autografts, allografts from cadavers may also be used as repair tissues. Allografts have the advantage of not requiring donor sites, reducing surgery duration and providing a more ample source of tissue (9,17,29,31). However, they have the potential to carry infection, cause an immunogenic response in the recipient, and are difficult to sterilize without damaging the biomechanical properties of the graft (9,17,29). Furthermore, the failure rate of allografts can be as high as three times that of autografts, especially in younger populations (9,31,37).

Alternatively, there are a few synthetic materials that have been unsuccessfully investigated for ACL repair, such as the Gore-Tex and Leeds-Keio prostheses (23). Although approved for limited use by the FDA, these materials invariably fail over time by mechanical wear and tear, leading to mechanical failure and loosening of the grafts, synovitis, and knee instability (17,23).

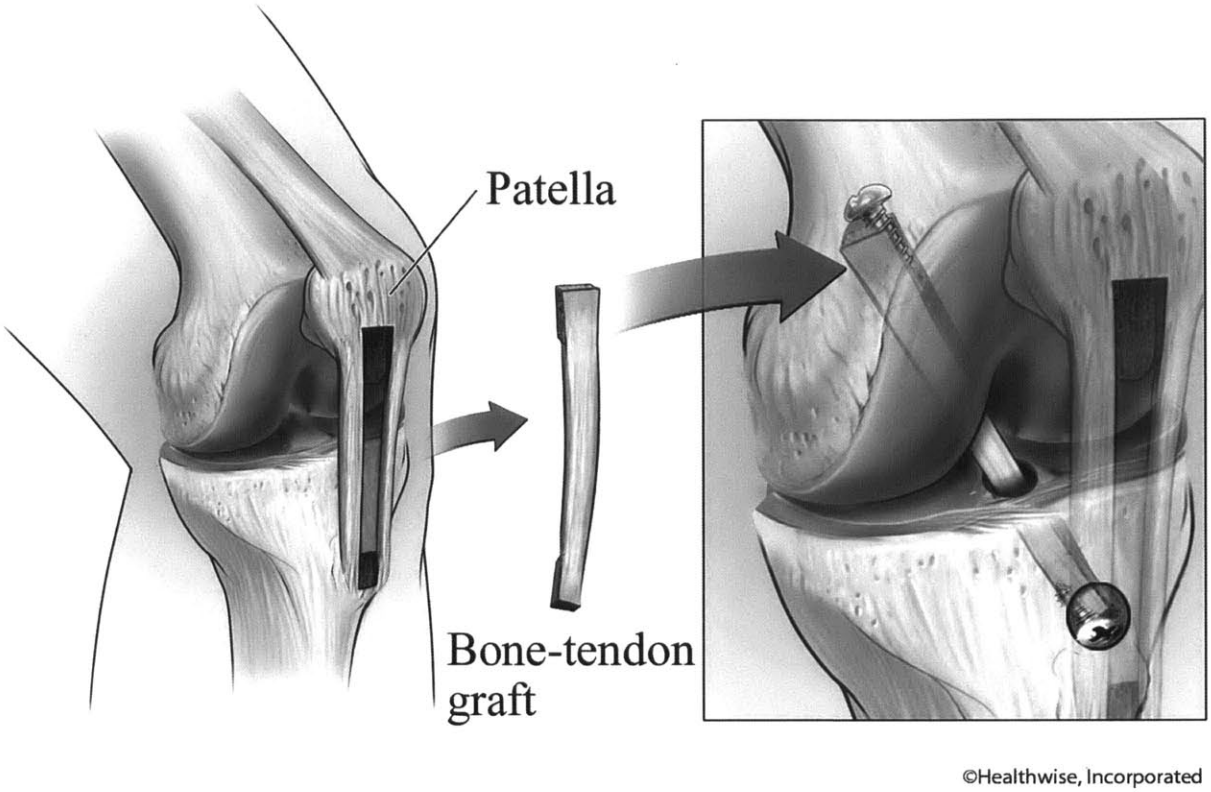

Figure 1.4. Patellar autograft diagram. Image courtesy of ©Healthwise, Inc., with permission.

1.4. Novel Approaches

Given the shortcomings of currently available options for ACL reconstruction and repair, the development of techniques that promote the healing of ligaments and tendons is of increased importance in Orthopaedics. Tissue Engineering and Regenerative Medicine have recently provided new pathways for potential tissue repair, where cell sources, biological regulators and biomaterial scaffolds can serve as instruments to improve tissue regeneration. Steadman and colleagues have developed the Healing Response Technique (HRT) to enhance primary ACL repair by an approach similar to cartilage microfracture surgery (10,38,39). The technique involves the microdrilling of holes at the proximal end of the injured ligament and through the ligament's origin, which should allow mesenchymal stem cells, biological regulators and clotting factors to reach the site of injury (10,38). A primary repair approach, where the torn ends are approximated and surgically repaired with a suture, is then performed on the ligament (10,38). Follow-up studies have been contradicting in demonstrating the success of the technique, with a

study by Steadman and colleagues reporting that HRT is effective in returning patients to normal levels of recreational activity with less than 9% of patients needing subsequent ACL reconstruction, while Wasmaier and group report reconstruction rates as high as 36% and did not see better outcomes when compared to conservative treatment options (40,41). Even though the technique shows promise, modifications and improvements are needed to ensure positive long-term outcomes.

Murray and colleagues have also developed a promising technique known as bioenhanced ACL repair (11). This approach utilizes a combination of collagen hydrogels or sponges and platelet-rich plasma (PRP) to create collagen-platelet constructs used to augment and enhance a primary ACL repair (11,42-47). The PRP serves as a source of biological factors and clotting factors required for wound healing, while the collagen sponges serve as a platelet activator and a vehicle to deliver and retain PRP at the injury site (45,46). The technique has shown positive results in artificially created ACL transections by improving the biomechanical functioning of primary ACL repairs, especially when supplemented with bone-to-bone suture fixation, which essentially acts as an additional scaffold providing structural support to the construct and stabilizing the knee (42,44,47,48). A drawback of this technique is the use of an artificial injury model, where the ligament transection is created cleanly and the ACL remnants are essentially intact, easily approximated and repaired immediately post-transection (42). Moreover, several groups have established that surgical transections are not clinically comparable to real ligament injuries (49-51). The technique has shown negative results when bioenhanced primary repair is delayed even for two weeks (52). It would be interesting to see the effectiveness of the technique in ACL injury models more similar to observed clinical scenarios, where the ACL stumps are severely damaged, retracted, inflamed, and reconstructive surgery generally delayed for two to four weeks (12,53-56).

Laurencin and group have developed yet another ACL engineering technique with promising potential (17,57). Three-dimensional, braided poly-L-lactide (PLLA) scaffolds seeded with primary ACL cells are used as biodegradable, synthetic allografts for ACL reconstruction (17,57-62). The grafts have shown appropriate tissue ingrowth, collagen and cellular penetration, vascularization, and short-term biomechanical stability (57). However, when tested *in vivo* in rabbits, the constructs failed biomechanically after 12 weeks (57). Goh and others have used a similar approach with silk or collagen scaffolds seeded with MSCs, achieving improved longer-

term biomechanical stability but still far from levels observed in native ACL (4,63-65). The method has encouraging features for ACL reconstruction, and with appropriate adaptations and combination with additional regenerative therapies may help advance the field of ligament repair.

1.5. Our Approach

As the complex biomechanical and sensory role of the ACL is better understood, increased interest has been placed in the primary repair of ACL injuries supplemented with localized regenerative medicine therapies (10,11,31,48). Sparing of the existing vascular supply, as well as the intricate array of neurosensory machinery, may help increase the long-term success of ACL repair and lead to better outcomes (10,28,66). The goal of this thesis is to establish the framework of a technique to create functional ACL tissue from autologous mesenchymal stem cells cultured in conjunction with primary ACL fibroblasts in a stable hydrogel matrix seeded on a resorbable polymer scaffold. The hydrogel serves as the cell delivery vehicle, ensuring homogeneous cell seeding of the polymer scaffold. The scaffold serves as the load-bearing material during the time of ligament regeneration. Our vision is to introduce a multi-component, combinatorial regenerative construct that may overcome the difficulties encountered by the ACL regenerative approaches introduced above. Two aspects of the thesis advocate the approach may have better outcomes. First, recent studies have shown that indirect and/or direct co-culture of MSCs and fibroblasts results in increased expression of tissue-specific markers, cell proliferation, DNA content and collagen production, and triggers differentiation of MSCs towards fibroblastic lineages (64,67-69). Second, current reports have shown that MSCs may not only provide cells for repair, but may also act as accessory cells that trigger regenerative responses in cells native to other tissues (70-72). Furthermore, MSCs have shown anti-inflammatory and immunomodulatory properties *in vivo* (73-75). Thus, combining MSCs and ACL fibroblasts may provide a double source of cells and regulators from which ACL can be regenerated (Figure 1.3). The thesis explores the effects of co-culture in ACL constructs and the behavior of the cells in two- and three-dimensional *in vitro* culture.

1.6. Research Aims

1. Characterization of tibial mesenchymal stem cells. Hypothesize that proximal tibial MSCs have the same differentiation potential as other bone marrow-derived MSCs.
2. Characterization of the co-culture effects of primary ACL fibroblasts and MSCs *in vitro*. Hypothesize that co-culturing MSCs with ACL cells leads to an enhanced regenerative response compared to either population alone.
3. *In vitro* development of a construct for primary ACL augmentation repair with a biodegradable scaffold based on poly(glycolic) acid: trimethylene carbonate (PGA:TMC) combined with a hydrogel seeded with autologous MSCs and primary ACL fibroblasts under dynamic tensile loading. Hypothesize that combining MSCs with primary ACL cells in a hydrogel loaded in a biodegradable scaffold enhances the regenerative response, particularly in tensile loading environments.

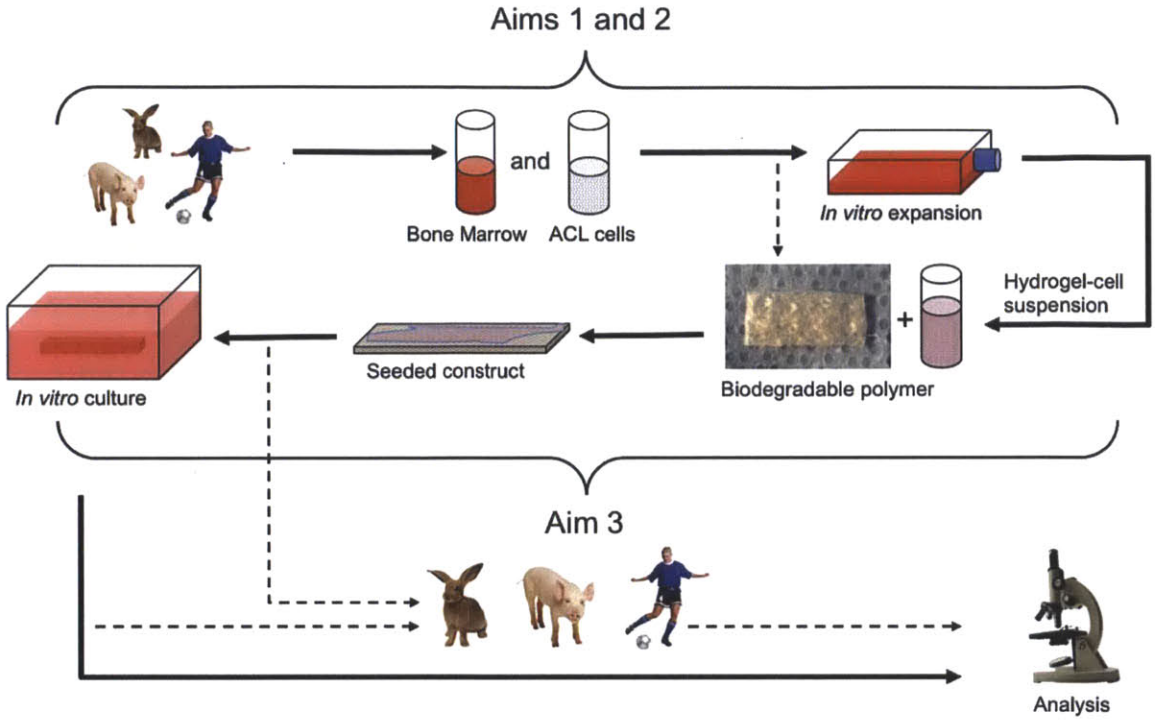

Figure 1.5. Research aims flowchart. Solid arrows represent accomplished steps. Dotted arrows represent future directions.

Chapter 2.

Feasibility of Tibial Bone Marrow-Derived Mesenchymal Stem Cells for Orthopaedic Tissue Engineering

2.1. Overview of Mesenchymal Stem Cells

Since their initial discovery by Friedstein and colleagues (76,77), mesenchymal stem cells (MSCs) have kindled vigorous interest from scientists in various fields, especially Tissue Engineering and Regenerative Medicine. The existence of an adult stem cell with the ability to differentiate into a wide variety of tissues not only bypassed ethical dilemmas faced by stem cell researchers dealing with embryonic stem cells, but also provided a potential source of autologous cells that could be seamlessly harvested and tolerated by patients in need of cell therapies, organ regeneration and/or transplantation (72,78).

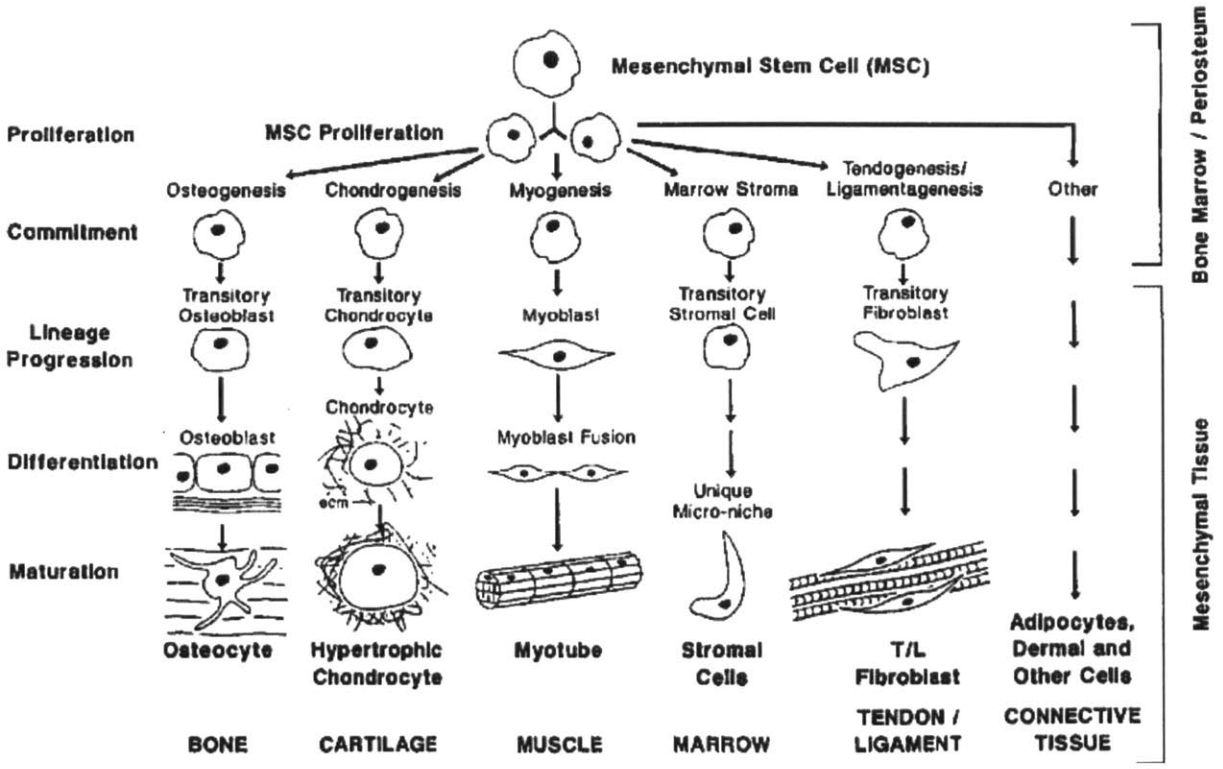

Figure 2.1. Mesenchymal stem cell differentiation. This image, "The Mesengenic Process", was published in references (72,79) © Elsevier. Reprinted with permission.

Although MSCs were first isolated from bone marrow (76), they have since been identified in practically every tissue in the body (70-73,80-86). Controversy about the true name, identity, meaning of the term, and behavior of MSCs is ongoing (reviewed in (87)). Nevertheless, the scientific consensus is that MSCs, with mesenchymal stem cell being the preferred denotation, are non-hematopoietic, multipotent, self-renewing stem cells made up of a mixed cell population with typical fibroblastic morphology capable of differentiating into a wide array of tissues, including bone, fat, cartilage, muscle, ligament, tendon and others (72,77,79,82,83,85,86,88) (Figure 2.1). Moreover, the literature has also described their ability to differentiate into tissues of neuroectodermal/ectodermal, and endodermal origins (84,85).

Even though complete knowledge of the biologic role of MSCs is still lacking, significant advances have been made in the understanding of MSC physiology (70,73,84,88). Initially thought of only as a stem cell source for the maintenance of tissues and/or the regeneration of damaged tissues *in vivo* (88), MSCs have more recently been observed to also act as secretory cells that release biological factors that can stimulate the recruitment and

proliferation of native tissue cells, enhance angiogenesis, while also inhibiting apoptosis and fibrosis (70-72,86,89). In addition, MSCs have also been observed to play a role in the regulation of immunologic and inflammatory responses (70,73-75,82,90). Some reports suggest MSCs have immunosuppressive characteristics, regulating dendritic cell, T-cell and B-cell functions (70,73,74,82); while others suggest MSCs secrete anti- and pro-inflammatory cytokines (70,90). Thus, MSCs may assist tissue regeneration in more ways than previously believed by providing raw stem cells for tissue replenishment, while also secreting local and systemic factors that can promote and enhance tissue repair (70-72,88).

2.2. Mesenchymal Stem Cell Sources, Isolation and Identification

As extensively reported by da Silva Meirelles and others, MSCs have been isolated from essentially everywhere in the body (70-72,83-86). However, the specific properties of MSC populations derived from different tissues vary depending on tissue origins (85). While the profile of surface markers observed is similar across MSCs from various tissue origins, their differentiation potentials and extent of differentiation are often very different (85). Further, others suggest their biosecretory activities can also be different and dependent on tissue of origin (71,91). These observations suggest that the source of MSCs for tissue engineering applications should be carefully assessed depending on the tissue being regenerated and the desired outcome (71,85,92).

Our interest in the regeneration of musculoskeletal tissues for Orthopaedic tissue engineering and eagerness to translate our technique to the clinic for surgical ligament repair advocates for bone marrow as the preferred source of MSCs. First, bone marrow MSCs (BMSCs) were the original population described by scientists, and is perhaps the best characterized and whose behavior is better understood (71,72,78). Second, BMSCs by default tend to go down their mesenchymal lineage, more easily differentiating into skeletal connective tissue cells than MSCs from other sources (71,84,87,88,93). In fact, Bianco and others (93,94) recognize bone marrow MSCs as "skeletal stem cells", underscoring their increased ease and potential in generating skeletal cells, including osteoblasts, chondrocytes, adipocytes and fibroblasts, with *in vivo* "natural" assays and without the need for artificial interventions (93,95,96). Though the use of alternatives sources for MSC harvest such as fat (97), peripheral

blood (97,98) or dental pulp (93) may increase convenience, experimental evidence suggests bone marrow-derived MSCs have the greater ability to differentiate into skeletal tissues (71,93,95,96). In fact, studies in marrow, adipose and peripheral blood imply bone marrow-derived MSCs have the greater potential for orthopaedic tissue engineering, and particularly ligament regeneration (92,99,100). For example, in a study with adipose-derived MSCs (ADMSCs) cultured *in vitro* with growth factors, the cells did not show a significant or stable increase in the expression of ligament-associated markers (92). Peripheral blood is also a theoretically attractive source of MSCs (97,98,101). However, some groups have questioned the true origin of peripheral blood-derived MSCs (PBMSCs), suggesting they are biologically different from BMSCs and very scarce (102), released or mobilized from other tissues into the circulation after injury or other *in vivo* physiological or pathological processes (85,103-105), or observed only when derived from cancer patients undergoing chemotherapy (94,106); while others have only had success in isolating PBMSCs when dealing with fetal or umbilical cord blood (88,107-109). Even more so, many groups have been unable to isolate MSCs from peripheral blood (85,105,110,111). These observations pooled together insinuate a lack of true understanding of the biology, origin and behavior of alternative MSC sources, reason enough to lead our group to focus on the well-characterized bone marrow MSCs.

Bone marrow is a form of reticular connective tissue composed of extracellular matrix, hematopoietic cells and stem cells, white blood cells, adipocytes, and MSCs among others (83). Bone marrow is located within bones, particularly in the pelvis and long bones. In order to obtain pure MSC cultures, the cells must be separated from the rest of the components of bone marrow. Typically, explant cultures are established by diluting bone marrow aspirates in nutritional cell media and allowed to grow in tissue culture flasks or dishes (83). MSC cultures result as the cells attach to the flasks and the rest of the marrow components are washed away with media changes (83). If increased purity or faster isolation is desired, density gradient centrifugation methods, where components are separated into layers based on their densities, have been used (112-115). Finally, commercially available syringe filters specific for bone marrow-derived MSCs have recently been used for diluted bone marrow aspirates (116). The device utilizes a bi-directional non-woven filter in a column to selectively trap nucleated cells during forward flow while allowing other marrow components to pass through, the nucleated cells are then harvested via reverse flow (116). The method has the potential to significantly

lower isolation times during surgical procedures, especially when compared to explant or density gradient centrifugation methods (116).

To date, no single marker has been discovered to uniquely identify MSCs. Because of this the International Society for Cellular Therapy (ISCT) published a guideline to establish the minimal criteria to define the identity of MSCs (117): (1) cells must adhere to tissue culture plastic; (2) cells must express surface markers CD105, CD73 and CD90, while not expressing CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; and (3) cells must differentiate to osteogenic, adipogenic and chondrogenic lineages in *in vitro* assays (117). It is important to note that this is a minimal criteria list, since MSCs are known to be positive and negative for a variety of additional non-specific surface markers, and are able to differentiate into a variety of additional tissues (73,83,84).

2.3. Tibial Bone Marrow-Derived Mesenchymal Stem Cells

Using MSCs for ligament tissue engineering necessitates the procurement of bone marrow from sites that would seamlessly integrate with a site-specific surgical procedure, avoid increasing patient discomfort and pain, and provide a sufficient amount of cells for efficient regeneration (114). Currently, the pelvis is the preferred site for bone marrow harvest, with the posterior superior iliac spine being the most common site ((118), reviewed in (119)). However, for upper and lower extremity procedures, interventions in the pelvis would require patient repositioning and a second surgical site (114). Thus, alternative sources of BMSCs have been of increased interest in regenerative Orthopaedics. Recent studies have explored the use of proximal humerus and distal femur bone marrow as a source of MSC-like cells for skeletal tissue regeneration (113,114,120). The studies are well performed, but fall short of fully characterizing the cell isolates as true MSCs, and fail to compare their technique to additional MSC isolation methods that could more easily integrate to a surgery (116).

Another source that is directly in the operative field during knee surgeries is the proximal tibia. Many orthopaedic procedures of the knee require tibial interventions, including autologous chondrocyte implantations (ACI) with tibial tubercle osteotomies (TTO) (121-123), and ACL reconstructions, where femoral and tibial tunnels are routinely created (30). Thus, the tibia is a source that has been unexplored and may offer an additional site to the distal femur for

MSC isolation. In collaboration with Dr. Andreas Gomoll from the Cartilage Repair Center in the Department of Orthopaedic Surgery at the Brigham and Women's Hospital, our laboratory pursued a clinical study with human tibial bone marrow. Our goal was to develop a simple and integrated protocol for tibial bone marrow harvest during ACL reconstructions and/or ACIs with TTOs, and to isolate and fully characterize the MSCs obtained from the aspirates for tissue engineering applications in Orthopaedics.

2.3.1. Preliminary Evaluation of Isolation Methodologies

In a preliminary study, we compared three methods of MSC isolation using sheep bone marrow: explant culture, density gradient centrifugation, and a more recently introduced protocol utilizing a bone marrow MSC syringe filter device (116). Iliac crest bone marrow aspirates post-euthanasia were obtained from 6 to 8 month old sheep via the tissue-sharing program of Institutional Animal Care and Use Committee (IACUC)-approved protocols at Harvard Medical School and the Brigham and Women's Hospital. The isolated cells were assessed for their differentiation potential.

2.3.1.1. Materials and Methods

Tissue harvest and cell isolation

Explant method

As previously described (99,124), 5-10 ml of BM from the iliac crest were aspirated with a 16-gauge needle attached to a syringe with 0.5 ml sodium heparin (APP Pharmaceuticals) and seeded in T-75 flasks (BD Falcon) at a 1 ml BM to 9 ml media ratio with DMEM (Gibco) with 10% FBS (Gibco) and 1% antibiotic mixture (BM DMEM). Bone marrow-derived mesenchymal stem cells were isolated by their ability to attach to tissue culture plastic (99,124).

Density gradient method

The density gradient Ficoll-Paque PREMIUM 1.073 (GE Healthcare), designed for optimal isolation of mononuclear cells (including MSCs) from blood and bone marrow, was used. MSCs were isolated following the manufacturer's protocol. Briefly, 2 ml of BM were diluted with 2 ml of sterile HBSS-- (Invitrogen). The final 4 ml volume of diluted marrow was carefully layered on top of 3 ml of Ficoll-Paque PREMIUM 1.073 in a sterile 15 ml conical tube (BD Falcon) and centrifuged at 400 x g for 35 minutes at 20°C. Carefully, the layer of mononuclear cells (~2 ml) was transferred to a new sterile 15 ml conical tube, resuspended with ~6 ml of HBSS--, and centrifuged at 500 x g for 15 minutes at 20°C. The pellet was washed once more by resuspending in ~6 ml HBSS--, and centrifuging at 500 x g for 10 minutes at 20°C. The cell pellet was then seeded in T-75 flasks with BM DMEM.

Filter device method

A bone marrow MSC Separation Device – Basic Set (Kaneka, Japan) was used as previously described (116), and following manufacturer's protocols. Briefly, 5-15 ml of BM were diluted in two equal volumes of HBSS--. The filter device was primed with 50 ml of HBSS- - to flush the air out of the column. The diluted bone marrow was then attached to the forward flow side of the filter and allowed to stand vertically for 3 minutes to separate the marrow fat from the dilution. The bone marrow fluid was the forward flushed through the filter at 6 ml/min into a biohazard disposal container. The filter was then washed with 30 ml of HBSS- - in forward flow direction at a rate of 6 ml/min. The filter was then reversed and 30 ml of BM DMEM were flushed in reverse flow at a rate of about 10 ml/sec into a sterile 50 ml conical tube. The harvested fluid was centrifuged at 260 x g for 6 min, and the cell pellet resuspended in BM DMEM and seeded in T-75 flasks.

All cells were grown to passages 2 or 3. Viability was determined by the trypan-blue (Sigma) exclusion method. MSCs were not allowed to reach more than 60-70% confluency before passaging. Culture medium was changed twice a week.

Osteogenesis

MSCs were seeded in 24-well plates (BD Falcon) at a density of 4.2 x 10^3 cells/cm^2 and cultured in BM DMEM until 50-70% confluency was reached. Medium was then replaced with

osteogenic differentiation media composed of a-MEM Basal Medium (Gibco) supplemented with dexamethasone, ascorbate, and b-glycerophosphate (Osteogenic Supplement, R&D Systems). Media was changed every 3 days for 2 weeks. Cultures were then washed carefully with HBSS--, fixed with 10% formalin (Sigma) for 45 minutes and rinsed with distilled water. Alizarin Red S staining solution was freshly made with 2g Alizarin Red S (EMD Chemicals) in 100 ml distilled water, adjusting pH to 4.1-4.3 with 0.1% NH_4OH. Cell monolayers were then covered with staining solution and incubated at room temperature in the dark for 45 minutes. Wells were then rinsed with distilled water and imaged using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

Adipogenesis

MSCs were seeded in 24-well plates (BD Falcon) at a density of 2 x 10^4 cells/cm^2 and cultured in BM DMEM until 100% confluency was reached. Medium was then replaced with adipogenic differentiation media composed of a-MEM Basal Medium (Gibco) supplemented with hydrocortisone, isobutylmethylxanthine and indomethacin in 95% ethanol (Adipogenic Supplement, R&D Systems). Media was changed every 3 days for 2 weeks. Cultures were then washed carefully with HBSS--, fixed with 10% formalin (Sigma) for 45 minutes and rinsed with distilled water. Wells were then covered with 60% isopropanol for 5 minutes and then stained with 0.3% Oil Red O staining solution for 15 minutes at room temperature. Wells were then rinsed with distilled water and imaged using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

Chondrogenesis

Approximately 250,000 MSCs were transferred to a 15 mL conical tube and centrifuged at 200 x g for 5 minutes at room temperature. Medium was discarded, cells then resuspended in 0.5 mL of chondrogenic differentiation medium and centrifuged at 200 x g for 5 minutes without discarding medium. Chondrogenic differentiation medium was composed of DMEM/F-12 basal medium (Gibco) supplemented with 1% ITS supplement (R&D Systems) and dexamethasone, ascorbate-phosphate, proline, pyruvate and TFG-b3 (Chondrogenic Supplement, R&D Systems). Pellets were then incubated at 37°C and 5% CO_2 for 2 weeks. Media was changed every 3 days for 2 weeks. Pellets were then washed carefully with PBS, fixed with

10% formalin (Sigma) for 45 minutes, and 5-7um frozen sections were obtained for staining. Slides were stained with Hematoxylin I solution (Richard-Allan Scientific) for 10 minutes, rinsed in tap water for 5 minutes, then incubated in 0.001% Fast Green (Fisher) solution for 5 minutes. 1% Acetic Acid (Fisher) was then added for 15 seconds, followed by 0.1% Safranin O solution (Fisher) for 5 minutes. Slides were rinsed and mounted for imaging using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

2.3.1.2. Results and Conclusions

Our goal for this preliminary evaluation of isolation methods was to ensure consistent results in MSC harvesting, particularly focusing on our success with the filter device. Our results showed that after 2 weeks of *in vitro* differentiation, all three methods successfully generated bone, fat and cartilage tissues (Figure 2.2). Alizarin Red S is a special stain that is used to establish the presence of calcium deposits within osteogenic cells; bright red coloration signals a positive stain (Fig. 2.2 A, B, C). Oil Red O is a fat-soluble dye used as a special stain for the identification of lipids within adipocytes; bright red vacuoles signal a positive stain (Fig. 2.2 D, E, F). Safranin-O is a special stain used to identify proteoglycans and sulfated glycosaminoglycans, major components of the cartilage ECM; a pink to dark red color signals a positive stain (Fig. 2.2 G, H, I).

The positive results of our preliminary study suggested to our team that for future MSC harvesting in the clinical setting, either the density gradient method or the filter device method would offer the most promise, as they require significantly less amount of time before a purified collection of MSCs can be obtained when compared to the explant technique.

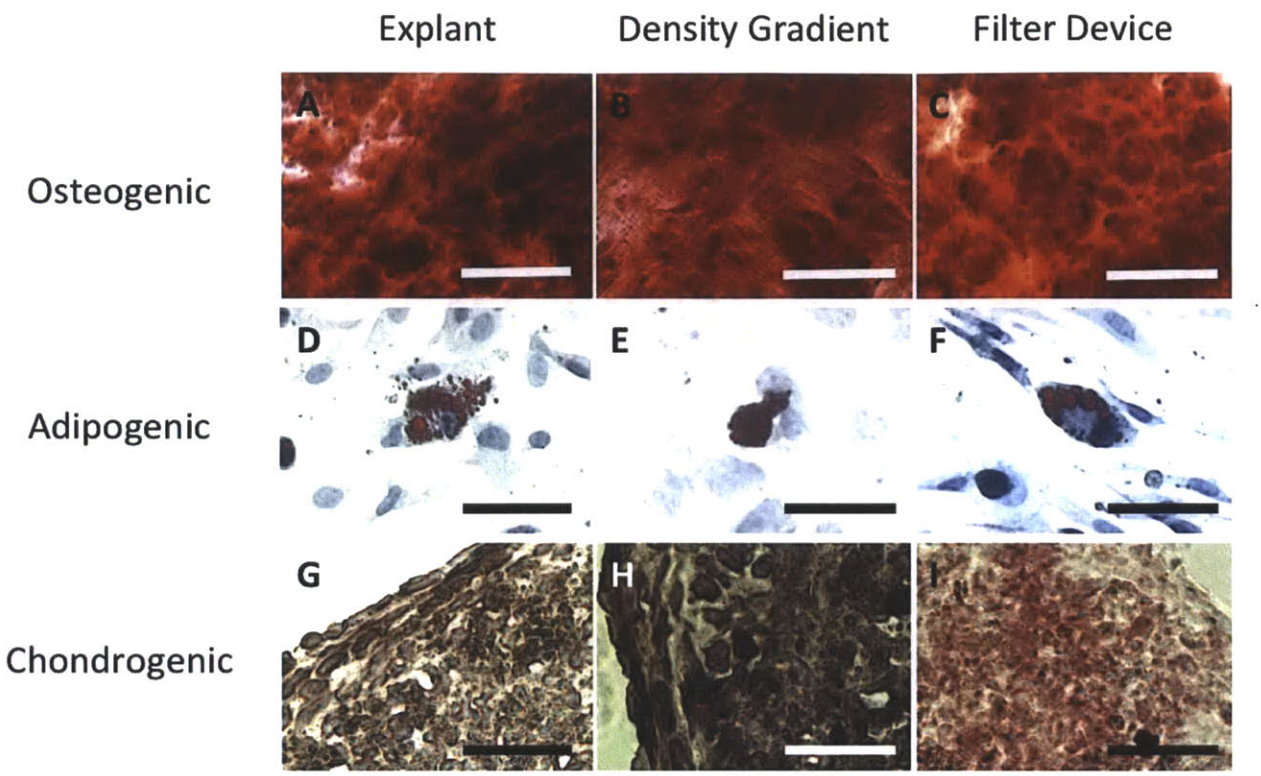

Figure 2.2. Sheep MSC differentiation assays. Each MSC isolation method (explant, density gradient and filter device) was assayed for cell differentiability to osteogenic, adipogenic and chondrogenic lineages. Panels **(A-C)** show osteogenic stains performed with Alizarin Red S (scale bar = 100 μm), red is a positive stain. Panels **(D-F)** show adipogenic stains performed with Oil Red O (scale bar = 50 μm), intracellular fat droplets in red signal a positive stain. Panels **(G-I)** show chondrogenic stains performed with Safranin O (scale bar = 50 μm), pink to dark red coloring signals a positive stain for cartilage proteoglycans and glycosaminoglycans.

2.3.2. Characterization of Human Tibial Bone Marrow-Derived Mesenchymal Stem Cells

Our ultimate clinical interest is to isolate MSCs from the proximal tibia for the regeneration of ligament, cartilage and other musculoskeletal tissues. To assess the properties of MSCs isolated from tibial bone marrow, an Institutional Review Board-approved clinical study was initiated with the collaboration of the Department of Orthopaedic Surgery at the Brigham and Women's Hospital. The objective of the study was to characterize the properties of MSCs obtained from the bone marrow of the proximal tibia to devise a single-stage procedure that could offer advantages to patients, including decreased morbidity and reduced costs.

Patients scheduled to undergo procedures that affect the proximal tibia were eligible for participation. These procedures included anterior cruciate ligament reconstruction (ACL), tibial tubercle osteotomy (TTO) and high-tibial osteotomy. These specific procedures were selected because during the normal course of the procedure the cortical bone of the proximal tibia has to be opened, thus providing easy access to the bone marrow, without causing additional trauma above and beyond that of the index surgical intervention. Sixteen patients aged 18 to 55 were enrolled and informed consented, since this patient cohort is the target population for eventual ligament and cartilage repair with the mesenchymal stem cell-based approaches being investigated in this thesis.

The tibial aspirates were processed via the density gradient centrifugation or with the filter device for MSC isolation, and the cells were characterized *in vitro* according to the minimal criteria set forth by the ISCT (117).

2.3.2.1. Materials and Methods

Cell isolation, the MSC differentiation assays, and the osteogenic and adipogenic staining assays were performed as described in the sheep preliminary study detailed in section 2.3.1.1.

Tissue harvest

Tibial bone marrow was harvested during ACL reconstructions or TTO procedures. After drilling on the tibia, a 14-gauge Angiocath Autoguard Shielded catheter (Becton Dickinson) was inserted into the drilled orifice. The guiding needle was removed, and a 10 ml syringe pre-coated with 5000 units of sodium heparin solution was adapted to the catheter. An assistant applied pressure to the surroundings of the catheter at the insertion site to prevent air leaks and 2 to 10 ml of bone marrow were suctioned into the syringe. The marrow was transferred to sodium heparin BD Vacutainer tubes, transported to the laboratory within 30-60 minutes of harvest and cell isolation performed. The harvests added less than 5 minutes to the entire surgical procedure.

Cartilage immunofluorescence staining

Cartilage pellets were washed carefully with PBS, fixed with 4% paraformaldehyde (Wako) for 20 minutes at room temperature, then washed twice with PBS for 5 minutes. Pellets were then placed in a cryomold (Sakura), embedded in OCT compound (Sakura), and rapidly frozen in a mix of dry ice and 100% ethanol. Serial 5-7 μm thick cross-sections were cut and mounted on Superfrost/Plus slides (Fisher). Slides were washed three times with PBS, then permeabilized and blocked with 0.3% TritonX-100 (Sigma), 1% BSA (Fisher), and 10% normal donkey serum (Jackson ImmunoResearch) for 45 min at room temperature. Slides were then incubated in goat anti-human aggrecan antibody (1:100, R&D Systems #962644) overnight at 4°C. Slides were then washed three times with PBS supplemented with 1% BSA for 5 minutes, then incubated for 1 hr with either donkey anti-goat FITC secondary antibody (1:200, JacksonImmunoResearch) or donkey anti-goat Alexa Fluor 555 (1:200, Invitrogen) at room temperature. DAPI (1:500, Sigma) counterstain was added for 10 minutes at room temperature. Slides were washed twice with PBS for 5 minutes, then mounted with Vectashield (Vector) and imaged with an Olympus IX70 fluorescent microscope and a SPOT digital camera and image processing software (Diagnostic Instruments).

Flow cytometry for surface marker identification

A BD Stemflow Human MSC Analysis Kit (BD Biosciences) was used with manufacturer's protocol. Briefly, human MSCs were detached from tissue culture flasks using Accutase Cell Detachment Solution (BD Biosciences) for 10 minutes at 37°C with gentle shaking, washed twice with sterile HBSS- - + 2% FBS, and resuspended in HBSS- - + 2% FBS at a concentration of 5 x 10^6 cells/ml. For each sample, seven 5 ml polystyrene FACS-ready tubes with cell-strainer cap were prepared with the following contents: tube (1) 5 μl Mouse Anti-Human CD90; (2) 5 μl PE Mouse Anti-Human CD44; (3) 5 μl PerCP-Cy 5.5 Mouse Anti-Human CD105; (4) 5 μl APC Mouse Anti-Human CD73; (5) Empty control; (6) 20 μl human MSC positive isotype control cocktail (mIgG1 for κFITC, κPerCP-Cy5.5 and κAPC), and 20 μl PE human MSC negative isotype control cocktail (mIgG1 for κPE and mIgG2a for κPE); (7) 20 μl human MSC positive cocktail (CD90 FITC, CD105 PerCP-Cy5.5, CD73 APC), and 20 μl PE human MSC negative cocktail (CD34 PE, CD11b PE, CD19 PE, CD45 PE and HLA-DR PE). 100 μl of the cell suspension was added to each tube and incubated in the dark for 30 minutes

on ice. The cells were then washed twice with HBSS- - + 2% FBS, and resuspended in 300 μl of HBSS-- + 2% FBS for analysis. An LSR Fortessa Cell Analyzer (BD Biosciences) was used.

2.3.2.2. Results

The human differentiation assays confirmed our belief that the differentiation potential of tibial bone marrow-derived MSCs is similar to that of hip-derived bone marrow MSCs (Figure 2.3). Both isolation methods, the density gradient and the filter device, produced plastic-adherent cells that when cultured in specific differentiation media yielded positive osteogenic (Fig. 2.3A and B, Alizarin Red S), adipogenic (Fig. 2.3C and D, Oil Red O), and chondrogenic (Fig. 2.3E and F, immunofluorescence with goat anti-human Aggrecan antibody) stains.

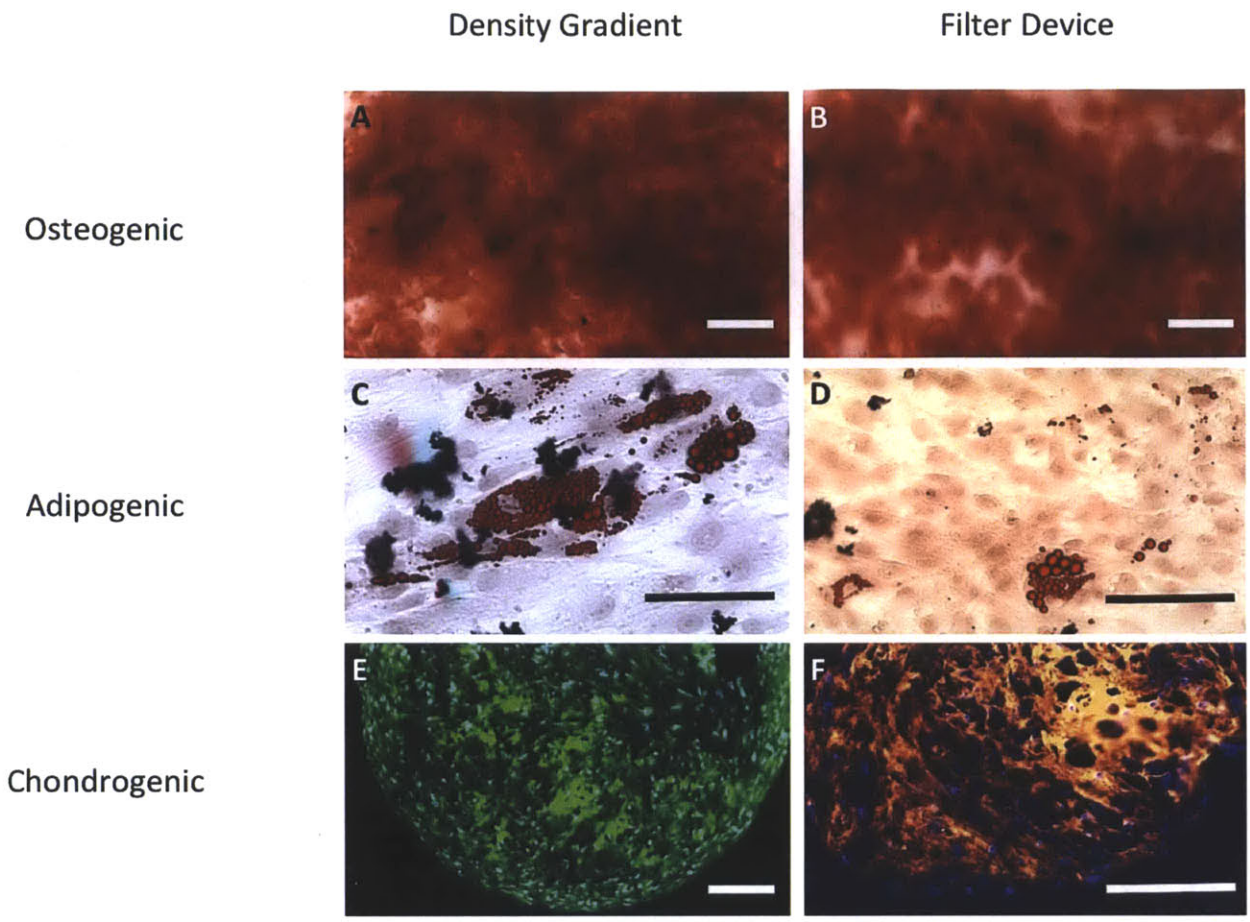

Figure 2.3. Human MSC differentiation assays. Density gradient and filter device isolations were assayed for cell differentiability to osteogenic, adipogenic and chondrogenic lineages. Panels **(A)** and **(B)** show osteogenic stains performed with Alizarin Red S (scale bar = 100 μm), red is a positive stain. Panels **(C)** and **(D)** show adipogenic stains performed with Oil Red O (scale bar = 100 μm), intracellular fat droplets in red signal a positive stain. Panels **(E)** and **(F)** show chondrogenic stains performed via immunofluorescence with goat anti-human aggrecan primary antibody, and FITC (green) or Alexa Fluor 555 (orange) secondary antibodies; DAPI nuclear stain is blue (scale bar = 100 μm).

In addition, flow cytometric analysis confirmed the presence and absence of the specified surface markers for human MSC identification as set forth by the ISCT guidelines (117) (Figure 2.4).

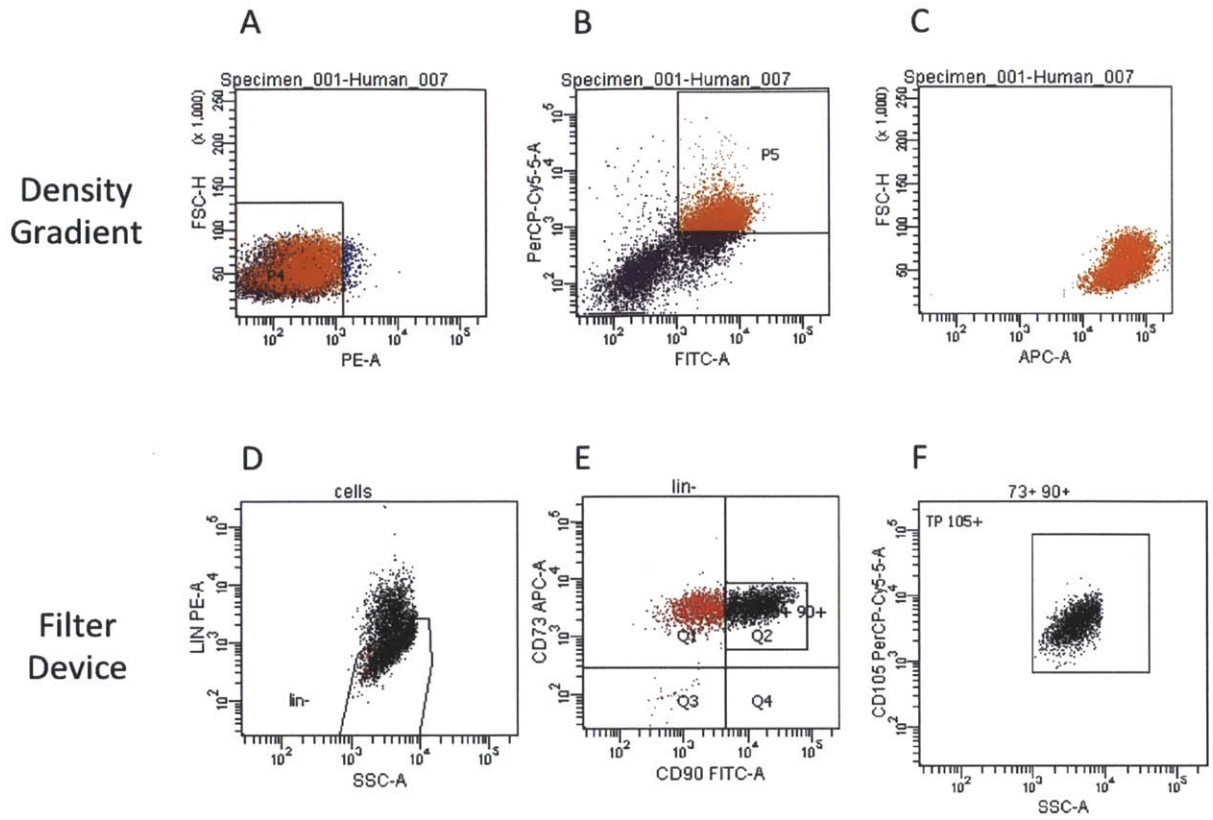

Figure 2.4. Flow cytometric analysis of tibial bone marrow-derived Human MSCs isolated via density gradient and filter device. Data confirm the presence of MSCs from both isolation techniques by surface marker analysis per the recommendations of the ISCT guidelines (117). Panels **(A)** and **(D)** show lineage negative cells for surface markers CD34, CD45, CD14, CD19 and HLA-DR for each isolation method. Panel **(B)** shows CD90+ and CD105+ cells from density gradient isolations. Panel **(C)** shows CD73+ cells from density gradient isolations. Panel **(E)** shows CD90+ and CD73+ cells, while panel **(F)** shows CD105+ cells, both from filter device isolations. Surface marker and fluorochrome pairings were as follows: PE (phycoerythrin) paired to CD34, CD45, CD14, CD19 and HLA-DR; APC (allophycocyanin) paired to CD73; FITC (fluorescein isothiocyanate) paired to CD90; PerCP-Cy 5.5 (Peridinin-chlorophyll proteins-cyanine 5.5) paired to CD105.

2.4. Conclusions and Next Steps

The identification of a MSC harvesting sites located close to the surgical site of common orthopaedic knee procedures is important in regenerative Orthopaedics, as it would improve surgical outcomes and reduce risks (113,114). As expected, our human tibial bone marrow study confirmed that MSCs are present in and have very similar properties to MSCs isolated from hip, humerus and femur marrow (113,114,124). More specifically, in contrast to other studies, our results characterized tibial marrow-derived MSCs in greater detail and followed the

guidelines established by the ISCT (113,114,117,124). Our observations suggest that marrow aspirates obtained from the tibia can be used to procure MSCs used in future tissue engineering techniques within regenerative Orthopaedics.

Our IRB-approved study is still active and currently enrolling patients to [1] optimize the intraoperative harvesting procedure for various types of surgeries, including TTOs and ACL reconstructions; [2] to determine the volume of harvested bone marrow that yields an optimal number of colony forming units of MSCs; and [3] to optimize the bone marrow processing timing for optimal isolation of viable MSCs. Furthermore, our team is considering expanding the study to include the characterization of distal femur bone marrow MSCs following the ISCT minimal criteria (117).

Chapter 3.

Effect on Ligament Marker Expression by Direct-Contact Co-Culture of Mesenchymal Stem Cells and Anterior Cruciate Ligament Cells (99)[†]

3.1. Abstract

Ligament and tendon repair is an important topic in orthopedic tissue engineering; however, the cell source for tissue regeneration has been a controversial issue. Until now, scientists have been split between the use of primary ligament fibroblasts or marrow-derived mesenchymal stem cells. The objective of this study was to show that a co-culture of anterior cruciate ligament (ACL) cells and mesenchymal stem cells (MSCs) has a beneficial effect on ligament regeneration that is not observed when utilizing either cell source independently. Autologous ACL cells (ACLc) and MSCs were isolated from Yorkshire pigs, expanded *in vitro* and cultured in multiwell plates in varying %ACL/%MSCs ratios (100/0, 75/25, 50/50, 25/75 and 0/100) for two and four weeks. Quantitative mRNA expression analysis and immunofluorescent staining for ligament markers Collagen-I, Collagen-III and Tenascin-C were performed. We show that Collagen-I and Tenascin-C expression is significantly enhanced over time in 50/50 co-cultures of ACLc and MSCs ($p \leq 0.03$), but not in other groups. In addition, Collagen-III expression was

[†] Chapter first published as an original article in Tissue Engineering Part A 18(23-24), 2549–2558, 2012, © Mary Ann Liebert, Inc., publishers. Included with permission.

significantly greater in MSC-only cultures ($p \leq 0.03$), but the Collagen-I to Collagen-III ratio in 50% co-culture was closest to native ligament levels. Finally, Tenascin-C expression at four weeks was significantly higher ($p \leq 0.02$) in ACLc and 50% co-culture groups compared to all others. Immunofluorescent staining results support our mRNA expression data. Overall, 50/50 co-cultures had the highest Collagen-I and Tenascin-C expression, and highest Collagen-I to Collagen-III ratio. Thus, we conclude that using a 50% co-culture of ACL cells and MSCs, instead of either cell population alone, may better maintain or even enhance ligament marker expression and improve healing.

3.2. Introduction

Injuries to the anterior cruciate ligament (ACL) represent one of the most common sports-related injuries and the most common ligament injury in the knee (17,18). In the United States there are at least 100,000 ACL repairs performed each year (19). For these reasons, the development of techniques that promote the healing and repair of ligaments and tendons is of increased importance in orthopedics.

Tissue engineering principles have recently been applied to the development of novel ligament regeneration techniques; the ideal cell source, however, has been a controversial topic within orthopedic tissue engineering. Initial studies, focused on defining the optimal individual cell source for ligament regeneration, are divided between the use of primary ACL cells (ACLc) and mesenchymal stem cells (MSCs) (18,21,125). However, new evidence suggests there are advantages to the use of a co-culture system. Recent reports have established that indirect co-culture of MSCs and ligament/tendon cells results in an increased expression of ligament/tendon markers Collagen type I (Collagen-I), Collagen type III (Collagen-III) and Tenascin-C in the MSCs (64,67,68). It was also observed that cell proliferation, DNA content and collagen production were all increased in MSCs as compared to non-co-cultured controls; the induction of differentiation of MSCs towards a ligament lineage was also noted (64,67-69). Direct cell-cell contact between MSCs and a variety of other differentiated cell populations has proven to be a key determinant of the fate and effect of MSCs in culture (126,127). It has been observed that cell-cell contact between MSCs and certain fibroblast populations can induce phenotypic changes in the fibroblast (126,127). Furthermore, recent studies have shown that MSCs have

anti-inflammatory and immunomodulatory properties *in vivo* (73-75). These observations have led some investigators to suggest that MSCs may aid tissue regeneration in more ways than previously believed (70-72). By introducing co-culture systems, scientists may be able to enhance the regeneration of tissues by exposing two or more cell populations to each other's regulatory molecules naturally and simultaneously, or at a sequence dictated by the cells themselves. All of these studies lead to the conclusion that MSCs can serve both as a cell source for tissue regeneration and/or as a source of regulatory cues for the differentiation of other cell types.

More recent studies have identified the presence of MSC-like cells in several tissues, including ligament and tendons (70-72,80,81). In the body, MSCs appear to function as support cells that can differentiate into specific cell types to regenerate injured tissues; and can promote regeneration by providing regulatory cues to native tissue cells that promote angiogenesis, stimulate progenitor cells from within the injured tissue, and reduce apoptosis and scar formation (71).

This study was based on the premise that ACLc and MSC co-cultures can initiate a regenerative response that could stimulate ACL tissue to enhance its own repair. Scientists in the field of ligament tissue engineering use the expression of Collagen-I, Collagen-III and Tenascin-C as a tool to evaluate the degree of ligament regeneration (4,64,67-69,128). Microstructurally, close to 94% of ACL tissue is composed of fascicles of collagen fibers, with the remaining 6% comprised of cells and additional extracellular matrix components, including Tenascin-C (2,129). Moreover, approximately 90% of collagen in ACL is type I, with Collagen-III comprising the remaining 10% (2-4). Our objective was to determine the effects of direct co-culture of a variety of ratios of ACLc and marrow-derived MSCs on the overall expression of ligament markers *in vitro* as a way to elucidate the optimal cell ratio for future ligament tissue engineering studies. We hypothesized that a co-culture of ACLc and MSCs would potentially enhance the expression of ligament markers Collagen-I, Collagen-III and Tenascin-C when compared to ACLc or MSCs cultured independently.

3.3. Materials and Methods

Tissue harvest and cell isolation

ACL tissue and bone marrow were harvested from 4-8 month old Yorkshire pig legs purchased from a local butcher house. First, the ACL was isolated by an improvement on the protocol first developed by Nagineni and adapted by others (21,130-132). ACL tissue was aseptically dissected and washed in sterile Hank's Buffered Salt Solution, Calcium and Magnesium free (HBSS--, Invitrogen) and then placed in a Petri dish (BD Falcon) with fresh HBSS--. Two No. 21 blade scalpels were used to gently scrape off remaining synovial tissue from the surface of the ligament. The tissue was transferred to a second dish with fresh HBSS-- and scraping was repeated gently. Subsequently, the tissue was transferred to a new dish with 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 15% fetal bovine serum (FBS, Gibco), 1% L-glutamine (Gibco), and 1% antibiotic mixture (Gibco) (ACL DMEM). The tissue was minced with scissors for approximately 10 minutes until all pieces were <1mm long axis length, and then digested in a mixture of 2.4 U/ml Dispase-II (Roche) and 10 mg/ml Collagenase D (Roche) solution for 60 min in a 37°C shaker. Next, the resulting cell solution was filtered through a 100 μm strainer (BD Falcon) and centrifuged at 260 g for 6 minutes. The ACLc obtained were resuspended in ACL DMEM and seeded at a density of $27x10^3$ cells/cm^2 in T-75 tissue culture flasks (BD Falcon).

Secondly, bone marrow was aspirated from the distal femoral end of the same pig leg as previously described (124). Briefly, 3-4 ml of BM were aspirated with a 16-gauge needle attached to a syringe with 0.5 ml sodium heparin (APP Pharmaceuticals) and seeded in T-75 flasks at a 1 ml BM to 9 ml media ratio with DMEM with 10% FBS and 1% antibiotic mixture (BM DMEM). Bone marrow-derived mesenchymal stem cells were isolated by their ability to attach to tissue culture plastic (124).

All cells were grown to passages 2 or 3. Viability was determined by the trypan-blue (Sigma) exclusion method. MSCs were not allowed to reach more than 60-70% confluency before passaging, while ACLc were allowed to reach 100% confluency. Culture medium was changed twice a week for all cell types.

Osteogenesis

MSCs were seeded in 24-well plates (BD Falcon) at a density of 4.2 x 10^3 cells/cm^2 and cultured in BM DMEM until 50-70% confluency was reached. Medium was then replaced with osteogenic differentiation media composed of a-MEM Basal Medium (Gibco) supplemented with dexamethasone, ascorbate, and b-glycerophosphate (Osteogenic Supplement, R&D Systems). Media was changed every 3 days for 2 weeks. Cultures were then washed carefully with HBSS--, fixed with 10% formalin (Sigma) for 45 minutes and rinsed with distilled water. Alizarin Red S staining solution was freshly made with 2g Alizarin Red S (EMD Chemicals) in 100ml distilled water, adjusting pH to 4.1-4.3 with 0.1% NH_4OH. Cell monolayers were then covered with staining solution and incubated at room temperature in the dark for 45 minutes. Wells were then rinsed with distilled water and imaged using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

Adipogenesis

MSCs were seeded in 24-well plates (BD Falcon) at a density of 2 x 10^4 cells/cm^2 and cultured in BM DMEM until 100% confluency was reached. Medium was then replaced with adipogenic differentiation media composed of a-MEM Basal Medium (Gibco) supplemented with hydrocortisone, isobutylmethylxanthine and indomethacin in 95% ethanol (Adipogenic Supplement, R&D Systems). Media was changed every 3 days for 2 weeks. Cultures were then washed carefully with HBSS--, fixed with 10% formalin (Sigma) for 45 minutes and rinsed with distilled water. Wells were then covered with 60% isopropanol for 5 minutes and then stained with 0.3% Oil Red O staining solution for 15 minutes at room temperature. Wells were then rinsed with distilled water and imaged using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

Chondrogenesis

250,000 MSCs were transferred to a 15mL conical tube and centrifuged at 200 x g for 5 minutes at room temperature. Medium was discarded, cells then resuspended in 0.5 mL of chondrogenic differentiation medium and centrifuged at 200 x g for 5 minutes without discarding medium. Chondrogenic differentiation medium was composed of DMEM/F-12 basal medium

(Gibco) supplemented with 1% ITS supplement (R&D Systems) and dexamethasone, ascorbate-phosphate, proline, pyruvate and TFG-b3 (Chondrogenic Supplement, R&D Systems). Pellets were then incubated at 37°C and 5% CO_2 for 2 weeks. Media was changed every 3 days for 2 weeks. Pellets were then washed carefully with PBS, fixed with 10% formalin (Sigma) for 45 minutes, and 7um frozen sections were obtained for staining. Slides were stained with Hematoxylin I solution (Richard-Allan Scientific) for 10 minutes, rinsed in tap water for 5 minutes, then incubated in 0.001% Fast Green (Fisher) solution for 5 minutes. 1% Acetic Acid (Fisher) was then added for 15 seconds, followed by 0.1% Safranin O solution (Fisher) for 5 minutes. Slides were rinsed and mounted for imaging using an Olympus IX70 microscope equipped with a SPOT digital camera and image processing software (Diagnostic Instruments).

Co-culture assay

Direct contact co-culture between ACLc and MSCs was performed using tissue culture treated multiwell plates (BD Falcon). Five groups: 100% ACLc/0% MSCs (n=4), 75% ACLc/25% MSCs (n=3), 50% ACLc/50% MSCs (n=4), 25% ACLc/75% MSCs (n=3), 0% ACLc/100% MSCs (n=4), were examined in multiwell plates seeded at 2,500 total cells/cm^2 for 14 and 28 days. A control with 50% ACLc/0% MSCs (total of 1,250 ACLc/cm^2, n=3) was also examined. ACL DMEM was used for all co-culture experiments and was changed twice per week.

Quantitative RT-PCR

Expression of ligament markers Collagen-I, Collagen-III and Tenascin-C was quantified by real-time PCR. Total RNA was isolated from cells freshly harvested from ACL tissue (n=3) and from each well at 14 and 28 days of co-culture using an RNeasy Plus Midi kit (Qiagen) in accordance with the manufacturer's protocol. Total RNA (1 μg per sample) was then reverse-transcribed in a 20 μl total reaction volume using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's specifications. The 20 μl samples were loaded onto a Mastercycler (Eppendorf) with the following program: 25°C 10 min → 37°C 120 min → 85°C 5 min. Next, 2 μl of a 1:10 dilution of the RT reaction product was subjected to quantitative PCR (qPCR) using an iQ SYBR Green Supermix kit in 20 μl reactions (Biorad). A C100 Thermal Cycler (Biorad) was used with the following program: 95°C 3 min, 1 cycle; 95 °C

10 sec → 64 °C 30 sec, repeat for 40 cycles total; 95 °C 10 sec → 65 °C 5 sec, 1 cycle. 18s rRNA was used as the housekeeping gene for normalization. Preliminary mRNA studies showed that 18s rRNA levels remained stable over time and across ACLc, MSCs and ACLc/MSCs co-cultures (data not shown). Custom forward and reverse gene-specific primers for Collagen-I, Collagen-III, Tenascin-C and 18s rRNA are shown in Table 3.1. Pig liver tissue was used as a negative control. Dissociation and amplification curve analysis was performed with the Biorad CFX Manager software. Gene expression was obtained using the Pfaffl method to account for individual reaction efficiencies using freshly harvested native ACLc as the calibrator sample (133). For freshly harvested native ACLc expression analysis, pig liver was used as the calibrator sample.

Immunofluorescence

Wells were fixed with 4% paraformaldehyde (Wako) for 20 min at room temperature, washed with PBS, then permeabilized and blocked with 0.3% TritonX-100 (Sigma), 1% BSA (Fisher), and 10% normal goat serum (Jackson ImmunoResearch) for 45 min at room temperature. Mouse primary antibodies for Collagen-I (1:250, Cat. no. C2456, Sigma), Collagen-III (1:200, Cat. no. ab6310, Abcam), and Tenascin-C (1:250, Cat. no. ab88280, Abcam) were added to respective wells and incubated overnight at 4°C. Goat anti-mouse Cy3 secondary antibody (1:500, Cat. no. 115-165-062, Jackson ImmunoResearch) was added for 60 minutes at room temperature. DAPI (1:500, Sigma) counterstain was added for 10 minutes at room temperature. Images of each well were then obtained with an Olympus IX70 fluorescent microscope equipped with an Olympus UM4-100 7A Cy3-710 fluorescent light filter, and a SPOT digital camera and image processing software (Diagnostic Instruments). Orange coloring in all images represents Cy3 staining.

Data analysis

Quantitative PCR was performed in triplicate per group per pig. Collagen I/ Collagen III and Collagen I/ Tenascin-C ratios within each sample were calculated using relative expression (2^{-CT}), since their primer efficiencies were all above 97%. No control genes were considered in this case, as the calculation of a direct ratio does not need normalization. Statistical significance was calculated using a student's T-test. P values <0.05 were considered significant.

Table 3.1. Custom Pig Primer Sequences for Quantitative RT-PCR

Target	Forward Sequence	Reverse Sequence
Collagen-I	5'-CCTGGCTCTAGAGGTGAACG-3'	5'-AGGATTACCCACAGCACCAG-3'
Collagen-III	5'-TTGGCCCTGTTTGCTTTTTA-3'	5'-TGGTTGACAAGATGAGAACAAAA-3'
Tenascin-C	5'-TTAAGTACGCGCCCATCTCT-3'	5'-CCTTCACAGCAGACACTCCA-3'
18s rRNA	5'-TCGCGGAAGGATTTAAAGTG-3'	5'-AAACGGCTACCACATCCAAG-3'

3.4. Results

Optimized primary ACL harvest

We find it important to note the difficulties we encountered in our initial attempts at harvesting ACLc using previously published methodologies, and the subsequent improvements we made to these protocols (21,130-132). First, we found that mincing the ACL tissue with scissors in ACL DMEM resulted in an increased number of viable cells recovered as compared to the published method of doing so in HBSS-- (data not shown). In addition, using only collagenase was not sufficient for digestion if done for 2 hours or less, and if done for more than 2 hours it resulted in cell suspensions with low viability (<60%). The combination of collagenase and dispase for 1 hour, as previously described by our laboratory for muscle tissue (134), yielded a high-quantity of healthy fibroblasts (an average of $4x10^6$ +/- $1.5x10^6$ cells per 1 pig ACL with 90%+ viability) in a significantly smaller amount of time. Thus, we have established an improved protocol for primary ACL cell isolation, where the midsubstance of a ligament is cleaned of non-ligamentous tissue, minced in ACL DMEM and digested in a solution of 0.3% Dispase-II and 1% Collagenase D for 60 minutes.

Cell characterization

The morphology of the ACLc isolated and used throughout the study is fibroblastic in nature, as is typical of ACLc (Figure 3.1 A and 3.1 C). In addition, cells freshly isolated from ACL tissue expressed ligament makers Collagen-I, Collagen-III and Tenascin-C, further

characterizing them as ACL fibroblasts (Figure 3.2 D). MSCs isolated from bone marrow also showed the expected fibroblastic morphology (Figure 3.1 B and 3.1 E), as well as the ability to differentiate into osteogenic, adipogenic and chondrogenic lineages (Figure 3.2 A-C).

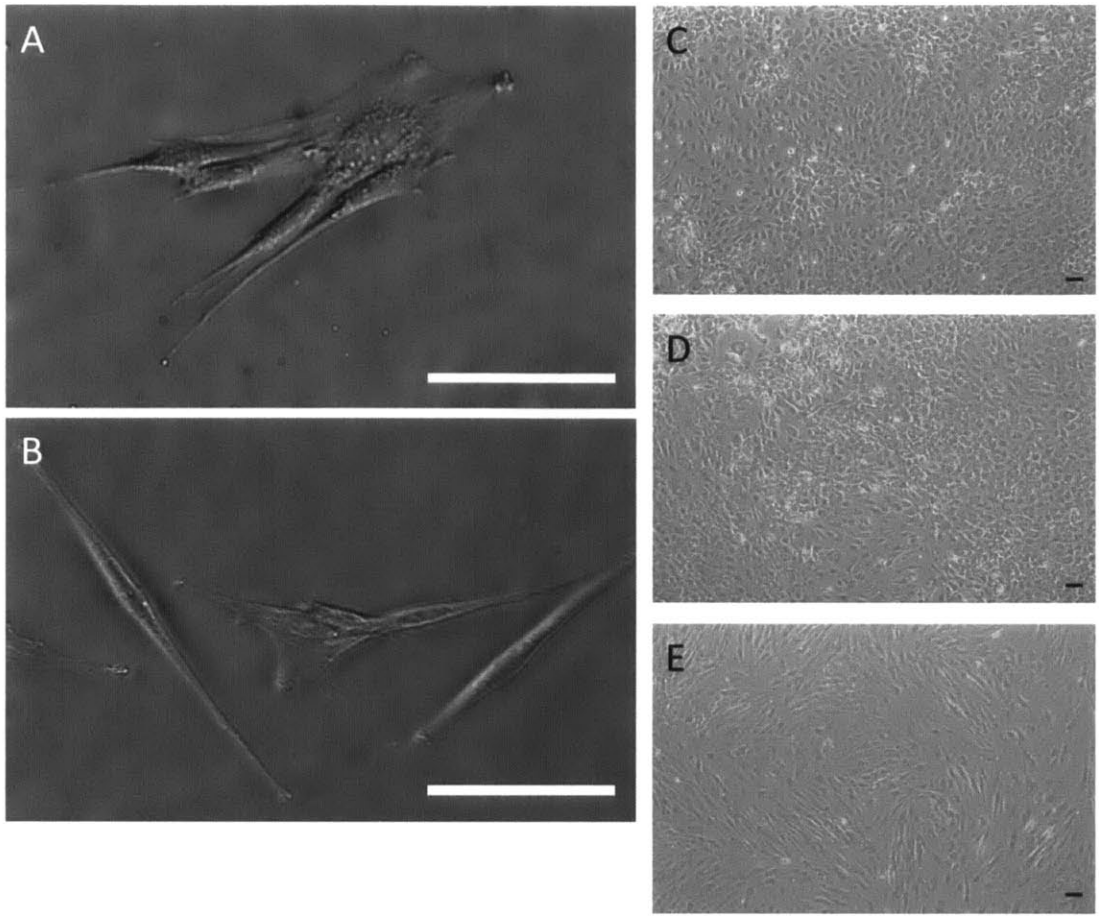

Figure 3.1. *In vitro* morphological characterization. **(A)** MSCs isolated from Pig bone marrow show fibroblastic morphology. **(B)** ACL fibroblasts isolated from Pig ligament. Day-14 unstained cultures of 100% ACLcs **(C)**, 50% ACLcs 50% MSCs, **(D)** and 100% MSCs **(E)**. Scale bar = 100 μm. ACL, anterior cruciate ligament; ACLcs, ACL cells; MSCs, mesenchymal stem cells.

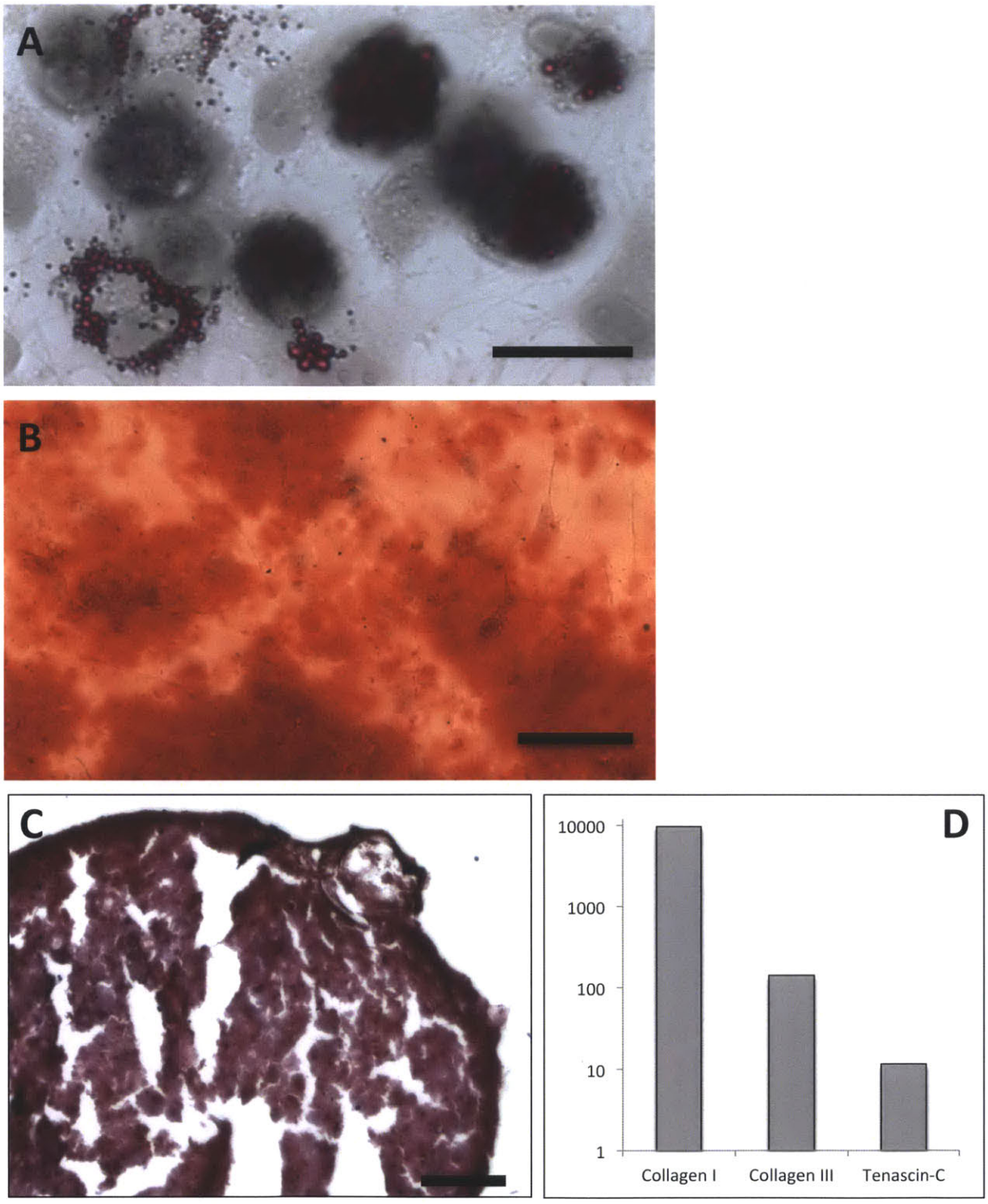

Figure 3.2. Fibroblast characterization. Pig MSCs differentiability to **(A)** adipogenic (scale bar = 25 μm), **(B)** osteogenic (scale bar = 100 μm), and **(C)** chondrogenic (scale bar = 100 μm) lineages. **(D)** Log_{10} of the fold expression of ligament markers from freshly harvested ACL fibroblasts calibrated with Pig hepatocytes.

Quantitative effect of co-culture on ligament marker expression

Results show that at day 14 the expression of Collagen-I was statistically similar across cultures ($p>0.05$, Figure 3.3 A). At day 28 the 50% ACLc/50% MSCs sample was the only one to show a significant increase in Collagen-I expression ($p\leq0.02$), and the level of Collagen-I expression was significantly higher than any other culture group ($p\leq0.01$). Collagen-III expression was significantly higher in the MSCs alone throughout the experiment ($p\leq0.03$), and expression remained relatively constant across groups from 14 to 28 days (Figure 3.3 B). At 14 days, Tenascin-C expression was significantly higher ($p\leq0.02$) only in ACLc alone compared to all other samples (Figure 3.3 C). By day 28, the 50% ACLc/50% MSCs sample was the only group that showed a statistically significant increase in Tenascin-C expression ($p\leq0.03$). Moreover, at 28 days ACLc alone and 50% ACLc/50% MSCs samples had significantly higher Tenascin-C expression compared to all other samples ($p\leq0.02$), and the difference in their expression at this time point was not statistically significant ($p=0.4$). Collagen-I to Collagen III and Collagen-I to Tenascin-C expression ratios for all samples were compared to published ratios for native ACL ligament (Figures 3.3 D and 3.3 E). In native ACL tissue, the Collagen-I to Collagen-III ratio is approximately 9:1, while the Collagen-I to Tenascin-C ratio is greater than 15.7:1 (2-4,135). The highest ratio of Collagen-I to Collagen-III was observed in the 50% co-culture group with a value of 5.8, while the lowest ratio was seen in the MSCs alone group at 0.8. The 75% ACLc/25% MSCs had the second highest Collagen-I to Collagen-III ratio at 5.4, however the overall expression of both markers in this group was markedly lower than the 50% co-culture group throughout the experiment. For Collagen-I to Tenascin-C ratios, the highest value was seen in the 25% ACLc/75% MSCs group at 6.5; while the lowest value was seen in the ACLc alone group at 2.2, with the 50% co-culture group a close second at 3.2. Primer efficiencies for qPCR gene expression analysis were 90.4% for 18s rRNA, 97.5% for Collagen-I, 97.6% for Collagen-III, and 98.7% for Tenascin-C.

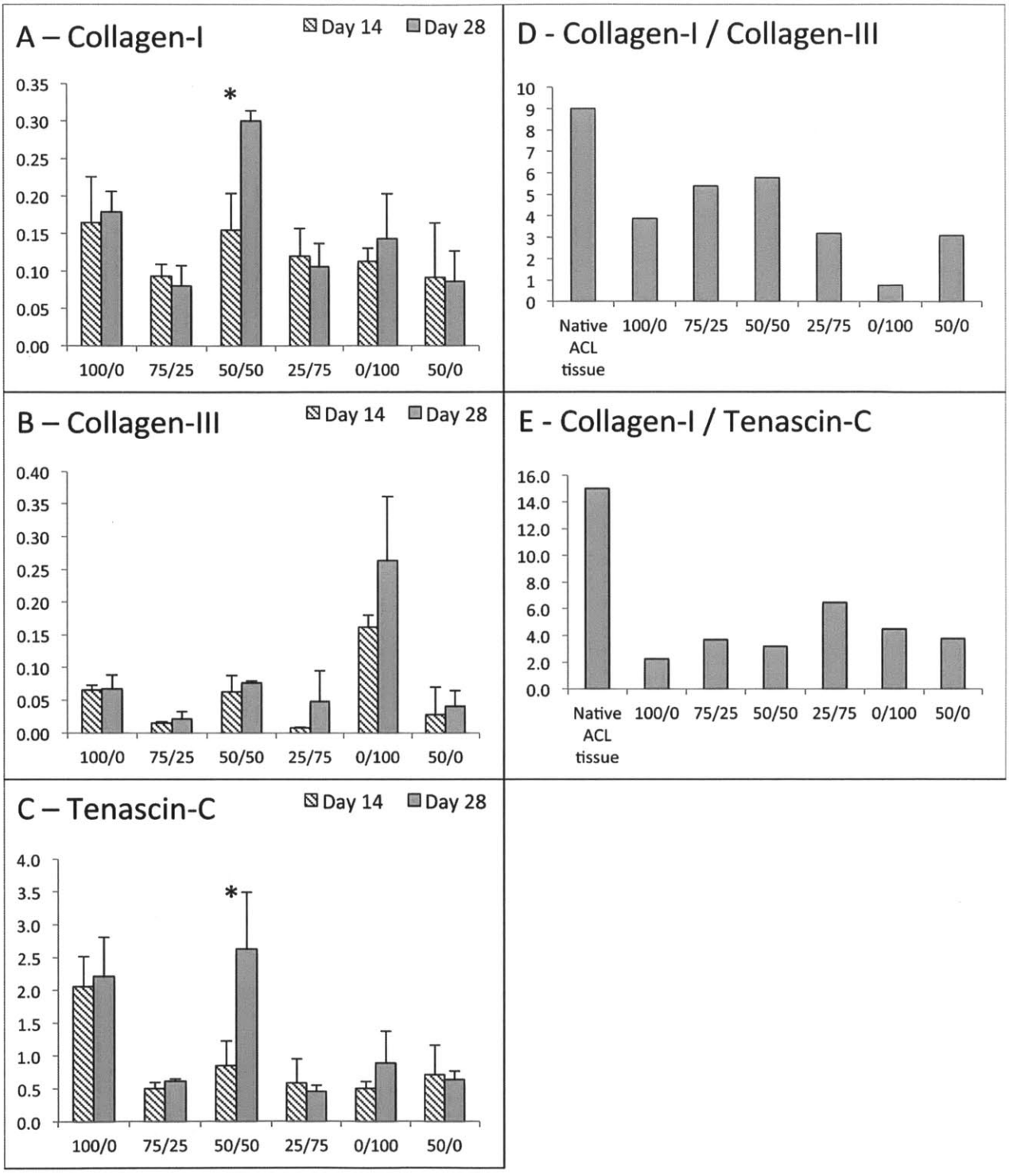

Figure 3.3. Fold expression of **(A)** Collagen-I, **(B)** Collagen- III, and **(C)** Tenascin-C at 14 and 28 days. **(D)** Collagen-I- to-Collagen-III ratios for native ACL tissue and all co- culture conditions. **(E)** Collagen-I-to-Tenascin-C ratios for native ACL tissue and all co-culture conditions. X-axis labels indicate the %ACLcs/%MSCs ratio during co-culture. mRNA expression was detected using quantitative PCR and normalized to 18s rRNA. For 100/0, 50/50, and 0/100, n=4, and all others n=3. * indicates statistically significant increase with $p < 0.05$. Means – SD shown. Collagen-I, Collagen type I; Collagen-III, Collagen type III.

Extracellular matrix ligament marker expression

At 14 days, Collagen-I staining was similar across all samples (Figure 3.4 A); by day 28, Collagen-I staining intensity was clearly increased in all samples, except in MSCs alone (Figure 3.5 A). At 14 days, Collagen-III staining was negligible in all samples (Figure 3.4 B); by day 28, only the MSCs alone sample showed increased Collagen-III staining intensity (Figure 3.5 B). At 14 and 28 days, Tenascin-C staining was more intense in ACLc alone and 50% ACLc/50% MSCs samples, while MSCs alone showed minimal Tenascin-C staining at both time points (Figures 3.4 C and 3.5 C). Unstained day 14 cultures for ACLc alone, 50% ACLc/50% MSCs and MSCs alone are shown in Figure 3.1 C-E. Native ACL tissue staining for the three markers is shown in Figure 3.4 D.

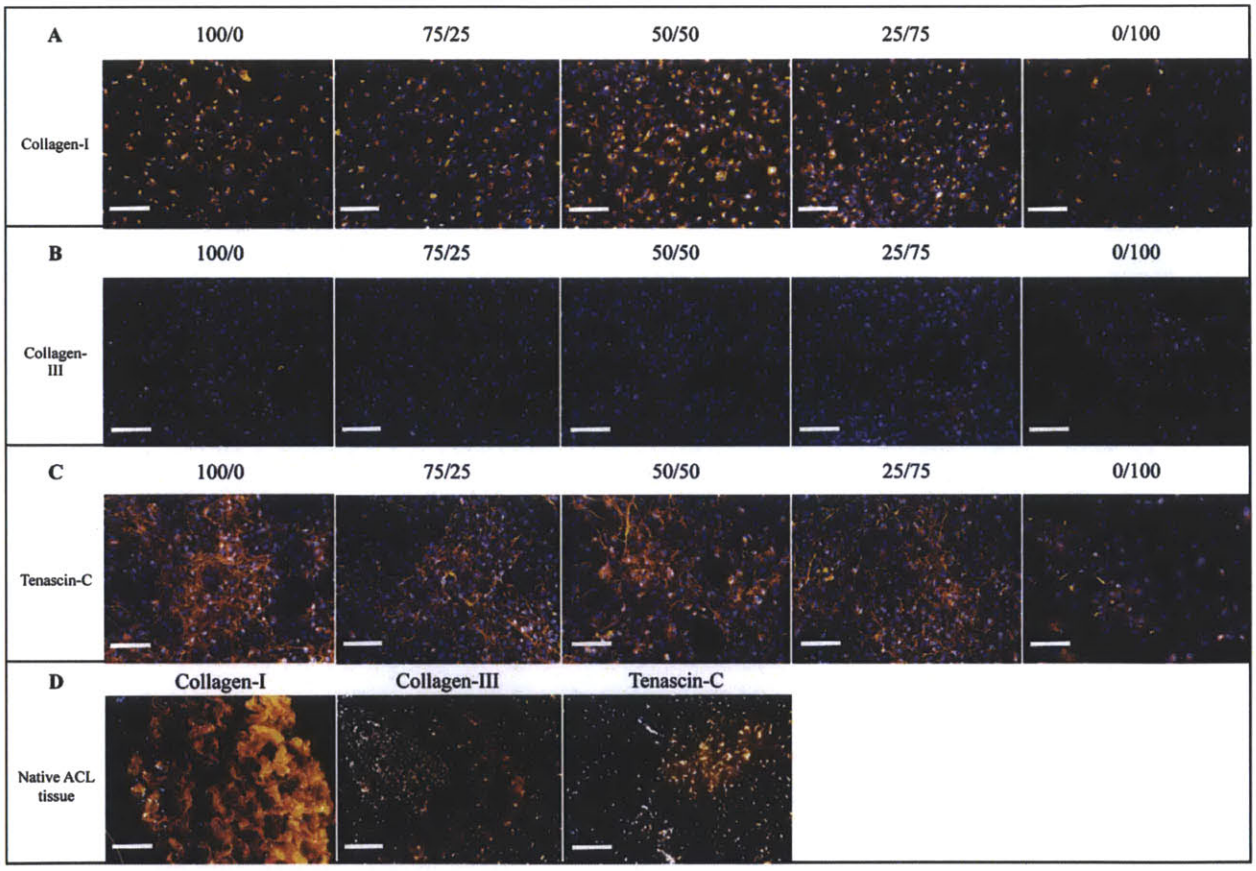

Figure 3.4. Ligament marker expression for **(A)** Collagen-I, **(B)** Collagen-III, and **(C)** Tenascin-C at 14 days. Column labels indicate the %ACLcs/%MSCs ratio during co-culture. **(D)** Native Pig ACL stains. Orange coloring represents primary antibody, blue coloring represents nuclear stain. Scale bar = 100 μm.

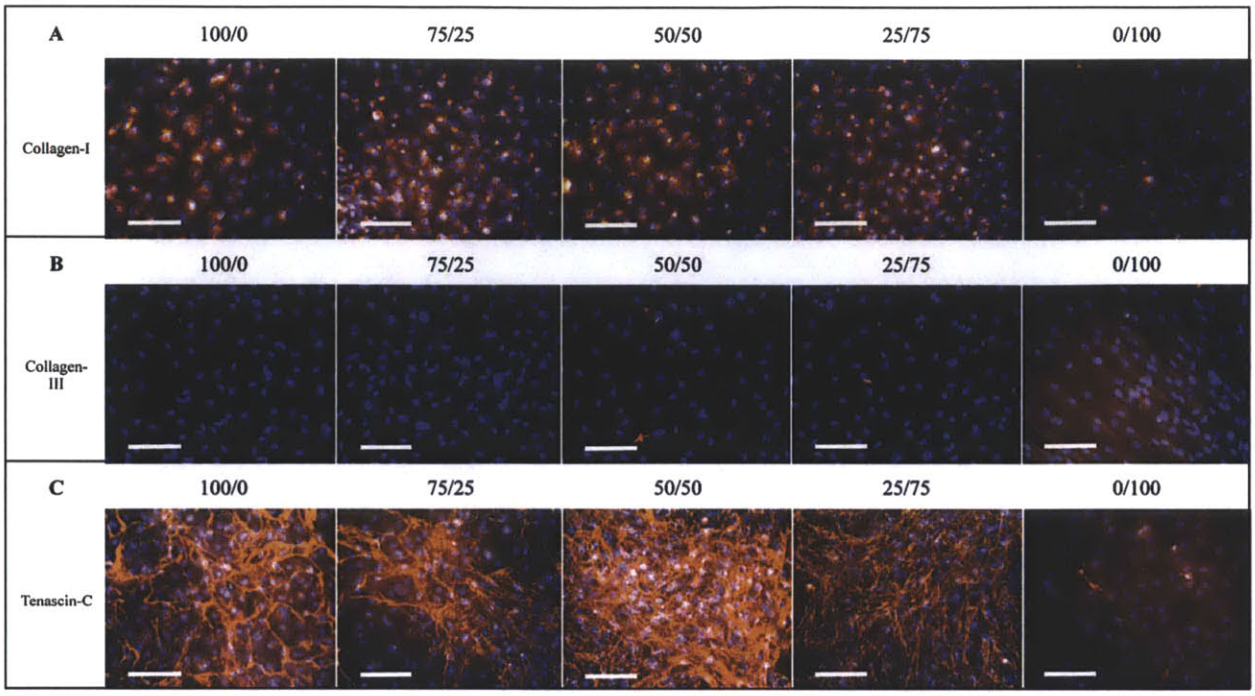

Figure 3.5. Ligament marker expression for (A) Collagen-I, (B) Collagen-III, and (C) Tenascin-C at 28 days. Column labels indicate the %ACLcs/%MSCs ratio during co-culture. Orange coloring represents primary antibody, blue coloring represents nuclear stain. Scale bar = 100 μm.

3.5. Discussion

A key component of the successful creation of a tissue-engineered ligament is the source of the cells from which regeneration is to occur. The goal of this study was to explore the effect of direct co-culture of several ACLc/MSCs ratios on the overall expression of ligament markers. Recent evidence suggests that co-culture systems may lead to enhanced regenerative responses, especially when MSCs are involved (64,67,68,126,127,136). However, the majority if not all of the studies focus on the differentiation potential of MSCs towards a specific lineage, and disregard the plethora of additional benefits MSCs can provide to a regenerative response (70-72). The ACL is a tissue that has limited regenerative potential, due in part to its restricted vascular supply and also to its inability to form a functional healing bridge between the injured or torn ends (7,11). Nevertheless, recent evidence suggests the tissue has the inherent capacity to heal (80), with MSC-like progenitor cells readily available in the tissue, although this resource must potentially be stimulated to induce a regenerative response.

Our results show that a direct co-culture of ACLc and MSCs leads to increased expression of ligament markers over 28 days. Collagen-I and Tenascin-C mRNA expression increased over time in 50% co-culture compared to ACLc or MSCs cultured alone, suggesting that *in vitro* extracellular matrix (ECM) formation of ACL cells may be enhanced in the presence of MSCs. Immunofluorescent staining supported our mRNA observations, with Collagen-I staining intensity becoming more widespread from 14 to 28 days, particularly in the ACLc and 50% co-culture groups; and Tenascin-C staining intensity increasing in ACLc alone and most remarkably on 50% co-culture. Collagen-I is the marker that stains most prominently in native pig ACL tissue, and the marker whose mRNA expression most remarkably increased when 50% ACLc/50% MSCs were co-cultured. This observation becomes more notable when considering that only half as many ACLc were seeded for the co-culture as compared to the ACLc alone group. The control with ACLc cells seeded in a well at the 50% density without MSCs did not show a Collagen-I expression response comparable to the 50% co-culture. In addition, Tenascin-C mRNA expression significantly increased over 28 days in 50% co-culture, a response not observed in any of the other groups. With the understanding that ECM deposition does not always follow patterns of mRNA expression, our gene expression and immunofluorescence data is complementary and suggests that MSCs in 1:1 co-culture with ACLc may enhance the expression of Collagen-I and Tenascin-C.

An unanticipated result from our study was the finding that the MSCs alone culture exhibited a significantly higher expression of Collagen-III throughout the experiment. Moreover, our finding that Collagen-III expression in ACLc-containing cultures remained relatively constant over time is consistent with previous reports (137). Collagen-III is a fibrillar component of the extracellular matrix of ACL that is universally located throughout the ligament but is most prominently found close to the attachment zones of the tissue (2,18). It comprises about 10% of all collagen content, and plays a key role during the early phases of ligament healing and during the ligamentization of tendon grafts undergoing remodeling after ACL reconstruction (2,115). Collagen-III is known for its ability to make strengthening intermolecular disulfide crosslinks in newly formed connective tissue ECM, and has been detected in high quantities at sites of injury during the early phases of the healing response in bone, ligament, and tendon (135). These responses during connective tissue healing may explain why we see MSCs alone as the group with the highest expression of Collagen-III, since mesenchymal cells that differentiate into bone,

cartilage and ligament cells tend to be the first to migrate to the injury sites of these tissues and produce the early matrix of the reparative response (135). These observations about Collagen-III suggest that MSCs could have a positive effect in healing during ACL regeneration. Perhaps, a significant presence of MSCs in a construct aimed at ligament regeneration may have a greater potential to initiate ligament healing and establish a structurally sound framework for the early ECM, while a similar presence of ACLc may be required for long-term deposition of the predominant and stronger Collagen-I. Taking all our conclusions into account, our results suggest that utilizing a 50% co-culture of ACLc and MSCs in future ACL tissue engineering studies may better maintain or enhance ligament ECM expression, and thus improve healing.

Native tissue studies have determined that about 90% of all collagen in ACL is type I, while the remaining 10% is type III, yielding a 9:1 ratio of Collagen-I to Collagen-III (2-4). This fact strengthens our conclusion that the 50% co-culture sample may be the best cell combination in future tissue engineering studies of the ACL, since it exhibited the highest Collagen-I to Collagen-III ratio of all samples at 5.8. Previous ligament healing studies have noted that, even after long-term follow-up, the ligament scar found at a site of injury exhibited close to a 30% decrease in normal collagen content and a significant increase in the relative amount of Collagen-III content (135). Thus, a ratio lower than the native level of 9 should not be unexpected in healing ligaments.

Tenascin-C is a glycoprotein found in tissues that experience high tensile and compressive stresses, such as ligament and tendons, and it is involved in the binding of cell surface receptors with the ECM (129). Even though its native levels in ACL tissue have not been determined to our knowledge, it is part of the 6% of ligament components that are not a form of collagen (94%) (2), making the true Collagen-I to Tenascin-C ratio in native ligament significantly higher than 94:6. For this reason our analysis estimated this native ratio to be at least 15. After tissue injury and during wound healing Tenascin-C levels have been shown to be transiently overexpressed (138,139). Because of this, high levels of the marker are not expected in native ligament tissue but are expected during a reparative response. Thus, the Collagen-I to Tenascin-C ratio would be expected to be large in native ligament and low in instances of ligament repair. Our mRNA analysis showed that the lowest ratios of Collagen-I to Tenascin-C were observed in the ACLc and 50% co-culture groups, 2.2 and 3.2 respectively. This result would imply that Tenascin-C is being overexpressed, which is expected in cells isolated from

ACLc that may detect a state of "injury" and are mounting a healing response. This observation also strengthens our support for the use of 50% co-cultures of ACLc and MSCs for tissue engineering ACL attempts.

A co-culture of ACLc and MSCs may lead to an enhanced regenerative response *in vivo* by providing not only a cell source from which cell differentiation can replenish an implanted scaffold, but also by the production of chemokines and cytokines that promote angiogenesis within the scaffolds, prevent scar formation, and awaken/recruit tissue-intrinsic progenitor cells (ACL-derived MSCs) to the scaffold to increase the regeneration. Furthermore, MSCs anti-inflammatory and immunomodulatory properties can provide a beneficial effect for biomaterial-based implants (84,90). Many of the problems caused by biodegradable scaffolds implanted in the body relate to the triggering of an immune response against the scaffold. MSCs could prevent the immune response and decrease related inflammation at the site by their anti-inflammatory effects, which in turn would protect the implant and enhance the repair. Ongoing studies in our laboratory are examining this effect in 3D scaffold culture, as well as attempting to elucidate the fate of MSCs in direct co-culture. An *in vivo* ACL regeneration model is also ongoing, where the immunomodulatory and anti-inflammatory roles of MSCs are being more directly assessed.

Chapter 4.

In vitro Development of Dynamic Three-Dimensional Co-Cultures of Mesenchymal Stem Cells and Anterior Cruciate Ligament Cells for Primary ACL Augmentation Repair

4.1. Rationale Behind 3D Co-Culture for Primary ACL Augmentation Repair

Anterior cruciate ligament (ACL) injuries are a common occurrence, particularly in athletes participating in team sports. The literature suggests an incidence greater than 1 in 3500 ACL injuries per year in the general population (24). Over the past decade, yearly ACL injuries and surgical reconstructions in the United States have been estimated at 200,000 and 100,000, respectively (9,18,21,22,24). Although ACL reconstructions have had great success (9,29-31), recent interest has shifted to the development of techniques that augment ruptured ACL and permit a primary repair (10,11,38,140,141). The shift is based primarily in the desire to [1] spare the remaining ligament innervation for proprioception at the knee joint, and [2] maintain the local vascularization, all in an attempt to improve long-term outcomes (9,10,28,56,140-143).

After an ACL tears, the ligament stumps have a tendency to retract (56,141), and are composed of inflamed and displaced tissue remnants (141,144). In order to perform a successful primary repair, biologically enriched ligament augmentation devices are likely required for success (11,140,141,145). Several groups have investigated the benefit of various biological enhancers to primary repair techniques, including Platelet-Rich Plasma (PRP) with collagen

sponges (11), and mesenchymal stem cell (MSCs) infiltration (10); however, the benefit of co-culture systems for primary ACL repair augmentation has yet to be investigated. Recently, our laboratory and others have shown that 1:1 co-cultures of MSC with primary ACL fibroblasts (ACL cells, ACLcs) have a beneficial and enhancing effect *in vitro* on the expression of ligament-associated markers and deposition of ligament extracellular matrix (97,99). Moreover, it has been observed that seeding MSCs in aligned scaffolds with or without dynamic tensional loading can stimulate MSC proliferation and differentiation towards a ligament lineage, with increased overall extracellular matrix deposition and enhanced expression of ligament-associated markers (146,147). Recent reports have shown that, in addition to promoting anti-inflammatory and immunomodulatory processes, MSCs may create a regenerative atmosphere surrounding a tissue injury that can enhance a healing response (70-75). Further, mononuclear cells including MSCs have been shown to add beneficial effects to other primary repair techniques (98). With these observations in mind, we wanted to explore the possibility of creating ligament augmentation constructs from MSC and ACL fibroblast co-cultures in 3D cell-hydrogel-polymer constructs. Our results in 2D suggest 1:1 co-cultures of MSCs and ACL fibroblasts may enhance ligament tissue engineering (99), hence confirmation of these observations in 3D is warranted.

Incorporation of cells into a hydrogel matrix for tissue engineering has been extensively investigated by our laboratory and others (124,148-152). One of the benefits of delivering cells in hydrogel suspensions is the creation of a 3D extracellular matrix-like environment around the cells that has been shown to help with the maintenance of cell phenotypes and the suitable deposition of tissue-specific ECM (148,149,151,153-155). Additionally, delivering cell co-cultures within a homogeneous hydrogel suspension allows for more uniform seeding of a solid biomaterial scaffold, and prevents cell loss to the surrounding medium *in vitro* or neighboring tissues *in vivo* (151,156-158). For our purposes, we selected a commercially available synthetic hydrogel composed of thiol-modified sodium hyaluronate and gelatin cross-linked with polyethylene glycol diacrylate (151,159). The hydrogel is stable *in vitro*, biocompatible, and has shown promise in orthopaedic regenerative applications (151,160).

For successful primary ACL augmentation repair, a construct must have sufficient mechanical stability to support joint function (44). To provide mechanical stability to our ligament augmentation construct, the hydrogel suspension was seeded on a biodegradable scaffold composed of a co-polymer of polyglycolic acid (PGA) and trimethylene carbonate

(TMC), known as polyglyconate (PGA:TMC). PGA is a rigid and crystalline biodegradable polymer used extensively in tissue engineering applications (124,152,161-166). It degrades primarily via hydrolysis and has a degradation time of between 4 to 12 months, depending on material configuration (164,166,167). TMC is a flexible, amorphous, non-acidic polycarbonate that is stable to hydrolysis and mostly undergoes enzymatic degradation (161,164,166-168). The block co-polymer PGA:TMC is FDA-approved and used commercially in Maxon™ sutures (Covidien), SURETAC III fixation devices (Smith & Nephew), and as SEAMGUARD® bioabsorbable staple line reinforcements and BIO-A® fistula plugs (W. L. Gore & Associates) (161,166,167). Polyglyconate is a customizable biomaterial in terms of interconnecting porosity, pore size, degradation rate and mechanical properties (161). It is also more biocompatible than many alternative synthetic scaffolds, such as poly-L lactide (PLLA) or polyglycolide (PGA), because of its decreased number of acid producing groups (161,167,169). Regarding degradation, polyglyconate has an intermediate rate of degradation compared to PGA or PLLA, with a degradation time of 6-12 months (161,165-167). This customizable biodegradation property, dependent on the percent composition of PGA or TMC in the co-polymer (161,170), is attractive for ligament augmentation, since the construct must be in place and functional long enough to support mechanical loading, while allowing native tissue ingrowth, maturation and load transfer to the regenerated ligament over time (171,172).

As tissue engineering began to be applied to ligament regeneration, investigators have been interested in the effects of mechanical stimulation on cell behavior (173,174). Several studies have observed the positive effect of mechanical tensional loads on ligament engineering, including enhanced biomechanical properties, cell proliferation and differentiation, matrix deposition and tissue orientation, among other benefits (68,69,146,173-184). In the present study our intention was to subject the constructs to dynamic tensional loading conditions, previously observed by others to be beneficial for ligament regeneration (173,177,178,183), to assess the response of ACL cell-MSC co-cultures in three-dimensions. Our intent was to mimic *in vitro* the active environment an augmentation construct would experience *in vivo* in a joint after implantation. This multi-component approach using co-cultures, hydrogels and dynamically loaded scaffolds is a feature that may allow our technique to succeed where others have failed (10,11,40,52,57). Our objective was to determine the *in vitro* effects of the MSC-ACL cell co-culture under dynamic tensional loading while seeded in three-dimensional constructs using

hydrogels as the cell delivery vehicle onto biodegradable and mechanically sound polymer scaffolds. We hypothesized that the 3D co-culture constructs under tensional loading would exhibit enhanced expression of ligament-associated markers. Our hope is to develop the framework of a technique that can successfully allow the primary repair of ACL injuries off-the-shelf and without the use of autografts or allografts.

4.2. Materials and Methods

Tissue harvest and cell isolation

ACL tissue and bone marrow were harvested from 6-8 month old Yorkshire pig legs purchased from a local butcher house. The ACL fibroblasts (ACLcs) and MSCs were isolated as previously described (99). Briefly, ACL tissue was aseptically dissected and washed in sterile Hank's Buffered Salt Solution, Calcium and Magnesium free (HBSS--, Invitrogen). No. 21 blade scalpels were used to gently scrape off synovial tissue from the surface of the ligament in a dish. The tissue was transferred to a new dish with 10 ml of Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 15% fetal bovine serum (FBS, Gibco), 1% L-glutamine (Gibco), and 1% antibiotic mixture (Gibco) (ACL DMEM), and minced with scissors for 8-10 minutes until all pieces were <1mm long axis length. Digestion of the tissue was performed in a solution of 2.4 U/ml Dispase-II (Roche) and 10 mg/ml Collagenase D (Roche) for 60 min in a 37°C shaker. The cell solution was filtered through a 100 μm strainer (BD Falcon) and centrifuged at 260 g for 6 minutes. The cells obtained were resuspended in ACL DMEM and seeded at a density of $27x10^3$ cells/cm^2 in T-75 tissue culture flasks (BD Falcon). Bone marrow was aspirated from the distal femur of the same pig leg as previously described (124). Briefly, 3-5 ml of BM were aspirated with a 16-gauge needle attached to a syringe with 0.5 ml sodium heparin (APP Pharmaceuticals), diluted 1:9 in DMEM with 10% FBS and 1% antibiotic mixture (BM DMEM), and seeded in T-75 flasks. Bone marrow-derived mesenchymal stem cells were isolated by their ability to attach to tissue culture plastic (124).

Cells were passaged using 0.25% Trypsin/0.9 mM ethylenediaminetetraacetate (EDTA) (Gibco) and seeded in 1:3 to 1:4 split ratios in T-175 flasks (BD Falcon). All cells were grown to passage 2. MSCs were not allowed to reach more than 60-70% confluency before passaging,

while ACL cells were allowed to reach 80-90% confluency. Culture medium was changed twice a week.

Biodegradable polymer scaffold

Scaffolds composed of self-cohered, aligned, non-woven fibers of 50% PGA:50% TMC were provided by W.L. Gore & Associates. Supplier-reported polymer density was approximately 8 mg/cm^2, fiber diameter ~30 μm, and porosity over 90% (Figure 4.1). 10 x 10 cm sheets with 1-2 mm thickness were sterilized by immersion in 70% ethanol for 20 min and UV light irradiation for 15 min, and allowed to dry overnight. For ligament regeneration, rectangular scaffolds were cut out of the sheet with the long axis of the rectangle along the direction of fiber orientation.

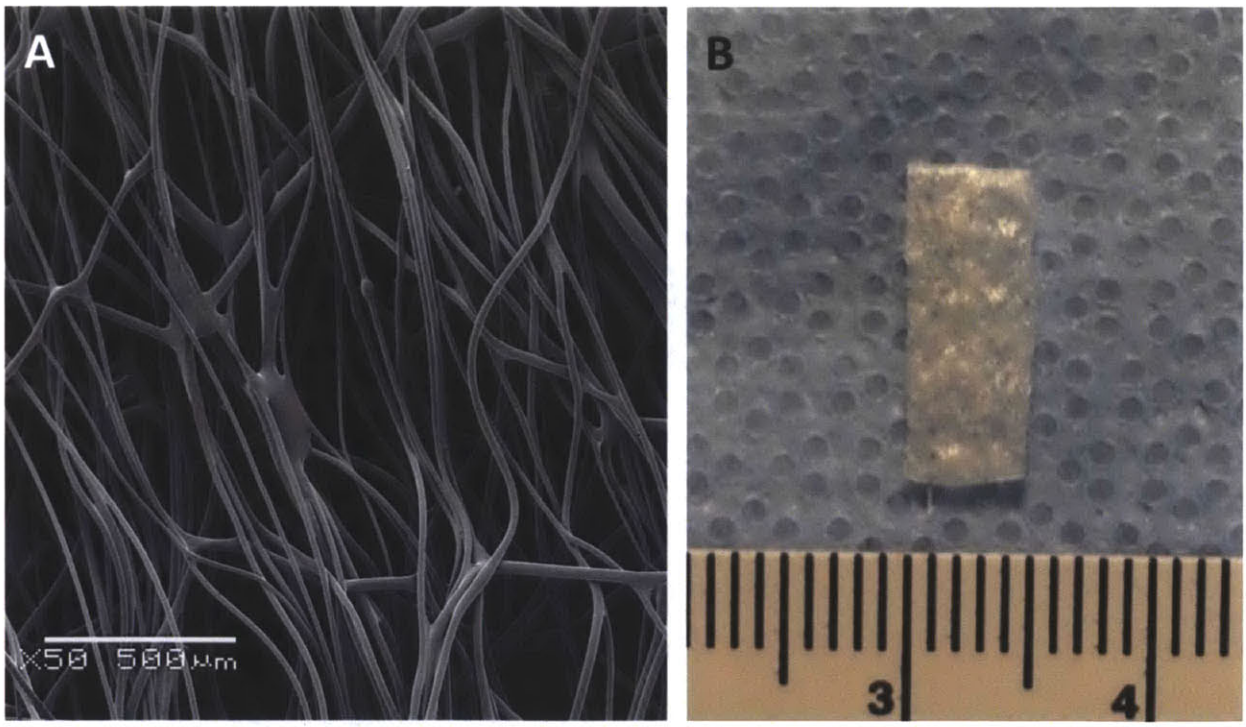

Figure 4.1. 50%PGA:50% TMC Scaffold. **(A)** Scanning Electron Microscope image (X50). **(B)** Gross appearance of unseeded, non-loaded scaffold. In both images fiber orientation is vertical.

Hydrogel reconstitution

Extracel™ hydrogel (Glycosan Biosystems) was reconstituted per manufacturer's protocol. Briefly, thiol-modified sodium hyaluronate, thiol-modified gelatin, and polyethylene

glycol diacrylate (PEGDA) were allowed to thaw to room temperature. Aseptically, 1.0 mL of degassed/deionized water was added to each, the hyaluronate and the gelatin, and allowed to dissolve for 30 minutes at 37°C with gentle shaking. Reconstituted hyaluronate and gelatin produced 1% (w/v) solutions. The clear, viscous solutions were mixed in equal volumes. The PEGDA was reconstituted with 0.5 mL of sterile degassed/deionized water, making a 2% (w/v) solution. Reconstituted PEGDA was immediately combined with the hyaluronate/gelatin mixture in a 1:4 volume ratio, mixed well by pipetting, and immediately mixed with the respective cell pellet. Within 5 minutes the cell-hydrogel liquid suspensions were seeded and adsorbed onto the PGA:TMC scaffolds. Gelation occurred within 15 minutes. Reconstituted Extracel™ has pH ~7.4 and a 0.4% w/v composition of thiol-modified hyaluronan and gelatin (159,185).

Scaffold seeding and dynamic culture

Passage 2 cells were collected using 0.25% Trypsin/0.9 mM EDTA, and viability determined with the trypan-blue (Sigma) exclusion assay. MSCs and ACLcs (A-M) were combined in 1:1 ratios in ACL DMEM and pelleted; pure ACLcs were also pelleted as a control. Cell pellets were mixed with reconstituted Extracel™ and micropipetted onto scaffolds at a density of ~50 million cell/cm^3. The hydrogel:scaffold volume ratio was ~0.8 to prevent initial hydrogel-cell suspension overflow from the scaffold. Upon gelation, within 15 minutes, the scaffolds were transferred to well plates (BD Falcon) and ACL DMEM was added carefully to prevent scaffold floating. A-M-seeded (co-cultured, n=15) and ACLcs-seeded (control, n=17) scaffolds were cultured in static conditions in ACL DMEM at 37°C and 5% CO_2 for 21 days. ACL DMEM medium was changed twice per week.

After 21 days, seven scaffolds of each seeding condition, A-M (n=7) and ACLcs (n=7), previously loaded onto custom made interchangeable stainless steel clamps, were secured via aluminum mounting block extensions onto a custom dynamic loading system that allows for automated, cyclic, uniaxial tensile strains to be applied to ligament constructs while incubated in nutritional media at 37°C (186) (Figure 4.2). Tensional loaded polymers were longer than unloaded polymers to ensure clamped portion was not cell-seeded; care was taken to ensure the polymer midsubstance (portion between clamps) had the same dimensions as all other samples before adsorbing the cell-polymer suspension. As previously described for drug transport within stretched muscle samples (186,187), the dynamic loading system has a LinMot® P01-

37x240/60x260 linear servo motor that drives the mechanical loading and can reach a peak force of 72 N, velocity of 0.45 m/s, and acceleration of 8.5 m/s^2. The position of the motor is precisely tracked by an internal sensor that has a 20 μm resolution and is controlled by proportional-integral-derivative logic enabling the execution of peak-to-peak displacements of 0.7 mm with +/-60 μm positional errors (186). Ligament constructs were cyclically loaded with 5% strain at 0.5 Hz for 60 minutes every other day for 7 days at 37°C in ACL DMEM. The loading regimen was adapted from previously published reports of physiologically relevant strain environments for ligament tissue engineering (146,177,178,180,183,184). When not under cyclic loading, the samples were incubated at 37°C and 5% CO_2. Medium was changed after each loading cycle. Static culture for these samples was 21 days, with a subsequent period of 7 days under intermittent, cyclic tensional loading for a total culture time of 28 days.

The unloaded samples, A-M (n=8) and ACLcs (n=10), were maintained in static culture at 37°C and 5% CO_2 for the same 7 days. Medium was changed every other day. Total static culture time for these samples was 28 days.

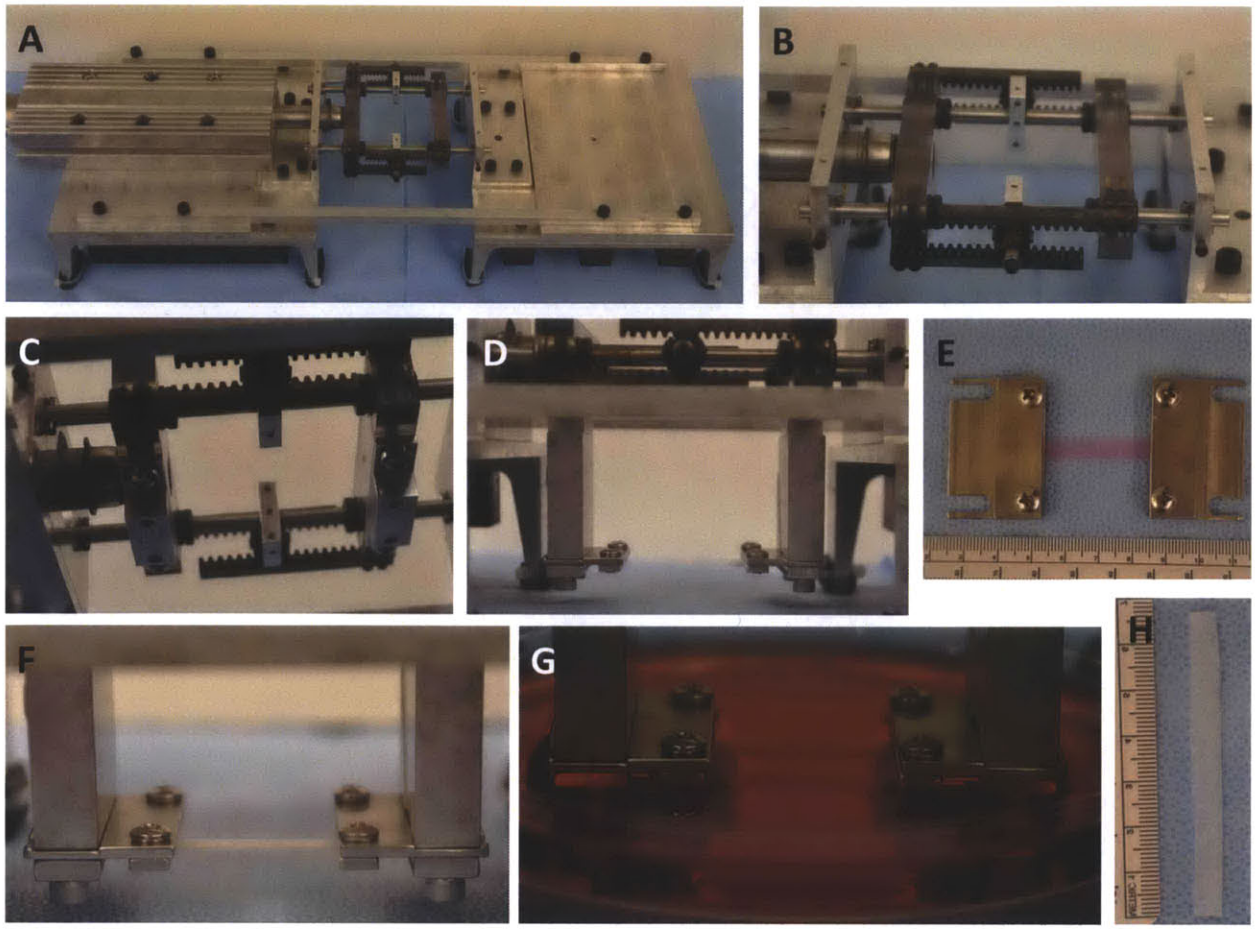

Figure 4.2. Uniaxial Tension Dynamic Loading System (186). **(A)** Linear motor for uniaxial tensional loading. **(B)** Dynamic carriage for synchronized bilateral symmetric elongation. **(C)** Inferior view. **(D)** Loading assembly with aluminum extensions and stainless steel clamps. **(E)** Stainless steel clamps with ligament construct. **(F)** Secured polymer on dynamic loading assembly. **(G)** Ligament construct submerged in media for cyclic loading. **(H)** Unseeded PGA:TMC polymer.

Quantitative reverse transcriptase-polymerase chain reaction

Expression of ligament-associated markers Collagen-I, Collagen-III and Tenascin-C was quantified by real-time PCR as previously described (99). Briefly, total RNA was isolated from co-cultured and control constructs with and without dynamic tensile loading (n=3 each) with an RNeasy Plus Midi kit (Qiagen) per manufacturer's protocol using a tissue homogenizer (VWR). 1 μg of total RNA per sample was reverse-transcribed in 20 μl reactions using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) per manufacturer's specifications. Next, 2 μl of a 1:10 dilution of the reaction product was subjected to quantitative PCR using an iQ SYBR Green Supermix kit in 20 μl reactions (Biorad). A C100 Thermal Cycler (Biorad) was

used with the following program: 95°C 3 min, 1 cycle; 95 °C 10 sec → 64 °C 30 sec, repeat for 40 cycles total; 95 °C 10 sec → 65 °C 5 sec, 1 cycle. Previously published forward and reverse gene-specific primers for Collagen-I, Collagen-III, Tenascin-C and 18s rRNA were used (Table 3.1)(99). Pig liver tissue was used as a negative control. 18s rRNA was used as the housekeeping gene for normalization. Dissociation and amplification curve analysis was performed with the Biorad CFX Manager software. Gene expression was obtained using the Pfaffl method to account for individual reaction efficiencies using freshly harvested native ACL cells as the calibrator sample (133).

Immunohistochemistry

Co-cultured constructs were fixed in 10% neutral buffered formalin (Sigma) overnight, paraffin embedded and sectioned. Slides were treated with a 1:10 dilution of Target Retrieval Solution (Dako) in distilled water for 10 min at 95-99°C. Non-specific blocking was performed with Protein Block serum-free solution (Dako) for 30 min at room temperature. Mouse primary antibodies for Collagen-I (1:200, Cat. no. C2456, Sigma), Collagen-III (1:100, Cat. no. ab6310, Abcam), and Tenascin-C (1:200, Cat. no. ab88280, Abcam) were added to respective slides and incubated overnight at 4°C. Goat anti-mouse Cy3 secondary antibody (1:500, Cat. no. 115-165-062, Jackson ImmunoResearch) was added for 60 minutes at room temperature. DAPI (1:500, Sigma) counterstain was added for 10 minutes at room temperature. Images were obtained with an Olympus IX70 fluorescent microscope equipped with an Olympus UM4-100 7A Cy3-710 fluorescent light filter, and a SPOT digital camera and image processing software (Diagnostic Instruments).

Data analysis

Quantitative PCR was performed in triplicate per group. Collagen I/ Collagen III and Collagen I/ Tenascin-C ratios within each sample were calculated using relative expression (2^{-CT}), since primer efficiencies were all above 97%. No control genes were considered in this case, as the calculation of a direct ratio does not need normalization. Statistical significance was calculated using a student's T-test. P values <0.05 were considered significant.

4.3. Results

Quantitative effect of 3D-dynamically loaded co-cultures

After a period of 28 days of total culture, with 7 days of dynamic loading at 5% strain, results show that loaded co-cultured constructs had significantly higher expression for all three ligament-associated markers compared to unloaded constructs (Figure 4.3). Dynamic loading significantly increased Collagen-I ($p \leq 0.01$, Fig. 4.3A), Collagen-III ($p \leq 0.03$, Fig. 4.3B), and Tenascin-C ($p \leq 0.03$, Fig. 4.3C) expression in A-M constructs compared to non-loaded A-M constructs. The ACLcs controls did not show a statistically significant difference between loaded and unloaded samples ($p \geq 0.05$) for all markers. Within non-loaded samples, ACLcs constructs had significantly increased expression compared to A-M constructs for Collagen-I ($p \leq 0.03$) and Collagen-III ($p \leq 0.04$), but not Tenascin-C ($p = 0.26$). Interestingly, all marker expressions were not statistically different among loaded A-M constructs and both, loaded and unloaded, ACLcs constructs ($p > 0.05$), even though half as many ACLcs were seeded in the A-M scaffolds compared to the ACLcs-only constructs.

Expression ratios between Collagen-I:Collagen-III, and Collagen-I:Tenascin-C were compared to ratios obtained in the literature from native ACL (Fig. 4.3D, E). Native ACL has a Collagen-I:Collagen-III ratio of 9:1, while the Collagen-I:Tenascin-C ratio is around 15:1 (2-4,99,135). Our results show that loaded and unloaded A-M constructs had Collagen-I:Collagen-III ratios of 8.8 and 10.4, respectively; while loaded and unloaded ACLcs construct ratios were 10.2 and 7.4, respectively. For Collagen-I:Tenascin-C, loaded and unloaded A-M constructs had the lowest ratios at 5.9 and 6.0, respectively; whereas loaded and unloaded ACLcs constructs had ratios of 8.2 and 11.4, respectively.

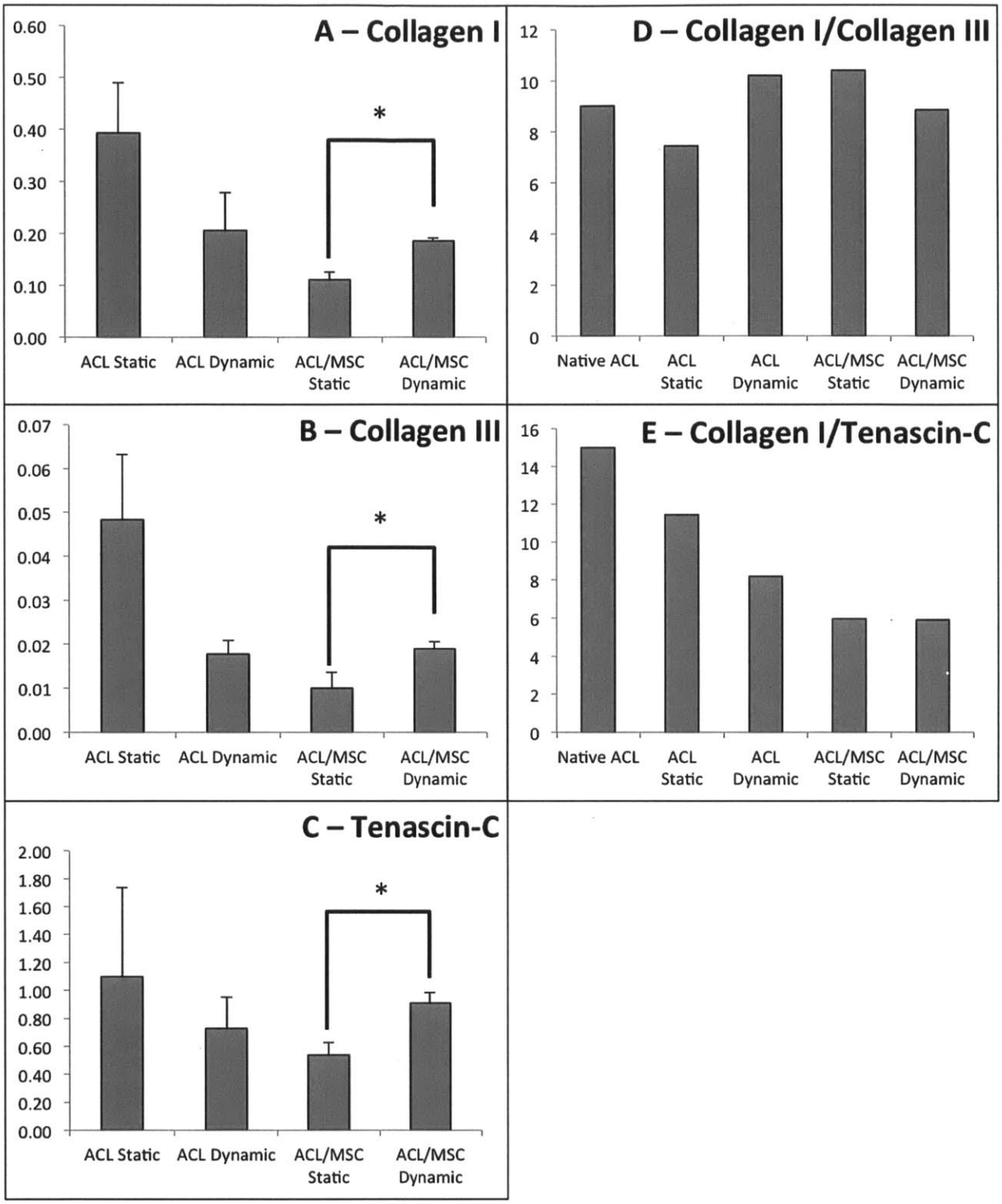

Figure 4.3. Fold expression of 3D co-cultures. **(A)** Collagen-I, **(B)** Collagen-III, and **(C)** Tenascin-C at 28 days under static and dynamic conditions. **(D)** Collagen I/Collagen III ratios. **(E)** Collagen I/Tenascin-C ratios. ACL/MSC cultures performed with a 1:1 ratio of each cell type at a density of 50 million cell/cm^3. ACL cultures performed with only ACL fibroblasts at the same density. mRNA expression was performed using quantitative PCR normalized with 18s rRNA. * indicates statistically significant increase with $p < 0.05$. Means ± SD shown.

Extracellular matrix deposition

Immunohistochemistry results showed that A-M co-cultured constructs had remarkably increased staining intensity when dynamically loaded (Figure 4.4). For all three ligament-associated markers, Collagen-I, Collagen-III and Tenascin-C, the intensity of the stain was brighter and more abundant for A-M loaded samples.

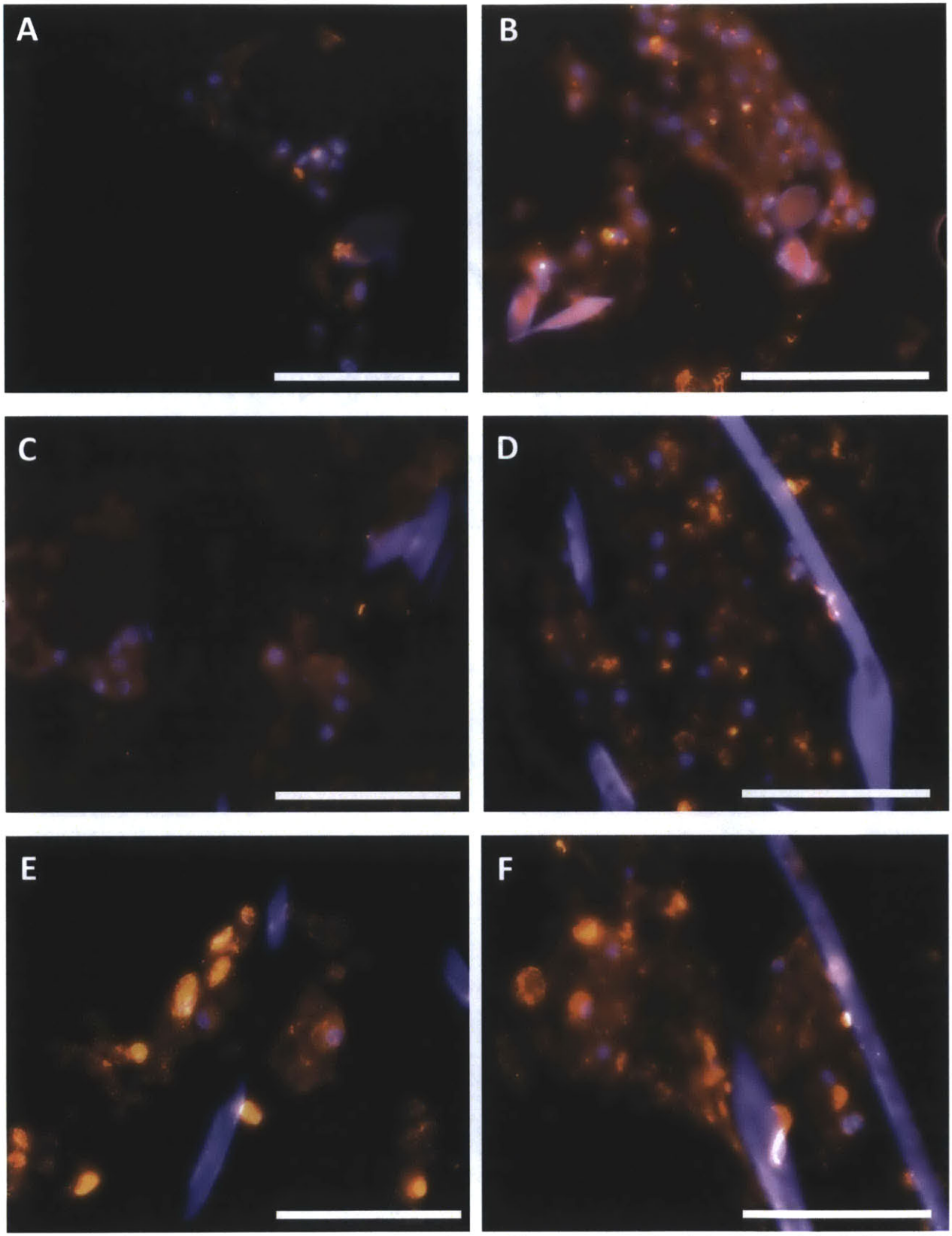

Figure 4.4. ACL/MSC construct marker expression. Collagen-I, **(A)** unloaded, **(B)** loaded; Collagen-III, **(C)** unloaded, **(D)** loaded; Tenascin-C, **(E)** unloaded, **(F)** loaded. Scale bar = 100 μm.

4.4. Discussion

The objective of the present study was to evaluate the effect of dynamically active environments on the *in vitro* response of A-M co-cultures in three-dimensional polyglyconate-hydrogel scaffolds. In order to develop ligament augmentation constructs that may be clinically useful, *in vitro* studies must subject potential constructs to environments similar to what would be experienced in the knee (188). Previous studies in our laboratory and others have shown that direct co-cultures of ACLcs and MSCs have an enhancing effect on the expression and deposition of ligament markers Collagen-I, Collagen-III and Tenascin-C (97,99). Before proceeding to *in vivo* animal studies, our group wanted to confirm this observation in 3D, and develop a potential construct for primary augmentation ligament repair.

Our results show that three-dimensional 1:1 A-M co-cultures under dynamic tensional loading exhibit increased expression of ligament-associated markers. All three ligament markers, Collagen-I, Collagen-III and Tenascin-C had statistically significant increases in expression over 28 days when compared to non-loaded co-cultured constructs. Notably, the loaded A-M constructs did not show a significant difference in marker expression to the loaded and unloaded ACLcs-only constructs, even though the A-M constructs had half as many ACLcs. As we have shown before in 2D (99), this observation suggests that the interaction between ACLcs and MSCs has an enhancing effect on the expression of the ligament-associated markers. Immunohistochemical staining supported the mRNA results, with more intense and abundant staining observed in loaded co-cultured constructs than unloaded samples. Taken together, these observations suggests that *in vitro* ECM deposition by ACL cells is enhanced when co-cultured with MSCs in three-dimensions, a result that supports our two-dimensional observations (99). Thus, in future tissue engineering and regenerative medicine applications for ACL repair, the use of 1:1 ACL cell-MSC co-cultures should be considered.

The 3D results observed in the current study directly follow the observations from our 2D study (99), with the exception of the response seen for Collagen-III. In two dimensions, the only group that exhibited significantly higher Collagen-III expression was the MSC-only cultures (99). The significant difference was seen from the beginning, and there was no statistically significant increase in Collagen-III expression from 2 to 4 weeks, even within MSC samples (99). In the current three-dimensional study in hydrogel-polyglyconate scaffolds, the dynamically stretched

A-M co-culture exhibited a statistically significant increase in Collagen-III expression when compared to the unloaded sample. This positive result is akin to observations by other groups studying 3D ACL and tendon regeneration (127,146,173,180), and 2D observations in ACL and MSC co-cultures with and without mechanical stimulation (68,69,182).

Previously, we showed that Collagen-I and Tenascin-C expression could be enhanced solely by direct contact 2D co-cultures of ACLcs and MSCs (99). During the present study, all three markers exhibited the increase, Collagens-I and III, and Tenascin-C. The 3D/ECM-like environment provided by the hydrogel and polyglyconate scaffold, combined with the dynamic loading, appear to have had an enhancing effect on the ligament marker expression and deposition, particularly for Collagen-III. Within tissue engineering, cell co-cultures in 3D scaffolds have shown promise in terms of cell proliferation, differentiation and potential for vascularization of the regenerated tissue (155). For ligament and tendon, hydrogel cell deliveries have shown positive effects (47,62,189). Murray and colleagues have observed enhanced ligament marker expression and matrix deposition, and improved biomechanical properties in primary ACL repair enhanced with collagen-PRP hydrogels and scaffolds (11,42,43,47,98). Nöth *et al.* also observed marker expression and deposition in Collagen type I hydrogels seeded with MSCs for ACL repair (189). More recently, Freeman and colleagues used a polyethylene glycol diacrylate-based hydrogel combined with PLLA to form ACL constructs (62). Their results showed that the composites supported cell growth and had favorable protein release profiles that can be used for future ACL repair (62). Analogously, a dynamic environment has been shown to enhance ligament marker expression, including Collagen-III, particularly in three dimensions (68,69,127,146,173,176,180,182). Thus, it is reasonable to conclude that the significant increase of Collagen-III expression in our 3D scaffolds, not observed in 2D, was a synergistic result of the three-dimensional and mechanically active environment.

Analyses of the Collagen-I/Collagen-III and Collagen-I/Tenascin-C ratios showed responses suggestive of repair, and further support our conclusions. The Collagen-I/Collagen-III ratios for ACL alone and co-culture samples, both loaded and unloaded, were at the level of that observed in native ACL tissue, around 9:1 (2-4,99), suggesting an ECM deposition response more similar to naturally three-dimensional native ligament. The Collagen-I/Tenascin-C ratios were lowest and similar in the loaded and unloaded co-culture samples, both around 6:1 and far from the 15:1 ratio observed in native ACL (2). This response is also anticipated, because

Tenascin-C expression is transiently up-regulated during connective tissue healing responses (138,139,190). Thus, the co-cultured samples exhibited Collagen-I/Tenascin-C ratios indicative of proportional Tenascin-C overexpression, and suggestive of an improved reparative response compared to ACL-only constructs. Interestingly, the Collagen-I/Tenascin-C ratio was moderately lower in dynamically loaded ACL-alone constructs compared to unloaded ACL-alone constructs, with ratios of 8.2 and 11.4, respectively. This observation in ACLcs-only samples further suggests that dynamic stimulation improves the healing response of engineered ligaments, as Tenascin-C was also proportionally overexpressed only under loading conditions, and is consistent with previous ligament tissue engineering reports (68,69,127,173,176,180,182).

The choice of a hyaluronan-based hydrogel combined with a polyglyconate scaffold was not deliberate. The use of hyaluronic acid for cartilage and ligament tissue engineering has been well documented (11,149,151). Hyaluronan is a naturally-occurring glycosaminoglycan found in a variety of tissues around the body, particularly connective tissues (149,151,191). It not only plays a role in joint lubrication, but also in cell proliferation, cell regulation, cell differentiation, as well as extracellular matrix modifications during development, injury and wound healing, and immune/inflammatory responses (151,192,193). As it applies to ligament, intra-articular hyaluronan injections have shown a positive effect during the healing of partial ACL tears in a rabbit model, improving angiogenesis, decreasing inflammation and enhancing Collagen-III synthesis when compared to saline controls (194). Furthermore, intra-articular delivery of hyaluronan does not appear to cause detrimental or degenerative changes in ACL tissue (195), and its use seems to be safe and beneficial in knee joint pathologies and procedures (196,197). In terms of tissue engineering applications, Li and colleagues tested the effect of self-assembled hyaluronic acid/gelatin coatings on polyethylene terephthalate ACL reconstruction grafts, showing that the coating increased the synthetic material's biocompatibility by allowing better cell adhesion and growth, and reducing inflammation (198). Their *in vivo* results showed that the coating significantly reduced the inflammatory response to the implanted grafts and allowed for neoligament regeneration with collagen deposition (198). Seo and colleagues tested a silk-collagen-hyaluronan scaffold for ligament tissue engineering, and found that the collagen-hyaluronan component improved angiogenesis and cell recruitment (199). The Extracel™ hydrogel used in our study has been shown to allow controlled release of growth factors, while also allowing adequate infiltration and proliferation of cells (159). For cartilage tissue

engineering, Extracel™ has been shown to significantly increase total collagen content and sulfated glycosaminoglycan retention (185). These observations motivated us to use a hyaluronic-acid based hydrogel for the delivery of cell suspensions to the polymer, as it would have a complementary positive effect within the knee joint when used in a primary augmentation repair approach for ACL. Our overall positive results in terms of ligament matrix marker expression and deposition support the use of the hydrogel for repair, although the particular effect of the hyaluronan/gelatin hydrogel on cell behavior should be further explored.

Regarding the structural polymer choice, various biomaterials have been used in the past with some success (reviewed in (22)). Particular success has been seen with PLLA and PLGA scaffolds, but their long-term mechanical shortcomings, observed problems with tissue integration, and overly slow degradation (57,146,200,201) motivated us to look for an alternative material. The polyglyconate scaffold component was pursued as a structural stabilizer of a potential primary augmentation ACL repair technique. Since polyglyconate is FDA-approved, obstacles for its use in clinical applications are less compared to other materials. Previous techniques in primary ACL repair have had relative success. Murray's bioenhanced augmented primary repair has only been successful when performed immediately after ACL transection (42); when the repair of ACL transection using the technique was delayed for 2 or 6 weeks, the results were significantly adverse (52). As the investigators point out, a likely reason for the bioenhanced technique failure is the stump retraction seen after ACL rupture (52,56). This retraction is why a primary repair with augmentation can be a viable option. The benefits of augmentation are well documented (66,140), and suture repair has shown mechanical improvements in joint function after ACL injury (48). Thus, a combinatorial approach in which our construct is used as an augmentation device in conjunction with a primary suture repair of a ruptured ACL could be promising. The seeded construct would fill the gap between the retracted stumps, serve as an enriched/regenerative bridge for cell, nerve and blood vessel migration between the stumps, and provide initial stability to the joint in conjunction with the suture (11,44). As previously established by Murray (44), bone-to-bone fixation with a suture would still be needed to further stabilize the joint while regeneration is underway. Even during rehabilitation the construct would be beneficial, as our current results and those of others show that cyclic tensional loads similar to physiologic ranges enhance the regenerative response (146,177,183,184,188). Polyglyconate is an ideal candidate as a scaffold, as it has shown

excellent biocompatibility while having adjustable biodegradable and biomechanical properties (161,170).

Recently, many groups have isolated MSC-like or adult stem cell-like cells directly from ACL (80,81,115,202,203). Cheng and colleagues isolated a clonal population of ligament stem cells by low seeding density expansion from ACL tissue (81,115). Their work shows that stem cells from ligament have very similar properties to donor-matched BMSCs, and even argue that ligament-derived stem cells have enhanced ligamentogenic properties compared to BMSCs (81,115). Steinert *et al.* isolated what they describe as true MSCs from ACL, but in contrast to Cheng, they conclude that ACL-derived MSCs have ligamentogenic properties comparable to those of BMSCs (80). Mifune and group isolated CD34+ ligament vascular stem cells from ACL, and though their properties are somewhat different to MSCs, they show potential for ligament repair and even improvement for current ACL reconstruction techniques (202,203). Though the evidence seems to suggest the existence of intrinsic stem cells in ACL, the inability for ACL to mount a self-healing response is still unexplained (11,80). This is where co-culture may provide a breakthrough. The regenerative potential of MSCs has been broadened by additional evidence of their vast biologic behaviors: their trophic activity that helps with the generation of vascularization, decreases scarring and fibrosis, and enhances tissue-intrinsic regenerative responses (71,72,89); their immunomodulatory activity (74,75,90); and their plasticity (72,77,79,82,83,85,86,88). Furthermore, several studies have shown the enhancing effects of co-cultures between MSCs and other cell types, including ACL fibroblasts (64,67-69,99,126,127). Our study in 2D (99) and the current results, combined with the additional attributes of MSC biology (71,72), provide a potential framework for enhancing primary ACL repair by [1] providing a source of stem cells (BMSCs) with ligamentogenic potential for differentiation, [2] delivering a cell source (BMSCs) known to have regenerative, recruiting and anti-fibrotic properties, and [3] a cell source (BMSCs) that can modulate the immune and inflammatory responses existing in an injured ACL and that can be triggered in the body by foreign implants, such as an ACL augmentation construct. The observation that dynamically loaded A-M constructs experience a significant increase in ligament markers is supported by evidence observed by other investigators (146), and backs the notion that co-culture with MSCs could incite an intrinsic ACL reparative response.

Murray and colleagues have also explored the potential benefits of mononuclear cell (including lymphocytes, macrophages, monocytes and MSCs) supplementation of primary ACL repair (97,98). They observed that 3D collagen constructs seeded with ACL fibroblasts and supplemented with platelet-rich plasma and peripheral blood mononuclear cells increased the expression of Collagen-I and Collagen-III after 14 days of culture (98). They further observed that peripheral blood MSCs and adipose-derived MSCs in co-culture with ACL fibroblasts enhanced cell migration, proliferation and ligament marker expression after 14 day cultures (97). These observations support our previous results (99), as well as our 3D conclusions. Nevertheless, in our opinion, the source of the MSCs should remain within the skeletal lineage. Extensive reports support the notion that the preferred source of mesenchymal stem cells for repair should be dependent on the targeted tissue, since MSCs from a particular source tend to show behaviors similar to their origin, and an improved ability to regenerate tissue similar to its source of origin (71,84,87,88,93,204). Xie and colleagues studied the effects of PRP on BMSC and ADMSC chondrogenesis (205). Even though PRP did trigger a chondrogenic-like response in both MSC sources, their results showed that BMSCs were superior in expressing chondrogenic markers and generating hyaline-like cartilage, as the ADMSCs generated more immature and fibrous tissue (205). These reports, along with the observation that even with artificial growth factor supplementation ADMSCs do not exhibit a consistent and reliable ability to regenerate ligament (92), support the use of BMSCs in future studies, as BMSCs have reliably and consistently been differentiated into ligament (18,68,69,127,146,173,176,180,181), and shown beneficial effects in terms of ligament regeneration (68,69,99,127,146,180).

In conclusion, our study shows that 3D hydrogel-scaffold constructs seeded with 1:1 co-cultures of ACLcs and MSCs have an enhancing effect on the expression of genes for ligament markers and the deposition of ligament ECM, especially under tensional loads. The next step in our technique development is to assess the biochemical and mechanical response of the 3D constructs in an *in vivo* animal model. Our hope is to establish a framework for a technique that can allow the primary augmentation repair of ACL tears off-the-shelf, and without the need for autografts or allografts.

Chapter 5.

Summary and Future Directions

5.1. Thesis Summary

The overall objective of this thesis is the establishment of a multi-component, combinatorial, tissue engineering framework for the development of a technique for primary augmentation repair of ACL injuries. As outlined in Section 1.6, the experimental work of this thesis was based on three specific aims.

Specific aim 1. *Characterization of tibial mesenchymal stem cells. Hypothesize that proximal tibial MSCs have the same differentiation potential as other bone marrow-derived MSCs.*

As discussed in Chapter 2, human tibial bone marrow aspirates yielded MSCs that have very similar properties to MSCs isolated from the iliac crest and other long bone sources. Furthermore, the filter device for BMSC collection showed promise for use in future clinical applications, as it will more quickly and efficiently deliver MSCs intraoperatively during regenerative orthopaedic procedures. The detailed characterization of the tibial MSCs and the positive differentiation results will allow our group to pursue *in vivo* animal studies and clinical studies where MSCs can be harvested closer to the injury site, simplifying surgical procedures, increasing patient comfort, and decreasing associated morbidities.

Specific aim 2. *Characterization of the co-culture effects of primary ACL fibroblasts and MSCs in vitro. Hypothesize that co-culturing MSCs with ACL cells leads to an enhanced regenerative response compared to either population alone.*

Chapter 3 discussed extensively the behavior of ACLcs and MSCs in monolayer cultures. We showed that during *in vitro* two dimensional culture, 1:1 ACLcs and MSC co-cultures experience a significantly increased expression of Collagen type I and Tenascin-C markers, and a Collagen-I/Collagen-III ratio closer to native ligament than other samples. The enhanced response of the co-cultures in terms of ligament marker expression and matrix deposition suggests that they may better preserve or even enhance ligament healing in potential orthopaedic tissue engineering applications.

Specific aim 3. *In vitro development of a construct for primary ACL augmentation repair with a biodegradable scaffold based on poly(glycolic) acid: trimethylene carbonate (PGA:TMC) combined with a hydrogel seeded with autologous MSCs and primary ACL fibroblasts under dynamic tensile loading. Hypothesize that combining MSCs with primary ACL cells in a hydrogel loaded in a biodegradable scaffold enhances the regenerative response, particularly in tensile loading environments.*

The discussion in Chapter 4 supports our 2D results in 3D environments, with co-cultures exhibiting enhanced ligament marker expression and matrix deposition. Moreover, the dynamic loading of 3D co-cultured samples with 1:1 ACLcs-MSC ratios resulted in significantly increased Collagen-III expression, something not observed in 2D. Thus, in dynamic 3D, all three ligament-associated markers had statistically significant increases in expression, an observation that was corroborated by immunohistochemical data. These results allow our group to conclude that for the development of a technique for primary ACL augmentation repair, the use of 3D co-culture systems of ACLcs and MSCs can be beneficial, particularly when used *in vivo*, where the constructs would experience dynamic tensional loadings during rehabilitation.

5.2. Future Directions

The next step in the development of our ACL repair framework is the evaluation of 3D co-cultured constructs in an *in vivo* animal model. Prior to pursuing a large animal model, we explored the co-culture response *in vivo* in a preliminary study in a subcutaneous rodent model. We implanted 1:1 Pig ACLcs:MSCs cell-hydrogel-scaffold constructs in subcutaneous pockets of Nu/Nu mice for 3 months. As before, the scaffolds were seeded at a density of 50 million total cells/cm^3. ACL-only and MSC-only seeded constructs were also implanted as controls. To avoid unexpected biocompatibility issues during the preliminary study, Pluronic® F-127 was used as the hydrogel. The hydrogel is similar to Extracel™, and our laboratory has more experience with it *in vitro and in vivo* (150,206). The constructs were cultured *in vitro* at 37°C and 5% CO_2 for 4 days prior to implantation. After 3 months *in vivo*, the implants were harvested, paraffin embedded, sectioned and stained for all three ligament markers using immunohistochemistry.

The results were remarkably similar to what we observed in 2D and 3D *in vitro* cultures (Figure 5.1). Collagen-I staining was similar and more intense in A-M and ACL-only samples, whereas the MSC-only sample had practically no positive stain (Fig. 5.1A, D, G). Collagen-III staining intensity was similar in all cell culture conditions, including MSC-only samples (Fig. 5.1B, E, H). Tenascin-C staining was noticeably more intense in A-M samples compared to ACL-alone samples; MSC-only samples stained weakly positive for Tenascin-C (Fig. 5.1C, F, I). Although we understand that a subcutaneous, static environment is drastically different than an intra-articular and dynamic environment, these preliminary results suggest that the co-culture response observed *in vitro* could be reasonably expected to happen *in vivo*, prompting our laboratory to consider a large animal model for ACL repair using our technique.

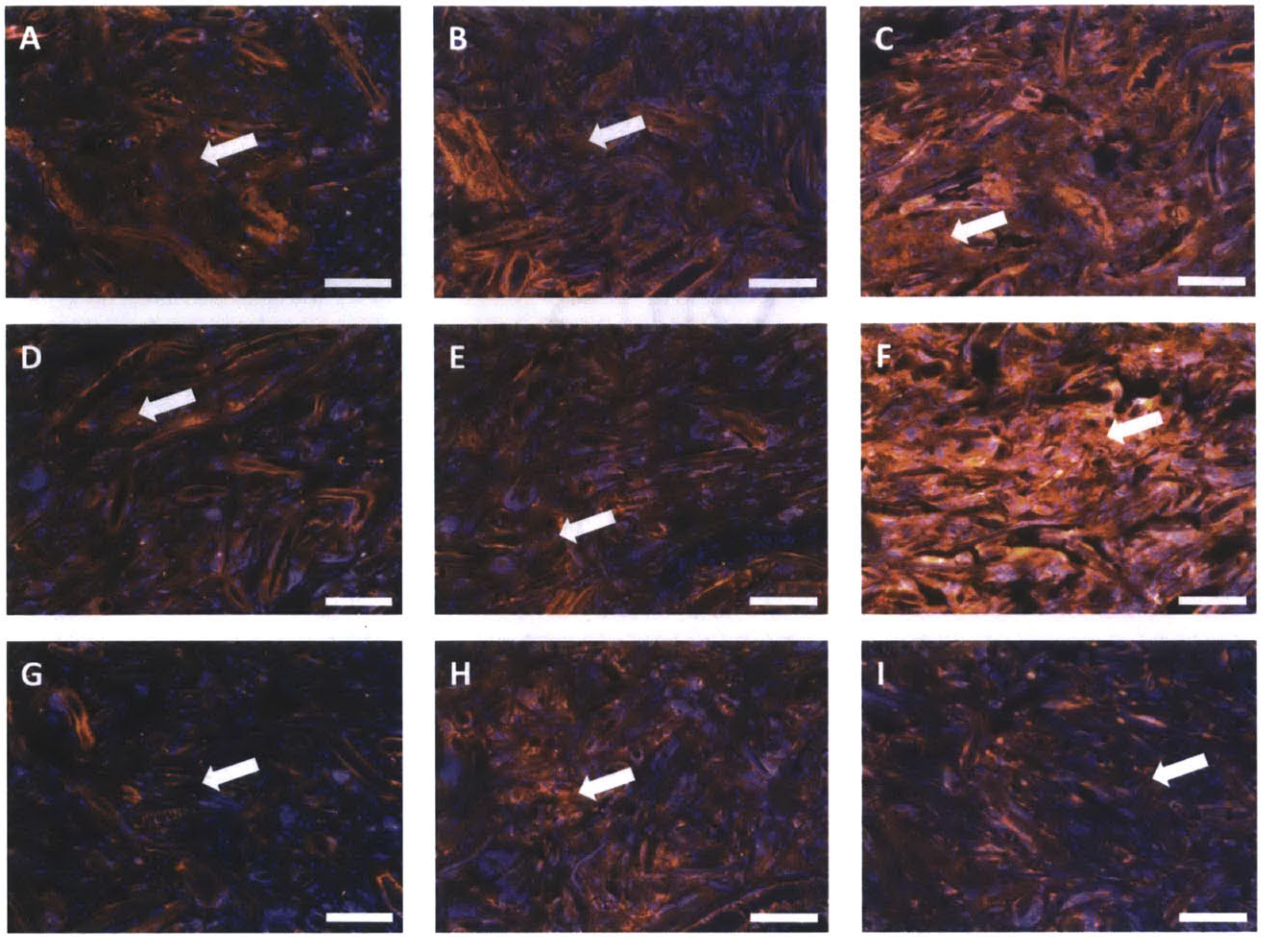

Figure 5.1. Ligament marker expression of *in vivo* ACL constructs. Pig ACL cells and MSCs were resuspended in Pluronic® F-127 hydrogel and seeded onto 50%PGA:50%TMC scaffolds. Three conditions were examined: ACL-only seeded constructs **(A, B, C)**; 1:1 ratio of ACL cells-MSCs **(D, E, F)**; MSC-only seeded constructs **(G, H, I)**. Constructs were cultured *in vitro* for 4 days, then implanted in subcutaneous pockets of Nu/Nu mice for 3 months. Upon harvest, constructs were stained with immunohistochemistry for ligament markers Collagen-I **(A, D, G)**, Collagen-III **(B, E, H)** and Tenascin-C **(C, F, I)**. Arrow points to location of positively stained matrix. Orange color represents Cy3 positive stain for primary antibody. Blue color represents DAPI nuclear stain. Scale bar = 100 μm.

The ideal animal model for an ACL injury and repair is debatable (42,48,57,100). Nevertheless, large animal models more closely resemble the environment experienced by an ACL construct in a human knee (42,100). Consequently, our aim is to conduct an *in vivo* pig or goat study of a delayed, full transection ACL injury model. The objective would be to use our 3D co-cultured cell-hydrogel-scaffold construct as an augmentation device to bridge the retracted stumps of the ACL rupture, and evaluate the regenerated ligament with biochemical, histological and biomechanical tools. In an *in vivo* model, the biomechanical properties of the construct post-

implantation become critical, as applicability in humans would depend on the ability of the implant to achieve mechanical stability in the joint close to, and preferably better than, current graft reconstructions. The study would ideally evaluate constructs at 3, 6, and 12 months, with 6 animals at each time point for each study condition.

Conditions to explore during an *in vivo* study should include the choice of hydrogel with which to deliver the cells in suspension to the polyglyconate scaffold. The Extracel™ hydrogel, based on hyaluronic acid and gelatin components, has shown positive results and good biocompatibility in rabbit models of cartilage repair (160). Its ability to cross-link *in situ*, without the need for temperature regulation or the addition of calcium ions, makes Extracel™ preferable for use in the operating room setting than more commonly used hydrogels, such as pluronic or calcium alginate (151,159,160).

Platelet-rich formulations are also intriguing, as their rich growth factor and cytokine content, as well as their anti-inflammatory and analgesic properties, appear to enhance healing of sports injuries and increase the speed of recovery (207-209). Several groups have described these various formulations and highlighted the benefits of using platelet-rich plasma (PRP) and/or platelet-rich fibrin (PRF) in sports medicine and for orthopaedic injuries (207-213). Moreover, the International Olympic Committee (IOC) recently published a consensus where the use of PRP for athletic injuries is not considered a "performance enhancer", and its use is currently permitted under IOC and World Anti-Doping Agency (WADA) regulations (214). For ACL repair, Murray and colleagues have shown that PRP can enhance primary repairs of ACL (11,42). Thus, it would be worth studying the effects of hydrogel enrichment with PRP on the regenerative response of our constructs, or exploring the use of PRF hydrogels as the cell delivery vehicle to the scaffold, instead of other hydrogels.

Another factor worth exploring in an *in vivo* model is whether dynamic tensional loading of the ACL constructs prior to implantation is beneficial. Given that the results of our 3D study showed that cyclic loads enhanced the expression of ligament markers and the deposition of ECM, pre-conditioning a construct with dynamic loading before implantation may lead to better tissue regeneration (215,216). On the other hand, pre-conditioning regiments would require *in vitro* cultivation, a step that would increase the risk of contamination, delay the timing for repair, and make more difficult any regulatory approval process for clinical use. Nevertheless, the effect of pre-conditioning our constructs prior to implantation warrants study.

Additionally, it would be interesting to evaluate *in vivo* whether ACL cells need to be seeded with the MSCs directly on the scaffold before construct implantation. ACL cells have been shown to migrate into various scaffolds, and MSCs have shown recruiting properties towards ACL cells (97,217,218), therefore the implantation of MSC-seeded constructs may trigger the migration of ACL cells into the construct from the augmented ligament stumps. This would render our technique more easily introducible into a clinical setting by avoiding the need to isolate ACL fibroblasts for scaffold seeding prior to implantation. Naturally, we would need to determine if a sufficient number of ACL cells migrate into the scaffold *in vivo*, if any; and if the ACL cell-MSC co-culture benefits reported in this thesis are still observed in such a "delayed co-culture" approach. It is possible that not enough cells migrate in a timely fashion into the scaffold, or that the delayed ACL cell-MSC ratio attained is not sufficient to trigger the observed co-culture benefits.

Finally, even though the use of primary ACL cells and MSCs has shown promise, exploring pioneering sources of cells for tissue regeneration would be desirable. Recently, our laboratory has identified the presence and isolation method of adult tissue-derived pluripotent stem cell clusters with the potential to differentiate across germ lines (219). These spheres are composed of a heterogeneous population of cells, and unlike embryonic or induced pluripotent stem cells, their pluripotency does not evoke ethical dilemmas, nor require genetic or biochemical manipulations (219). Currently, we have started to explore their application for tissue engineering, including cartilage and ligament; as well as their somatic plasticity under stress environments (submitted manuscript). Perhaps their use in regenerative Orthopaedics will one day spare the need to invasively harvest cells, and instead small blood or mucosal samples would suffice to obtain cell sources for repair.

5.3. Glimpse Into the Future of ACL Repair

Our laboratory believes that there are three key requirements for an ideal, clinically useful tissue-engineered ACL construct (220): [1] a cell harvest procedure that is easy and minimally invasive, and yields sufficient cells for polymer seeding without the need for extensive *ex-vivo* expansion; [2] a scaffold that is both biocompatible and biodegradable, circumventing inflammatory responses from the host, and providing biomechanical support

similar to that of native tissue until the ligament is regenerated; and [3] the ability of the implant to continue to grow, especially when treating pediatric patients and young athletes. With these points in mind, we developed the framework discussed throughout this thesis.

In the ideal future, an augmented primary ACL repair would be an arthroscopic, one-stage procedure using intraoperatively harvested cells delivered in off-the-shelf products. Possible approaches have been described by various investigators (28,140,141). In our vision, the surgeon would arthroscopically harvest bone marrow with available kits, filter the marrow with an MSC filter device similar to the one described in this thesis, and make a cell-hydrogel suspension in the OR with a hydrogel. Standard length polymer scaffolds could then be cut to fit the size of the patient and seeded with the cell-hydrogel suspension. An augmented primary ACL repair would then be performed by connecting the remaining stumps using the scaffold and sutures as a bridge. A bone-to-bone suture would then be added for mechanical stability as previously described (44). This vision assumes that ACL fibroblasts would naturally, or with a biological trigger, migrate out of the stumps into the scaffolding bridge, and an enhanced regenerative response similar to the one observed in our studies would ensue (26,97,99,217,221).

A final thought about Sports Orthopaedics. Though surgical interventions, and soon regenerative approaches, are often the best solution to orthopaedic injuries, the ultimate goal of the field should be to develop means to better prevent injuries. Training programs that target the prevention of injuries in athletes have shown a considerable reduction in the risk of ACL injury (222), and should be further developed and implemented. The best treatment of an ACL injury is preventing the injury from happening in the first place.

Tissue engineering and Regenerative Medicine is a relatively new field in science, with its birth occurring somewhere around the mid-1980's (223-225). Despite numerous challenges (223,224), the field has come a long way, with clinical trials ongoing for dermatologic, orthopaedic, vascular, ophthalmologic, genitourinary and respiratory tissues (225). As knowledge expands and new techniques are developed, organ regeneration in the clinic will become commonplace. Even though we understand that the framework developed in this thesis is far from becoming a clinical reality, we believe it represents the foundation and beginnings of a technique with great potential.

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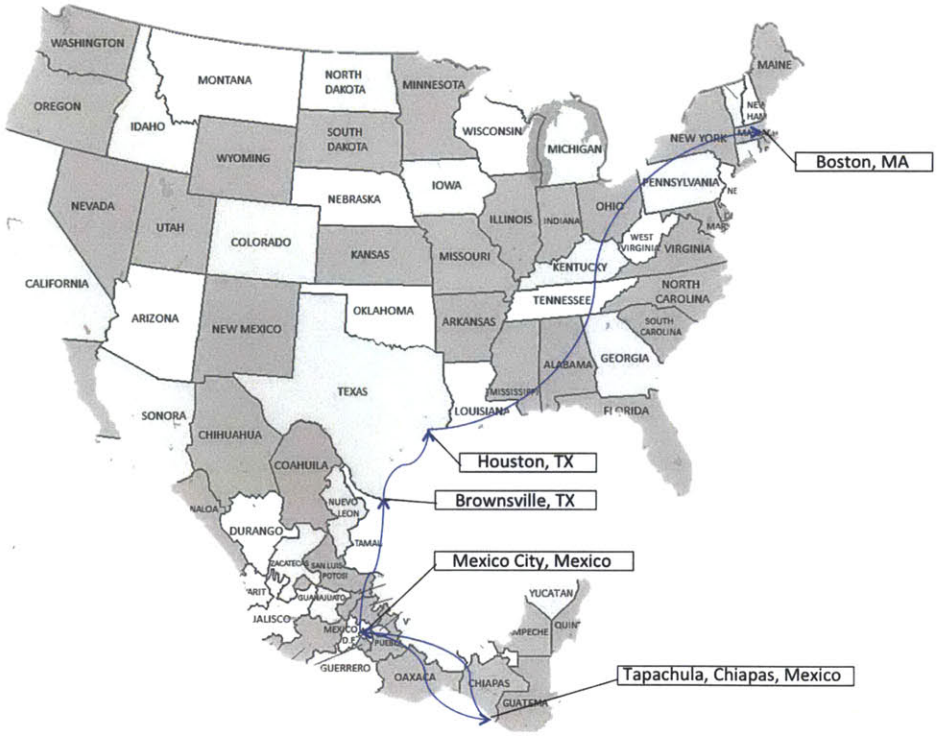
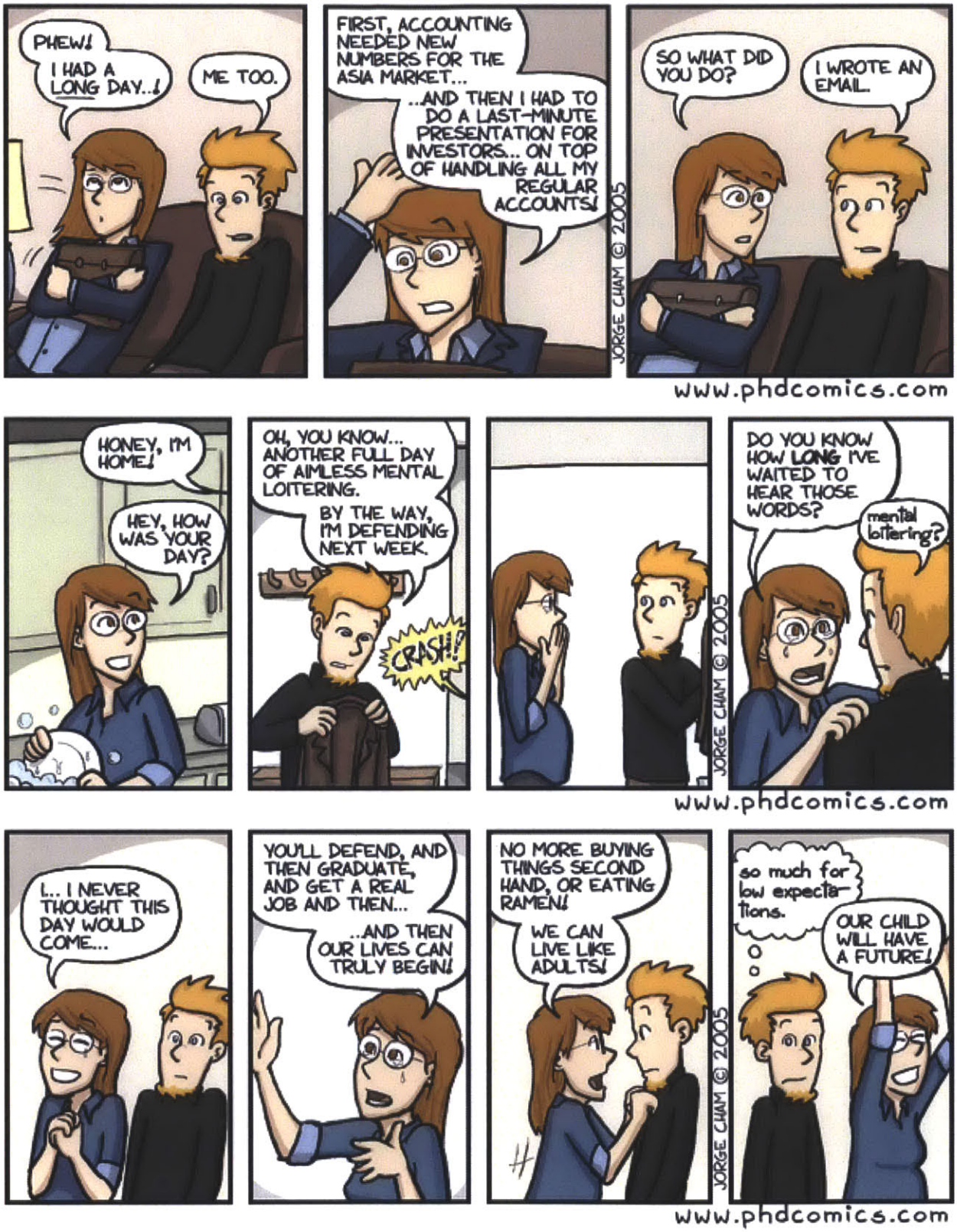
Biographical Note

José Antonio Canseco was born in Mexico City, Mexico, and was raised in Tapachula, Chiapas, the southernmost state in Mexico, and Brownsville, TX. From 1999 to 2003, he attended Rice University, where he studied Bioengineering and earned a Bachelor of Science degree, *cum laude*. After graduation, he moved to Boston and became an MD student in the Harvard-MIT Division of Health Sciences and Technology (HST) at Harvard Medical School. Two years into his medical studies, José joined Dr. Charles A. Vacanti's Laboratories for Tissue Engineering and Regenerative Medicine at the Brigham and Women's Hospital, where he completed a two-year research assistantship in cartilage and ligament regeneration as part of his HST curriculum. He then completed his 3rd year medical rotations at the Massachusetts General Hospital, during which José realized the need for rigorously trained physician-scientists with a focus on translational research and engineering. Tissue Engineering and Regenerative Medicine surgeon-scientists were needed in the operating room, and José wanted to train to fill that vacant niche. Therefore, he joined the Medical Engineering and Medical Physics PhD Program through HST at MIT, returning to Dr. Vacanti's laboratory to complete doctoral studies in Biomedical Engineering and focusing on the regeneration of anterior cruciate ligament, cartilage repair, and mesenchymal stem cell biology. During graduate school, José was also a Teaching Fellow at Harvard University for undergraduate and graduate courses in Anatomy & Physiology, Cell Biology and Drug Development, receiving numerous distinguished teaching awards. Upon finishing his graduate studies, José received a Cycle II funding award for the completion of medical school by the Harvard Medical School MD-PhD Program, recognizing his research accomplishments and dedication to a career as a physician-scientist.

José will receive his PhD in Biomedical Engineering from the Massachusetts Institute of Technology in 2013, and his MD from Harvard Medical School in 2014. He will pursue residency training in Orthopaedic Surgery, followed by a fellowship in Orthopaedic Sports Medicine. His ultimate goal is to become a Sports Orthopaedic Surgeon at a large academic medical center, where he can have an active clinical practice while continuing his work in Orthopaedic tissue engineering, hoping to bridge the gap between research innovations and patient care.





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