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Transgenic Mouse Model: Examination of Healing, Development and Mechanical Response of Cells

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Transgenic Mouse Model: Examination of Healing, Development and Mechanical Response of Cells

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ABSTRACT

Tracking real-time changes in Col1 and Col2 gene expression could speed up the FTE process and could lead to new strategies to create zonal insertions in repair tendons. Hence double transgenic mice with fluorescent promoters for Col1 and Col2 gene expression were created and used to examine natural healing of patellar tendons, development of knee structures and the mechanical response of primitive MSCs as well as specialized chondrocytes and fibroblasts.

The natural healing study sought to determine how full thickness, mid-substance punch defect injuries affect spatial and temporal patterns of Col1 and Col2 gene expression and biomechanics compared to age-matched, normal patellar tendons. Col1 expression in injured tendons was highest at two weeks after injury. No detectable Col2 gene expression was seen in the mid-substance healing wound site at any time point. Natural healing resulted in inferior repair properties at both 4 weeks and 6 weeks post injury compared to contralateral sham and age-matched tendons.

Monitoring fluorescent changes in murine knees from embryonic through early post-natal development indicates that Col2 expression precedes Col1 expression. There were temporal and spatial changes in the expression of two related growth factors, TGF-β1 and BMP-2, and their downstream markers, pSmad1,5,8 and pSmad2,3 respectively.

Exposing specialized and primitive cell-scaffold constructs to dynamic ***compressive strains*** produced temporal changes in gene, protein expression and biomechanics. Compression of ***chondrocyte-agarose scaffolds*** significantly increased ECFP fluorescence, Col2 mRNA expression, type II collagen content and aggregate modulus. ECFP fluorescence and Col2 mRNA expression were found to be positively correlated. Compression of ***MSC-agarose constructs*** significantly increased ECFP fluorescence, type II collagen content and aggregate modulus. Finally, 28 days of compressive stimulation of ***fibroblast-agarose***

constructs produced significant decreases in GFP-T fluorescence as well as increases in aggregate modulus. The absence of ECFP expression in any of the fibroblast-agarose constructs indicates that cells were not upregulating the Col2 gene.

In a similar way, ***tensile stimulation*** of specialized and primitive cells altered both gene expression and tensile stiffness. While tensile stimulation of ***MSC-collagen constructs*** increased GFP-T fluorescence, Col1 gene expression and linear stiffness, tensile stimulation of chondrocyte-collagen constructs decreased ECFP fluorescence and linear stiffness. Absence of GFP-T expressing cells in the chondrocyte-collagen constructs indicates that the cells were not upregulating the Col1 gene.

In summary, natural healing of patellar tendon results in inferior tissue even after 6 weeks of healing. Murine knees exhibit temporal and spatial variations in Col1 and Col2 gene expression. In-vitro mechanical stimulation studies show that specialized cells respond more rapidly and to a greater extent than MSCs when stimulated with signals that they normally experience in-vivo. MSCs respond more rapidly and to a greater extent than specialized cells when they are stimulated with signals they do not normally experience in-vivo. Future studies must identify appropriate mechanical cues to control/modulate MSC, chondrocyte, and fibroblast differentiation in-vitro so that multiple cellular phenotypes can be spatially and temporally produced, resulting in the synthesis of functional extracellular matrices after surgery.

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This dissertation is dedicated to my parents and my son, Nishyanth.

“Imagination is more important than knowledge. Knowledge is limited. Imagination encircles the world.”

Albert Einstein
In an Interview by George Sylvester Viereck,
for the October 26, 1929 issue of
The Saturday Evening Post

ORGANIZATION OF DISSERTATION

This dissertation is organized as a collection of several journal manuscripts, each with the customary sections, including a summary, materials and methods, results and discussion. The presentation of these manuscripts is integrated to produce a coherent dissertation with well-defined objectives and clearly stated conclusions.

Chapter 1 presents the background of tendon injuries and the different approaches to their treatment. This is followed by the specific aims and hypotheses of the current work.

Chapters 2 through 9 present 7 journal manuscripts. The first manuscript presented in Chapter 2, describes Col1 and Col2 gene expression and biomechanics of natural healing of punch defects in the patellar tendons. The second manuscript presented in Chapter 3 examines Col1 and Col2 gene expressions and TGF-β1 and BMP-2 expressions during normal growth and development of knee structures. Chapter 4 presents the development and validation of a bioreactor capable of delivering precise compressive signals. This chapter is part of a bigger manuscript on bioreactors from our lab. The fourth, fifth and sixth manuscript in Chapters 5, 6 and 7 respectively describe in-vitro studies that investigates the effect of compressive stimulation on chondroyctes, MSCs and fibroblasts respectively. The seventh and the eighth manuscript in Chapters 8 and 9 describe in-vitro studies that investigate the effect of tension on MSCs and chondrocytes respectively. Chapter 9 presents discussion of the studies above and Chapter 10 present recommendations for future research in this field.

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Chapter 1

Background and Literature Review

1.1. Frequency and Significance of Musculoskeletal Injuries

Soft tissue (e.g. tendons, ligaments) injuries represent about half of the 32 million musculoskeletal injuries that take place each year in US [4]. Orthopaedic doctors treat about 14.5 million patients with ligament, tendon sprains and joint dislocations [5]. Statistics also reveal that the incidence of tendon and ligament disorders is high. For example Achilles tendon, rotator cuff, and patellar tendon disorders are reported to be around 100,000 per year, 51,000 per year and 42,000 per year respectively [6-8]. Anterior cruciate ligament injuries are reported to be around 125,000 per year [9]. Such injuries also continue to escalate each year [4]. Such injuries can produce serious disability and discomfort to the patients.

The cost associated with soft tissue injuries are substantial, amounting to over $30 billion in 1998 [4] which includes both direct (hospitals, physicians etc; 86%) and indirect (lost productivity, morbidity etc; 12%). With the incidence of injuries rising each year the associated cost rises as well. Hence the impact associated with injuries is substantial. Successful repair of such soft tissue injuries should not only return their functionality but also their normal structure and composition. Hence knowledge of these tissues in their normal state is essential.

1.2. Tendon Function

Tendons connect bone to muscle and function to effect the movement of lower limbs, upper extremities and head by transmitting forces from the muscle [4, 10-12]. They carry compressive forces when wrapped around bone like a pulley [13-15]. Tendons also serve as shock absorbers, energy storage sties and serve to maintain posture through their proprioceptive properties [12, 14].

1.3. Structure and Composition of Tendons

Tendons, dense fibrous connective tissues, can be described as biphasic materials composed of both solid and fluid components. Tendons are highly hydrated structures in that 65% of the tissue's wet weight is accounted by water [3, 16-17]. Type I collagen is the primary protein of the tendon accounting for not only 60% to 85% of the dry weight but also for 95% of collagen types present [3, 16-18]. Type II collagen is primarily found in the fibrocartilage regions of the tendon insertions into bone and is also present in tendons that wrap around bone [12-15]. Elastin accounts for about 2% of the dry weight and the rest of the tendon mid-substance is made up of proteoglycans, glycoproteins (such as fibronectin, thrombospondin) and other collagen types such as type, III, V, IX, X, XI and XII [3, 12, 17-18]. These minor collagens in tendons have unique functions. For example type V collagen has been shown to regulate the fibril diameter of type I collagen [18-20]. Type III collagen has been shown to be important during early healing and then is believed to then decrease as new type I collagen is synthesized and organized [21-22]. Collagen types IX, X and XII exist with type II collagen in the insertions [18, 23-25] and thought to minimize the stress concentrations when loads are transmitted from soft tissue into bone [18, 26]. The predominant proteoglycan in tendon is decorin [3, 17]. Decorin along with fibronectin is thought to regulate collagen fibril diameter [18, 27-28].

Collagen in the tendon is arranged in hierarchical levels of increasing complexity beginning with the tropocollagen molecule [3]. Soluble tropocollagen molecules first form cross-links to create insoluble collagen molecules which then aggregate to form collagen fibrils, then fibers, primary bundles and secondary bundles, and finally the whole tendon (Fig.1.1) [29-31]. Scanning and transmission electron microscopy reveals that the fibrils are 100-500 nm in diameter and consist of microfibrils which are 3.6 nm in diameter [29-31]. A collagen fiber is the smallest tendon unit that can be tested mechanically and is visible under light microscopy [29-

31]. Although collagen fibers are oriented longitudinally, fibers also run transversely forming spirals and pleats[12].

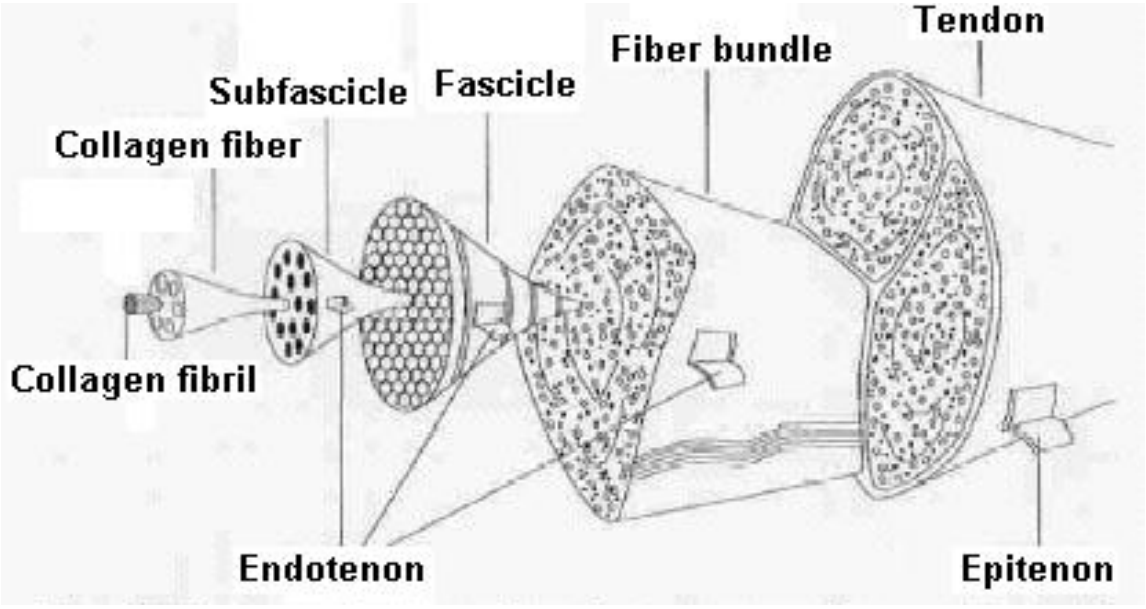

Fig. 1.1. The organization of the tendon structure from collagen fibrils to the entire tendon [3]

Such extracellular matrix of the tendon is synthesized and maintained by the tissue's cells, including tenocytes and tenoblasts (immature tendon cells) [3]. These cells account for about 90-95% of the cellular elements of the tendon [1, 3, 12, 16]. Tenoblasts are spindle-shaped and have numerous cytoplasmic organelles, reflecting their high metabolic activity [12]. As they mature, tenoblasts become elongated and transform into tenocytes with lower nucleus to cytoplasm ratio, less cytoplasmic organelles and less metabolic activity than tenoblasts [3]. The other 5-10% of the cells includes the fibrochondrocytes at the insertion sites, synovial cells on the tendon surface and endothelial cells and smooth muscle cells in the endotenon and epitenon [1, 3, 16]. In pathological conditions, other cells such as inflammatory cells, macrophages, myofibroblasts can also be observed [3].

1.4. Biomechanics of Tendons

Tendons transmit force from muscle to bone and act as a buffer by absorbing external forces to limit muscle damage [32-34]. Tendons exhibit high tensile strength [33-37]. Tendons

are viscoelastic tissues that display 20-40% stress relaxation but rather modest creep [38-39]. The stress-strain mechanical behavior of tendon (Fig. 1.2) depends on the number and types of intramolecular and intermolecular bonds or crosslinks in collagen [33-34, 40]. Unstretched collagen fibers and fibrils in tendons display a crimped configuration [33-34, 41]. When the tendon is strained upto 2%, flattening of the crimp pattern occur leading to the initial concave portion (toe region) [33-34, 42-44] (Fig. 1.2). Beyond this point, the intramolecular sliding of the

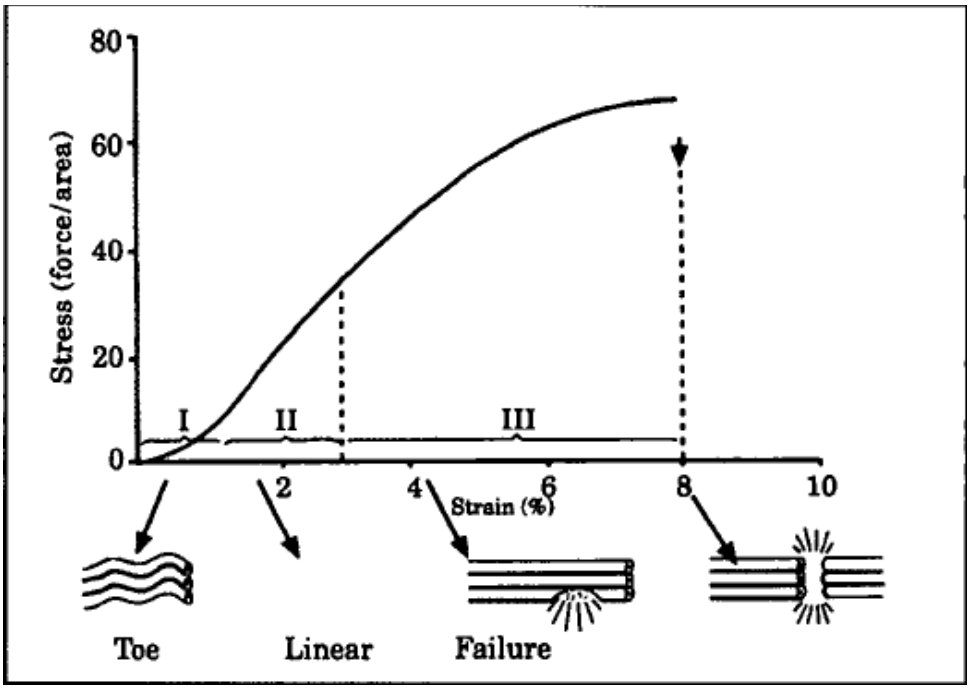

Fig.1.2. Typical stress strain curve for tendon [1]

collagen triple helices which causes a more parallel fiber orientation, results in tendon deforming in a linear fashion [33-34, 45-46]. If the strain remains <4-5%, the tendon deforms in an elastic fashion, returning nearly to its original length when un-loaded [33-34, 47]. Between 5%-8% strain, microscopic failures occur, and beyond 8% strain, macroscopic failure occur because slippages of lateral adjoining molecules. As the strain, increases the gap between the molecules increases eventually leading to rupture [33-34, 36, 43, 48-49]. After this, complete failure occurs rapidly, and the fibers recoil into a tangled bud at the ruptured end [32-34]. This structure-function relationship indicates that for repairs to be successful one most recreate the collagen structure found in normal tissues.

The material properties of tendon allow them to tolerate stresses that correspond to forces as high as 15 times body weight. Tendons possess ultimate tensile strength, moduli and strain at failure in the range of 60-100 MPa, 500-800 MPa and 13-22% respectively [50-53]. The above parameters serve as normal benchmarks when evaluating tendon repair.

1.5. Tendon injury

Tendon injuries can be classified as either acute or chronic and either direct or indirect [54-55]. Direct acute tendon injuries occur due to trauma, sports and lacerations by sharp objects. Indirect tendon injuries occur due to acute tensile overload and repetitive microtrauma. These indirect injuries generally occur in the musculotendinous junction (sprains, strains and rupture), or to the osteotendinous junction (avulsion fractures or bone detachment) [55]. These indirect injuries predominate over mid-tendon ruptures because healthy tendon can withstand higher tensile loads than the muscle or most bone junctions [54-59].

1.6. Tendon healing

1.6.1. Tendon healing in animal model

Inducing distinct, but reproducible injuries offers an ideal way to study tendon healing in an animal model. The two most common types of tendon injury models studied in the animal models have been the complete and partial transections [55]. Complete transections with a scalpel better simulate the loss of function seen clinically, despite being more local than the fiber disruption observed at surgery [55, 60]. The advantages of complete transection models are that they are generally reproducible in location, pattern, and extent of damage [55, 60] and create a gap that must be closed between the two free ends generally with the help of sutures. This closure of gap is essential for a variety of reasons: 1) to minimize scar tissue formation, 2) hasten collagen synthesis, and 3) avoid the introduction of non-tendinous tissue between the two ends [55, 61]. One major disadvantage in this model, however, is that the resulting repair biomechanics are strongly influenced by suture type, injury location and knot location [55]. Investigators have also created complete transection models without sutures, allowing tendon

healing to commence by gap formation. However, gap dimensions and gap shape affect repair biomechanics. As expected, the rate and extent of natural healing slow as the gap increases in length. The tensile strength of gaps exceeding 3 mm in length does not improve over time [55, 62-63]. These types of gaps are ideal for studying the benefits of cell based tissue engineering but only if they have the initial inherent strength to tolerate the expected in-vivo forces.

Partial transection models can also be created in the middle half or along one side of the tissue. Although they are unlikely to occur clinically, injuries in the central half of the tissue leave the edges of the tissue intact, circumventing the need for suture repair and allowing for immediate postoperative tendon mobilization [55, 64-66]. In addition, partial injuries have the advantage of not requiring sutures to reduce gap formation. The resulting gaps in the central region of the tissue also permit the quality of the newly synthesized tissue to be examined separately from the remaining intact tissue. This is why we in our lab have elected to choose this model to study tendon tissue engineering modalities.

1.6.2. Phases in Tendon Healing

Injured tendons heal by a cascade of events which can be classified into three phases; 1) Inflammation, 2) Proliferation, and 3) Remodeling [1, 16, 33-34, 55]. These phases, which are briefly described below, have characteristic cellular, temporal and biomechanical characteristics. These phases overlap and their duration can vary greatly due to location of injury or disease [1, 33-34, 55, 67-68]. Understanding this biology of tendon repair is essential to devise strategies to effectively repair them.

1.6.2.1. Inflammation

The first step in the healing process is the inflammatory phase in which hematoma occurs in the injured sites resulting in blood clots. This process is then followed by the activation of vasodilators, platelets and the release of pro-inflammatory chemicals from circulating mast cells [1, 33-34, 55, 69-70]. These inflammatory cells are attracted to the injury site in the first 24 hours after injury. These cells help in the breakdown of blood clots and in the digestion of

necrotic tissues [34, 70]. New fibroblasts are then recruited to the injured site. Angiogenesis promoting factors are also released to initiate growth of capillary networks within the wound [34, 55, 70-72]. This phase is characterized by increases in DNA, fibronectin, glycosaminoglycans, water, and collagen type III content [55, 67-68, 73-74].

1.6.2.2. Proliferation

The injury site in this phase is composed of a disorganized matrix of granulation tissue with fibroblasts as the predominant cell type, though a small number of macrophages and mast cells still exist. These fibroblasts are engaged in active matrix synthesis as evidenced by an increase in their endoplasmic reticulum [55, 73]. This phase is characterized by peak increases in Type III collagen and DNA concentrations [34, 70].

1.6.2.3. Remodeling

During this phase, the scar tissue becomes more translucent. Fibroblasts decrease in size and show less collagen and glycosaminoglycan synthesis [34, 55]. Collagen fibers also begin to reorient along the long axis of the tendon [75]. As the scar matures, the ratio of type III to type I collagen decreases and increases occur in collagen crosslinks, glycosaminoglycan, water, and DNA content [55, 76]. However, this process occurs slowly, in that healed tendon can take up to a year to approach the functional strength of uninjured tissue [55, 67, 77-78].

1.6.3. Intrinsic vs. Extrinsic Healing

The tendon healing biology is dominated by two theories, extrinsic and intrinsic healing [33-34, 55, 76, 79-81]. The central tenet in extrinsic healing, is that tendon cannot heal on its own. Tendon repair is accomplished by the formation of adhesions, the infiltration of inflammatory cells and fibroblasts, and an extratendinous blood supply [33-34, 55, 80-85]. In the case of intrinsic healing, it is believed that tendon can heal on its own without the formation of adhesions. This is accomplished by the proliferation of epitenon and endotenon cells within the tendon and with the help of an intratendinous blood supply [71, 79, 81, 86-97]. While both theories seem credible, there is no clear evidence as to which theory is correct. It is plausible

that tendon heals by a combination of both extrinsic and intrinsic factors. Which factor dominates might likely depend on tendon type and location, the magnitude of tendon trauma, availability of synovial fluid and a blood supply and degree of tendon mobilization [79, 86, 98].

1.7. Conventional Treatment Techniques

Conventionally, either conservative or surgical methods or tendon replacements have been used to repair tendon injuries. Conservative management involves rest and pain relief, and can include injection of a variety of drugs, including corticosteroids and physiotherapy [99]. This type of treatment can result in prolonged treatment times, possible weakness in the affected area, recurrent injury, and partial loss of function even after extensive rehabilitation [99]. Conservative methods will not work if the injury is serious.

Surgical methods involve repairing the damaged tissues using sutures and when damage is extensive, replacing the damaged tissue with grafts or replacements. There are a variety of different suture techniques used clinically such as Lin locking, Savage, Kessler, Becker, modified Kessler, Tajima, and epitenon suture method [55-57, 77, 100-101]. However these suture techniques have resulted in mixed results [102-104].

Autografts and allografts have been used as tendon replacements. However preclinical studies in animal models using autografts reveal the potential shortcomings of this treatment approach. While short-term studies show improvements in autograft maximum force, longer-term studies reveal no significant further improvements in strength [105]. Another important drawback in using autografts to replace damaged tendon is the creation of a second morbidity site that heals slowly and incompletely. The best example of this is the use of bone-patellar tendon-bone (BPTB) autografts to reconstruct the anterior cruciate ligament (ACL). While the central-third portion of patellar tendon remains the gold standard to reconstruct the ruptured ACL [106] with a success rate approaching 90% [107], harvesting these graft can lead to patellar tendon rupture [108], transverse patellar fractures and medial or lateral patellar subluxation and dislocation [109] in patients. Animal studies also support these clinical

concerns. These wound sites may never return to normal function, even after prolonged periods [110-111]. For example, even 21 months after removing the goat's central PT, the maximum force and maximum stress of the tendon repair tissue are still only 49% and 35% of normal [111]. After 6 months, the maximum force of the healing canine PT defect were about 60% of normal [112]. Moreover, the modulus of this PT repair site continued to decline from almost 90% of normal initially, to 32% and 16% at three and six months, respectively [112]. Histologically studies have demonstrated that the harvest site is not normal. The harvest site was filled with disorganized collagenous scar tissue composed of small collagen fibrils, is more cellular and vascular than the normal tissue [110-112]. It is obvious from all these studies that even after long periods of time, the repair tissue that develops after harvest of a central bone patellar tendon bone (BPTB) autograft is inferior to normal tissue not only in terms of its structural and material properties, but also in terms of its histological and ultrastructural appearance. It is for these reasons that surgeons not only avoid harvesting the same graft when revising a failed ACL reconstruction, but are looking for new and less invasive alternatives.

While allograft reconstruction avoids the need to create a secondary morbidity site, these grafts also pose a risk of transmitting infectious diseases such as hepatitis and HIV to the recipient. Sterilizing techniques such as ethylene oxide or gamma radiation have been used to minimize the risk of disease transmission [113-118]. Though the freeze-dried ethylene oxide-sterilized allografts have been used in various animal models and in humans [113-114, 118], it has some drawbacks. For example ethylene oxide sterilization has been shown to completely degrade the graft and to cause the formation of femoral cysts in patients [118]. Also the effectiveness of this type of sterilization against HIV is under doubt [118]

Gamma radiation of greater than 3 Mrad are necessary to sterilize grafts [115]. However, these high levels of gamma irradiation also significantly reduce allograft strength [116-117]. While delivering a 2 Mrad dose to the graft did not alter its properties, frozen BPTB grafts which were exposed to 3 Mrad of gamma irradiation showed 27% and 40% reductions in maximum

force and strain energy to maximum force respectively. Should the BPTB allografts require even higher dosages of irradiation to avoid any risk of disease transmission, even more significant, dose-dependent reductions can be expected in both initial structural and material properties [115-116]. These reductions in strength were accompanied by significant reductions in hydroxypyridinium crosslink density [116]. The problems associated with treating these allografts have led researchers to look at synthetic graft materials.

1.8. Prosthetic/Biologic Tendon Replacement

Both prosthetic (polyester [119-120], Gore-Tex prosthesis (polytetrafluoroethylene) [121-123] silicone [124-126] and carbon fibers [127-133]) and biologic materials (collagen [134-135]) have been used as tendon replacements. Prosthetic replacement systems have fallen out of favor in orthopaedic practice because of these following problems. Use of these prosthetics resulted in 1) high incidence of rupture or loosening in patients [136-137], 2) in not fully replacing the function of damaged/torn tissues [120], 3) induced growth [127-131] with the characteristics of a prolonged inflammatory reaction and a disorganized ECM [132, 136], 4) having a finite lifetime as their mechanical properties degraded over time [99], 5) raising questions about their long-term bio-compatibility [124-126], 6) producing particles which accumulated in lymph nodes which in some cases inhibited fibroblast growth [133]. These problems with synthetic grafts have lead researchers to seek our tendon replacements that are biological in nature.

Because of the above-mentioned problems with synthetic scaffolds it would, therefore, be desirable to have a functional tendon replacement that is biologic in origin, which not only integrates into the host but also does not induce inflammatory reaction [138]. Scaffolds made from collagen have been used as biologic tendon replacements [134-135]. Use of collagen scaffolds to treat Achilles tendon gap defects led to tissue ingrowth [135]. The collagen scaffold was completely resorbed by 10 weeks post implantation. The initial strength of these tendon repairs were only 11% of normal, but increased to 80% by 20 weeks [134]. However use of

such biological replacements has some drawbacks. 1) Long term evaluation of repairs show that the strength of the repairs dropped to about 66% of normal [134]. 2) Collagen scaffolds cross-linked with glutaraldehyde resulted in the inducing inflammatory response and resulted in the formation of a fibrous capsule [135]. 3) The strength of the repairs were not significantly different from the repairs achieved by simply reattaching the Achilles tendon [134-135]. 4) Factors such as age, cell proliferation capacity affect the resultant repair from these biologic materials [139-140]. New concepts such as tissue engineering may now offer potential cell-assisted repair approaches to overcome such impairments.

1.9. Tissue Engineering as a Possible Alternative to Conventional Tendon Repair

Given these difficulties with using certain biological and synthetic scaffolds to improve tendon repair, it is not surprising that investigators are using cell-based tissue engineering approaches to treat tendon injuries. Tissue engineering offers an attractive alternative to these clinical problems and has been studied in a variety of animal models. Tissue engineering has been defined as *"a field of science that makes use of biological and/or biocompatible synthetic materials in conjunction with cells to create new tissues or biologic substitutes to serve as functional tissue replacements"* [141].

Investigators have attempted to enhance the healing response of tendons through the use of engineered scaffolds mixed with either differentiated cells such as tenocytes or undifferentiated cells such as mesenchymal stem cells. Several groups have explored tenocyte seeded constructs [142-143]. While these constructs have shown to improve healing[144], still concerns exist. For example factors such as cell seeding density, adhesion, viability, and extracellular matrix production might play a role in the determining the mechanical properties of the resultant tissue engineered constructs [51, 145-146]. There are concerns about cell source because of the creation of a secondary morbidity site to harvest the autologous cells and the time needed for cell expansion in-vitro.

A second approach is the use of mesenchymal stem cells (MSCs) to create tissue engineered constructs [51, 147-156] . The main drawback with the use of MSCs is the question about controlling differentiation. Differentiation towards the fibroblast phenotype can be achieved through the use of growth factors as well as mechanical stimulation [151-155]. Although MSC-seeded scaffolds placed in a tissue defect do improve healing [156] the tissue stiffness and strength do not yet meet in-vivo loading demands [157-159] and questions of safety remain since ideal conditions to create the desired ECM is not fully understood. Similar to the use of tenocytes factors such as scaffold, cell seeding, adhesion, viability, and extracellular matrix production also play a role. Researchers continue to investigate the optimal combination of scaffold, cells and stimulation (either chemical or mechanical or a combination of both) to augment tendon healing. What must also be included, however, is a better understanding of the functional environment into which these constructs are to be placed at surgery.

1.10. Functional Tissue Engineering

Despite the potential advantages of tissue engineering, investigators now recognize the need to adapt the field to include tissue function in the design of tissue engineered repairs. This has resulted in the creation of a new branch of tissue engineering called functional tissue engineering (FTE) [159] whose goal is to enhance tissue stiffness and extracellular matrix synthesis and organization as well as shorten the time needed to fabricate these engineered tissues. “FTE emphasizes the importance of biomechanical considerations in the design and development of cell and matrix-based implants for soft and hard tissue repair” [160]. Functional Tissue Engineering ideally seeks to regenerate or at least functionally repair damaged and diseased tissues, in order to ensure their normal mechanical performance after surgery. Our group first described these criteria for all load-bearing tissues. We emphasized the need to record normal in-vivo tissue forces for different activities of daily living (ADLs) to serve as design parameters for new generations of reparative tissue constructs [156, 160-161]. FTE also emphasizes the need to mechanically precondition the tissue in culture so as to acclimate these

constructs to their future in-vivo loading regimes. Numerous investigators have cited the beneficial effects of applying FTE principles to various types of cell-seeded constructs and tissue models [162-170]. Our group and others have shown the beneficial effects on linear stiffness and collagen expression after applying dynamic, uniaxial tensile as well as compressive strains to constructs seeded with mesenchymal stem cells (MSCs) and chondrocytes, respectively [167, 170-173]. We have also demonstrated that mechanically stimulating MSC-sponge constructs in culture with aspects of the in-vivo strain signal and then implanting these constructs in patellar tendon defects significantly improves the resulting repair biomechanics 12 weeks after surgery [171]. As a result, FTE principles and strategies hold much promise in advancing tendon tissue engineering toward the ideal goal of regenerating normal tissue with normal properties in the functional range of loading.

1.11. Challenges Still Remain in Using FTE to Improve Tendon Repair

At the same time, tissue engineers still face major challenges in creating a truly functional tendon mid-substance and insertion into bone. 1) Restoring the zonal insertion site. Using FTE principles, we have already been able to introduce mechanically-stimulated MSC-collagen sponge constructs to improve repair of rabbit central-third patellar tendon defects. While the 12-week repairs actually match normal PT tangent stiffness up to 150% of peak in-vivo forces [171], these 12-week repairs still do not recreate the complex fibrocartilaginous zonal insertions of the tendon into bone. Overcoming this challenge requires a new strategy that better controls cell phenotype and matrix expression and assembly. 2) Native tendon biomechanics and biochemistry. These need to be better characterized. These will then serve as mechanical and biological benchmarks against which we can compare our repairs. 3) Scaffold material. There is no universally agreed upon biomaterial to use as a tendon FTE scaffold. Either current scaffolds need to undergo tests specific to tendon structure-function or investigators need to develop new tendon-specific scaffolds. 4) Cell and culture conditions. Cell type, seeding conditions, and mechanical and chemical culturing conditions need to be

determined in order to optimize the environment for cells in culture. 5) Design parameters to judge successful outcomes. Methods to assess the effectiveness of tissue engineering approaches also need to be developed. We must ascertain whether the tendon has been truly regenerated or at least functionally repaired and at long-enough time periods after surgery.

1.12. Need for New Strategies to Improve Tendon Repair

Although these FTE constructs created in our lab matched or exceeded mechanically-based tissue engineered design parameters, parameters other than mechanical are also critical to restore. Clearly we need new strategies to create repair tendons with zonal insertions and to speed the FTE process. Below are several proposed strategies.

1) One strategy to create insertions is to introduce primitive cells such as bone marrow-derived mesenchymal stem cells or MSCs into biological scaffolds and then precondition these MSC-based constructs to produce tendon like cells in the mid-substance and fibrocartilage-like cells near the attachments. We might accomplish by using either hybrid biomaterials or different stimulation methods (mechanical and or chemical) in the mid-substance vs. ends.

2) Another possible strategy would be to use specialized cells (fibroblasts or chondroctyes) in different regions of the constructs and then apply mechanical strains and other culture conditions to create constructs with tendon-like cells in the mid-substance and fibrocartilage-like cells in the insertions.

3) Still a third strategy for tissue engineers would be to understand tendon mid-substance and insertion biology during normal growth and development and then seek to mimic these events during natural healing or with cell-based therapy. However, our knowledge of these biological processes in different regions of the tissue is still quite limited. Understanding these events could lead to more meaningful therapies that accelerate repair to both the tendon mid-substance and its zonal insertion into bone.

Unknown in this process are the functional loads acting on the tendon during development. The lack of such data hampers our ability to select appropriate mechanical

signals to properly stimulate the cell-based tissue engineered constructs (TECs) in-vitro. Tissue engineers may ultimately need to identify those mechanical signals that cause the TEC to recreate not only regional variations in collagen architecture but also spatial changes in cell phenotype that promote synthesis and maintenance of appropriate extracellular matrix (ECM).

Systematically monitoring relevant signaling remains a very complex and costly tissue engineering process. Currently, this in-vitro stimulation process is slow and serial in nature. This process is also difficult to optimize because various culture conditions like cell and scaffold type as well as environmental stimuli (e.g. mechanical and chemical) must be tested to understand their interactions and how various combinations affect the synthesis of appropriate ECM. These extra steps in the design phase also make tissue engineering quite expensive and time consuming. If these cell-based approaches are to eventually become clinical tools for the orthopaedic surgeon, what is likely required is a novel approach to determine the effects of these environmental stimuli (e.g. specific loads and strains) on cell phenotype in real or at least near-real time.

One example of how this strategy might be applied relates to regional differences in collagen types in tendon. Since Types I and II collagen are major structural proteins found in the tendon mid-substance and insertion site, respectively, we need to understand spatial and temporal expression of these genes in normal, injured and repairing tendons as well as in tendon TECs in culture. Developing a real-time technology to monitor these genes would aid in this effort.

1.13. Use of Fluorescent Proteins as Markers for Collagen I and II Gene Expression

We and others have been linking fluorescent proteins to various gene promoters to visualize expression of genes in real time. Such promoter-fluorescent protein markers have been used to examine embryonic and skeletal development, to distinguish cells in different stages of differentiation, and to conduct cell culture studies [174-178]. We have chosen to

initially use this fluorescent technology in the mouse model to track expression of two important structural genes, Collagen I and II, in real time during development and healing.

The mouse containing the transgene pOBCol3.6GFPtpz was acquired courtesy of David Rowe, University of Connecticut Health Center. This transgene contains a 3.6 Kb fragment of the rat Col1a1 promoter, enhancer sequence and GFP-T. GFP-T expression in these mice is evident in skin, tendon and osseous tissues and indicates upregulation of the Col1 gene [176].

To monitor Col2 expression in the tendon, we have utilized ECFP as a reporter. Plasmid pCol2-ECFP was derived from the expression gene containing the mouse type II collagen promoter and enhancer [179] (provided by W. Horton, Northeastern Ohio College of Medicine). The β-gal gene was replaced with ECFP (Clontech, Palo Alto, CA). Orientation of the ECFP was confirmed by sequencing, and ECFP function was tested in cellulo by transient transfection of chondrocytes in a primary culture from rabbit cartilage. The pCol2-ECFP construct was injected into mice blastocysts. The founder mice identified by PCR showed high levels of expression based on fluorescence in cartilaginous tissue as viewed through a fluorescence stereomicroscope (MZFL3, Leica, Germany)

Mice transgenic for either pOBCol3.6GFPtpz or pCol2-ECFP were then bred to produce double transgenic animals. No apparent phenotypic differences were observed between the doubly and non-transgenic mice. GFP-T fluorescence was apparent in ossifying tissues and ECFP fluorescence was observed in cartilage (Fig. 1.3). This novel reporter mouse has permitted us to visualize, in near real time, either a fibroblast program or a chondrocyte program within a population of cells. Using these mice we have been analyzing Col1 and Col2 gene expression levels during in-vivo development, injury and repair. Our ability to also quantify these gene expression levels in near-real time in culture holds the potential for screening constructs in culture to determine how gene expression patterns change with alterations in cell and scaffold type as well as environmental stimuli). Such technology could ultimately speed development of new repair therapies.

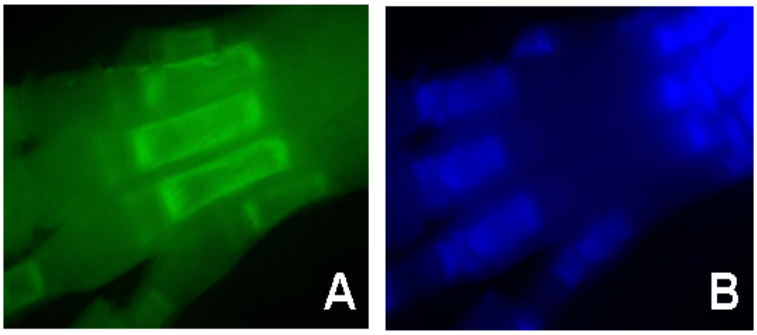

Fig. 1.3. Newborn doubly transgenic mouse foot. GFPT fluorescence is apparent in ossifying tissue (A), while ECFP fluorescence is apparent in cartilage (B).

Using these double transgenic mice, the first part of my dissertation has been focused on observing gene expression during healing by inducing punch biopsy injuries in the patellar tendon of the doubly transgenic mouse and then examining spatial and temporal changes in Col1 and Col2 gene expression in and around the injury site. While the small size of the mouse patellar tendon precludes us from confidently reproducing central one-third defect injuries that occur clinically during harvest of ACL grafts, the punch defect in a superficial PT involves minimal surgery and is thus less invasive than injuries that might be induced in the ACL or in other deeper tendon models. Next I focused on understanding Col1 and Col2 gene expression patterns in normal development and natural healing. I examined these patterns in embryonic to early post natal tendon development.

Using cells from these transgenic mice, the second part of my dissertation research has focused on how mechanical signaling of cell-based TECs in culture affects the plasticity of primitive MSCs and specialized chondrocytes and fibroblasts. I decided to de-couple the complex signals acting on tendon by initially investigating how controlled compressive and tensile displacements influence near real-time Col1 and Col2 gene expression in two cell-based constructs of simple shapes. Although these cylindrical- and rectangular-shaped constructs do not resemble actual tendon geometry of the tendon, these simplifications allow me to carefully

apply these controlled displacement patterns to the constructs in a simple fashion that allows their individual effects to be quantified.

1.14. Specific Aims and Hypotheses

When adult tendons are injured, the body repairs the tissue by a complex wound healing cascade that results in the formation of a scar [55, 67-68, 81, 96, 180-182]. The mechanical properties of this scar tissue have been shown to be inferior when compared with normal tissue [55, 181, 183-185]. While natural healing of tendons has been studied [55, 67-68, 81, 96, 180-185], little knowledge currently exists about spatial and temporal patterns of Col1 and Col2 gene expressions during natural healing after injury to tendons. This lack of knowledge is the basis for our first set of research questions. ***How does introducing a controlled injury in the mid-substance of the PT affect Col1 and Col2 gene expression over time? How does this injury affect repair biomechanics over time?*** We chose to study the effect of injury to the patellar tendon of the doubly transgenic mouse because the patellar tendon is easily accessible unlike other tendons. Also the patellar tendon inserts proximally into the patella and distally into the tibia, which will facilitate mechanical testing of the small mouse tendon after injury. In order to address the research questions above, we developed the following specific aim and hypotheses.

Specific Aim 1: Create a punch defect in the mid-substance of the PT in a 10-week old DT mouse that has nearly achieved skeletal maturity. Evaluate how earlier time intervals after injury (1, 2, and 4 weeks) change Col1 and Col2 gene expression and how later time intervals post surgery (4 and 6 weeks) influence repair biomechanics. Compare these results to gene expression patterns and biomechanics in the contralateral shams and age- matched PTs.

Hypothesis 01: Compared to age-matched PT and sham injuries in the contralateral PT, a punch defect injury increases Col1 gene expression at 1 and 2 weeks post surgery which then declines at 4 weeks post surgery as healing enters the remodeling phase.

Hypothesis 02: A punch defect injury produces no detectable Col2 gene expression in the mid-substance healing wound site.

Hypothesis 03: Repair structural and material properties of the injured PT increase over time but remain significantly lower than structural and material properties for paired sham controls and age-matched controls.

Hypothesis 04: Structural and material properties of shams will not be different from the structural and material properties of age-matched controls.

Results from the natural healing study showed increases in Col1 gene expression in the injured PT mid-substance from 1 to 2 weeks with a subsequent decrease by 4 weeks. This temporal pattern seems to follow the temporal phases of healing. Results also indicate that the quality of repair is inferior. Hence strategies are necessary to improve the quality of repair and one such strategy is tissue engineering. Recently, tissue engineers have turned to developmental biology as a way to try and improve the repair of tendons [186]. The premise for this new approach is that understanding normal growth and development of tendons might help in designing tissue engineering constructs that facilitate later repair. In particular, understanding similarities in expression of extracellular matrix genes and growth factors might allow tissue engineers to develop more effective treatments to augment adult tendon healing. "*A bio-mimetic approach to tissue engineering or emulation of some aspects of normal tissue development and remodeling, could be a key to future success in the field of tissue engineering*" [186]. This need to understand the expression of extracellular matrix genes and growth factors during normal growth and development led to our next research question. ***How does embryonic to early post-natal development affect Col1 and Col2 gene expression levels in the murine patellar tendon?*** Two growth factors TGF-β1 and BMP-2, [187-189] and their signaling pathways through PSmads [187, 190] have been shown to be important during normal knee development. Treating tendon fibroblasts with TGF-β increases the phosphorylation of smad2/3 (194), suggesting that TGF-β signaling is mediated through pSmad2,3 [190]. BMP signaling has

been shown to be mediated through pSmad1,5,8 [187]. Details of control of normal tendinogenesis by TGFβ1 and BMP-2 are not known, nor when and where pSmad2,3 and pSmad1,5,8 are expressed. Determining these spatiotemporal activation patterns during tendon development could provide cues to enhance tendon healing using TECs. These led to the next set of research questions. ***How does normal knee development affect spatial and temporal expression levels of TGF-β and pSmad2,3? How does normal knee development affect spatial and temporal expression levels of BMP-2 and pSmad1,5,8?*** In order to address these questions we developed the following specific aims and hypothesis.

Specific Aim 2: Determine how murine patellar tendon (PT) development from embryonic (E17.5) through early post-natal (P1, P3 and P14) ages influences expression of both the Col1 and Col2 genes, as well as TGF-β1 and BMP-2, and their upstream signaling molecules, pSmad1,5,8 and pSmad2,3, respectively.

Hypothesis 05: Col1 and Col2 will be expressed at similar times during PT development in utero.

Hypothesis 06: Col1 and Col2 gene expressions increase until P3 with a subsequent decrease by P14.

Hypothesis 07: TGF-β1 will be expressed in the mid-substance of the PT while BMP-2 will be expressed in the PT insertions, femur and tibia at all ages.

Hypothesis 08: pSmad1,5,8 expression will mirror TGF-β1 expression.

Hypothesis 09: pSmad2,3 expression will mirror BMP-2 expression

Results from growth and development studies of knee structures indicate that there are temporal changes in Col1 and Col2 gene expressions and that there are regions in the PT insertions which have co-expression of Col1 and Col2. These observations lead me to the following research questions***. Can such temporal changes in gene expression be recreated during in-vitro culture using both primitive (MSC) and specialized cells (chondrocytes,***

fibroblasts)? Can such co-expression of Col1 and Col2 also be recreated during in-vitro culture in primitive and specialized cells?

Col1 and Col2 gene expression can be modulated in-vitro by the use of either mechanical or chemical stimulation. Since mechanical stimulation has been the focus of the tissue engineering efforts in our lab, I decided to use tensile and compressive signals to modulate collagen gene expression in cells.

The first set of in-vitro studies were designed to evaluate how compressive stimulation on MSCs, chondrocytes and fibroblasts affect Col2 and Col1 gene expression levels. Researchers have studied the effects of compressive stimulation of, chondrocytes [148, 165, 168-169, 191-197], MSCs [198] and fibroblasts [199]. Dynamic compression of chondrocyte-agarose constructs increased type II collagen synthesis, gene expression, synthesis of GAG as well as compressive mechanical properties [148, 165, 168-169, 191-197]. Preliminary studies performed in our lab using chondrocytes from single transgenic ECFP+ mice show that dynamic compression (10% peak strain at 1 Hz for 1 hr followed by a 1 hr rest period repeated for 6 hours/day) induces significant increases in ECFP Relative Fluorescence Units (RFUs) (Col2 gene expression) at both day 7 and 14 compared to non-stimulated controls.

Groups have examined the effects of compressive stimulation on mesenchymal stem cells. Angele et al [198] found that applying 14 and 28 days of cyclic hydrostatic pressure to TECs containing human marrow-derived mesenchymal progenitor cells significantly increased both proteoglycan and collagen contents. Cyclic compressive loads (sinusoidal waveform of 0.33 Hz to a peak stress of 7994 Pa over a period of 4 hours/day) applied to TECs composed of mesenchymal progenitor cells in 3-D scaffolds significantly increased chondrogenic differentiation [200]. Static and dynamic compressive loads applied to TECs containing embryonic limb-bud mesenchymal cells in agarose increased chondrogenic differentiation [201-202]. Cyclic compressive displacements applied to constructs formed using rabbit bone-marrow derived mesenchymal stem cells in agarose induced chondrogenesis in-vitro [203]. Investigators

have also shown that dynamic compressive displacements applied to rabbit MSC-agarose constructs elevated Col2 and aggrecan expression levels. While chondrogenesis does occur in MSCs the amount of the matrix formation and the mechanical properties are lower than that produced by chondrocytes under the same conditions [204]. These studies taken together with studies from the previous paragraph provide evidence that application of compressive stimulation increases Col2 gene expression in chondrocyte and MSC constructs.

Investigators have also examined the effects of compressive stimulation of fibroblast seeded in 3-D constructs. Application of 10% compressive strain to human periodontal ligament fibroblasts in-vitro [205] also decreases Col1A1 RNA expression as well as type I collagen and fibronectin protein expression. Cyclic hydrostatic pressure applied to tendon fibroblasts expanded from rat Achilles tendon has also led to increased Col2 and aggrecan mRNA expression with an associated decrease in Col1 mRNA expression [206]. All of these studies provide evidence that compression decreases Col1 gene expression levels and increase Col2 gene expression levels in fibroblasts.

Given these findings, we then sought to determine if compressive stimulation 1) would increase Col2 gene expression, protein and compressive properties, in doubly transgenic MSCs and chondrocyte constructs and 2) decrease Col1 gene expression and increase Col2 gene expression in fibroblast constructs. These studies motivated several research questions***. Does dynamic compression of murine MSC and chondrocyte constructs increase ECFP expression? Does this increase in ECFP expression correspond to increases in Col2 mRNA expression in these constructs at the same time interval? Do these expected increases in ECFP and qRT-PCR expression levels lead to an increase in Type II collagen protein content and later increases in aggregate modulus of these constructs? Can dynamic compression of fibroblasts drive these cells toward a chondrogenic phenotype?***

In order to address these questions we developed the following specific aims and hypotheses.

Specific Aim 3: Examine how dynamic compression (0% vs. 10% peak strain at 1 Hz for 1 hr followed by a 1 hr rest period repeated for 6 hours/day) of TECs composed of double transgenic chondrocytes cultured in 3-D agarose gels affects ECFP expression, Col2 mRNA expression, type II collagen content and aggregate modulus of the constructs after 0, 7, 14, 21 and 28 days of stimulation.

Compared to non-stimulated controls at equivalent time points in culture, dynamic compressive stimulation of chondrocyte-agarose TECs

Hypothesis 10: Increases ECFP fluorescence.

Hypothesis 11: Increases Col2 mRNA expression.

Hypothesis 12: Increases type II collagen content.

And

Hypothesis 13: Increases aggregate modulus.

Also we propose that,

Hypothesis 14: ECFP fluorescence as quantified by relative fluorescence units (RFUs) and Col2 mRNA will be linearly correlated.

Specific Aim 4: Examine how dynamic compression (10% peak strain at 1 Hz for 1hr followed by a 1hr rest period repeated for 6hours/day) of constructs containing double transgenic MSCs in 3-D agarose gels affects the cell's ECFP expression, type II collagen content, as well as the construct's aggregate modulus after 0, 7, 14, 21 and 28 days of stimulation. Compare Col2 gene expression levels, type II collagen content and aggregate modulus in these MSC-agarose constructs to those observed in chondrocyte-agarose constructs exposed to identical conditions

Compared to non-stimulated controls at equivalent time points in culture, dynamic compressive stimulation of MSC-agarose TECs

Hypothesis 15: Increases ECFP fluorescence.

Hypothesis 16: Increases type II collagen content.

Hypothesis 17: Increases aggregate modulus.

Compared to chondrocyte seeded constructs exposed to similar conditions, MSC seeded constructs will have

Hypothesis 18: Delayed ECFP fluorescence.

Hypothesis 19: Lower ECFP fluorescence.

Hypothesis 20: Lower type II collagen content.

Hypothesis 21: Lower aggregate modulus.

Specific Aim 5: Examine how dynamic compression (10% peak strain at 1 Hz for 1 hr followed by a 1 hr rest period repeated for 6 hours/day) of TECs containing double transgenic murine fibroblasts in agarose gels affects ECFP and GFP-T expression levels and aggregate modulus after 0, 7, 14, 21 and 28 days of stimulation.

Compared to non-stimulated controls at equivalent time points in culture, dynamic compressive stimulation of fibroblast-agarose TECs

Hypothesis 22: Decreases GFP-T fluorescence.

Hypothesis 23: Increases ECFP fluorescence.

Hypothesis 24: Increases aggregate modulus.

The second set of in-vitro studies were designed to evaluate how tensile stimulation of MSCs and fibroblasts affect Col1 and Col2 gene expression levels. Researchers have studied the effect of tensile stimulation on MSCs and chondrocytes. Our lab and others have applied FTE principles to create tendon TECs by mechanically stimulating mesenchymal stem cells (MSC) seeded in collagen constructs in tension. Juncosa-Melvin et al [171] demonstrated that two weeks of mechanical stimulation applied to rabbit MSC-collagen sponge constructs in culture improved their stiffness and also significantly improved PT repair biomechanics at 12 weeks post surgery. Juncosa-Melvin et al [167] also showed that mechanically stimulating these constructs in culture not only improved repair linear stiffness and modulus, but also increased Col1 and Col3 gene expression levels. Altman et al [173] also showed that mechanically stimulating bovine stem cell-collagen gel constructs for 21 days in culture increased Col1 and

Col3 gene expression levels. These studies suggest that applying a tensile stimulus to our murine MSC-seeded collagen constructs might also increase Col1 gene expression levels.

Several modes of mechanical stimulation, including compression, shear, and hydrostatic pressure has been shown to modulate chondrocyte matrix synthesis, but the effects of tensile strains on chondrocytes have not been widely explored. Oscillatory tension (0-10%)is known to decrease Type II collagen synthesis in chondrocyte-seeded constructs [207]. What is not known is how tension applied to murine chondrocytes affects Col2 and Col1 gene expression levels.

Given these findings, we then sought to determine if tensile stimulation would increase Col1 gene expression levels in double transgenic murine MSCs seeded in collagen constructs and decrease Col2 gene expression levels in double transgenic chondrocytes seeded in agarose. This motivated several research questions**. *Does tensile stimulation of murine MSCs seeded in collagen sponges increase GFP-T expression? Does tensile stimulation also increase Col1 mRNA expression in these MSC-collagen constructs by quantitative real time RT-PCR, a conventional measure for assessing gene expression*? *Does such mechanical stimulation increase linear stiffness within these cell-based constructs? How does tensile stimulation of TECs containing chondrocytes seeded in collagen sponge affect both Col1 and Col2 mRNA expression?***

In order to address these questions we proposed the following specific aims and hypotheses.

Specific Aim 6: Examine how applying a tensile stimulus (0% vs. 2.4% peak strain at 1 Hz for 20 seconds followed by a 100 second rest period repeated for 5 hours/day) to a TEC containing double transgenic MSCs cultured in 3-D collagen sponge scaffolds affects GFP-T expression, Col1 mRNA expression and linear stiffness after 0, 7, and 14 days of stimulation.

Compared to non-stimulated controls at equivalent time points in culture, tensile stimulation of MSC-collagen TECs

Hypothesis 25: Increases GFP-T fluorescence.

Hypothesis 26: Increases Col1 mRNA.

And

Hypothesis 27: Increases linear stiffness.

We also propose that

Hypothesis 28: ECFP fluorescence as quantified by relative fluorescence units (RFUs) and Col2 mRNA will be linearly correlated.

Hypothesis 29: Tensile stimulation will not induce ECFP fluorescence.

Specific Aim 7: Examine how a tensile stimulus (2.4% peak strain at 1 Hz for 20 seconds followed by a 100 second rest period repeated for 5 hours/day) applied to a TEC containing double transgenic chondrocytes cultured in collagen sponges affect ECFP and GFP-T expression after 0, 7, 14 , 21 and 28 days of stimulation.

Compared to non-stimulated controls at equivalent time points in culture, dynamic compressive stimulation of chondrocyte-collagen TECs

Hypothesis 30: Decreases ECFP T fluorescence.

Hypothesis 31: Increases GFP-fluorescence.

Hypothesis 32: Decreases linear stiffness.

These proposed studies are intended to improve our understanding of the in-vitro behavior of tissue engineered constructs. This knowledge is critical to our ability to control/modulate differentiation of MSCs, chondrocytes and fibroblasts in-vitro into desirable cell phenotypes. This level of control should help us achieve our goals of preconditioning TEC's to rapidly create functional tissue engineered [FTE] constructs for complex structures like tendons that insert into bone.

Chapter 2

Spatial And Temporal Relationships Of Collagen I And II Gene Expression And Repair Biomechanics In Healing Patellar Tendons Of Double Transgenic Mice

The objectives of this study is to examine the spatial and temporal changes in Col1 and Col2 gene expression and repair biomechanics during the healing of murine patellar tendons. 10 week mice carrying fluorescent transgenes for Col1 and Col2 gene were used. A 0.75mm full thickness punch defect was created in the mid-substance of the patellar tendon of 27 mice. The contralateral limb served as sham controls. Another 27 animals served as non-operative controls. Col1 and Col2 gene expression were examined at 1, 2 and 4 weeks post surgery. The patellar tendons were tested biomechanically at 4 and 6 weeks post surgery. Our results indicate that swelling and disorganization decreased with healing time. Col1 gene expression after injury increased by 2 weeks and then decreased in intensity by 4 weeks. No Col2 expression was seen in the mid-substance of healing tendons. Force-elongation and stress-strain curves demonstrate that healing tendons while not different at 4 and 6 weeks were still significantly weak than sham and normal tendons. In the future, this repair model and models like it should allow researchers to develop a roadmap for tissue engineering and cell therapy that more closely simulates normal development, potentially leading to regeneration rather than repair of tendon injuries.

2.1. Introduction

Injuries to tendon account for nearly half of the 33 million musculoskeletal injuries seen by physicians in the United States alone each year [4]. Epidemiologists estimate at least 100,000 Achilles tendon (AT) injuries [5] and 75,000 to 125,000 anterior cruciate ligament (ACL) injuries [208] are diagnosed and treated annually in the US alone. Patellar tendon injuries constitute a significant problem in a wide variety of sports [209-212]. While rupture of patellar

tendons is relatively infrequent, the incidence of such ruptures increases in older, active patients [213-218]. The cost of such musculoskeletal injuries account for over $30 billion of health care dollars spent each year [1]. Hence the impact associated with these injuries is substantial.

Successful orthopaedic surgical procedures should result in the return of normal tissue properties. Unfortunately, surgical repairs often exhibit inferior biomechanical properties compared to the native structures [138, 219-238]. Though knee cruciate ligament reconstructions using tendon grafts are 90% successful [219], studies suggest that these grafts achieve only a small fraction of their original strength and remain susceptible to re-injury [138, 220-222]. Moreover, the defect created in the patellar tendon as a result of the graft harvest fills slowly and can maintain a gap for up to 27 months [223]. This healing defect in the tendon exhibits inferior maximum stress at 3 months post surgery (36% of normal) which appears to decline by 6 months to 14% [224]. Animal studies have demonstrated that biomechanical and biochemical properties of the harvest site may never return to normal. even after prolonged healing time [111, 224, 239-240]. Healing tendons in the sheep recover only about 80% of their original strength in about a year [225, 227, 241]. This tendon repair site remains vulnerable to re-injury [228] and is still abnormal by ultrasound even 10 years after surgery [229]. Over 50,000 rotator cuff repairs are performed each year [230-231], but failure rates are high [242], reaching 40% due to muscle contraction, decreased range of joint motion, neurovascular damage, or postoperative alteration of normal shoulder mechanics [232-233]. Even fetal tissues which have show scarless repair after tendon healing after injury, exhibit inferior biomechanics [243]. Results from all these studies clearly indicate that more effective repair strategies are needed.

Tissue engineering [244] a cell-based therapy is one such strategy. Tissue engineering approaches have recently been used to improve repair of tendon defects [171, 245-248]. However, despite these developments in tissue engineering, we know less about the molecular biologic response in healing tendons. This is especially true for Collagen I and II gene expression, two critically important structural genes upregulated in musculoskeletal tissues like

tendon and its insertion into bone, respectively. As will be shown, we have bred double transgenic mice with fluorescent promoters for Collagen I and II gene expressions in order to track cellular response during the early phase of tendon healing.

In this study, we compared spatial and temporal patterns of Col1 and Col2 gene expression in normal patellar tendon vs. patellar tendons subjected to full thickness, mid-substance punch defect injuries. We hypothesized that relative to normal tendon expression patterns, mid-substance injury would: 1. significantly increase collagen I gene expression in the wound site by one week after injury. 2. Then decrease collagen I expression by 4 weeks post injury. 3. Produce no detectable collagen 2 gene expression in the mid-substance healing wound site. 4. Result in inferior repair stiffness at both 4 weeks and 6 weeks post injury compared to contralateral sham tendon stiffness and external normal tendon stiffness. 5. Higher nuclear aspect ratio at 1 and 2 weeks post injury compared to contralateral sham and age-matched controls.

2.2. Materials And Methods

Experimental Design

This study was approved by the University of Cincinnati IACUC. Fifty four (54) double transgenic (DT), 10-week old male mice were assigned to either a surgery group (n=27) or a non-operative age-matched control group (n=27) [249]. In each surgery animal, we created a full-thickness, half-width (0.75 mm) punch defect injury in the left patellar tendon mid-substance. These defects were wide enough to create reproducible injuries allowing our healing results to be related to those from a previous study [249]. A sham punch defect injury was performed in the contralateral right PT mid-substance. Nine (9) of the 30 surgery animals were assigned for Col1 and Col2 gene expression at 1, 2 and 4 weeks post surgery (n=3 for each time point) The remaining 18 animals were assigned for biomechanics at 4 and 6 weeks post surgery (n=9 for each time point). Of the 27 age-matched controls, three (3) animals each were assigned to

examine Col1 and Col2 expression at 11, 12 and 14 weeks, and nine (9) animals each were assigned to measure biomechanics at 14 and 16 weeks of age.

Surgical and Post-Operative Activity

Each animal was initially anaesthetized with 5% isoflourane and maintained under a 3% flow during surgery. Both hindlimbs were aseptically prepared prior to the surgical procedure. Using loupes at 2.5X magnification, an anterior skin incision was made over each patellar tendon and the edges of the patellar tendon were identified. Medial and lateral borders of the tendon were then longitudinally incised. Jeweler's forceps were initially used to create a pocket beneath the patellar tendon. A plastic-backed, modified #15 knife blade was then slipped beneath the full substance of the tendon (Fig. 2.1.A) [249]. Using a 0.75mm punch (Shoney Scientific, Waukesha, WI) a full-thickness defect was created in the mid-substance of the left tendon, using the knife blade as a support, so as not to injure tissues beneath the tendon (Figs. 2.1. B&C) [249]. The modified knife blade was also slipped behind the sham patellar tendon but no punch defect was created (Fig. 2.1.A). Both the injury and sham sides were closed using a 5-0 nylon suture. The subjects were monitored as they recovered from anesthesia and then permitted ad-lib activity in a cage.

Sacrifice

The mice were euthanized using CO_2 following protocols established by IUCAC. Hindlimbs were harvested and then prepared for either histology or biomechanics.

Histology

Surgery, sham and non-operative tissues were dissected to isolate the tendon mid-substance and insertions into bone (BTB). Tissues were then fixed in 4% paraformaldehyde overnight. The bone ends then fixed in 0.5M EDTA/PBS decalcifying solution for 7 days. The specimens were then soaked for 15 minutes in OCT and transferred to 30% sucrose where they were soaked for 1 additional day. All fixation steps were performed at 4^0C.

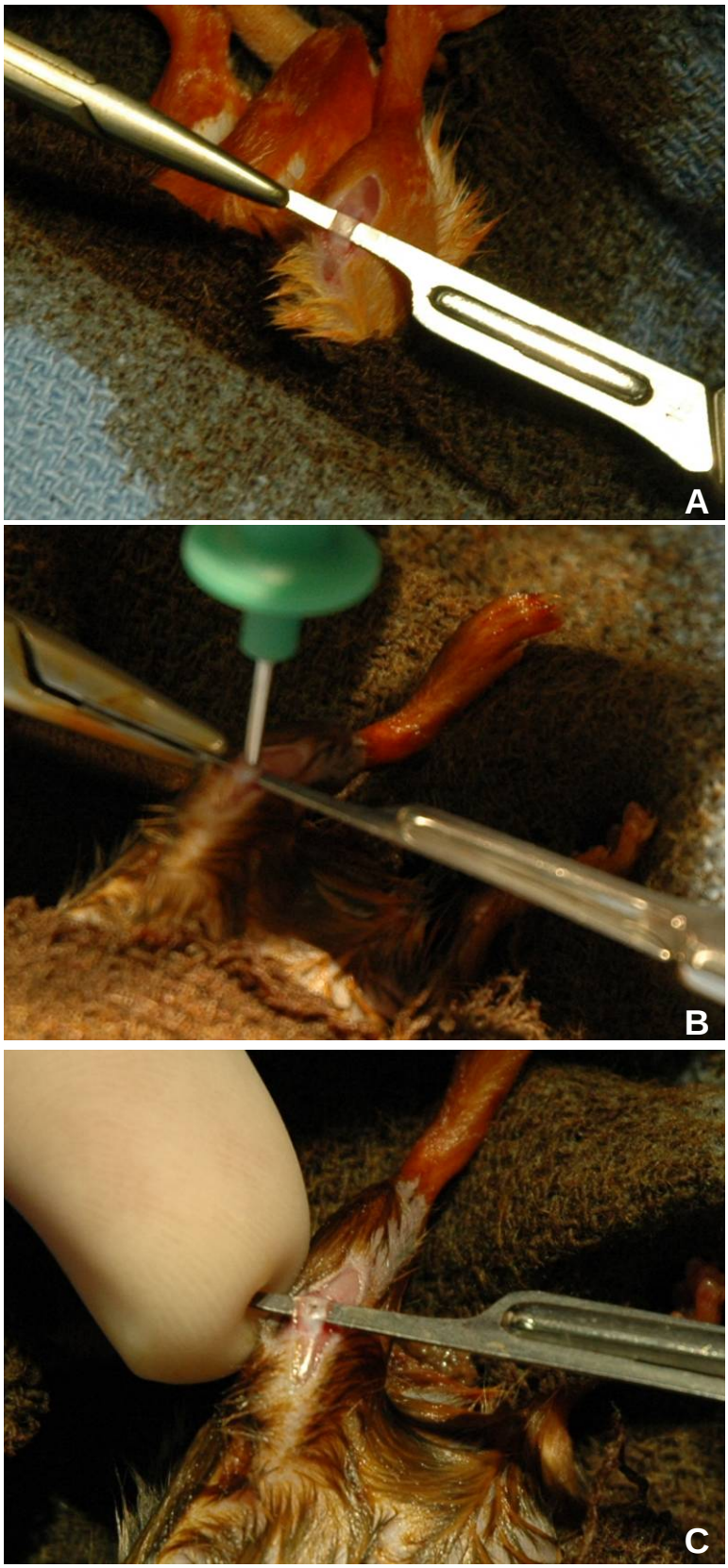

Fig 2.1. A punch defect injury was created in the mouse patellar tendon. A) A modified blade was first inserted beneath the tendon to provide support; B) a mid-substance injury was then made using a 0.75mm biopsy punch; C) revealing the mid-substance tendon injury. Only step A was performed in the contralateral sham tendon.

Specimens were then immersed in disposable base molds filled with embedding medium frozen on dry ice and stored at -80^{0}C. The bone-tendon-bone units were then sectioned in the sagittal plane (25 μm) and examined under an inverted fluorescent microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany). Filters for GFP-T (XF104-2, Omega) and ECFP (XF114-2, Omega) were selected to identify regions of Collagen I and Collagen II gene expression, respectively. Sections were also visualized using a rhodamine filter set (11002VZ, Chroma) to rule out auto-fluorescence. Sections were stained with H&E and nuclear aspect ratio was quantified using stereological sampling technology as described previously [250-251].

Biomechanical Analysis

Each injured, sham and unoperated control patellar tendon assigned for biomechanics was dissected and cleaned, leaving only the patella, patellar tendon, and tibia as one unit. The struts on either side of the patellar tendon were removed in order to leave a repair width of approximately 0.75mm. Tendon width and thickness were quantified from digital images in order to compute the cross-sectional areas of the normal and repair tissues.

The tibia was then embedded in polymethylmethacrylate in a custom-designed gripping fixture and secured in place with a metal pin (Fig. 2.2, [249]). The patella was then secured in a custom-designed, cone-shaped gripping fixture (Fig. 2.2, [249]). Each specimen was immersed in a Plexiglas chamber containing PBS (37^{0}C, 7.4).

The grips were then secured to a load cell above and an actuator below in a materials testing system (100R; Testresources, Shakopee, Minnesota). After ensuring vertical alignment, each specimen was preloaded to 0.02 N and then preconditioned for 50 cycles using a sinusoidal waveform (1% peak-to-peak strain at 1Hz). Each test specimen was then elongated to failure at a constant strain rate of 0.1%/s [249]. Force and grip-to-grip elongation were continuously recorded during the failure test. Linear stiffness was computed from the linear region of the force–elongation curve. Modulus was computed from the linear region of the

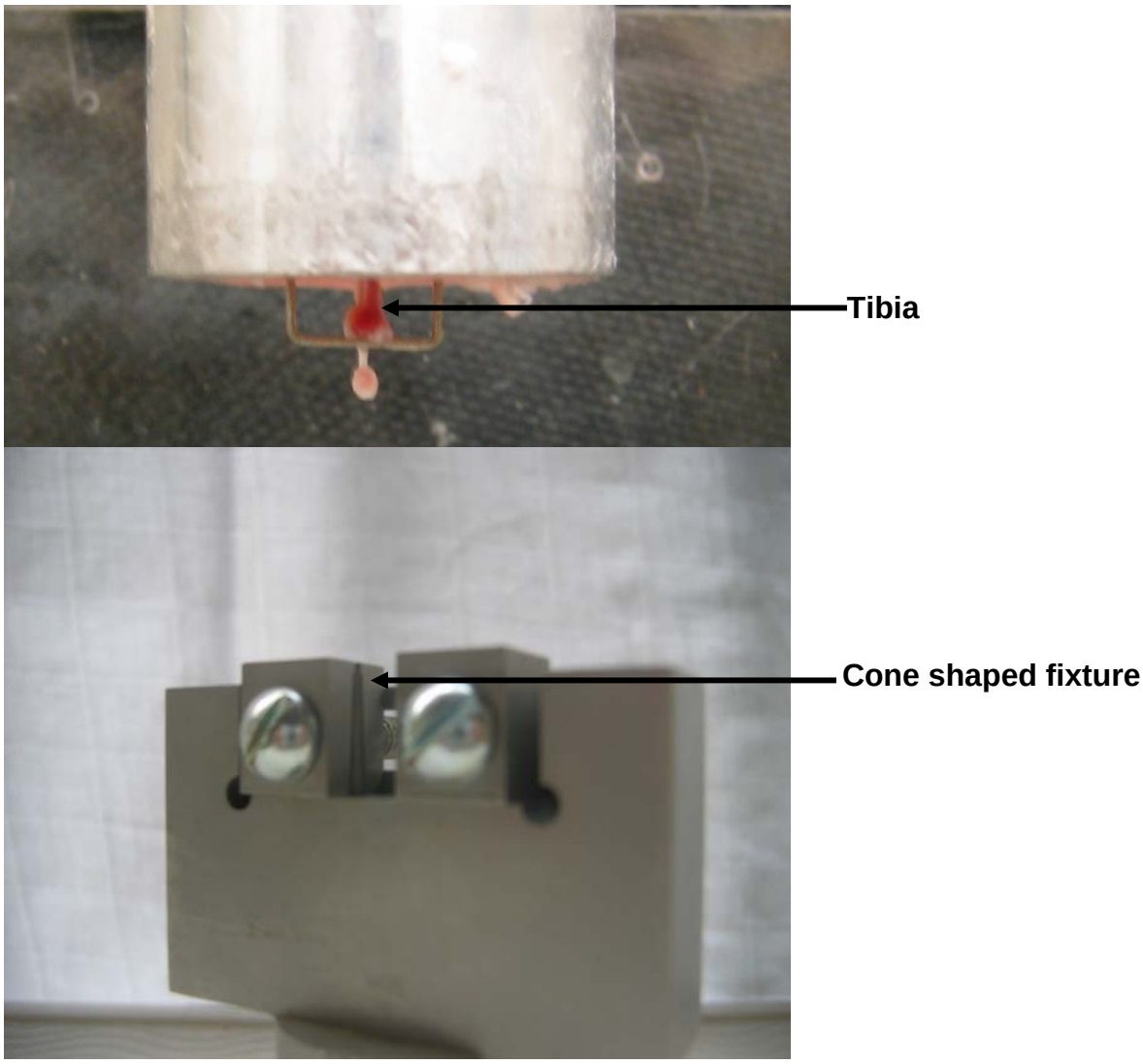

Fig 2.2. Tibia embedded in PMMA and held in place by a pin and cone shaped fixture for securing the patella. Our thanks to Dr. Lou Soslowsky for his laboratory's assistance with designing these grips.

stress-strain curve. Maximum force and elongation to maximum force were also recorded as well as the strain energy to maximum force (the area beneath the force-elongation curve to maximum force). Maximum stress, strain to maximum stress, and strain energy density to maximum stress was also calculated.

Statistical Analysis

Eight biomechanical parameters (linear stiffness, maximum force, elongation to maximum force, strain energy, modulus, maximum stress, strain to maximum stress, and strain energy density), nuclear aspect ratio and GFP-T average intensity were compared among treatment groups at each time interval and across time intervals using a three-way ANOVA. Significance was set at $\alpha = 0.05$.

2.3. Results

Gene Expression in Unoperated Patellar Tendon and Knee

Normal fluorescence patterns in the PT and knee were similar for animals of the same age. A typical example of fluorescence pattern for 12 week group is shown (Fig. 2.3C). These patterns were also similar across the three age groups (11, 12 and 14 weeks) and there were no difference in intensity measured quantitatively (Table 2.1). Mild GFP-T fluorescence was evident along the entire PT soft tissue substance for all three age groups (Figs. 2.4A-C). Areas of GFP-T fluorescence were relatively uniform in intensity in the tissue but not observed at all tendon locations in the section. GFP-T fluorescence was slightly more intense in the cortical bone regions of the femur, tibia and patella. This fluorescence was even more intense in the trabecular regions in all bones, especially in the 14 week-old animals. ECFP fluorescence was restricted to the femoral, tibial and patellar cartilage surfaces as well as to the proximal femoral and distal tibial growth plates (e.g. Fig. 2.4B for 12 week-old mouse). No ECFP fluorescence was present elsewhere in the cortical or trabecular bone of the patella, tibia and femur, however.

Bilateral Expression Patterns 1 Week after Injury (11 week-old mouse)

Fluorescence patterns in the PT and knee were similar for different 11 week-old animals. One week after injury, Col1 gene expression appeared to be lower than normal expression in the punch defect wound site as well as along the entire tendon mid-substance (Fig. 2.5A).

However, creating the punch defect resulted in swelling of the entire tendon and not just in the defect region at 1 week (Fig. 2.5A). This swelling was greater than that observed in either the contralateral sham (Fig. 2.6A) or its age-matched control (Fig. 2.4A). The healing tissue also appeared quite disorganized (Fig. 2.5A). Although ECFP expression was not observed within the tendon body, faint ECFP expression was seen in the insertional regions (Fig. 2.5A). Compared to injured tissues, sham controls at one week post surgery showed mildly increased

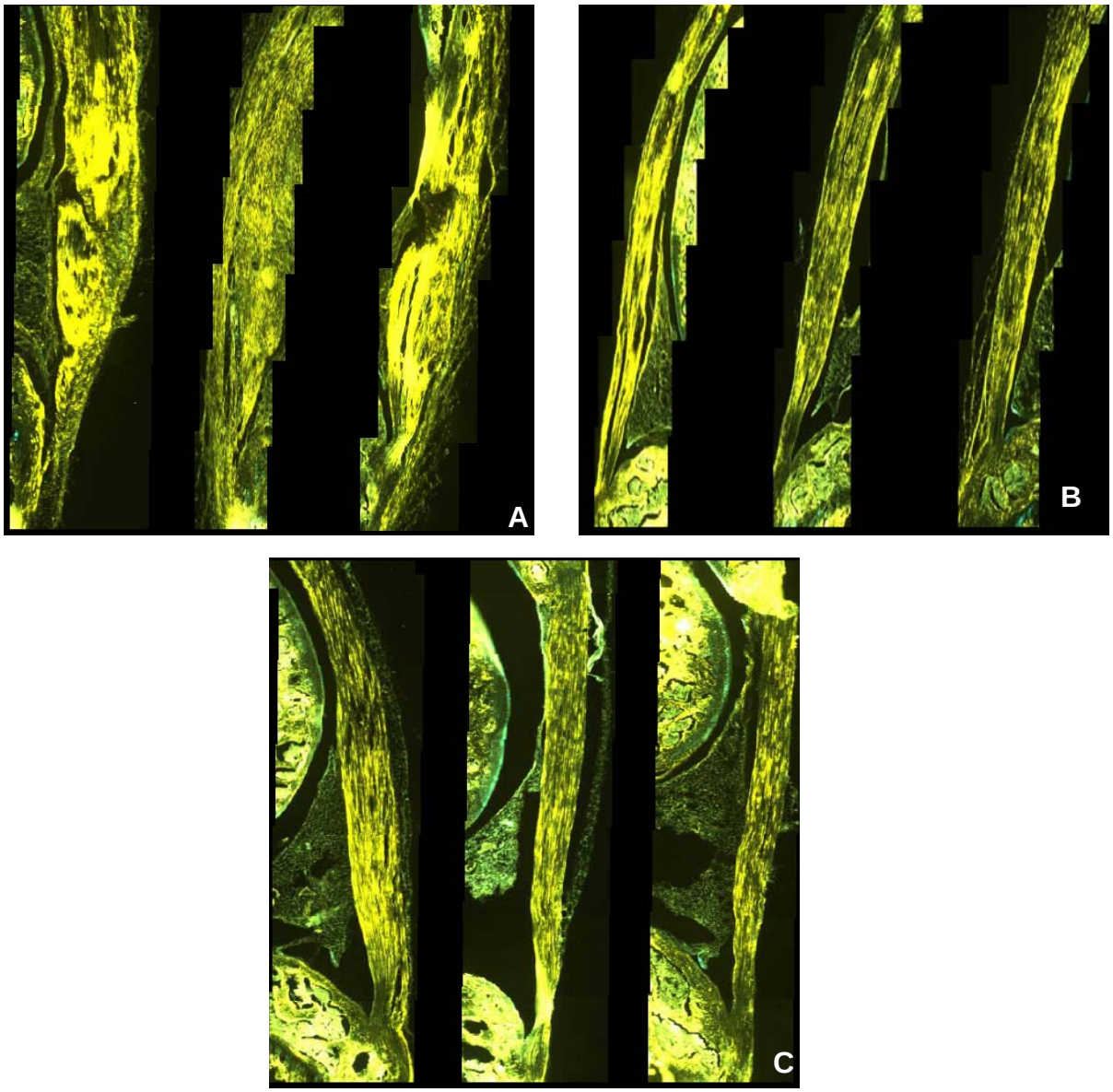

Fig 2.3. GFP-T fluorescence patterns are similar in different animals in A) 2 week post surgery B) corresponding shams C) 12 week age-matched controls

Col1 expression in the insertion sites and on the posterior surface (Fig. 2.6A). However, these shams showed no swelling and a more parallel fiber orientation compared to the injured tissue (Fig. 2.6A). Average GFP-T intensity was significantly lower in injured tendons than sham and age matched controls (Table 2.1, $p<0.05$).

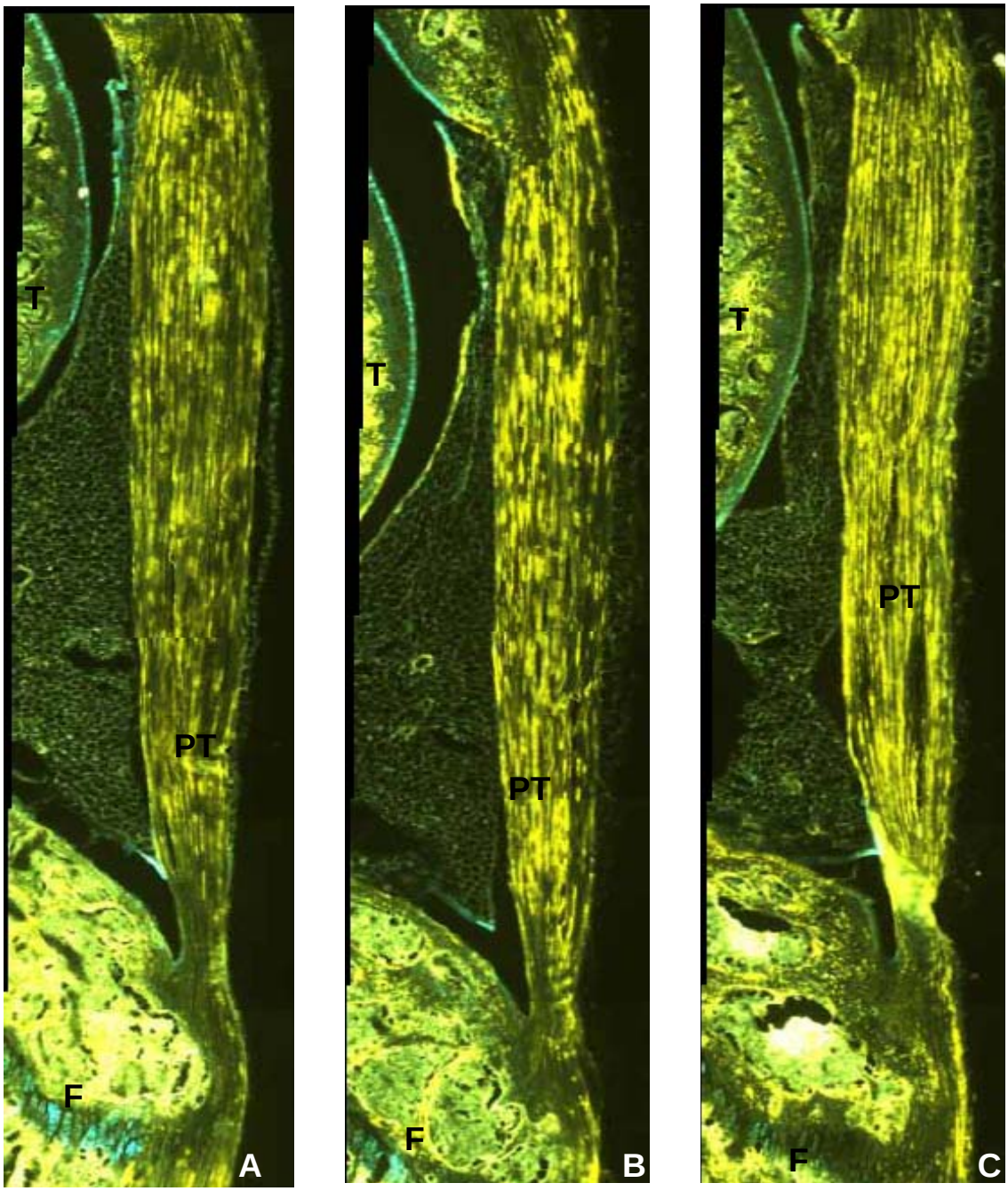

Fig 2.4. Typical GFP-T and ECFP expression patterns in: A) 11, B) 12, and C) 14 week-old, age-matched controls. GFP-T expression in evident in the mid-substance of the tendon and ECFP expression is evident in the articular cartilage of the condyle and in the growth plate of the tibia at all ages. F-Femur, T-Tibia, PT- Patellar Tendon.

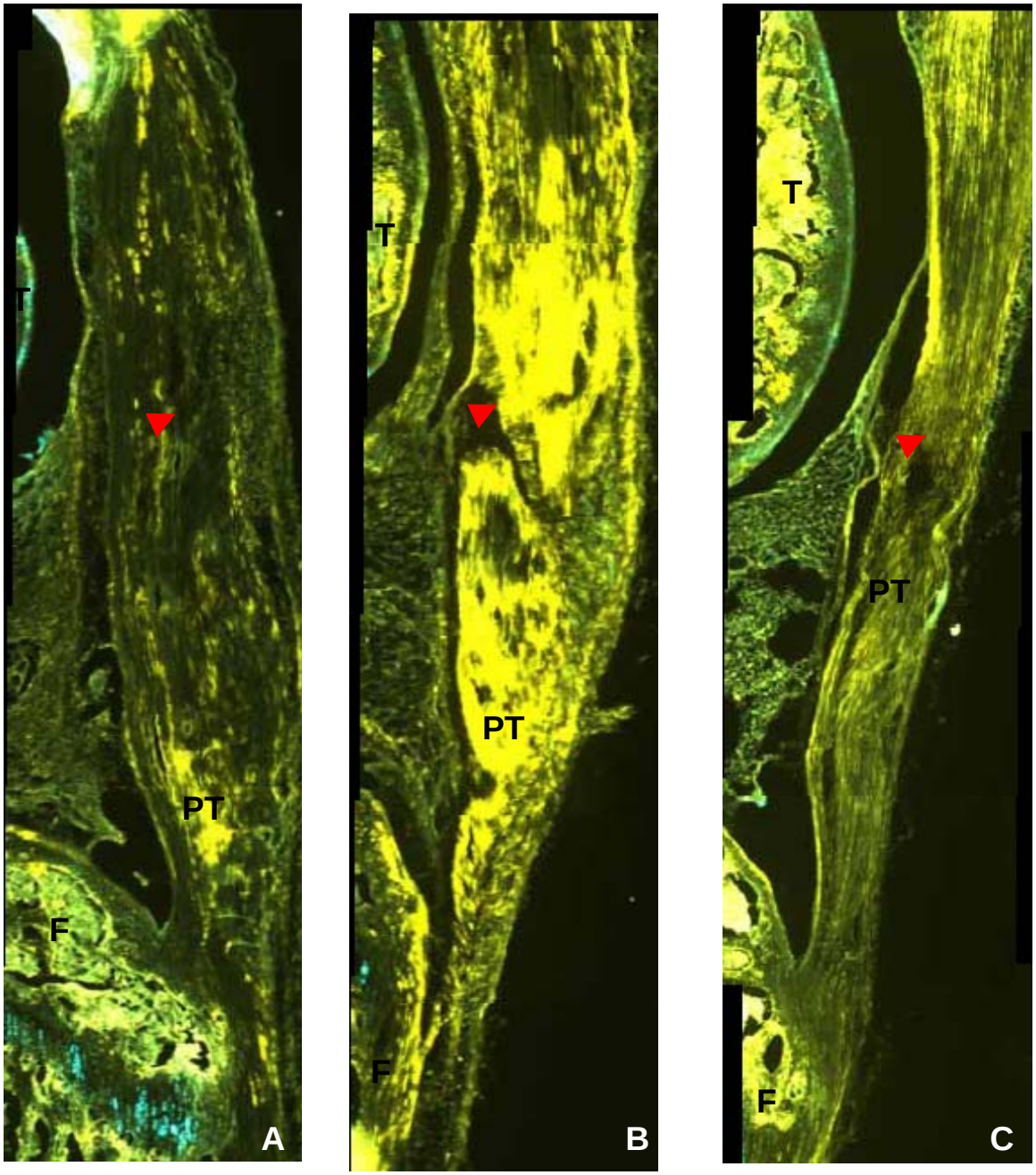

Fig 2.5. Typical GFP-T and ECFP expression patterns in healing tendons at A) 1, B) 2, and C) 4 weeks of natural healing (corresponding to 11, 12 and 14 week-old animals). Each arrow indicates the region where the punch defect was originally created. GFP-T expression is highest at 12 weeks. Edema of the tendon is especially evident at both 1 and 2 weeks of natural healing. F-Femur, T-Tibia, PT- Patellar Tendon.

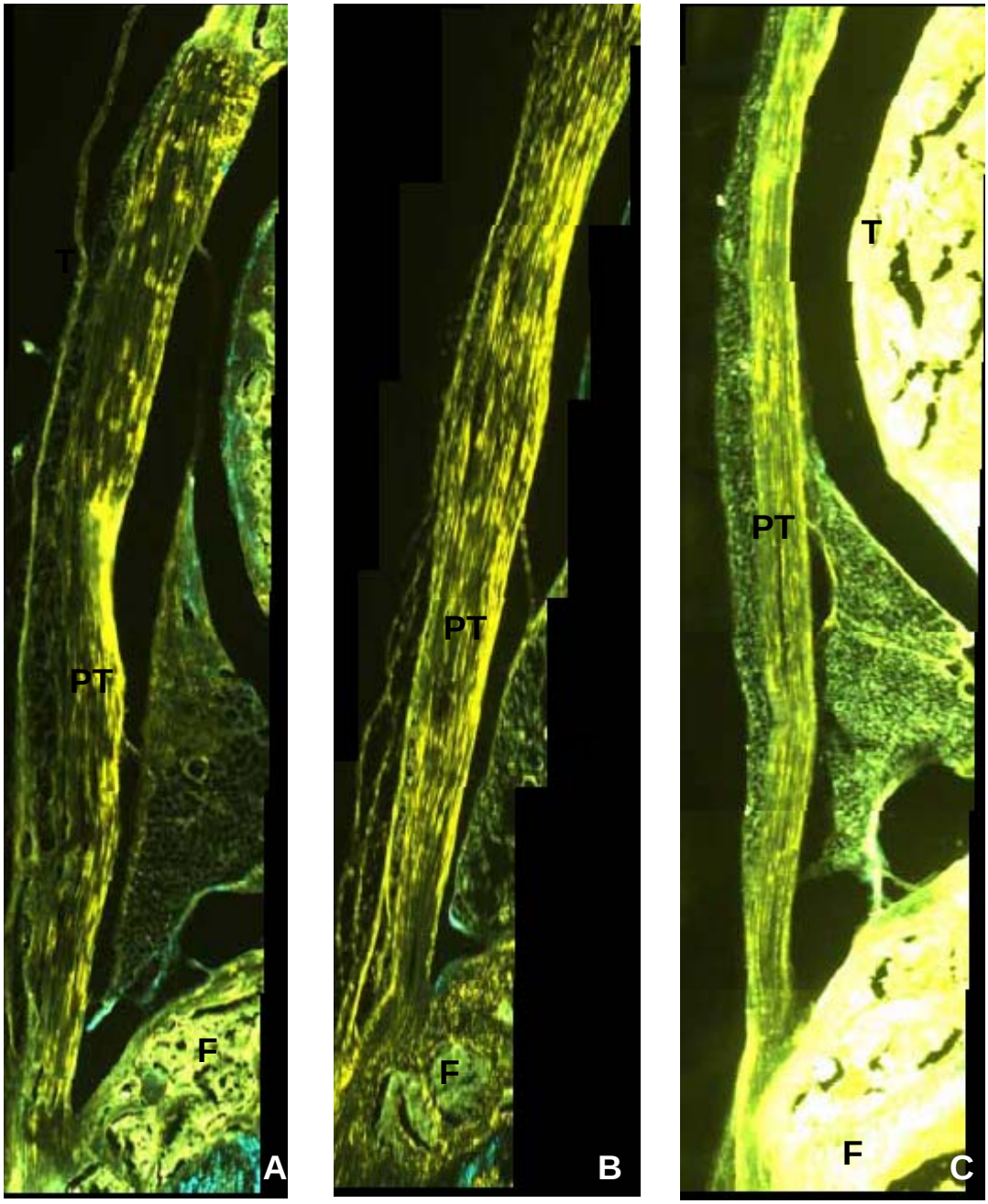

Fig. 2.6. Typical GFP-T and ECFP expression in contralateral sham tendons at A) 1, B) 2, and C) 4 weeks after surgery. Note the local increases in GFP-T expression levels on the posterior surfaces of the tendons. However, no swelling is obvious as a result of the procedure. F-Femur, T-Tibia, PT- Patellar Tendon.

Bilateral Expression Patterns 2 Weeks Post Injury (12 week-old mouse)

Fluorescence patterns in the PT and knee were similar for different 12 week old animals (Figs. 2.3A&B). Compared to results at 1 week, Col1 gene expression in the 2-week injured tendon was typically elevated all along the tendon mid-substance (e.g. Fig. 2.5B). These increases were particularly notable just proximal and distal to the cut edges of the punch defect site (Fig. 2.5B). The injured tendon showed a much more well-aligned fiber orientation with much less swelling compared to one week (Fig. 2.5B). Sham tendons (Fig. 2.6B) showed similar fiber orientation to age-matched controls (Fig. 2.4B) but with slightly elevated Col1 gene expression (Fig. 2.6B). Faint ECFP expression was noted in the insertional region of the injured and sham tendons but no ECFP expression was observed in either tendon mid-substance (Figs. 2.5B&2.6B). Average GFP-T intensity was significantly higher in injured tendons than sham and age matched controls (Table 2.1, $p<0.05$).

Bilateral Expression Patterns 4 Weeks post Injury (14 week-old mouse)

Once again fluorescence patterns in PT and knee were similar. By 4 weeks post injury, typical GFP-T expression had diminished all along the tendon (Fig. 2.5C), although it was still elevated compared to 1-week injured tendons (Fig. 2.4A). Collagen fibers were also more aligned compared to earlier time intervals. The injured tendon showed only modest thickening and/or swelling, and these changes were more restricted to the vicinity of the wound site (Fig. 2.5C). Sham PTs were relatively normal in appearance, but showed slight thickening (Fig. 2.6C). ECFP expression was only mildly elevated in the insertion regions of both the injured and sham tendons. Average GFP-T intensity was not different between injured tendons than sham and age matched controls (Table 2.1, $p>0.05$).

Nuclear aspect ratio

Nuclear aspect ratio in age-matched controls at 11, 12 and 14 weeks were similar (Table 2.2, $p> 0.05$). Similarly the nuclear aspect ratio in shams at 11, 12 and 14 weeks were similar (Table 2.2, $p>0.05$). No difference was seen between age-matched and shams at

corresponding time points (Table 2.2, p> 0.05). 1 week injured tendons had significantly higher aspect ratio than both 2 week and 4 week injured tendons and corresponding sham and age matched controls Table 2.2, p<0.05). By 4 weeks the injured tendons had ratios comparable to that of controls.

Healing Biomechanics 4 and 6 Weeks after Injury

Force-elongation and stress-strain curves for injured, contralateral sham, and external age-matched control PTs are shown in the 14- and 16-week old animals at 4 (Figs. 2.6A & 2.7A) and 6 weeks post injury (Figs. 2.6B&2.7B), respectively. Note the similar curve shapes and amplitudes to failure between the sham and control tendons for each age group. Also note the

	1 week	**2 weeks**	**4 weeks**
Injury	22.3±4.6^	184.1±5.2*^	41.6±10.2**
Sham	45.63±4.6	43.6±7.6	48.7±9.4
Age-matched	48.8±2.3	50.4±7.2	45.3±9.8

Table 2.1. Average GFP-T intensity was highest at 2 weeks post injury. * Significant difference in GFP-T intensity in 2 week injured PT from both 1 and 4 weeks. ** Significant difference in GFP-T intensity compared to both 1 week and 2 weeks ^ Significant difference in GFP-T intensity between injury and sham at corresponding time points.

	1 week	**2 weeks**	**4 weeks**
Injury	0.567±0.3*^	0.41±0.3**	0.36.±0.25
Sham	0.32±0.2	0.27±0.3	0.31±0.19
Age-matched	0.29±0.12	0.31±0.22	0.29±0.16

Table 2.2. Average nuclear aspect ratio was highest at 1 weeks post injury. * Significant difference in aspect ratio in 1 week injured PT from both 2 and 4 weeks. ** Significant difference in aspect ratio in 2 week injured PT from 4 weeks. ^ Significant difference in aspect ratio between injury and sham at corresponding time points.

similar appearance of these sham and control curves for the 14 vs. 16 week animals. No significant differences in structural and material properties were found among the sham and control tendons at the two time intervals (Table 2.3; p>0.05). The force-elongation and stress-strain curves for the injured tendons from each of the two time intervals were also lower than the corresponding age-matched controls and contralateral shams. The structural parameters measured from these curves revealed significantly lower stiffness, maximum force, strain

energy, and elongations to maximum force for the injured vs. age-matched shams and external controls (Table 2.3, $p<0.05$). The material properties measured from these curves revealed significantly lower modulus, maximum stress, strain energy density and maximum stress for the injured vs age-matched shams and external controls (Table 2.3, $p<0.05$). Time after injury (4 vs. 6 weeks) did not significantly affect any of the structural properties (stiffness, maximum force, strain energy to maximum force, elongation to maximum force) or computed material properties (modulus, maximum stress, strain energy density to maximum stress, or strain to maximum stress) ($p>0.05$). Repair stiffness and maximum force were 54.5% and 42% of sham control values at 4 weeks post injury ($p<0.05$) and 57.6% and 52% of sham values at 6 weeks post injury, respectively ($p<0.05$; Table 2.3). Repair modulus and maximum stress were 46.1% and 34% of sham control values at 4 weeks post injury ($p<0.05$) and 39% and 51.7% of sham values at 6 weeks post injury, respectively ($p<0.05$; Table 2.3).

2.4. Discussion

The fields of musculoskeletal tissue engineering and developmental biology can benefit from murine models in which cells fluoresce either GFP-T or ECFP in response to changes in Col1 and Col2 gene expression, respectively. While others are using immunohistochemistry and in situ hybridization to track changes in gene expression, this method offers an advantage in that local intracellular changes in these patterns can be observed during healing as well as during normal growth and development. In fact, our group has already observed these patterns in the DT mouse during embryonic to early post-natal development (Chapter 3).

With this information, we can now contrast how these developmental patterns compare with current methods for healing soft tissues and their insertions into bone. Substantial differences between healing and development can also motivate promising new and novel therapies where, e.g. cell-based approaches might better mimic sequences of developmental gene expression patterns.

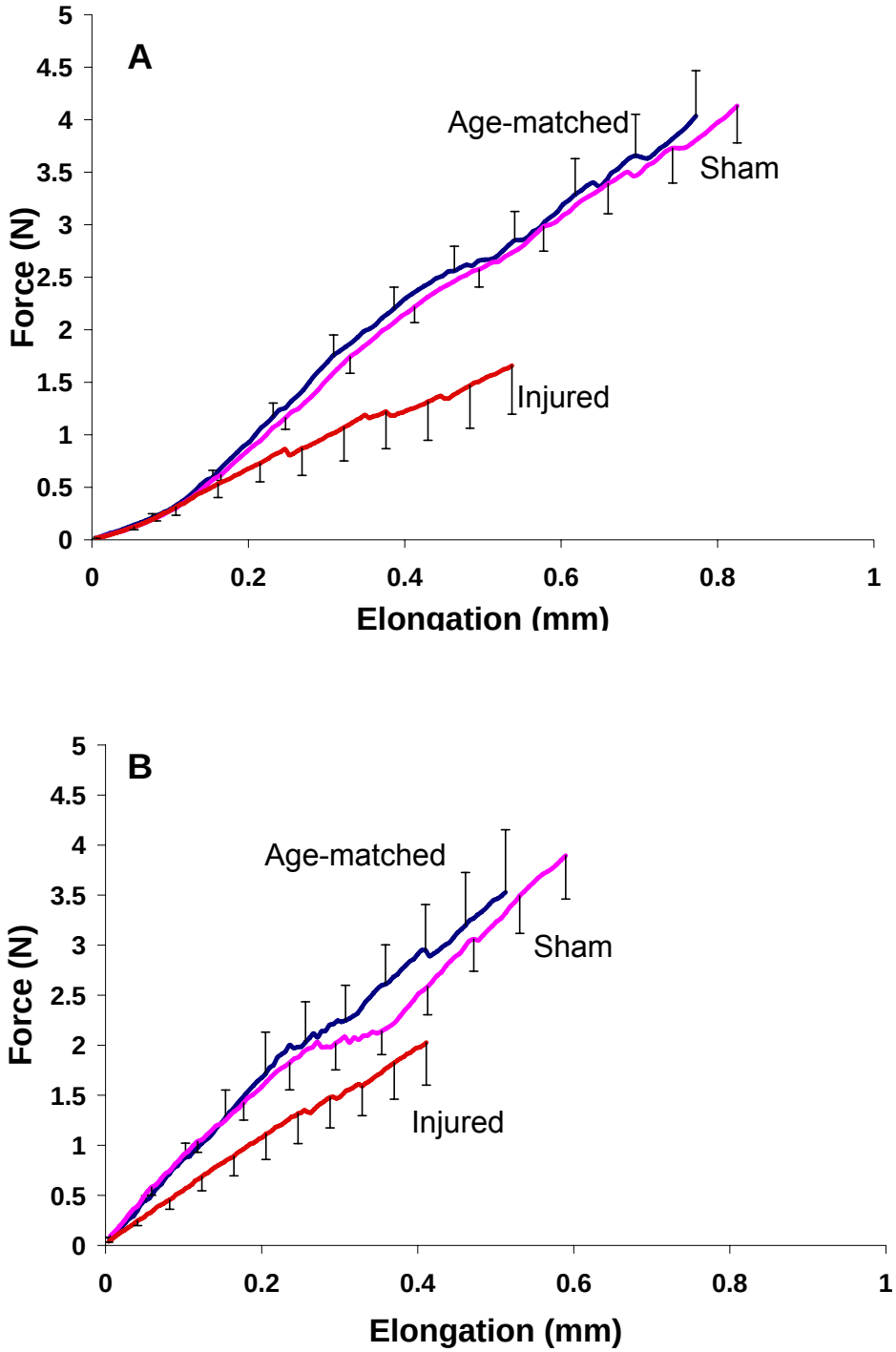

Fig. 2.7. Compared to age-matched controls and contralateral sham injuries, punch defect injuries to the central patellar tendon produced lower force-elongation curves at A) 4 weeks and B) 6 weeks post injury.

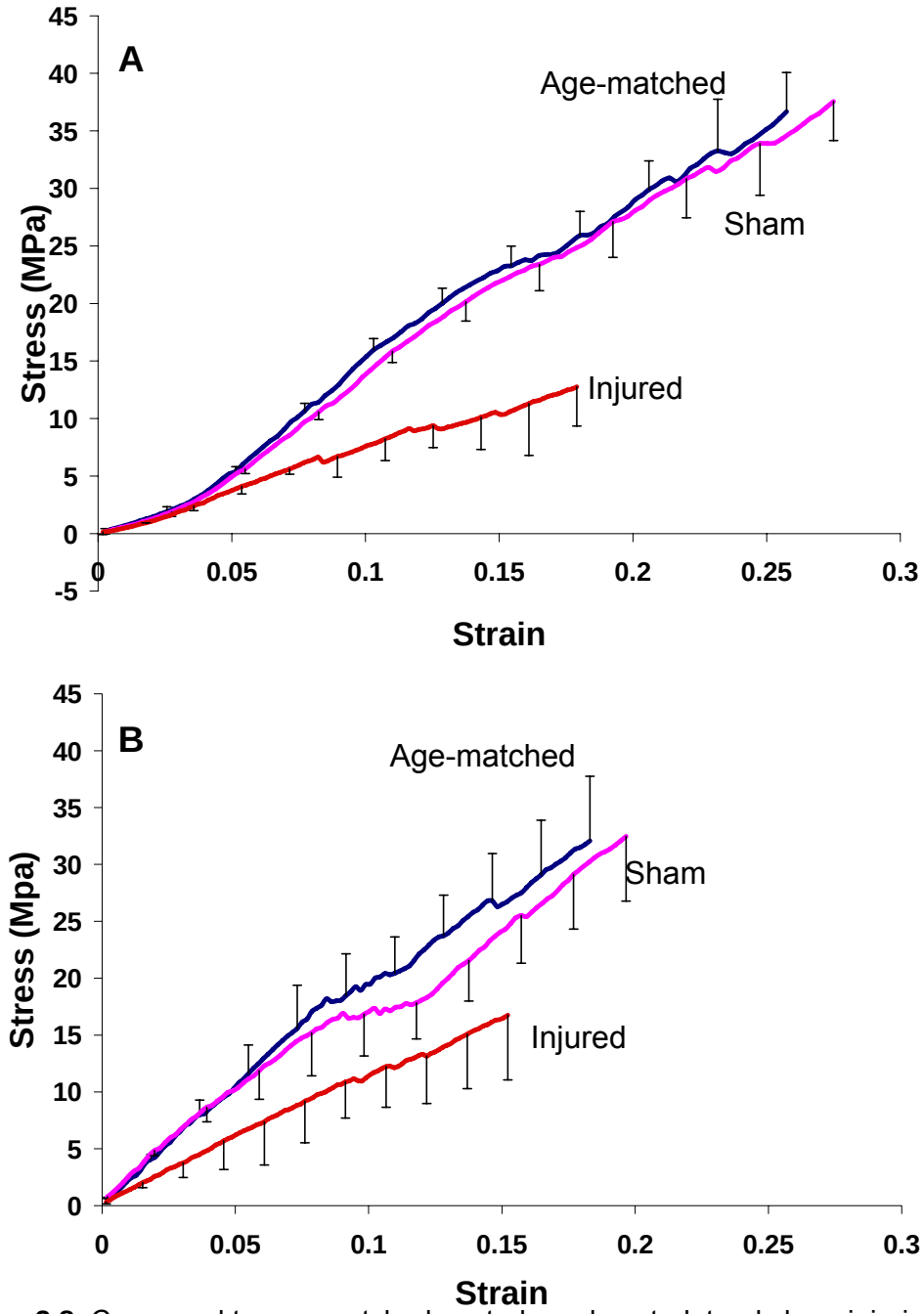

Fig. 2.8. Compared to age-matched controls and contralateral sham injuries, punch defect injuries to the central patellar tendon produced lower stress-strain curves at A) 4 weeks and B) 6 weeks post injury.

Such models are particularly important given the frequency of tendon injuries, the inconsistent results observed following classical repair of these injuries, and the lack of biological and biomechanical knowledge about the local cellular milieu in the healing wound site over time. Hence knowing both spatial and temporal expression of important structural genes

like Collagen I and Collagen II after tendon injury and healing will be critical to designing more effective engineered constructs that accelerate and enhance the repair process. Our model also takes advantage of key transgenic technology for tracking both Col1 and Col2 expression in response to controlled mid-substance tendon injuries.

Variable	Age-Matched		Sham		Repair	
	14 weeks	16 weeks	14 weeks	16 weeks	14 weeks	16 weeks
Structural Properties						
Max Force(N)	4.03±0.53	3.53±0.62	4.13±0.35	3.89±0.43	1.74±0.43*	2.02±0.42*
Stiffness(N/mm)	8.79±1.34	9.3±1.1	7.94±0.96	9.58±0.11	4.33±1.1*	5.52±0.86*
Strain Energy (N/mm)	1.67±0.37	1.47±0.33	1.78±0.26	1.50±0.23	0.47±0.16*	0.49±0.16*
Max Elongation (mm)	0.77±0.13	0.51±0.11	0.82±0.08	0.59±0.07	0.54±0.05*	0.41±0.05*
Material Properties						
Max Stress(MPa)	36.7±4.9	32.1±5.6	37.6±3.2	32.46±3.6	12.8±3.3*	16.8±3.52*
Modulus (MPa)	239.8±36.7	234.7±27.7	216.6±26.4	239.6±17.8	99.9±23.4*	93.9±22.6*
Strain Energy Density (N/mm) per mm^3	5.11±1.1	4.57±1.0	5.40±0.78	4.67±0.69	1.21±0.41*	1.49±0.49*
Max Strain (%)	25.7±4.4	19.7±4.4	27.5±2.8	19.6±2.6	25.1±1.9*	16.2±2.3*

Table 2.3. Structural and material properties (mean±SEM) for the patellar tendon healing tissues compared to values for contralateral sham and age-matched controls. * Statistically different from shams ($p<0.05$).

GFP-T expression in the tendon mid-substance of the normal 11, 12 and 14 week-old DT mice indicates upregulation of Col1 gene expression by tenocytes along the entire tissue structure. The fact that these patterns are similar for both the external controls and shams across time suggests that gene expression has stabilized over this range of ages. The fact that some regions in the tendon did not show GFP-T expression may mean that cells were either not fluorescing at the time point of evaluation (note that our GFP-T reporter has a 24-hour half life) or just beginning to show expression which could not be detected.

Observed GFP-T fluorescence intensity is likely more an indication of which cells are up-regulating Col1 expression and not necessarily the level of expression by any one cell. In situ hybridization studies are still needed to determine the actual number of cells showing increased expression and their degree of upregulation. The minimal detectable ECFP fluorescence observed in the insertion sites of the normal PT likely indicate that cells that maintain this fibrocartilage zone are not upregulating this gene. This is in contrast to what is known to occur by in-situ hybridization during growth and development [252].

Our current study documents the temporal changes in Col1 and Col2 gene expression levels following punch defect and sham injuries. We have shown that a baseline level of collagen I gene expression (Col1) is present one week after injury, suggesting that the response to this injury is not as immediate as one might expect. The increases in swelling, disorganization and Col1 gene expression by two weeks are dramatic and surprising, however. The fact that these changes in structure and Col1 expression are not localized to the injury site is also surprising and emphasizes how an entire tissue is affected by a local punch injury, possibly as a result of the disruption of vascular supply and biomechanical tension to the tendon. The subsequent reduction in swelling and gene expression by four weeks post injury suggests that the tissue is already undergoing an early repair process.

While early healing is associated with inferior biomechanical properties, we did observe a partial return in biomechanical stiffness and maximum force compared to external controls and sham values. This improvement in structural biomechanical properties between 4 and 6 weeks is quite encouraging. Unfortunately, we cannot provide an underlying reason for this improvement since our experimental design did not include an assessment of either gene expression or tissue structure at 6 weeks post injury. However, based on these changes between 2 and 4 weeks combined with our 6-week biomechanical results, we can speculate that gene expression levels likely undergo further decreases toward control levels with corresponding reductions in tissue thickness and more parallel alignment of collagen fibers.

Despite these patterns of change, tendon properties are still not normal even after 6 weeks of healing.

Our study supports findings that type I collagen protein is essential in the tendon repair process as are type III collagen and many glycosaminoglycans and growth factors (e.g. FGF and TGF-β) [253]. Once collagen fibers are injured, repair mechanisms occur where macrophages and inflammatory cells degrade collagen after which collagen I fibrils and fibers reconstitute the defect site [67, 81, 96, 110, 112, 180, 182, 254]. The temporal pattern of collagen repair and remodeling has been also been documented[254]. This study concludes that collagen protein is synthesized and already beginning to assemble by four weeks post injury [254]. To meet this timeline, recent studies by Berglund et al [255] conclude that Col1 mRNA is expressed in the healing tendon after only 12 to 24 days of healing. Galatz et al [253] found even earlier changes during tendon repair with increases in Col1 mRNA at the insertion sites by 10 days post injury. Lin et.al found by immunohistochemistry that by 2 weeks post injury, Col1 protein expression is greatest in wild type mice but dramatically reduced in IL6 or IL4 deficient mice [249]. This result suggests that IL6 and/or IL4 are necessary to upregulate Col1 gene expression during healing in tendons. Our results showing increased GFP-T expression (reflective of Col1 gene expression) by 14 days post defect injury also agree with findings by Loiselle et.al who found that Col1 mRNA peaked at 14 days during the healing of murine intrasynovial tendon adhesions [256].

While we did not specifically examine healing biomechanics at longer periods, it is not surprising that even after 63 days of healing, murine intrasynovial tendon adhesions still produce healing tissue with inferior maximum strength [256]. In fact, our study also supports the healing of rabbit PT window defects at 4 weeks, where inferior maximum force, maximum stress, stiffness, modulus, strain energy and strain energy density were seen in healing rabbit PT window defects [257]. Assembling the results of all of these studies with our own emphasizes the fact that healing produces inferior tissues. In fact, these inferior healing

biomechanical values combined with our structural and genetic findings strongly suggest the value of novel techniques like cell-based therapies to accelerate and improve this repair process.

Our study has some limitations. 1. We did not quantify Col1 and Col2 mRNA levels by correlating qRT-PCR to our fluorescence levels in our histological images. We have previously shown strong positive correlations between fluorescence and PCR in tissue engineered constructs containing murine cells that fluorescence Col1 and 2 genes [162-163]. 2. We did not study the expression of markers important for tendon healing such as TGF-β, BMP, etc. Future studies are now underway now to study these upstream markers. 3. The biomechanical results for the healing regions may have included some adjacent strut tissue. The inclusion of adjacent tissue was unavoidable due to the extremely small size of the repair area. Thus, our biomechanical results may have overestimated the true properties of the repair tissue alone. Such a small size increases the difficulty of inducing repeatable injuries in the murine model. Future studies, particularly those involving tissue engineering, may require using larger animals if genetic markers can be incorporated. 4. Sectioning effects could have disrupted our histological sections and could have influenced the intensity of the GFP-T and ECFP fluorescence.

In this study, we were able to document temporal and spatial patterns of collagen I and collagen II gene expression during the healing of a central third mid-substance patellar tendon defect in a novel double transgenic murine model. To our knowledge, this is the first time that local expression of both genes has been documented in a tendon at the cellular level. We further document the modest genetic and biomechanical healing that occurs after punch defect injury. In the future, this repair model and models like it should allow researchers to develop a roadmap for tissue engineering and cell therapy that more closely simulates normal development, potentially leading to regeneration rather than repair of tendon injuries.

Chapter 3

Col1 and Col2 Gene Expression During the Development of Murine Knee Joints

Recently, tissue engineers have turned to developmental biology to try and improve the repair of tendons and ligaments. The premise for this new approach is that describing normal growth and development of tendons and ligaments might help in designing tissue engineering constructs and in facilitating later repair. In particular, understanding temporal changes in extracellular matrix gene expression and growth factor expression might allow tissue engineers to develop more effective treatments to augment adult tendon and ligament healing. Unfortunately, investigators have identified very few relevant markers and growth factors expressed during normal tendon and ligament development. What are needed are near real-time markers that can monitor both spatial and temporal changes in gene and/or protein expression. To that end, we have created double transgenic mice with two distinct fluorescent markers, Green Fluorescent Protein-Topaz (GFP-T) and Enhanced Cyan Fluorescent Protein (ECFP), to track changes in Col1 and Col2 gene expression, respectively. We have monitored these fluorescent changes in knee tendons and ligaments from embryonic (E17.5) through early post-natal (P1, P3 and P14) development. We found that these structures form early, that Col1 and Col2 are expressed in utero and that both become localized, organized and enhanced shortly after birth. Our GFP-T and ECFP fluorescence expression indicates that Col2 expression precedes Col1 expression and rapid changes occur in spatial expression in the tissue mid-substance and insertion sites. These developmental changes in Col1 and Col2 expression also induce temporal and spatial changes in the expression of two related growth factors, TGF-β1 and BMP-2, respectively and their upstream markers, PSmad1,5,8 and pSmad2,3. Replicating spatial and temporal expression patterns of these important markers could represent one important way to speed and improve tissue engineered repairs after tendon injury.

3.1. Introduction

Soft tissue (tendon, ligaments etc) injuries represent almost 50% of all musculoskeletal injuries that occur in the US each year [5, 258]. These include frequent injuries to the patellar tendon (42,000), Achilles tendon (100,000) and rotator cuff (51,000) as well as ruptures to the anterior cruciate ligament (125,000) [6-9]. Inadequate healing of these injuries places patients at risk to dysfunction and disability.

Surgeons have traditionally tendon and ligament disorders by reducing inflammation, restoring flexibility, and performing surgical repair [6, 259-260]. Many repairs/reconstructions have been attempted (e.g. sutures, resorbable biomaterials, autografts, and allografts) with varying success [9, 105-107, 110, 112, 261-264]. Such problems with conventional techniques have led to new strategies such as tissue engineering and functional tissue engineering.

Our group helped to create a new branch of tissue engineering called Functional Tissue Engineering (FTE) [265]. Those working in FTE seek to determine forces and deformations during activities of daily living (ADL) in order to establish design goals for increasing extracellular matrix synthesis, improving tendon organization, shortening remodeling time, and enhancing biomechanical stiffness. These investigators also use bioreactors to mechanically stimulate tissue engineered constructs or TECs in-vitro with aspects of in-vivo tissue force or deformation [167, 171, 265]. Based on these results, FTE principles and strategies now hold much promise for advancing tendon tissue engineering toward the ideal goal of regenerating normal tissue properties in the functional range of loading. For example, when our group has used FTE principles to repair central-third rabbit PT defects using MSC-collagen constructs, we have been able to generate repairs that match normal tendon stiffness up to 150% of peak in-vivo forces after 12 weeks of healing [171]. Despite these successes in restoring repair stiffness and strength, these 12-weeks repairs still did not regenerate normal zonal insertions into bone. Furthermore, our group has still not identified the mechanical and chemical stimulation patterns in culture that optimize cell-based tendon repair. Thus, to improve both the mechanical function

and the structure of these repairs, we need new strategies that more rapidly and fully recreate all aspects of normal tissue biology, structure and function.

FTE could benefit from a more thorough understanding of how tendons normally develop and grow. Currently, our knowledge of normal tendons growth and development is quite limited as well as how these events compare with natural tendon healing and repair in the adult [252]. Tendon structures, which are known to undergo early regulatory changes around birth, are likely controlled by TGF-β and BMP which in turn are under the control of the downstream pSmad signaling pathways. Such knowledge would be invaluable and could lead to repair strategies that might someday regenerate the tendon mid-substance and insertion site after injury.

Developmental cues could help tissue engineers spatially arrange and mechanically or chemically stimulate cells in the TEC to more efficiently synthesize and maintain an appropriate extracellular matrix (ECM).Unfortunately, this process can be slow, incremental and difficult to optimize. Investigators must test various culture conditions (cell and scaffold type, mechanical and chemical stimuli) to determine if appropriate ECM has been synthesized. This process can also become quite expensive and time-consuming as well. If these cell-based approaches are to eventually become clinical tools for the orthopaedic surgeon, we need a more novel, real-time approach that would permit us to rapidly determine how these environmental stimuli (e.g. specific loads and strains) affect cell phenotype at a reasonable cost.

Types I and II collagen are major structural proteins in the tendon mid-substance and insertion site, respectively. We need to understand how genes that synthesize these proteins are spatially and temporally expressed in normal, injured, and repairing tendons, as well as tendon cell-based constructs. We also need a more real-time technology to monitor expression of these genes. In this regard we have created double transgenic mice in which two critically important structural genes, Col1 and Col2, are linked to intracellular Green Fluorescent Protein–Topaz (GFP-T) and Enhanced Cyan Fluorescence Protein (ECFP), respectively. Cells in these

mice fluoresce green and/or cyan depending on which collagen genes are activated, permitting us to monitor expression patterns for all of these tendon states.

Thus our objectives in this initial study were to track changes in Col1 and Col2 gene expression, respectively, in the knee patellar tendon mid-substance and insertions from embryonic (E17.5) through early post-natal (P1, P3 and P14) development. We hypothesized that: 1. Col1 and Col2 would be expressed at similar times in utero. 2. Expression of both genes would be localized, organized and enhanced shortly after birth. 3. Post-natal development would further induce temporal and spatial changes in both of these structural genes. 4. Such spatial and temporal changes in Col1 and Col2 expression would mirror that of two growth factors, TGF-β1 and BMP-2, respectively. 5. Development would also induce temporal and spatial changes in the expression of corresponding upstream signaling markers, pSmad1,5,8 and pSmad2,3 respectively.

3.2. Materials and Methods

Experimental Design

We assigned four animals each from different litters of double transgenic mice to one embryonic (E17.5) and three early post-natal time points (P1, P3, P14). The time points selected for studying changes in Col1 and Col2 expression were based on a prior study [252] where investigators monitored expression of extracellular matrix and growth factor genes during development of the supraspinatus tendon-to-bone insertion from E13.5 to 14 days. Knees were harvested and sectioned from each of the 4 animals at each time point. ECFP and GFP-T fluorescence were imaged in the same sections to indicate those cells that showed Col2 and Col1 expression, respectively. ECFP and GFP-T fluorescence were imaged in the same sections to indicate those cells that showed Col2 and Col1 expression, respectively. Adjacent sections were then stained with antibodies for TGF-β1 and BMP-2. Different adjacent sections

were also stained with antibodies for pSmad1,5,8 and pSmad2,3. Different adjacent sections were also stained with H&E.

Harvest, Sectioning and Preparation of Hind Limbs

Animals were sacrificed by CO_2 asphyxiation in accordance with IACUC protocol. The entire hindlimb was removed from each hip. The skin was then removed to expose the underlying soft tissues. Care was taken while dissecting the muscle tissue to avoid damaging the patellar tendon and the knee ligaments. Each limb was fixed for 2 days in freshly prepared 4% paraformaldehyde which was dissolved in PBS and adjusted to pH 7.4 with NaOH. Fixed limbs were then placed in disposable base molds filled with frozen embedding medium devoid of any air bubbles. Molds were wrapped in aluminum foil and stored at -80^{o}C. Hindlimbs were then cut in sagittal section (20μm thick) in a microtome. Sections were then stored at -80^{o}C until they were ready for staining and imaging.

Immunohistochemistry and H&E staining

Non-specific binding sites were first blocked using 4% BSA in 0.1%Triton for 1 hour. Histological sections were incubated overnight at 4^{o}C with 10mg/ml of one of the following: a) primary monoclonal anti-TGF-β1 and 2 (sc-146, Santa Cruz Biotechnologies, Santa Cruz, CA); b) anti-BMP-2 and 4 (sc-6267, Santa Cruz Biotechnologies, Santa Cruz, CA); c) anti-pSmad 1, 5, and 8 (9511, Cell Signaling, Beverly, MA); or d) anti-pSmad 2,and 3 (sc-11769-R, Santa Cruz Biotechnologies, Santa Cruz, CA). Sections were then stained with secondary antibody conjugated with CY5 (Jackson Immuno Research Laboratories, West Grove, PA) at a concentration of (1:200) for 1 hr.

For H&E sections were allowed to air dry to remove all moisture, washed in PBS and stained in immersed in hematoxylin and eosin for atleast 2 mins. The sections were finally cleared in xylene and coverslips were mounted.

Imaging the Knees

H&E sections were imaged in a light microscope (Zeiss, San Diego, CA). Fluorescence were examined in the confocal microscope(Zeiss, San Diego, CA) with filter cubes for GFP-T, ECFP and CY5 (Omega). Images were taken at 10X magnifications.

Quantification of Fluorescence

Custom Matlab code and a stereological sampling strategy[250-251] were used to quantify both the ECFP and GFP-T fluorescence in the images. One way ANOVA with Tukey's HSD was performed to determine if there were significant differences in either ECFP or GFP-T fluorescence among ages. Significance was set at $p<0.05$.

3.3. Results

GFP-T (Col1) and ECFP (Col2) fluorescence patterns in the patella-patellar tendon-tibia complex were similar among animals of the same age but from different litters. At all four ages, intracellular ECFP expression was robust in the subchondral bone regions and GFP-T expression was seen in the patellar mid-substance (Fig. 3.1A-D). ECFP expression was most intense in the bone ends at E17.5 and P3, decreasing both at P1 and at P14 (Fig. 3.1 & Table 3.1). GFP-T expression was quite evident in the patellar tendon mid-substance at P3 and most intense at P14 (Fig. 3.1 & Table 3.1). Cells expressing both ECFP and GFP-T fluorescence were seen in the insertions at both P3 (not shown) and P14 (Fig 3.2A-D).

	E17.5	P1	P3	P14
ECFP	94.6±16.5	46.1±5.2*	74.6±10.23	51.3±8.7*
GFP-T	12.3±4.6	15.4±7.2	38.7±9.8^	155.4±12.45^ #

Table 3.1. Average EFCP/GFP-T intensity at all four ages. * Significant difference in ECFP intensity from both E17.5 and P3. ^ Significant difference in GFP-T intensity from E17.5 and P1. # Significant difference in GFP-T intensity from P3.

Tissues showed both spatial and temporal differences in histological appearance. Between E17.5 and P3, cell number increased dramatically (Fig 3.3). Collagen fibrillogenesis also increased from P3 to P14, resulting in dramatic increases in tissue thickness (Fig 3.3).

Collagen fiber alignment also increases with age as evidenced by the changing birefringence in the micrograph in Figure 3.3. The insertion region of the tendon showed some of the most

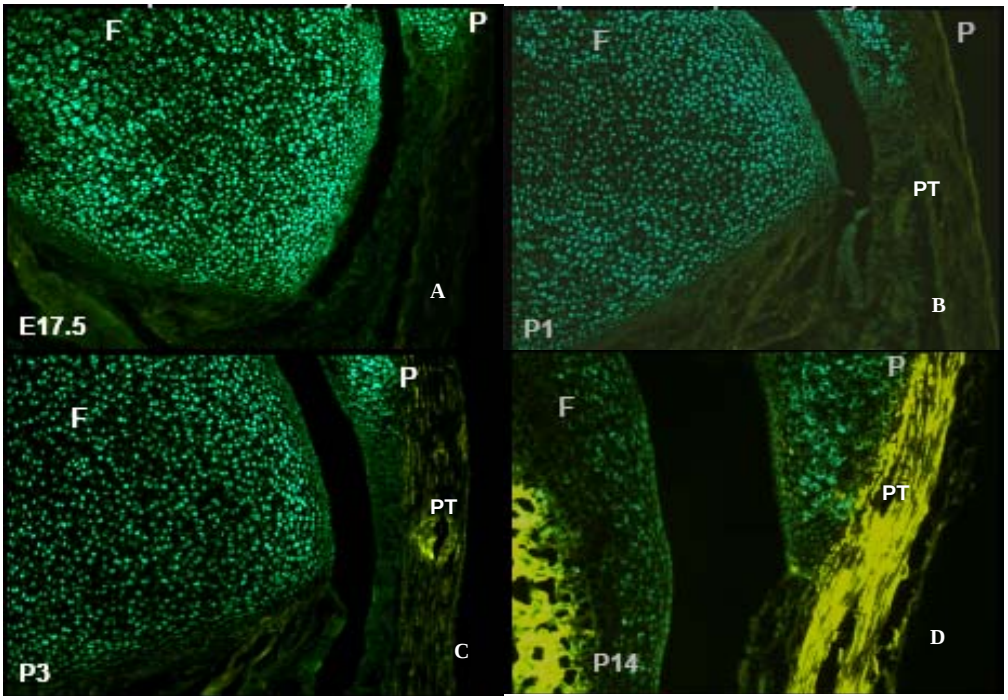

Fig. 3.1. Typical GFP-T and ECFP fluorescence expression in sagittal sections of murine knees at A) E17.5, B) P1, C) P3 and D) P14. GFP-T expression appears most intense at P14 while ECFP expression is most intense at E17.5 and P3. Magnification is 10x for all sections. F-Femur, P-Patella, PT-Patellar Tendon**.**

striking differences. Initially, the fibers at the distal end of the patellar tendon appeared to be adjacent to the developing tibial cartilage ("I" in E17.5, Fig 3.3). However, by P1 (day of birth), this region had incorporated into the proximal tibia and by P14, the birefringent insertional fibers extended throughout the insertion site (Fig 3.2).

Immunohistochemistry revealed differences in expression of growth factors and downstream signaling molecules across age groups. BMP-2 was evident in the patellar tendon mid-substance at E17.5 and P1 (Fig 3.4). BMP-2 was also quite pronounced in the proximal tibia by P14 (Fig 3.4). However, no BMP-2 could be found in any location at P3. Similarly, pSmad1,5,8, downstream marker of BMP signaling, was seen in the patellar tendons at all ages (Fig 3.5). TGF-β1 (Fig. 3.6) and pSmad2,3 (Fig. 3.7) (downstream of TGF-β1 signaling) were also seen in the patellar tendons at all ages.

3.4. Discussion

Our double transgenic reporter mouse model is a novel tool to study Col1/Col2 gene expression during the development of knee structures. We can visualize cells engaged in

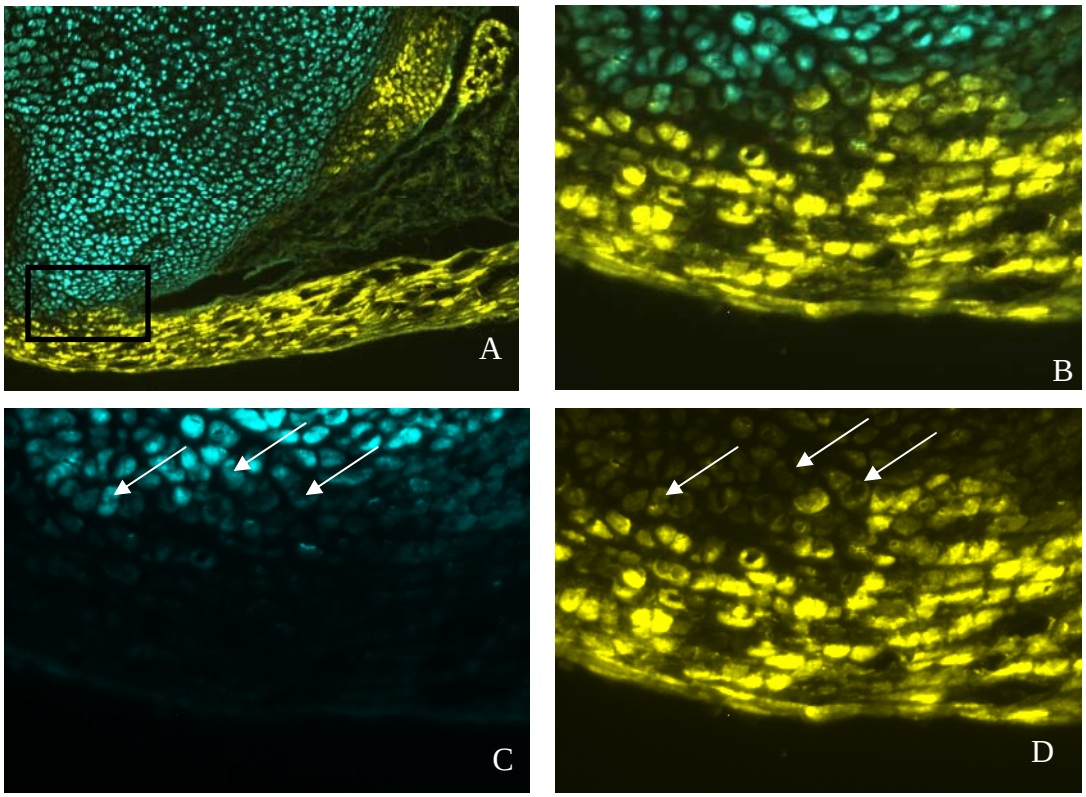

Fig. 3.2. A) ECFP and GFP-T expression at P14, B) Magnified view of the insertions showing both ECFP and GFP-T expression , C) Only ECFP expression, D) Only GFP-T expression. Note the presence of cells expressing both ECFP and GFP-T as indicated by white arrows. F-Femur, P-Patella, PT-Patellar Tendon**.**

chondrogenesis, osteogenesis and tendinogenesis in near real time. In particular, our ability to analyze gene expression in near real time in culture has the potential to speed the development process by permitting the investigator to easily screen constructs for appropriate gene expression as culture conditions (cell and scaffold type and environmental stimuli) are changed. The 24hr half-life of the fluorescent proteins also permits us to monitor changes in expression over somewhat longer time periods without the fear of “missing” very rapid changes in gene expression.

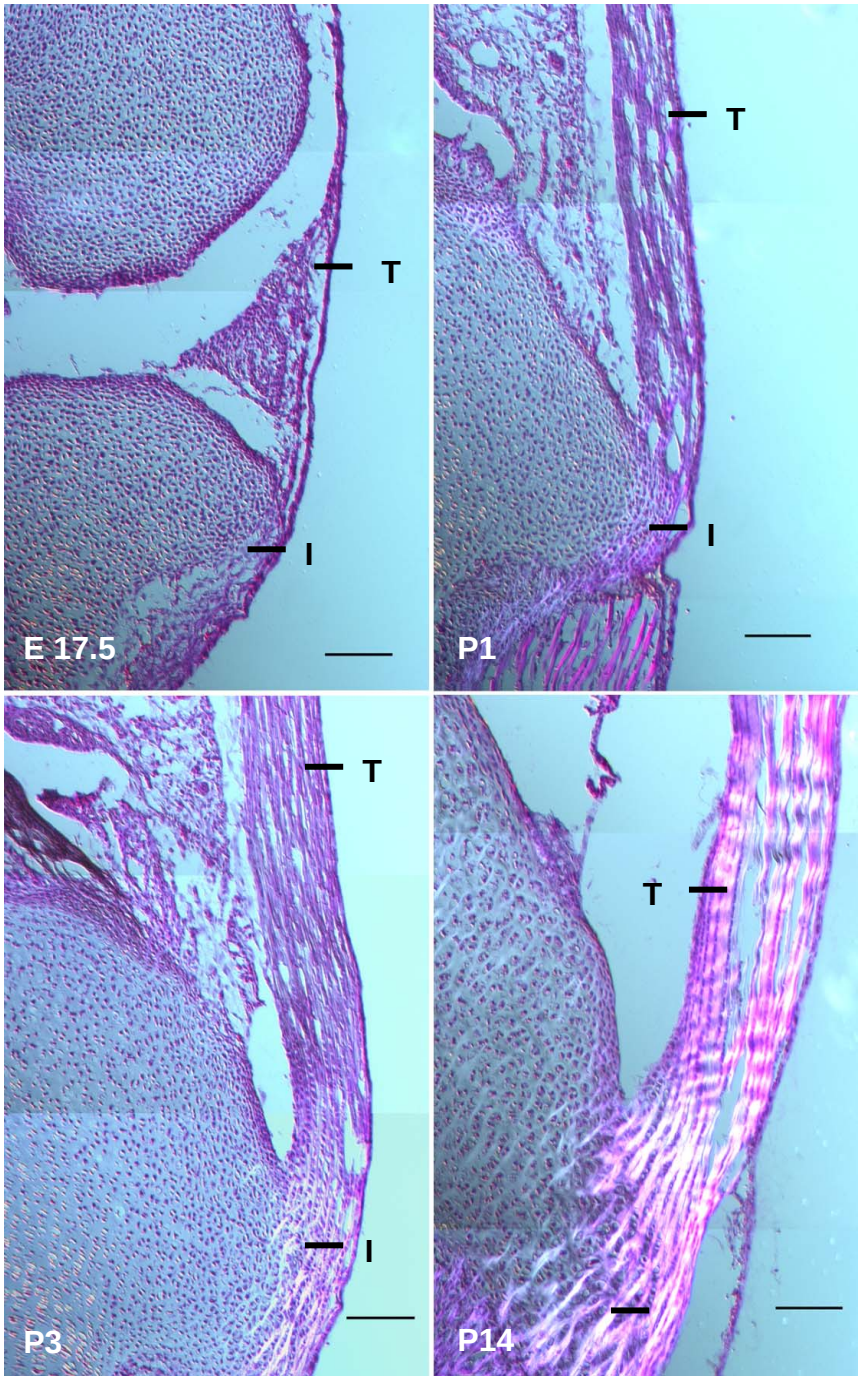

Fig. 3.3. Shown are birefringent images of patellar tendon morphology at the tendon mid-substance (T) and tibial insertion (I) at four time intervals of development (E17.5, P1, P3 and P14). Note the crimped appearance and integration into the tibia at P14.

GFP-T and ECFP expression show well-defined spatial and temporal patterns during embryonic and early post-natal development. Our ECFP/GFP-T fluorescence results show that developing knee structures (including the patellar tendon and insertions) undergo rather

repeatable and predictable spatial and temporal changes in Col1/Col2 gene expression. The greater intensities in ECFP (Col2) expression at E17.5 and P3 compared to P1 and P14

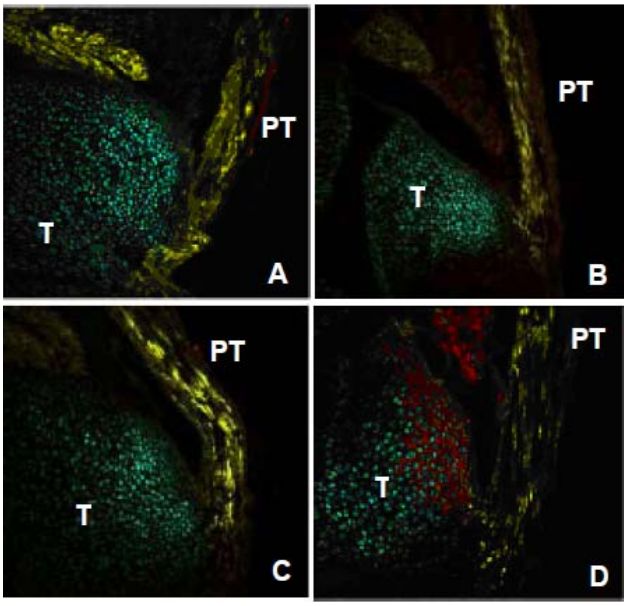

Fig. 3.4. BMP-2 expression in sagittal sections of murine knees at A) E17.5, B) P1, C) P3 and D) P14. BMP-2 is evident in the PT at E17.5 and P1 and prominent in the tibia at P14. T-Tibia, PT-Patellar Tendon. Red represents staining for BMP-2, Cyan represents Col2 expressing cells, Yellow represents Col1 expressing cells.

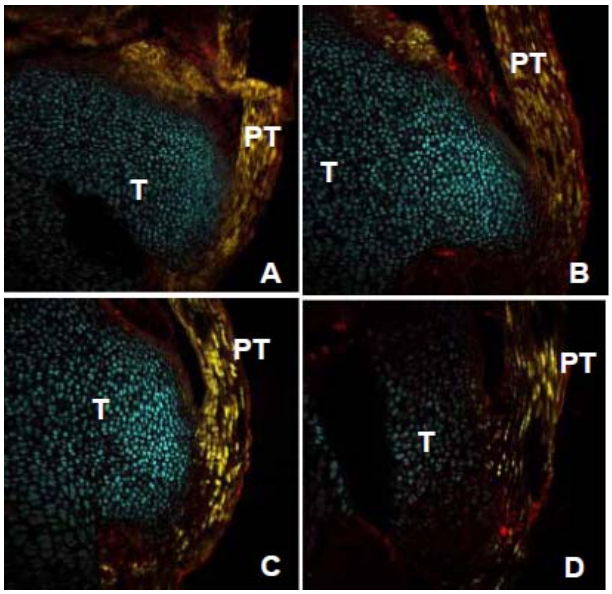

Fig. 3.5. pSmad1,5,8 expression, downstream of BMP-2 expression in sagittal sections of murine knees at A) E17.5, B) P1, C) P3 and D) P14. Unlike BMP-2, pSmad1,5,8 expression is seen in patellar tendons at all ages. Red represents staining for BMP-2, Cyan represents Col2 expressing cells, Yellow represents Col1 expressing cells.

suggest that tendon and ligament structures undergo early regulatory changes around birth which could be controlled by upstream signaling events. The appearance of GFP-T (Col1) expression between E17.5 and P1 and its further enhancement by P3 raises questions as to whether the same cells switch their mode of gene expression either by internal programming or through mechanical and/or chemical factors.

The increasing intensity and improving alignment of GFP-T expressing cells in the patellar tendon during development could offer strategies for improving the integrity of tissue engineered constructs in culture. In sagittal sections, we observed that GFP-T expressing cells increased fluorescence levels and were already well-aligned with the tendon axis by P3.

Evidence in the literature suggests changes in Col1 gene expression during early development but these changes have not been localized to the cellular level before. Regarding alignment, the fact that rows of tendon cells typically orient before collagen fibrillogenesis [14, 266] suggests that cell orientation strongly influences the organization of the ECM. Such knowledge about

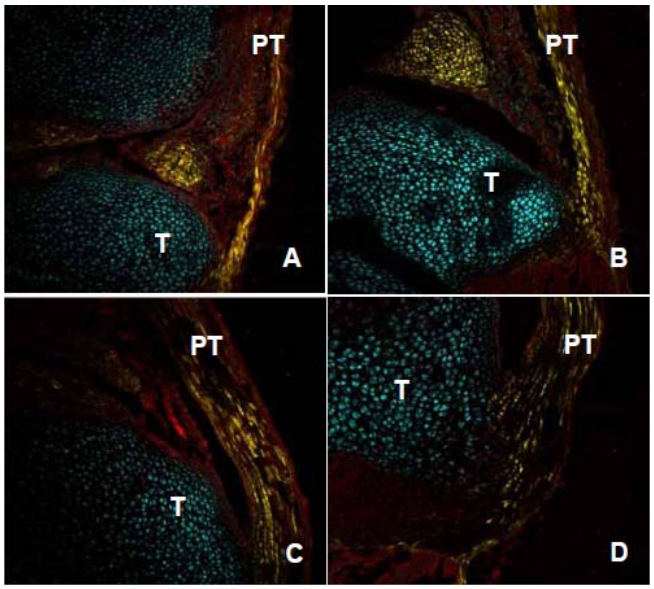

Fig. 3.5. TGF-β1 expression in sagittal sections of murine knees at A) E17.5, B) P1, C) P3 and D) P14. TGF-β1 is seen in patellar tendons at all ages although it is more pronounced at E17.5 and P3. T-Tibia, PT-Patellar Tendon**.** Red represents staining for BMP-2, Cyan represents Col2 expressing cells, Yellow represents Col1 expressing cells.

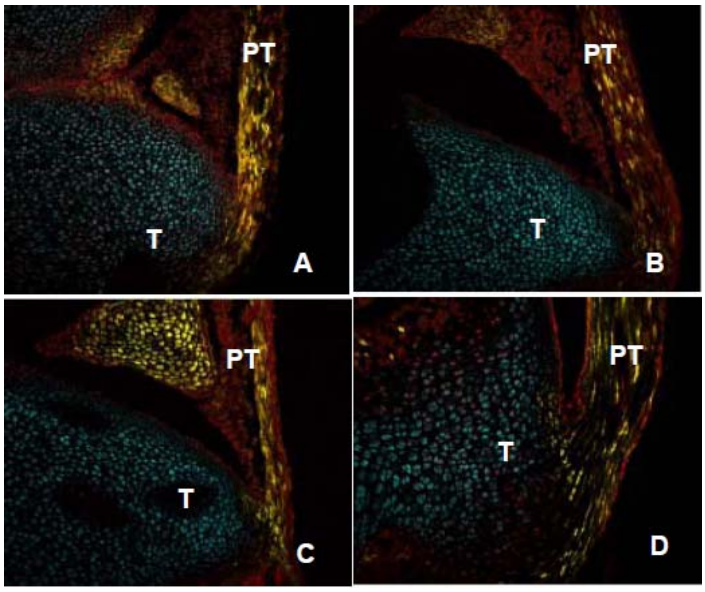

Fig. 3.6. pSmad 2,3 expression downstream of TGF-β1 in sagittal sections of murine knees at A) E17.5, B) P1, C) P3 and D) P14. Similar to TGF-β1. pSmad 2,3 is seen in patellar tendons at all ages. T-Tibia, PT-Patellar Tendon. Red represents staining for BMP-2, Cyan represents Col2 expressing cells, Yellow represents Col1 expressing cells.

organization and gene expression of cells in the developing tendon could be quite useful if strategies could be devised for mimicking these temporal and spatial events by applying tensile stimuli to cells within tissue engineered scaffolds in culture and then determining their effects on repair after surgery.

While creating a tendon or ligament mid-substance is now quite conceivable, recreating a complex tendon or ligament insertion into bone will require more sophisticated approaches. Our group recently found that mechanically stimulating rabbit MSC-collagen scaffold constructs in culture resulted in well-organized and biomechanically competent tendon ECM 12 weeks

after surgical implantation [171]. However, our group [171] and others [62, 267-269] have not been successful in regenerating the complex tendon or ligament zonal insertion site into bone after injury. The fact that Col2 gene appears to be expressed before Col1 gene during joint development suggests the need to investigate whether triggering cells to also express Col2 early after injury might increase our chances of recapitulating a zonal insertion. The fact that Col1 and Col2 genes are also co-expressed by cells in the insertional region during development emphasizes the value of closely monitoring these spatial and temporal changes during development and then driving expression of both structural genes in the adult healing attachment site. Given this early post-natal co-expression, animal models like our double transgenic mouse or use of in situ hybridization [252] could be quite valuable ways to monitor the developmental process so as to learn how to better repair and maybe someday regenerate soft tissue insertions into bone.

Previous studies in the literature indicate that regions of immature and even adult tendons and ligaments can convert to fibrocartilage at their sites of attachment to bones, similar to our insertional findings during embryogenesis. For example, a tendon or ligament can synthesize cartilage matrix proteins where it passes over bony surfaces [26, 270-271], presumably due to the high compressive and shear loads. Such adaptation reflects the common ancestry of connective tissue progenitors and cartilage-forming cells. Our data shows similar findings in that by P1, the distal end of the patellar tendon incorporates into the proximal tibia through differentiation from the tendon side and/or growth of cartilage up to the tendon boundary. These results suggest that to recreate insertion sites during tendon repair, investigators should monitor and attempt to enhance co-expression of Col1 and Col2 genes which is likely lacking in the current set of FTE tendon constructs.

These changes in GFP-T/ ECFP fluorescence around birth suggest that knee structures undergo regulatory changes which could be controlled by either biologic or physical factors. A recent study of rat supraspinatus entheses indicates that physical factors do not play a

significant role in enthesis maturation in the first 14 days postnatally, implying that biologic factors may drive early postnatal development [272]. Thus our Col1/Col2 gene expression patterns could be a result of differential expression of biological factors, especially BMP-2 and TGF-β1.

Immunohistochemistry did reveal differences in growth factor patterns across age groups. For example, BMP-2 was only evident in the PT mid-substance until P1 and in the tibia at P14. BMP signaling has been suggested to control regional differentiation in tendon. Generally BMP signaling is inhibited in the tendon mid-substance, allowing tendon fiber formation, but is activated at the insertion site, allowing fibrocartilage formation[273-274]. The appearance of BMP-2 in the tendon mid-substance is contrary to what we expected. It has been suggested that BMP-2 might act as a regulator of TGF-β1 signaling which is important in the mid-substance [274]. The absence of BMP-2 in the tendon mid-substance beyond P1 is also significant, suggesting that BMP-2 is likely important only during early development of the insertions and that other members of the BMP family could play important roles during later development.

pSmad1,5,8 expression did not mirror that of BMP-2 expression. Since only the BMP family acts through the pSmad1,5,8 pathway, other members of the BMP family are also active during development. Future studies are necessary to determine which BMPs are expressed at these developmental time points. Once these BMPs have been identified, these should be used to stimulate TECs in culture to see if such stimulation improves not only the in-vitro mechanical properties of TECs but also the resulting in-vivo repair mechanical properties.

Unlike BMP-2, TGF-β1 was seen in the patellar tendons at all ages. TGF-β1 has been shown to have a central role during morphogenesis and tissue formation by influencing the differentiation of connective tissue precursors into tendon forming cells [275]. Thus this expression of TGF-β1 can help to explain the increases in Col1 gene expression we observed. Also the appearance of TGF-β1 at all time points suggests that this growth factor is likely

important not only during early development, but also during later development of the tendon mid-substance. This suggested role of TGF-β1 during development taken together with results in the literature showing improvement in collagen synthesis [276-277] and improvements in healing of tendon mechanics [278] makes this a key factor to be investigated during the development of tissue engineered constructs for tendon repair.

pSmad2,3 expression mirrors that of the TGF-β1 expression. This suggests that the expressed TGF-β1 acts on the cells in the tendon mid-substance and further emphasizes the importance of TGF-β1 in the formation of tendon mid-substance and possibly delivery of this growth factor locally to TECs.

Recreating tendon zonal attachment sites after injury is another important problem facing tendon tissue engineers [62, 171, 267-269]. Our growth and development results motivate new strategies to solve this problem. Our results showing: 1) appearance of Col2 before Col1 during development, 2) early co-expression of Col1 and Col2 in insertion regions, and 3) differences in expression of BMP-2 vs.TGF-β1, suggest that to recreate insertions during tendon repair, investigators should monitor and attempt to enhance the co-expression of Col1 and Col2 in the end regions of tendon constructs. Researchers should also strive to regionalize the expression of BMP-2/TGF-β1, if these are lacking in the current set of FTE tendon constructs.

Our study is not without limitations. We only monitored GFP-T and ECFP fluorescence as early indicators for Col1 and Col2 gene expression in sections of the murine knees. While these fluorescent markers may appear to be subjective measures, we have previously shown positive significant correlations between relative fluorescence units or RFAs and qRT-PCR for Col1 and Col2, respectively [2, 163, 279]. We did not measure actual Type I or Type II collagen protein expression from these sections. However, we have shown in culture that Type II collagen protein is present within 7 days after upregulation of Col2 gene expression [2, 163, 279]. Intracellular fluorescence is more an average intensity rather than a quantity precisely

describing local cellular expression. Thus, our results indicate more about how many cells are actually upregulating either Col1 or Col2 rather than the degree to which expression is changing in each cell. This limitation is also in part a result of the 24 hour half life we chose for each fluorescent reporter. We are currently planning in situ hybridization studies to determine individual cell expression levels. Although our double transgenic model should allow us to track changes in Col1 and Col2 expression in near real time, currently we must sacrifice animals and section knees to image the fluorescence. Once we can identify powerful in vivo optical technologies that can visualize at the cellular level, we should be able to image the entire knee with the required magnification and conduct cost- and time-efficient longitudinal studies that eliminate assignment of animals for gene expression patterns at different ages and healing times.

In summary, we have used a murine model to study the development of collagenous soft tissue knee structures. We have shown that during normal development of tendons there are temporal and spatial changes in the expression of Col2 and Col1, BMP-2 and TGF-β1, and pSmad1,5,8 and pSmad2,3. Understanding differences in structural gene expression among developing structures as well as normal and healing adult tissues should allow us to formulate treatments for augmenting and accelerating tendon and ligament healing. Replicating rapid temporal changes in ligament and tendon gene expression and growth factors may also be important when mimicking key events in culture so as to create functional tissue engineered structures. Future studies will look at the expressions of upstream markers for Col1 (e.g., scleraxis, tenomodulin and thrompospondin) as well as for Col2 (e.g., Sox 9) in hopes of recapitulating more developmental events and dramatically improving the rate of soft tissue and insertional healing after injury and disease.

Chapter 4

Development And Validation Of A Bioreactor For Delivering Compressive Mechanical Stimulation To Functional Tissue Engineered Cartilage

External mechanical stimuli play a critical role in the natural development of musculoskeletal soft tissues, suggesting that delivering mechanical signals to constructs in culture that mimic aspects of in-vivo signals might create more “functional” tissues before surgery. Unfortunately, it has been difficult to identify or build bioreactors that can apply the desired mechanical stimulation to engineered load-bearing structures like articular cartilage and meniscus. To that end, we have designed and fabricated a one-step process bioreactor combining the culture and stimulation phases in order to create more functional tissue engineered cartilage. Our culture chamber, housed within an electromagnetic testing system (Model ELF 3200, Bose Corp, Eden Prairie, MN), sustains a long-term cell culture environment that is comparable to conditions created in standard incubators. Cells cultured for up to two weeks in this one-step bioreactor show comparable viability to cells cultured in standard incubators. A custom mechanical fixture within the chamber can stimulate up to twelve constructs and record tissue reaction loads in half of these, providing real-time calculation of construct stiffness for optimizing our stimulation conditions. The testing system with the custom fixture delivers repeatable and simultaneous dynamic compressive signals. Preliminary results show that compressive stimulation of chondrocyte-agarose constructs in our reactor for 14 days significantly increases collagen II gene expression. Using this bioreactor, we hope to speed the creation of more functional tissue engineered cartilage and menisci for future pre-clinical and clinical use.

4.1. Introduction

Cell-based tissue engineering strategies [280] have gained in popularity as an alternative approach to repair and replacement of injured and diseased soft tissues and organs, especially with the problems faced with conventional repair techniques[281-289]. Initially, tissue engineers simply mixed cells and scaffolds to create tissue engineered constructs for implantation in-vivo [51, 148, 150, 246-248, 290]. Recently, however, the field of tissue engineering has evolved as researchers began to recognize the importance of restoring mechanical function in-vivo. In 2000, a new branch of tissue engineering called functional tissue engineering (FTE) was advanced to account for this need for restoring function [265, 291-293]. The central tenet of FTE is the need to measure the loads and strains acting on tissues in-vivo during normal activities and to then use these as design parameters and requirements for the tissue engineered constructs. FTE also suggests that these mechanical stimuli could be used to "precondition" constructs during the in-vitro culture phase in order to acclimate them to their future in-vivo loading regimes.

With the emergence and growth of FTE, researchers have been striving to develop bioreactors which can stimulate cell/ cell-constructs with complex in-vivo profiles. Early investigators used spinning flasks and rotating vessels to apply hydrodynamic pressure and rotational forces [294-295]. Others used flexible membranes deformed by air pressure to stretch cells in monolayer and in 3-D culture[296-298] or to compress tissue explants[299]. Still, the paucity of commercially-available bioreactors has led many investigators to develop specialized systems that are typically housed within standard cell culture incubators [300-302]. Despite these efforts, these systems often lack the ability to apply complex dynamic in-vivo stress/strains and/or loads/displacements required to effectively stimulate FTE constructs. In

the case of cartilage, it has been shown that it experiences peak stresses of 2-8MPa,[303-304] and strains of about 3% to 20% [305-306] during various activities.

To achieve these FTE demands, we contend that a bioreactor should satisfy four design principles [2]. Bioreactors should: 1) Sustain a cell-culture like environment during periods of mechanical stimulation to ensure adequate cell viability; 2) Have the ability to stimulate multiple constructs with either identical or individual waveforms to reduce development times; 3) Should deliver precise displacements to compliant TECs, including signals that mimic in-vivo activities of daily living; and 4) Should be capable of monitoring reaction loads and adjusting displacement patterns based on these loads.

The pneumatic bioreactor used in our lab for over 10 years satisfies just two of the above mentioned design principles. The pneumatic system housed in a standard incubator sustains a TEC in culture with adequate cell viability. The pneumatic system also stretches five silicone dishes with individual waveform patterns, each dish having wells to accommodate four TECs. However, our pneumatic system is also regulated by pressure differential and displacement stops to limit peak strains. These characteristics mean that the signal we deliver to the TEC is not very precise and cannot mimic complex in-vivo deformation patterns. In addition, this system lacks the instrumentation required to monitor construct force in individual TECs.

To satisfy more of our bioreactor design requirements, we identified an electromagnetic testing system (ElectroForce 3200; BOSE Corp., Eden Prairie, MN) that could not only deliver a wide variety of dynamic, controlled-displacement waveforms with micron precision, but could also import future in-vivo displacement data to drive the actuator as we move toward our ultimate goal of using physiologic signals to stimulate engineered tissue in-vitro. We also sought a multi-station system that could support multiple load cells to allow for real-time monitoring of the forces applied to our engineered tissues. However, since it was not feasible to

place this system within a standard incubator, we instead chose to create and sustain a cell culture environment within the testing system.

Therefore, our objective in this study was to develop a bioreactor within an electromagnetic testing system that 1) maintains cell culture conditions and 2) could apply precise and repeatable dynamic displacements to multiple tissue engineered constructs and provide load feedback in real time. The following work describes our validation of the culture environment and applied displacements within our system as well as preliminary data on the performance of this system for loading cells in 3-D constructs.

4.2. Materials and Methods

Environmental chamber

A chamber made of plexiglass was adapted to fit within the electromagnetic system. The chamber (ported chamber and saline accessory bath) provided by BOSE (BOSE Corp., Eden Prairie, MN) served as the environmental chamber of the bioreactor. The bottom of the chamber can be filled with a 2L water bath. To maintain the temperature and the humidity within this chamber we heated this water bath using a feedback system I (Fig. 4.1A) [2].

The CO_2 levels within the chamber are controlled at 5.0% by using an external CO_2 controller (Model 3057; ThermoForma, Marietta, OH). This controller gets feedback from a sensor placed in the environmental chamber. CO_2 from the tank reaches the environmental chamber through a series of steps. First CO_2 flows from the tank into this external controller. From there CO_2 flows into an antechamber containing a blower. This antechamber is attached to the environmental chamber (Fig. 4.1B)[2]. The blower then forces the CO_2 into the environmental chamber. A CO_2 sensor mounted within the environmental chamber constantly measures CO_2 levels. When the measured level falls below the specified limit (5.0%), the controller injects CO_2 until the specified limit is achieved.

Apparatus for applying compressive loads

A fixture consisting of two parallel anodized aluminum plates are used to apply precise compressive waveforms from a single actuator shaft of this electromagnetic dynamic testing

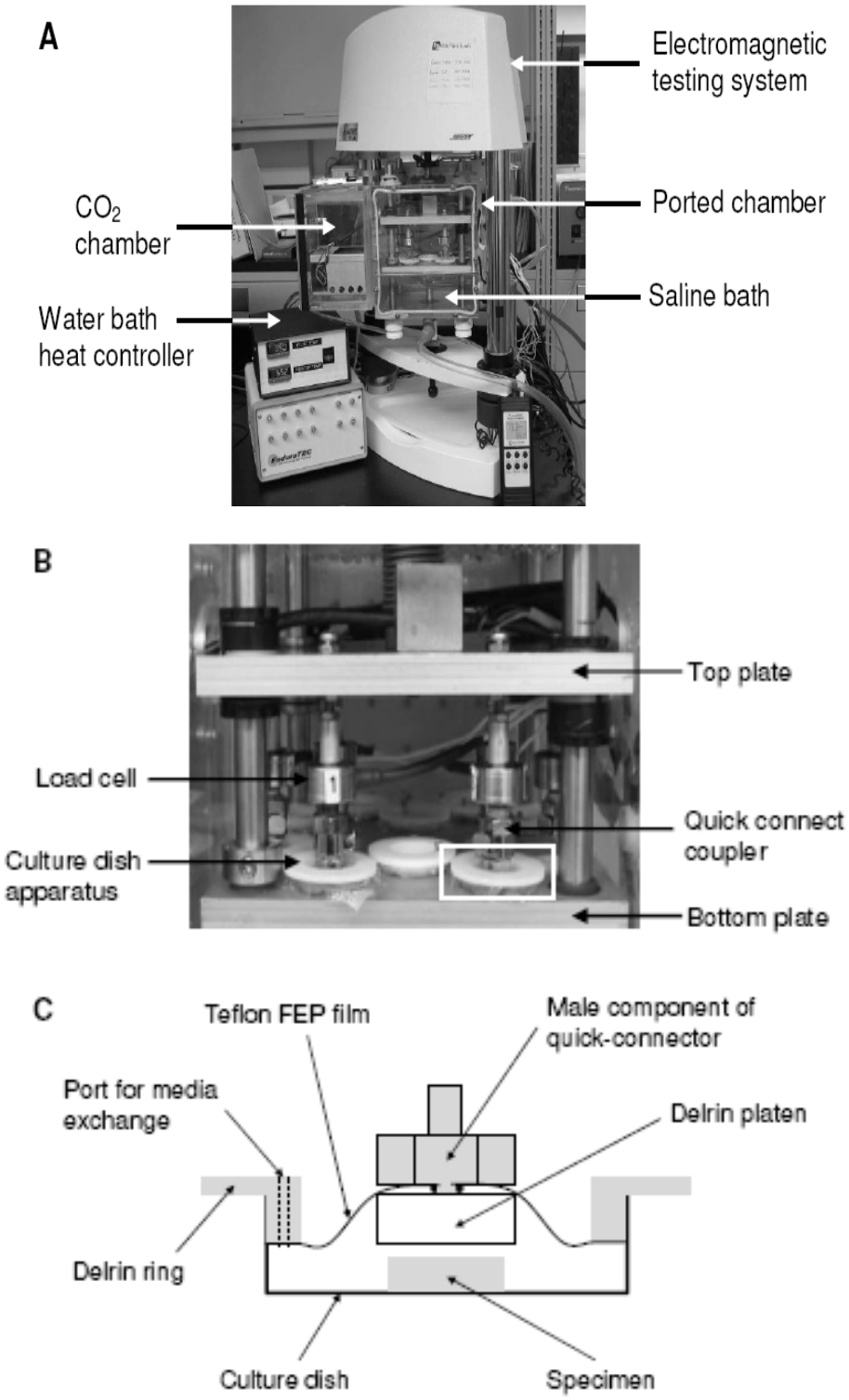

Fig 4.1. A) Compressive stimulus bioreactor. B) Environmental chamber and loading apparatus within the bioreactor. C) Schematic of culture dish with delrin disk, ring, and film assembly [2].

system (Fig. 4.1A). The top plate is attached to the actuator shaft while the bottom plate is attached to the vertically-adjustable arm mounted to the bottom of the chamber. These two plates fit within the environmental chamber and moves on bearings along stainless steel rods that maintain proper alignment of these plates (Fig. 4.1B)[2]. The bottom plate can securely house up to twelve 35 mm dia. tissue culture dishes. Six thin aluminum rods with adjustable screws and containing submersible load cells (Model 31, 25 lb capacity; Honeywell Sensotec, Columbus, OH) extend downward from the top plate and are positioned above the dish locations.

A quick-connect coupler system was designed to facilitate rapid loading and removal of the constructs (MC1002, Colder Products, St. Paul, MN). The female component of this coupler is attached to the free ends of rods that extend downward from the top plate. Male component of this coupler is attached to a delrin disk (8 mm thick x 15 mm dia) through a 4" x 4" piece of 100 Å thick Teflon FEP film (DuPont, Circleville, OH). This film is secured on top of the 35mm culture dishes via a custom delrin ring. This ring also has small threaded hole for media exchange (Fig. 4.1C)[2]. This film prevents media evaporation, reduces the risk of contamination and does not impede actuator motion. This film is also permeable to O_2 and CO_2. This coupler system allows easy rotation of specimen groups to increase the number of constructs that can be stimulated daily. The couple system also allows each dish to be removed easily from the system for observation. Once this coupler is assembled, constructs can then be subjected to compressive stimuli by activating the system actuator, which in turn can move the top plate and press the delrin disk onto the specimen within the dish.

Cell sources

Two types of murine cells were used in different studies to validate our bioreactor. For the cell viability study, mesenchymal stem cells (MSCs) of 6-8 week old mice wild type mice were used. For the compressive stimulation study, rib chondrocytes from six double transgenic newborn mice created in our laboratory were used. These cells have two colorimetric

promoters, enhanced cyan fluorescent protein (ECFP) and Green Fluorescent Protein (GFP-T), which are activated when the type II collagen gene and type I collagen genes are expressed, respectively. The creation of the double transgenic mice is described elsewhere[163].

Characterization of the bioreactor environment

A hygrometer (Model 11-661-18; Fisher Scientific) was used to measure both air humidity and temperature within the environmental chamber. CO_2 levels were measured using both a feedback sensor on the controller and independent Fyrite gas analyzer (Model 11-7029; Bacharach). A traceable digital thermometer (Model 11-661-9; Fisher Scientific) was used to measure the temperature of 5 ml of culture media within a tissue culture dish placed in one of the stations on the bottom plate. Time needed for the environmental conditions to reach steady cell-culture conditions was first monitored. The chamber door was then opened for five minutes to simulate dish exchanges, and conditions were again monitored to determine the time required to re-establish the steady state.

Measurement of axial displacements among bioreactor stations

A sonomicrometry system (Sonometrics Corp., Ontario, Canada)[307] was used to measure the relative displacements among stations located at all corners of the top plate (i.e. adjacent to the stainless steel rods). One piezoelectric peg crystal was mounted at the center of the delrin disk attached to the coupler end, and another was mounted in the center of a tissue culture dish. The disk and dish were immersed in water to allow for communication between the ultrasound crystals. The top plate was moved vertically using input sinusoidal waveforms of 0.1 and 1.0 mm amplitudes at 1 Hz for ten cycles. The chosen peak amplitudes define the range over which we expect to stimulate tissue engineered constructs. Relative crystal distances were measured and compared to the actuator displacement recorded by the internal LVDT of the electromagnetic system (Model ElectroForce 3200 system, Bose Corp.).

Determining cell viability/proliferation

The MSC isolation and expansion protocol up to P2 are described elsewhere [162]. Marrow-derived murine MSCs at P2 were plated onto 35-mm tissue culture dishes at a concentration of 100,000 cells per dish. Each dish was then covered with Teflon film and secured via the delrin ring to maintain sterility. Controls were placed in a standard incubator (Model 370; Thermo Electron Corp., Marietta, OH). Media was replaced every two days.

Cell proliferation and viability were examined after 3, 7 and 14 days of culture. At each time point, three dishes were removed from both the bioreactor and standard incubator and cell proliferation was determined using an MTT assay (Vybrant MTT Cell Profileration Assay Kit, Invitrogen, Carlsbad, CA). We performed the assay in accordance with manufacturer's protocol[162]. The absorbance of formazan was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2, Molecular Devices), and absolute cell numbers were obtained by plotting the obtained absorbance values on a known standard curve.

Mechanical stimulation of chondrocytes cultured in 3-D

Double transgenic newborn mice were euthanized by CO_2 following Institutional Animal Care and Use Committee (IACUC) protocols. Chondrocytes were first harvested from the ribs of six mice using an established procedure[308]. The cells were cultured in monolayer until passage 3, suspended in media (BGJb + 10% fetal calf serum), and mixed with equal volumes of 4% (w/v) agarose (Sigma) to obtain 2% agarose with $10x10^6$cells/ml. This mixture was cast between sterile glass plates from which 8 mm dia x 3 mm thick cylindrical constructs were created using a sterile biopsy punch. For each animal, three constructs were created for each treatment group. Cells were acclimated in the constructs for two days before stimulation. Constructs to be stimulated were subjected to cyclic sinusoidal compression at 10% peak strain at 1 Hz for 1 hour followed by a 1 hour rest period for 6 hrs/day in the bioreactor r[302]. Non-stimulated constructs were cultured in the same bioreactor without stimulation. Media was changed daily. At 0 and 14 days in culture, constructs were visualized in a fluorescence

microscope (Zeiss Axiovert) with a filter set (XF114-2, Omega) for cyan fluorescence (ECFP), indicative of Col2 gene expression. Other filter sets for green and red fluorescence (XF104-2, Omega and 11002VZ, Chroma) were also used to rule out autofluorescence. Constructs were washed thoroughly in PBS and then mechanically homogenized using a glass mortar and Teflon pestle, and ECFP was quantified in Relative Fluorescence Units (RFUs) using a spectrophotometer (Spectra Max M2, Molecular Devices) with excitation/emission/cutoff wavelengths of 436 nm/486 nm/475 nm, respectively. RFU values were normalized to day 0 values for all samples. Differences in RFUs were examined using a mixed effects model (SAS proc mixed) with time in culture and compression as fixed factors and animal as a random factor with α=0.05.

4.3. Results

Characterization of the environment

Increasing the temperature of the 2L water bath in the bottom of the environmental chamber to 39°C increased the media temperature to 37°C and the chamber air temperature to 32°C. This increase in media temperature occurred within 100 min of heating the water bath (Fig. 4.2). Relative humidity (RH) within the chamber reached 75% within 80 min, which was comparable to levels measured in the standard incubator in our lab using the same hygrometer (70-76%). CO_2 levels reached a 5% steady state within 14 minutes as measured by both sensors. Opening the chamber door for five minutes minimally affected the environment (Fig. 4.3), as media temperature was only

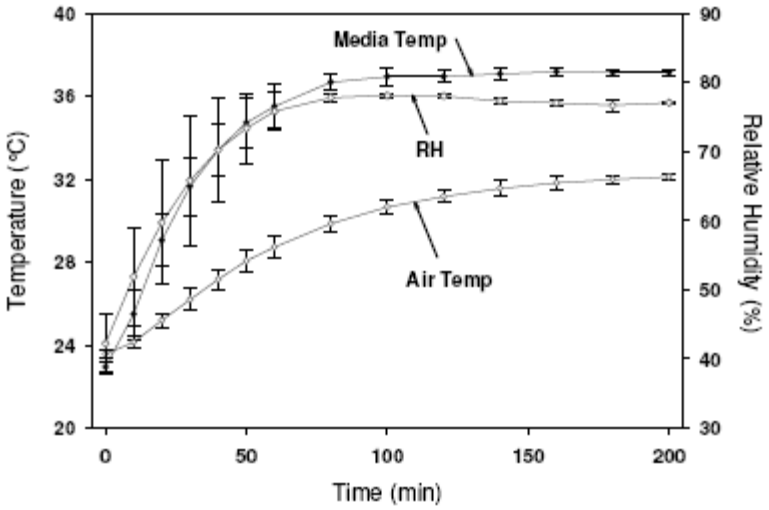

Fig 4.2. Media temperature, air temperature, and relative humidity within the bioreactor reach levels appropriate for cell culture within 100 minutes (n=3, mean ± std dev).

reduced by 1°C and relative humidity was restored to approximately 70% within 10 minutes of closing the chamber door.

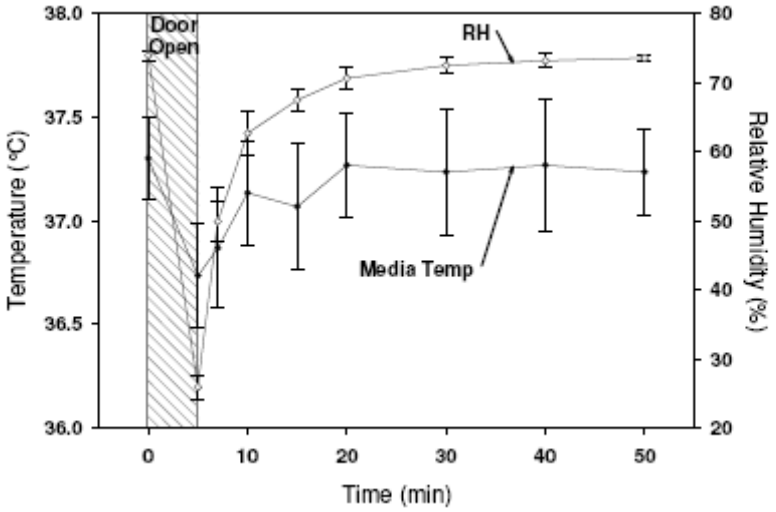

Fig 4.3. Media temperature and chamber relative humidity are restored to appropriate levels within 20 minutes after opening of chamber door for five minutes (n=3, mean ± std dev).

Characterization of axial displacements

The relative crystal motions were nearly identical for both input waveforms. Differences in peak-to-peak amplitudes were ≤ 0.014 mm between all stations for both waveform amplitudes. The crystal motions were in phase with the input motions throughout the motion path.

Cell proliferation/viability

The number of viable, proliferative cells within the bioreactor was not significantly different from that of cells cultured in

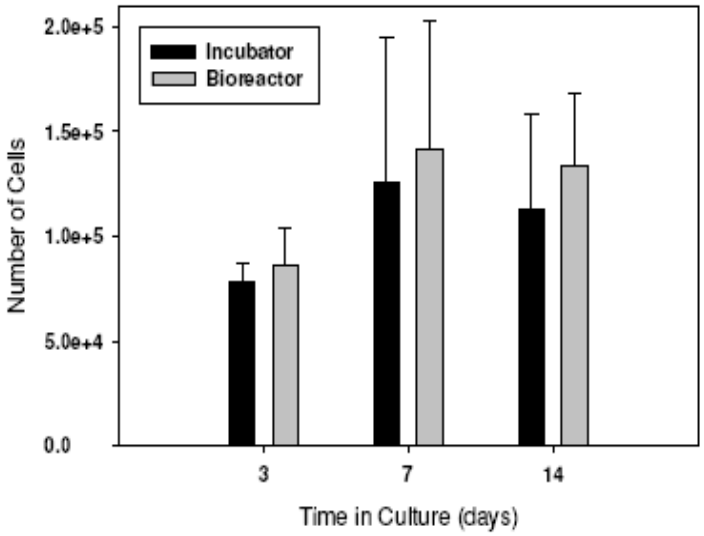

Fig 4.4. Viability of cells cultured in the bioreactor is comparable to that of cells cultured in a standard incubator over 14 days (n=3, mean ± std dev).

the standard incubator at 3, 7, and 14 days of culture ($p>0.25$; Fig. 4.4). Compared to 3 day cultures, cell numbers increased by 60.8% and 63.8% after 7 days of culture in the incubator and bioreactor, respectively. Compared to 7 day cultures, cell numbers decreased slightly by 10.3% and 5.6% in both the incubator and bioreactor, respectively.

Mechanical stimulation of chondrocytes cultured in 3-D

ECFP fluorescence was observed both in monolayer and in 3-D culture at day 0 and was maintained in all constructs at 14 days. Fluorescing cells in both stimulated and non-stimulated groups showed a rounded morphology. Quantitative RFU data (Fig. 4.5) showed that ECFP expression significantly increased with both stimulation and culture time. Stimulated constructs had 14% higher RFU levels than their non-stimulated counterparts ($p=0.002$). RFUs in non-stimulated constructs significantly increased by approximately 127% after 14 days compared to day 0 controls, with an even greater 158% increase in the stimulated group ($p<0.001$).

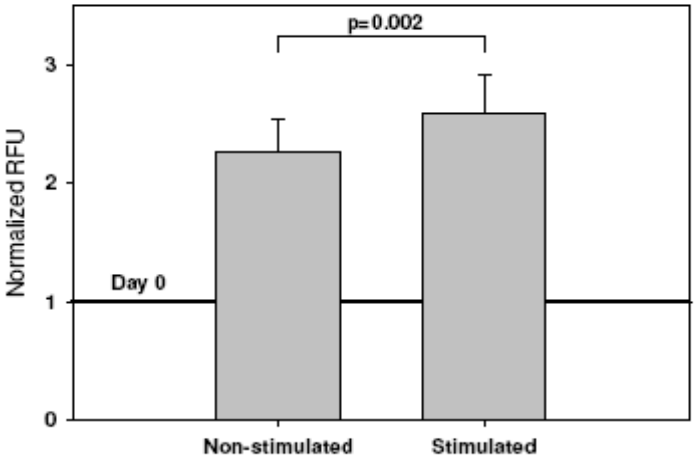

Fig 4.5. ECFP RFUs increase with mechanical stimulation and culture time (n=18, mean ± std dev).

4.4. Discussion

The benefits of mechanical stimulation have been both recognized and observed in a variety of tissue models [162-170]. Hence researchers have been designing bioreactors [170, 300-301, 309] to stimulate constructs using variety of stimulation profiles. We in our lab have created and validated a novel bioreactor for applying compressive stimulation to constructs. This type of bioreactor can be applied to cartilage, meniscus, disc and interface tissue engineering. In this bioreactor we have shown that we can 1) maintain and sustain cell-culture conditions. 2) apply precise displacements 3) stimulate 12 constructs at a time 4) improve Col2

gene expression in murine chondrocyte-constructs. With the instrumented load cells we can also measure the reaction forces. Thus our bioreactor satisfies all the four design criteria set forth for FTE bioreactors.

Our bioreactors have certain advantages when compared with the ones currently being used [170, 300-301, 309] .The first is the ability to calculate stiffness in real time by recording load vs displacement data during culture. The stiffness value can then be compared against normal tissues thus providing biomechanical feedback for adjusting the stimulation to better "challenge" the constructs and to accelerate their maturation in culture for more rapid delivery at surgery. The second advantage is the flexibility of our control system allows for easy manipulation of the various stimulation parameters, including waveform shape, frequency, and amplitude, and it can also receive imported in-vivo displacement profiles when these are available. Other systems may be limited in this capacity. There is also much potential for continued improvement, including the integration of systems for controlling nutrient supply and continuous media exchange and the addition of actuators and/or alteration of indenter shapes to create shear deformation/loading conditions. Ultimately, the concepts generated and employed in the construction of this bioreactor can be applied to the development of a more complex system that can reproduce most or all of the 3-D motions needed to simulate more physiologic joint motions.

Using our novel bioreactor, we plan to embark upon cartilage and fibrocartilage FTE in a similar fashion by optimizing the in-vitro stimulation profiles to create a more mechanically viable engineered tissue before surgery.

Chapter 5

3D In-vitro Effects Of Compression And Time In Culture On Aggregate Modulus And On Gene Expression And Protein Content Of Collagen Type II In Murine Chondrocytes

The objectives of this study were to determine how culture time and dynamic compression, applied to murine chondrocyte-agarose constructs; influences construct stiffness, expression of Col2 and type II collagen. Chondrocytes were harvested from the ribs of six newborn double transgenic mice carrying transgenes that use enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for expression from the Col2a1 and Col1a1 promoters, respectively. Sixty three constructs (8mm dia x 3mm thick) per animal were created by seeding chondrocytes ($10x10^6$ per ml) in agarose gel (2% w/v). Twenty eight constructs from each animal were stimulated for 7, 14, 21 or 28 days in a custom bioreactor housed in an electromagnetic system. Twenty eight constructs exposed to identical culture conditions but without mechanical stimulation served as non-stimulated controls for 7, 14, 21 and 28 days. The remaining seven constructs served as Day 0 controls. Fluorescing cells with rounded morphology were present in all constructs at all five time points. Compared to time-matched, non-stimulated controls, 7, 14, 21 and 28 days of stimulation significantly increased Col2 expression by ECFP fluorescence and mRNA expression by quantitative RT-PCR. Col2 gene expression in both stimulated and non-stimulated constructs showed initial increases up to day 14 and then showed decreases by day 28. Compared to time-matched controls, stimulation significantly increased type II collagen content at 21 and 28 days. Compared to time-matched controls, stimulation significantly increased aggregate modulus at only 28 days. There was a significant increase in aggregate modulus in stimulated constructs between day 0 and 7, and between day 21 and day 28. This study reveals that compressive mechanical stimulation is a potent stimulator of Col2 gene expression that leads to measurable

but delayed increases in protein (type II collagen) and then biomechanical stiffness. Future studies will look at the effects of components of the mechanical signal in culture and address the question of whether such in-vitro improvements in the tissue engineered constructs actually enhance repair outcome after surgery.

5.1. Introduction

Articular cartilage injuries are quite prevalent and if left untreated, can lead to long-term osteoarthritis [310-313]. In the US alone, nearly 21% of the adult population suffers from osteoarthritis [314] resulting in over $128 billion in direct and indirect costs[315]. Tissue engineering [244] is an appealing conceptual alternative when conventional repair techniques (e.g. arthroscopic techniques, periosteal/perichondral grafts, autograft/allograft osteochondral transplantation and prostheses) [316-322] prove unsatisfactory.

Tissue engineering approaches have evolved as investigators recognize the importance of mechanical function. Researchers mix cells with scaffolds to create tissue engineered "constructs" or TECs that can be stimulated in culture before surgical implantation (e.g. to fill tissue defect sites) [246-248]. However, these TECs often lack the inherent mechanical stiffness to tolerate large in-vivo forces such as those acting on articular cartilage. To address this problem, investigators have been applying principles of Functional Tissue Engineering (FTE) [159-160, 323] to precondition constructs using aspects of in-vivo tissue forces and deformations [157-158] to create new generations of reparative tissues [167, 170-171, 324-325]. Such preconditioning improves: 1) construct material properties by increasing synthesis of extracellular matrix proteins like collagens I and II [167, 170, 324-327]; and 2) repair biomechanics after implantation into defect sites [171, 324].

Unfortunately, tissue engineered cartilage constructs often require months of intermittent mechanical conditioning to achieve mechanical properties and protein composition even remotely approaching those of native cartilage [327]. For example, Mauck et.al [326] determined that tissue engineered cartilage constructs required a full 8 weeks of dynamic

loading in culture in order to achieve 75% and 25% of a normal tissue's Young's modulus and unconfined dynamic modulus, respectively. Even with these mechanical improvements, total collagen content was still only 12% of native tissue values [326]. While such long development cycles may be satisfactory in a research setting, these intervals are not practical for patients in a clinical setting.

To shorten the development cycle and reduce production costs, new technologies are needed to rapidly and repeatedly assess the evolving TEC in culture. During the early development period, tissue engineers should be able to non-destructively assess the TEC in culture to avoid creating inappropriate collagen types (e.g. Type I vs. Type II collagen in a TEC that could adversely affect the repair of articular cartilage). For example, if investigators could monitor how mechanical signaling influences Col2 gene expression in near real time, they could dramatically improve their ability to synthesize Type II collagen protein. Such technologies could also allow the tissue engineer to determine which mechanical signals most increase Col2 gene expression and compressive stiffness.

To address these needs, we have developed two innovative technologies. 1) We have bred specialized double transgenic (DT) mice that carry transgenes that use enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for Col2a1 and Col1a1 expression, respectively. ECFP and GFP-T each have a half life of 24 hours and serve as visual reporters for cells that express the genes for Types II and I collagen, respectively. ECFP linked to Col2a1 offers the potential to ultimately track in near real time how various stimuli (mechanical and/or chemical) influence Col2 gene activity in culture. 2) We have also designed and fabricated a bioreactor to deliver compressive displacement profiles with micron precision to constructs in an incubator setting over four-week intervals. This bioreactor is described elsewhere [2].

Using these two technologies, our objectives in this study were to test four hypotheses related to compressive stimulation of double transgenic chondrocyte-agarose constructs. We

hypothesized that compared to controls, compressive stimulation significantly increases: 1) ECFP fluorescence and Col2 mRNA expression; 2) Type II collagen production; and 3) TEC aggregate modulus up to 28 days in culture. We also hypothesized that 4) ECFP fluorescence and Col2 mRNA expression are positively correlated.

5.2. Materials and Methods

Experimental Design

Rib chondrocytes were obtained from six newborn DT mice (one cell line per mouse). Sixty-three cell-agarose constructs (8 mm dia. x 3 mm thick) were created per animal using one cell density (10 x 10^6 cells/ml) [169] in a 2% w/v ratio to maintain chondrocyte phenotype [328] and to achieve a power of 85% to detect treatment-related differences if they existed. We compared the responses of twenty eight (28) stimulated (S) constructs and twenty eight (28) non-stimulated (NS) controls at 7, 14, 21 and 28 days in culture (seven (7) per time period). These results were also compared to findings from seven (7) additional NS controls per cell line at day 0. S constructs all received the same compressive stimulation profile (sinusoidal displacement pattern @ 1Hz to 10% peak strain for one hour followed by 1 hour of rest, repeated three times a day) adapted from Mauck et al [169]. The seven (7) S and seven (7) NS constructs at each time point were assigned as follows: one for ECFP fluorescence in a spectrophotometer (measured in Relative Fluorescence Units (RFU))[329], one to evaluate changes in mRNA expression for Col2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) [167], one for confined compression to determine aggregate modulus [330], one for cell viability using an MTT assay [331], and three constructs for Type II collagen content using ELISA [332]

Chondrocyte isolation and expansion

Creation of the murine DT model is described elsewhere [163] . Newborn mice were euthanized by CO_2 using protocols approved by the Institutional Animal Care and Use

Committee (IACUC). Chondrocytes were harvested from the rib cages using previously-published procedures [333]. Briefly rib cages from each mouse were repeatedly washed in PBS and digested in 2.5 ml of Protease (Sigma P-8811; 2mg/ml) for 30 minutes in an incubator (Steri-Cult Model 3033, Forma Scientific). After incubating overnight in Collagenase B solution (Sigma; 1mg/ml), the digest was washed with media (phenol red free DMEM, 10% FBS, pen/strep), filtered (70μm) to remove remaining bone pieces, sorted by flow cytometry to exclude non-ECFP cells and plated in a 100mm tissue culture dish (Falcon) and incubated for one day at passage 0 (P0). Intracellular cyan fluorescence was evident in these chondrocytes when visualized in a fluorescence microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany) equipped with ECFP and GFP-T (Omega) filter sets. The monolayer chondrocyte culture was then trypsinized using 0.25%/EDTA and replated at $1x10^6$ cells per 100mm plate at P1 and cultured for 7 to 10 days. Chondrocytes were passaged until P3. No intracellular GFP-T expression was seen in cultured chondrocytes from P0 through P3.

Construct preparation

Chondrocytes at P3 were suspended at $20x10^6$ cells/ml in media (BGjB, Invitrogen-Gibco BRL/Life Technologies Inc., Gaithersburg, MD) containing 1% antibiotic/antimycotic and 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville,GA). Cell suspensions were mixed in equal volumes with 4% (w/v) agarose (TYPE VII, low gelling temperature, Fisher Scientific) and PBS to produce a cell-gel solution of 10×10^6 cells/ml in 2% (w/v) agarose. This solution was then cast for 20 minutes between sterile glass plates separated by 3-mm spacers. Disks (8mm diameter) were cored with a sterile dermal punch and each cultured in 5 ml of media in a multi-well plate for 2 days (henceforth denoted as “day 0”) in a standard incubator (Steri-Cult Model 3033, Forma Scientific) to allow cells to adjust to their new culture environment. Media was replaced daily.

Construct preparation for mechanical stimulation

All constructs were transferred from multi-well plates to individual 35 mm glass bottom tissue culture dishes (P35G-0-10-C, MatTek Corp, Ashland, MA) which were covered with Teflon FEP film (100Å; DuPont, Circleville, OH) by means of a custom delrin ring. The film, permeable to O_2 and CO_2 but not to water vapor, ensured sterility and prevented evaporation. The dish design is provided elsewhere (see Fig. 5.3c; [2]).

Dynamic compressive stimulation

Constructs were subjected to dynamic compression in a custom-built bioreactor housed in an electromagnetic testing system (ElectroForce 3200; BOSE Corp., Eden Prairie, MN) [2]. Briefly, this bioreactor consisted of twelve testing stations and a chamber to sustain a cell culture environment (37^oC, 5% CO_2, ≥75% relative humidity). Six of the twelve stations were equipped with loading rods to stimulate the constructs and the remaining six were devoid of loading rods and housed the NS controls.

Before starting dynamic compression, the environmental chamber of the bioreactor was aseptically cleaned and the system's culture environment was allowed to achieve steady state culture conditions. Culture dishes were then placed into each holding station. Consistent with previous patterns[169], constructs were subjected to the compressive strain profile described above. Feeding media was replaced daily. At specified time points, dishes containing treated and control constructs were removed from the bioreactor.

Fluorescence Microscopy

At each time point after the end of stimulation, the constructs subjected to RFU analysis were washed in PBS for 1 hour with gentle shaking to remove media and then visualized for ECFP and GFP-T fluorescence in a fluorescence microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany). To rule out cell auto-fluorescence, constructs were also visualized for rhodamine using an 11002VZ (Chroma) filter set.

Spectrophotometric Analysis

After imaging, constructs assigned for gene expression measurements were mechanically homogenized using a glass mortar and teflon pestle. The homogenate was suspended in 1ml of PBS and pipetted into a black bottom microplate (200µl per well in five wells). ECFP fluorescence in these digests was quantified in Relative Fluorescence Units (RFUs)[329] reading the microplate in a spectrophotometer (Spectra Max M2, Molecular Devices) using excitation/emission wavelengths of 436 nm/486 nm, respectively, with a cut off filter of 475 nm. ECFP RFU was normalized to day 0 values for all samples.

Real-time quantitative RT-PCR

RNA extraction, conventional gene expression analysis and real-time quantitative PCR (qRT-PCR) were performed according to published protocols [167]. Mouse specific primers were used for Type II collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. Before use in the experiment, all primers were extensively tested under conventional and real-time quantitative RT-PCR conditions to ensure: 1) they were specific for each gene, and 2) that we obtained a single clean band when running the electrophoresis gel (with the associated base pair size for each gene). The absolute amount of the corresponding gene mRNA in each construct was obtained from the corresponding gene standard curve. Expression was normalized by calculating the ratio between type II collagen and GAPDH genes for each sample. GAPDH was used as the housekeeping gene, because GAPDH did not change with treatment conditions when we mechanically stimulated cell-scaffold constructs in tension containing mesenchymal stem cells from 10 week old double transgenic mice and from skeletally mature NZ white rabbits [163, 167].

Determination of Cell Viability

At each time point, cell viability in constructs was determined using an MTT assay (Vybrant MTT Cell Profileration Assay Kit, Invitrogen, Carlsbad, CA), in which live cells reduce MTT to a strongly pigmented formazan product to provide colorimetric indicators of cell viability,

following the manufacturer's instructions. Briefly the cell-agarose constructs were homogenized using a mortar and pestle. The homogenate was incubated for 4 hours in an incubator in 500µl of 12mM MTT solution. This was followed incubation for 18 hours in 5ml SDS-HCL to completely dissolve the formazan. The absorbance of formazan was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, CA) [331]. The viability results were normalized to day 0 constructs.

Determination of Type II Collagen Content

A native Type II collagen detection kit (Chondrex Inc, Catalog #6009, Redmond, WA) was used to quantify Type II collagen. The three constructs from each treatment group and animal were pooled. Each construct was washed in PBS for 1 hour with gentle shaking to remove media. The constructs were then lyophilized, their dry weight measured, and incubated in cold water at 4^{o}C. Constructs were subjected to pepsin digestion and elastase digestion following manufacturer's protocols. ELISA was measured according to these protocols [332].

Biomechanical evaluation of the agarose-cell constructs

Aggregate modulus of the cell-agarose constructs was determined using confined compression testing. Constructs were placed in cryovials and stored at - 80ºC. Prior to testing, each tissue specimen was thawed in room temperature phosphate buffered saline (PBS) for 10 minutes. A 6-mm diameter core was created from the center of each construct and its height measured using a light-force micrometer (Model ID-C1012CE, Mitutoyo Corp., Japan). Each sample was placed into a 6-mm diameter x 4.3-mm deep well and subjected to a constant contact stress of 0.066 MPa until equilibrium displacement was achieved. During testing, specimen height and applied load were continuously measured by a fine resolution LVDT (± 0.25", Sensotec Corp., Columbus, OH, U.S.A.) and load cell (5lb Sensotec Corp., Columbus, OH, U.S.A.), respectively. Tissue aggregate modulus (H_A) was calculated using the formula $H_A = (Fo/\pi a^2) / (u/h)$ where Fo is applied compressive force, a is specimen radius, h is specimen thickness and u is equilibrium displacement [330].

Statistical Analysis

We determined the presence of treatment-related differences among values for all response measures (ECFP RFUs, Col2 mRNA, aggregate modulus, cell viability and Type II collagen content) using a two-way ANOVA with Tukey's HSD post hoc testing (SAS software). We chose animal as a random factor, culture time and compressive stimulation as fixed factors, and response measures as dependent variables [334]. Significance was set at $p < 0.05$.

5.3. Results

Qualitative inspection of constructs showed cells with rounded morphology, distributed homogenously in the constructs. Fluorescence microscopy revealed ECFP fluorescence at day 0 in all 3-D constructs which was maintained in 7, 14, 21 and 28 day constructs (Fig. 5.1). No qualitative differences were apparent in the amount or intensity of ECFP fluorescence among the images for non-stimulated and stimulated constructs at the four time points (Fig. 5.1 A-G). Only cyan fluorescing cells were observed in the sections examined. No cells in any of the treatment groups expressed GFP-T and hence we did not measure RFUs or perform qRT-PCR for GFP-T and Col1a1 expression, respectively. No autofluorescence was detected in samples using rhodamine filter sets.

Quantitative results showed that applying a dynamic compressive displacement significantly increased ECFP expression in the 3-D constructs. Compared to time-matched, NS controls, compressive stimulation significantly increased RFU values by 1.34 fold,1.8 fold,1.27 fold and 1.24 fold at 7,14, 21 and 28 days, respectively ($p<0.05$; Fig. 5.2A). There was a significant increase in RFUs in S constructs between day 7 and 14, and a significant decrease between day 21 and day 28 ($p<0.05$; Fig. 5.2A). There was a significant decrease in RFUs in NS between day 21 and day 28 ($p<0.05$; Fig. 5.2A).

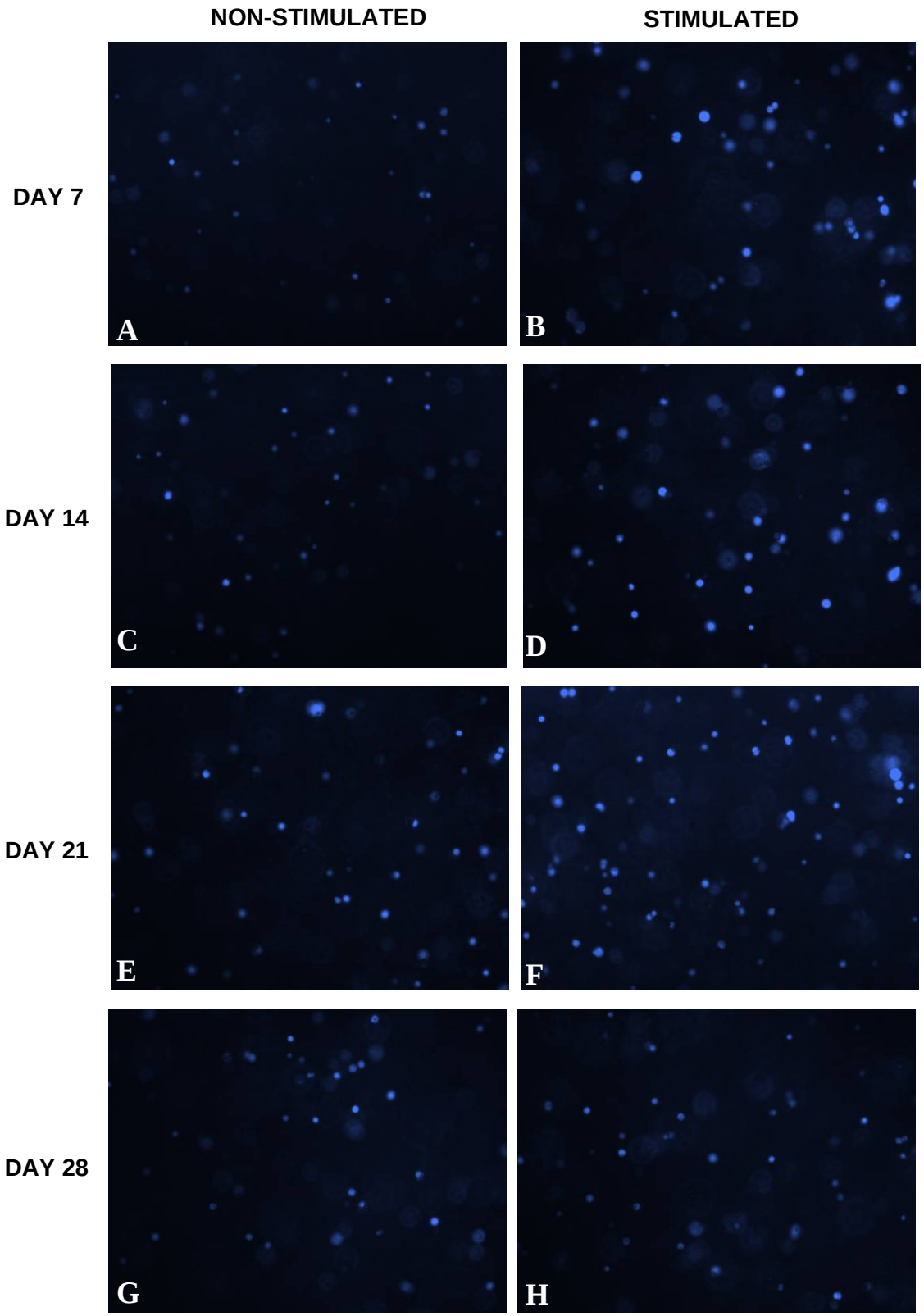

Fig. 5.1. Rounded ECFP-fluorescing cells in non-stimulated (A, C, E & G) and stimulated (B, D, F & H) constructs at day 7 (A & B), day 14 (C & D), day 21 (E & F) and day 28 (G & H).

Dynamic compressive displacement also significantly increased Col2 mRNA by qRT-PCR. Compared to NS controls and relative to GAPDH, compressive stimulation significantly

increased mRNA expression by 1.5 fold, 1.32 fold,1.36 fold and 1.45 fold at 7, 14, 21 and 28 days, respectively ($p<0.05$; Fig. 5.2B). There was a significant increase in Col2 mRNA in S constructs between day 7 and 14, and a significant decrease between day 21 and day 28 ($p<0.05$; Fig. 5.2B). There was a significant increase in Col2 mRNA in NS constructs between day 7 and day 14 and significant decrease between day 21 and day 28 ($p<0.05$; Fig. 5.2B). There were also no significant differences in GAPDH values among treatment conditions.

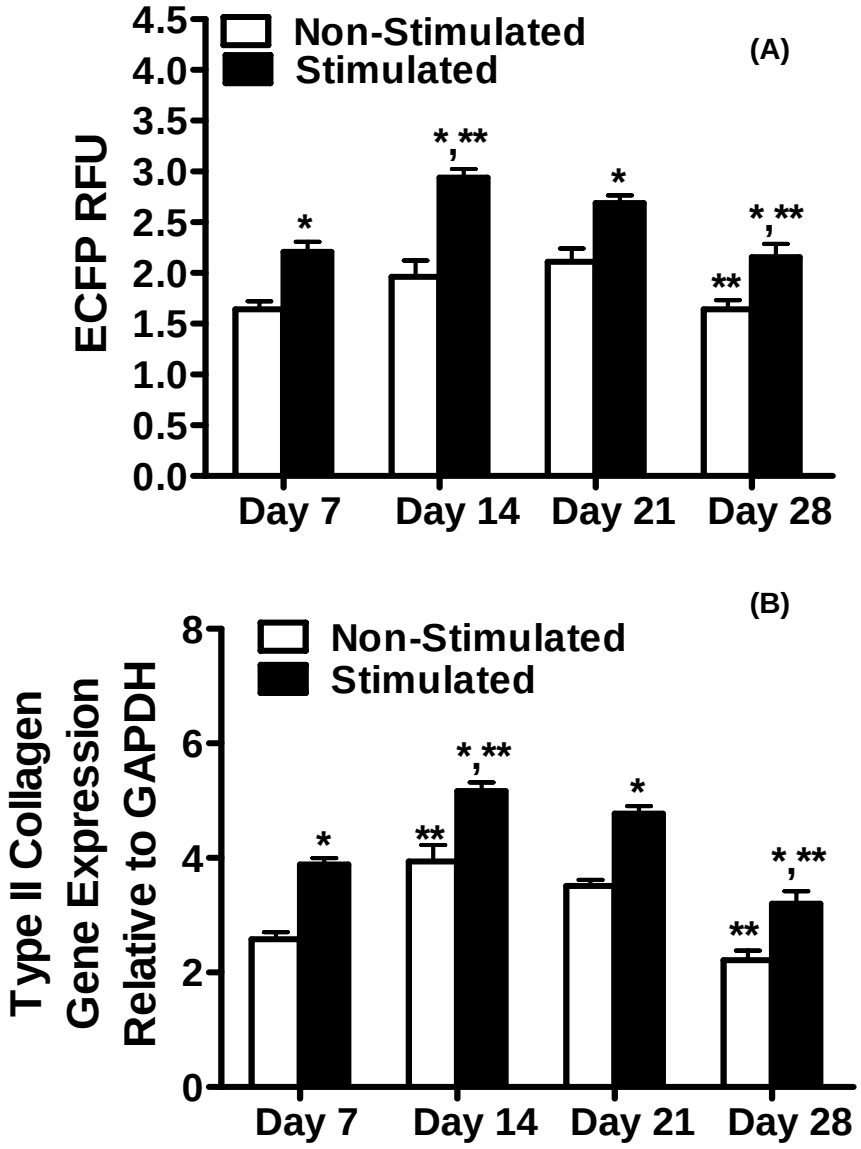

Fig. 5.2. Dynamic stimulation increases (A) ECFP expression by Relative Fluorescence Units (RFU) and (B) Col2 mRNA expression by qRT-PCR at all four time periods of observation. * Significant increase compared to non-stimulated control at same time point ($p< 0.05$). ** Significant difference when compared to same treatment at previous time point. Data represented as mean + one standard deviation. N=6 for all groups.

The results from qRT-PCR and fluorescence analysis were also positively correlated (Fig. 5.3, r^2 = 0.92 with a slope for the linear regression curve of 2.09).

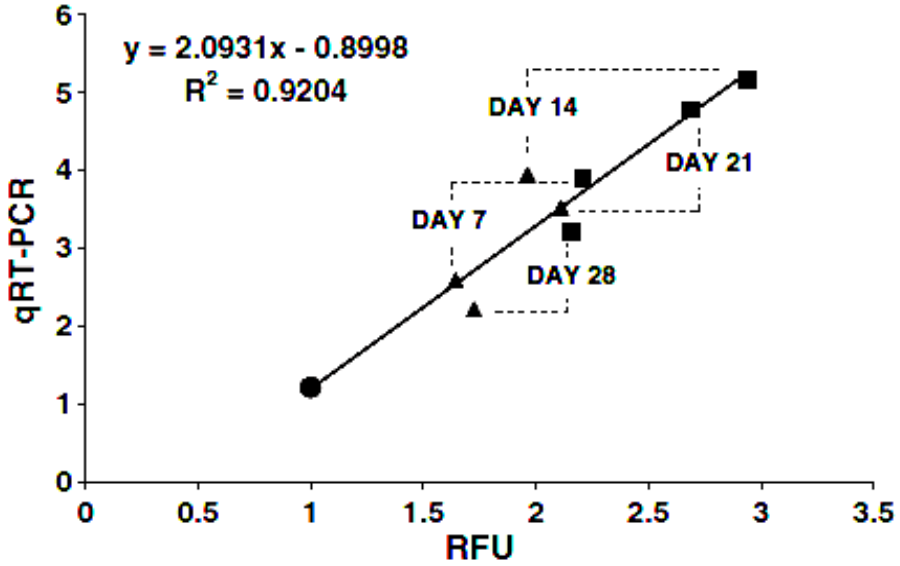

Fig. 5.3. RFU fluorescence of ECFP expression and qRT-PCR are positively correlated over the four time periods of evaluation (days 7, 14, 21 & 28). (Slope = 2.09; r^2=0.92). Circle denotes Day 0 constructs, triangles indicate non-stimulated constructs, and squares denote stimulated constructs.

Compressive stimulation also increased Type II collagen content of S constructs, but only after 21 and 28 days of stimulation. Compared to NS controls, S constructs showed 1.40 fold and 1.24 fold increases in Type II collagen content at 21 and 28 days, respectively ($p<0.05$; Fig. 5.4). There was a significant increase in type II collagen content in S constructs between day 14 and 21, and between day 21 and day 28 ($p<0.05$; Fig. 5.4).

Compressive stimulation increased the aggregate modulus of the chondrocyte-agarose constructs, but only after 28 days of stimulation. After this period of treatment, S constructs showed a 1.67 fold increase in aggregate modulus compared to NS controls ($p<0.05$; Fig. 5.5). There was a significant increase in aggregate modulus in S constructs between day 0 and 7, and between day 21 and day 28 ($p<0.05$; Fig. 5.5).

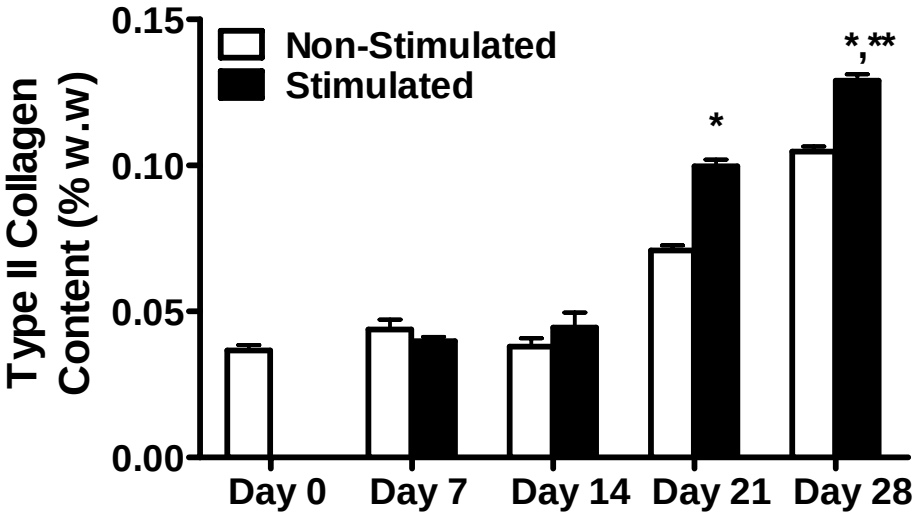

Fig. 5.4. Type II collagen content of constructs increased significantly after 21 and 28 days of dynamic compressive stimulation. * Significant increases compared to non-stimulated controls at same time point ($p < 0.05$). ** Significant increase when compared to same treatment at previous time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups. w.w denotes wet weight.

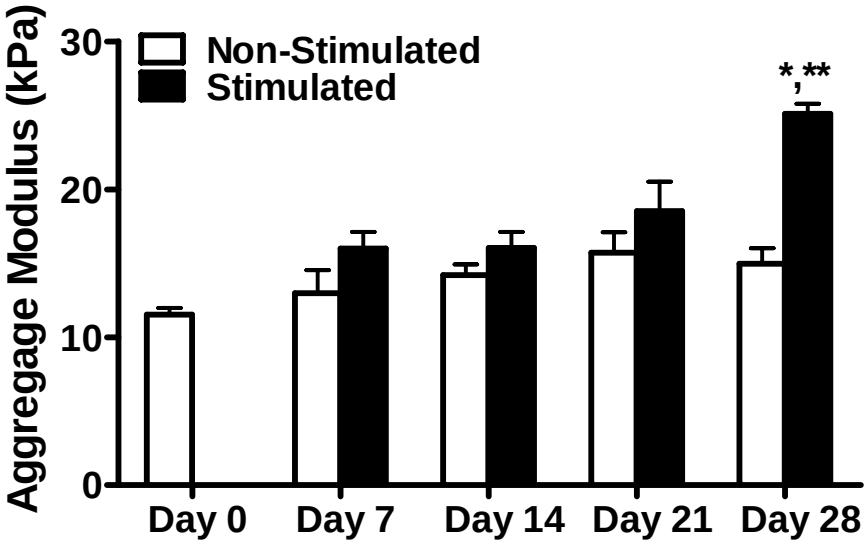

Fig. 5.5. Aggregate modulus of constructs increased significantly after 28 days of dynamic stimulation. * Significant increase compared to non-stimulated control at same time point ($p < 0.05$). ** Significant increase when compared to same treatment at previous time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups.

No significant changes occurred in cell viability due to mechanical stimulation or for increasing time in culture ($p>0.05$; Fig. 5.6). Day 28 constructs showed 8% lower cell viability than day 0 constructs.

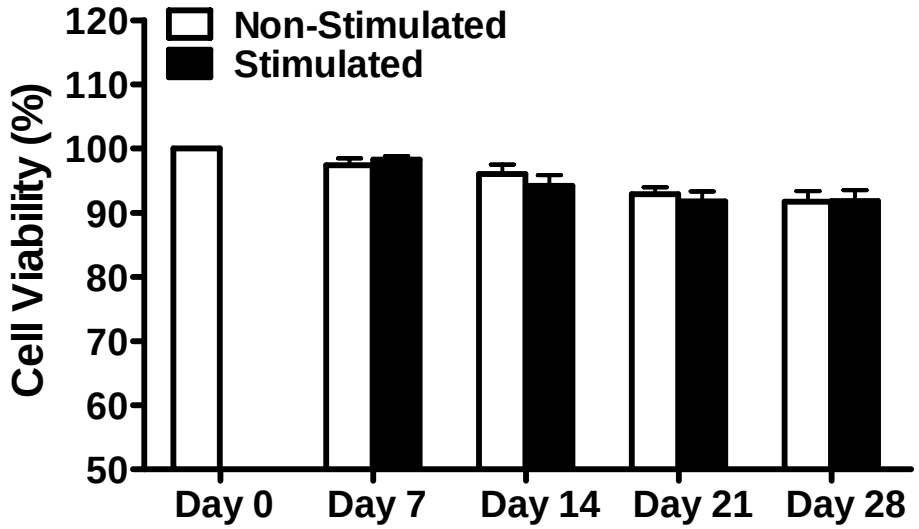

Fig. 5.6. No significant differences were measured in cell viability between stimulated and non-stimulated constructs after 0, 7, 14, 21 and 28 days ($p>0.05$). Cell viability exceeded 90% at all time periods in culture. Data represented as mean + one standard deviation. N=6 for all groups.

5.4. Discussion

Tissue engineers could benefit from new methodologies that rapidly and non-destructively assess the effects of various stimuli on expression of important structural genes. We chose to track changes in Col2 gene expression since Type II collagen, the primary structural protein found in articular cartilage, effectively resists ion-induced internal pressures as well as lateral strains created by compressive forces in cartilage [335]. We achieved this goal in the murine model by linking the Col2 gene to a fluorescent protein with a 24-hour half life that would allow us to detect near real-time changes in expression. While a protein with a shorter half life might have provided a "transducer-like" biological response to changes in mechanical signal, we also wanted to ensure that we did not miss observing these changes when they occurred.

Effects of Compressive Stimulation and Time on Gene Expression

Our current study using these transgenic chondrocytes was, in part, designed to examine the individual and combined effects of dynamic compression and time in culture on ECFP and Col2 gene expression. Both spectrophotometric analysis to measure ECFP RFUs and qRT-PCR to monitor Col2 mRNA expression showed similar patterns over time in culture (Figs. 5.2A and 5.2B). While NS constructs displayed modest temporal changes in RFUs (relative to Day 0 values), mechanical stimulation significantly enhanced fluorescence both compared to controls and between Day 7 and Day 14 (Figs. 5.2A, 5.7A and 5.7B). Very similar temporal and stimulation patterns were also observed by qRT-PCR (Fig. 5.2B), supporting our first hypothesis that compressive stimulation significantly increases ECFP fluorescence and Col2 mRNA expression.

Our results mirror the findings of two shorter-term studies but directly contradict other published reports showing no changes in collagen gene expression after mechanical stimulation. De Croos et.al [336] found significant increases in Col2 mRNA expression 12 hours after exposing bovine chondrocyte-calcium phosphate constructs to 1 kPa compressive pressure at 1 Hz for 30 minutes. In a similar way, Xie J et.al [337] found that 24 hours of continuous dynamic compression applied to rabbit chondrocytes seeded in microporous elastomeric scaffolds of poly(L-lactide-co-epsilon-caprolactone) significantly increased Col2 gene expression. By contrast, Hunter and coworkers found no change in Col2 gene expression when constructs containing bovine chondrocytes seeded in collagen gels were subjected to dynamic compression (± 4% strain @ 1 Hz for 24 hrs) [338]. Demarteau et al also showed no alterations in Col2 mRNA levels when human articular chondrocytes were seeded in PEGT/PBT (polyethylene glycol terephthalate/polybutylene terephthalate) foams and exposed to dynamic compression (6 cycles of sinusoidal deformation to 5 % peak strain at 1Hz followed by a 10 h rest period for 3 days) [339]. Mauck et.al [192] actually observed decreases in type II collagen promoter activity when bovine chondrocytes (transfected with pCol2-LUC promoter reporter

plasmid) were seeded in 2% w/v agarose. Mauck subjected these TECs to 10% cyclic compressive deformation at 1 Hz for either 60 or 180 mins, after which they observed decreases in Type II collagen activity 24 and 72 hours post stimulation. The differences seen among all of these studies could be attributed to different experimental conditions such as the choice of biomaterial, cell source, stimulation profile, and time of evaluation. For example, it is worth noting that our results are longer-term, showing stimulation-induced increases in Col2 mRNA expression and RFUs that persist after 28 days of stimulation. Future studies could benefit from tracking changes over longer time periods as well as systematically varying and controlling other factors to determine their importance in the tissue engineering fabrication process.

Numerous studies have sought to explain how dynamic compression increases Col2 gene expression. One study found that such increases occur through transcriptional activation, possibly through the Sp1 binding sites residing in the proximal region of the Col2a1 gene promoter [340]. Others suggest that certain phenomena like cell/tissue strain, fluid pressurization and flow, electrokinetic phenomena, convective transport, and release of cytokines/growth factors can trigger the complex chain of events that modulate the production of ECM proteins like Type II collagen [341-343]. Although not a specific objective of the current study, we do plan to study several of these mechanisms and how they might increase Col2 gene expression and ultimately Type II collagen production.

Our results also support our fourth hypothesis that ECFP fluorescence and Col2 mRNA expression are positively correlated (r^2=0.92; Fig. 5.3). These correlations are similar to GFP-T vs. Col1 mRNA results from a previous study in our lab where MSCs from double transgenic mice were seeded in collagen sponge scaffolds and exposed to tensile stimulation[163]. Both sets of findings are encouraging for tissue engineering applications and suggest that tracking fluorescence changes are a reasonable surrogate for monitoring changes in mRNA expression by qRT-PCR.

Effects of Stimulation and Time on Type II Collagen Content and Aggregate Modulus

Our results support our second and third hypotheses that compressive stimulation significantly increases Type II collagen production and TEC aggregate modulus. Although Type II collagen content relative to mRNA expression is delayed in both construct types, mechanical stimulation significantly enhances and that the amount of measured protein. While Col2 gene expression peaked between 7 and 14 days and then declined (Figs. 5.2B, 5.7A and 5.7B), Type II collagen content showed significant increases at 21 days with further enhancement at 28 days. Both the S and NS constructs showed these significant effects (Figs. 5.4, 5.7A and 5.7B) with S constructs showing further increases in protein content at these later time periods. The fact that changes in Col2 expression occur prior to increased Type II collagen content is not unexpected. However, the fact that this delay may be as long as 14 days in culture and that mechanical stimulation can enhance protein production by as much as 25-33% are rather surprising findings and likely specific to the cells, scaffold and media conditions chosen for these experiments. It is also worth noting that increased Type II collagen content at 21 and 28 days may have: 1) hampered diffusion of nutrients, inducing small decreases in cell viability (Fig. 5.6); and 2) switched cells to more of a "maintenance mode," thereby decreasing Col2 gene expression (Fig. 5.2B). These conclusions are only speculations at this time and will be the subject of further work by our group.

Our study also demonstrated further delays in biomechanical effects of mechanical stimulation. While Type II collagen content was elevated by twenty one (21) days, an additional seven (7) days of dynamic compression (28 days total) was required to significantly improve aggregate modulus (Figs. 5.5, 5.7A and 5.7B). Again, these effects are likely dependent on the cell, scaffold, and mechanical signals imposed. One might also question whether factors like freezing the constructs before testing could have affected our biomechanical results. Freezing was required because multiple constructs had to be tested each day, each test required five hours to complete, and we had only two confined compression testers available. To directly

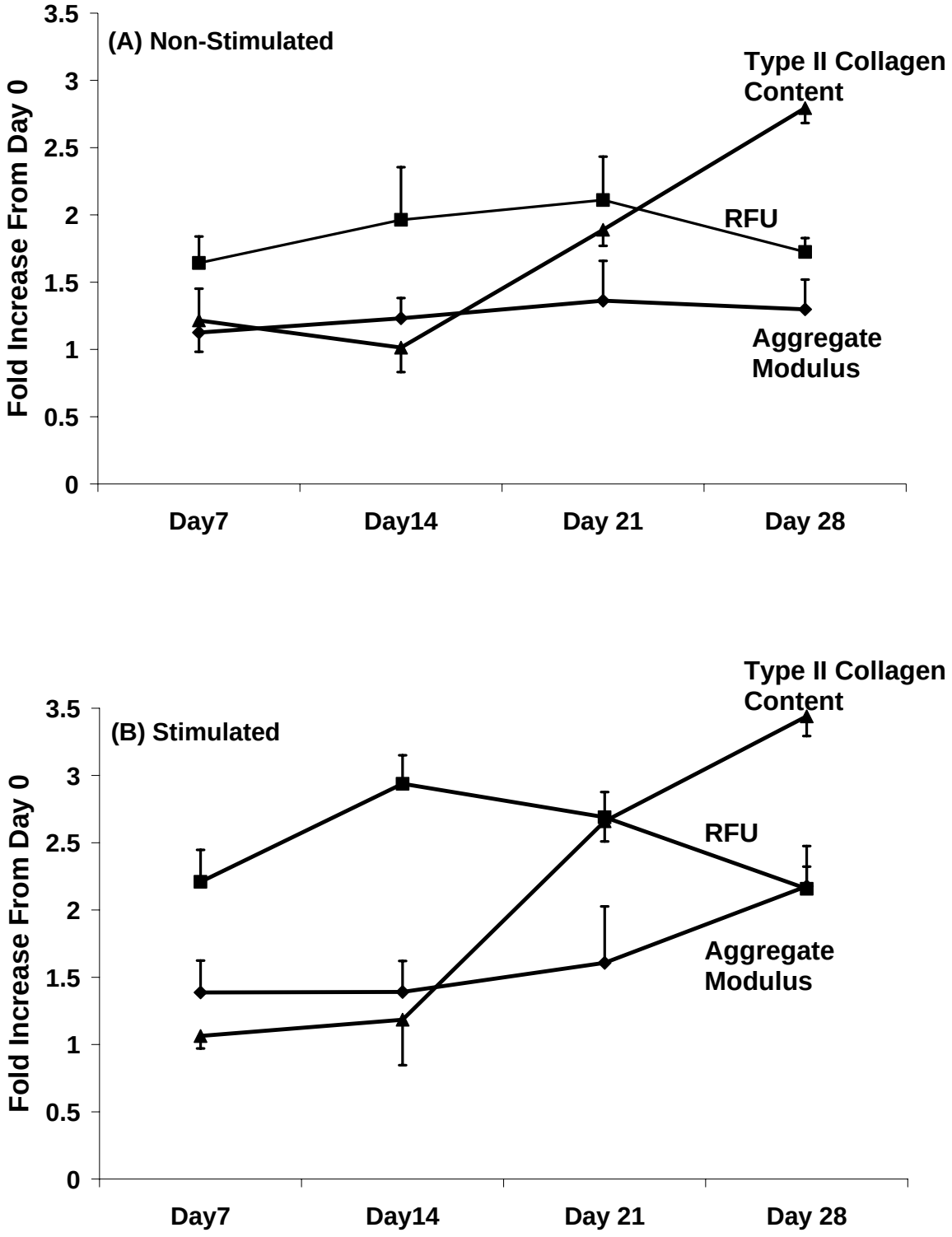

Fig. 5.7. Fold increase from day 0 in (A) non-stimulated constructs, (B) stimulated constructs. Note the earlier increase in Col2 expression, followed by delayed increase in type II collagen content, then followed by an increase in construct modulus. Data represented as means ± one standard deviation. N=6 for all groups.

examine potential freezing effects, we did culture two chondrocyte-agarose constructs from each of five cell lines (from 5 mice) for seven (7) days without mechanical stimulation and then froze one of each pair. Unconfined compression testing revealed no significant effect of freezing on aggregate modulus between groups ($p>0.05$). Our lab has also previously shown that freezing does not affect mechanical properties of MSC-collagen constructs [344].

Our improvements in construct aggregate modulus are similar to those of Mauck et al [169, 326], who stimulated bovine chondrocyte-agarose TECs to 10% cyclic compressive deformation. This stimulus profile significantly increased equilibrium aggregate modulus compared to time-matched, non-stimulated constructs. However, these studies also showed that aggregate modulus of non-stimulated constructs actually decreased between day 28 and day 55. These observations taken together with our results suggest that time in culture and dynamic stimulation can each significantly increase the matrix structure and biomechanics of chondrocyte-agarose constructs but that new strategies may be required to sustain these improvements in longer-term culture.

Overarching conclusions from the current study

Among the most important findings in our study are the temporal and stimulation-induced relationships among gene expression, protein expression and biomechanics. Most studies in the literature have examined the effects of stimulation on only one or two of these response measures. Instead, we chose to simultaneously monitor treatment-induced effects on all three measures. The increases we observed in Col2 gene expression at 7, 14, 21 and 28 days only culminated in increased Type II collagen content after 21 and 28 days and increased aggregate modulus after 28 days. Gene expression must obviously precede protein deposition, but any biomechanical benefits arise only after the protein is assembled into a more functional matrix. Additional studies are needed to corroborate these temporal findings in the murine model and in other model systems and to determine if gene and protein expression patterns might serve as predictors of biomechanical response, both in-vitro and ultimately in-vivo.

Using a murine chondrocyte source to develop tissue engineered constructs offers certain advantages and disadvantages. Although repairing cartilage defects in the mouse model using FTE principles remains a distinct challenge, murine cells offer molecular tools that are not currently available in higher models such as rabbits, goats and sheep. Such biological tools also exist in the human model, but human chondrocytes are not readily available. Performing in-vitro experiments in the mouse that can be successfully correlated to results in higher models may someday permit translation to humans. Finally, the murine model is an ideal candidate to evaluate signaling pathways involved in mechanotransduction. For example, our group is already examining the effects of mechanical stimulation on BMP/FGF signaling pathways with and without inhibitors.

Our study is not without limitations. 1) We did not track changes in expression of ECFP with passage number. Reduction in ECFP levels might indicate that these chondrocytes are dedifferentiating, hence prolonging any future increases in gene and protein expression and subsequent construct biomechanics. It has been shown previously that chondrocytes can rapidly dedifferentiate after even one passage and could exhibit more of a fibroblastic phenotype with increased Col1 expression [345-346]. However, none of the cells in the current study showed any GFP-T fluorescence up to P3, a marker that would indicate Col1a1 expression. In future studies, we plan to use our double transgenic cells to monitor the effects of cell passage number and loading type on both Col2a1 and Col1a1 gene expression, Type II collagen content and construct biomechanics. 2) We did not track real-time changes in Col2a1 expression in the intact construct. These structures are currently too thick and opaque to perform such non-destructive imaging. We continue to seek novel imaging technologies to observe such changes in cellular fluorescence that would permit us to perform longitudinal studies on the same constructs over time. 3) It was necessary to pool fluorescence from all cells extracted from constructs undergoing the same experimental condition. Thus, we can report only average rather than specific or local effects of compression on individual cells. 4) We

used juvenile chondrocytes because they could be easily extracted from the ribs of the newborn mice and because of our interest in examining how developmental biology might be applied to adult tissue healing. We also plan to study how adult chondrocytes respond to these stimuli. 5) Autofluorescence of agarose gel fragments could increase overall RFU values. In this study, we assumed that these changes were constant across groups. 6) While Type II collagen content and biomechanics significantly increased after 21 to 28 days in culture, these response measures do not directly indicate the degree of collagen assembly or alignment. 7) We did not specifically track changes in the production of proteoglycans like aggrecan. Increases in aggrecan content could reflect improvements in a construct's aggregate modulus. In the future we intend to track the production of proteoglycans. 8) We performed only confined compression testing on the constructs. Future studies should apply other testing modes like unconfined compression or indentation. 9) We applied only one compressive stimulus pattern to the constructs. We still need to systematically vary components of the mechanical signal so as to optimize gene and protein expression as well as construct biomechanics in the shortest possible time interval. 10) Other forms of stimulation (e.g. chemical stimulation with TGF-β) may also induce increases in these response measures. In the future, we intend to contrast results using these methods with those from our current study.

In conclusion, our results reveal that both compressive stimulation and time in culture increase Col2 gene expression, Type II collagen content and aggregate modulus in constructs formed from murine chondrocytes seeded in agarose gels. The transgenic and bioreactor technologies that we report hold much promise in optimizing tissue engineering methodologies for replacement of damaged and diseased tissues like articular cartilage and fibrocartilage. In particular, we hope to soon identify which profiles of environmental stimulation (mechanical, chemical, etc.) result in the most rapid and greatest increases in Col2 gene expression levels. Such innovative technologies will be needed to discover promising tissue engineering treatments to speed the repair of damaged and diseased tissues.

Chapter 6

Compressive Loading Applied To Double Transgenic MSC-Agarose Constructs Alters Collagen II Gene Expression, Collagen II Protein Expression And Aggregate Modulus

The objectives of this study were to determine how long-term dynamic compression, applied to murine MSC-agarose constructs, influences construct stiffness, collagen 2 gene expression and synthesized type II collagen protein. MSCs were harvested from the long bones of six ten-week old double transgenic mice carrying transgenes that display enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for expression from the Col2a1 and Col1a1 promoters, respectively. Fifty-four constructs (8mm dia x 3mm thick) per animal were created by seeding MSCs ($10x10^6$ per ml) in agarose gel (2% w/v). Twenty-four constructs from each animal were stimulated for 7, 14, 21 or 28 days in a custom bioreactor housed in an electromagnetic system. Another twenty-four constructs were exposed to identical culture conditions but without mechanical stimulation and thus served as non-stimulated controls at 7, 14, 21 and 28 days. The remaining six constructs served as Day 0 controls. Fluorescing cells with rounded morphology were present in only the day 14, 21 and 28 constructs. Compared to time-matched, non-stimulated controls, 21 and 28 days of stimulation significantly increased Col2 expression as measured by ECFP fluorescence, type II collagen content, and aggregate modulus. There were also significant increases in Col2 expression and type II collagen content in stimulated constructs between day 21 and day 28. Future studies will examine how components of the in-vitro mechanical signal affect these same response measures and address the question as to whether such in-vitro improvements in the tissue engineered construct enhance repair outcome after surgery.

6.1. Introduction

Articular cartilage injuries heal poorly because of the lack of blood supply and present a significant clinical problem [347-349]. These problems have motivated some surgeons to implant osteochondral allografts which can fill a cartilage defect with mature tissue, but which also raise concerns about disease transmission, immune response and proper height and integration into the defect [350-362]. Others have used cell-based therapies such as autologous chondrocyte implantation for the repair of cartilage defects [363-364]. This approach has provided patients with some pain relief but concerns remain about the structural integrity and integration of the cells and periosteal flap initially and the synthesized matrix in the longer term [348, 365]. Consequently, researchers are now investigating various chondrocyte-seeded scaffold constructs [366-369] in the laboratory. However, a major limiting factor using this approach is whether sufficient autologous chondrocytes can be harvested. This problem is especially evident in older and osteoarthritic patients who exhibit decreased chondrogenic capacity [370-371], proliferation rates, collagen synthesis and sensitivity to cytokines and growth factors [372-374]. Studies have also indicated that chondrocytes from these OA patients show degenerative alterations and DNA damage [375].Using autologous chondroctyes also poses the risk of donor-site morbidity [376].

Mesenchymal stem cell (MSC)-based therapies have the potential to overcome some of the disadvantages of chondrocyte-based therapies. MSCs are a promising alternative cell source for cartilage repair due to their ease of isolation and expansion and their chondrogenic differentiation potential [151, 376-380]. For example, MSCs undergo chondrogenic differentiation in the presence of growth factors [381-386]. Dynamic compressive loading and hydrostatic loading also enhance MSC differentiation along a chondrogenic lineage [198, 387-393]. In the absence of growth factors, dynamic compression of constructs containing bone marrow stromal cells stimulates Collagen II gene expression [390]. Dynamic hydrostatic loading in the physiological range also upregulates chondrogenic gene expression [392-393] and

protein synthesis [198, 392]. Taken together, these studies support the advantages of using dynamic loading to stimulate MSC constructs for cartilage tissue engineering.

Our laboratory has developed novel transgenic mouse and bioreactor technologies to study loading-induced effects on TECs containing cells like MSCs. We have created double transgenic mice in which the Col1 and Col2 genes are linked to intracellular Green Fluorescent Protein- Topaz (GFP-T) and Enhanced Cyan Fluorescent Protein (ECFP), respectively [162-163]. Cells from these mice fluoresce green when the Col1 gene is activated and cyan when the Col2 gene is activated. Using MSCs from these mice we have shown that tension upregulates Col1 expression [163] while compression of chondrocytes upregulates Col2 expression [162]. We have also demonstrated that gene expression by fluorescence and qRT-PCR are positively correlated [162-163]. Finally, we have created and validated bioreactors capable of providing micron-level precision waveforms to stimulate constructs in compression and tension [2].

The objective of the current study is to utilize these technologies to examine the effects of dynamic compression on 3-D tissue engineered constructs (TECs) containing murine double transgenic MSCs in agarose scaffolds. By depositing these cells in scaffolds and placing the constructs in our bioreactor, we sought to test the following hypotheses: that compared to the results for non-stimulated TEC controls, compressive stimulation up to 28 days in culture increases; 1) the amount of ECFP fluorescence (an indicator of Col II gene expression); 2) the amount of Type II collagen content; 3) TEC aggregate modulus.

6.2. Materials and Methods

Experimental Design

MSCs were obtained from the bone marrow of six (6) ten-week old double transgenic mice as previously described [163, 394-395]. Fifty four cell-agarose constructs (8 mm dia. x 3 mm thick) were created per animal using one cell density of 10 x 10^6 cells/ml in a 2% w/v agarose scaffold [162]. Twenty-four (24) TECs were stimulated (S) and the other twenty-four (24) TECs were non-stimulated (NS). Six (6) S and six (6) NS TECS were assigned to each of

four time periods of assessment (7, 14, 21 and 28 days in culture). The remaining six (6) constructs served as day 0 NS controls. S constructs all received the same compressive stimulation profile (sinusoidal displacement pattern @ 1Hz to 10% peak strain for one hour followed by one hour of rest, repeated three times a day) [162, 366]. The six (6) S and six (6) NS constructs at each time point were assigned as follows: one for ECFP fluorescence in a spectrophotometer (measured in Relative Fluorescence Units (RFU)) [162, 396], one for confined compression to determine aggregate modulus [162, 330], one for cell viability using an MTT assay [162, 397], and three constructs for Type II collagen content using ELISA [162, 398].

Mesenchymal stem cells

In accordance with protocols established by University of Cincinnati's Institutional Animal Care and Use Committee (IUCAC), mice were euthanized by CO_2. Femur and tibia were isolated and were scraped to remove all the soft tissues. Bones were then transected under sterile conditions and placed in modified centrifuge tubes. 400g of centrifugation for one minute was used to extract the bone marrow [163, 394-395]. These bone marrow cells were initially plated at concentration of 7.5 x 10^6 cells/100 mm dishes and fed media with supplements (MesenCult™ Basal Medium for Mouse Mesenchymal Stem Cells and Mouse Mesenchymal Stem Cell Stimulatory Supplements, Stem Cell Technologies, Vancouver, BC) [163, 394-395]. The se dishes were washed with phosphate buffered saline (PBS, Gibco BRL/Life Technologies Inc., Gaithersburg, MD) after two days to remove non-adherent cells, and fed fresh media [163, 394-395]. After seven to ten days in culture, cells at P0 were trypsinized and replated at 1 x 106 cells/100mm dishes and cultured for one additional week [163, 394-395]. This process was repeated until cells reached P4 [163, 394-395].

Construct Creation

To produce a cell-gel solution of 10 x 106 cells/ml in 2% (w/v) agarose, MSCs were suspended at 20x10^6 cells/ml in media (BGjB, Invitrogen-Gibco BRL/Life Technologies Inc., Gaithersburg, MD) containing 1% antibiotic/antimycotic and 10% fetal bovine serum (FBS;

Atlanta Biologicals, Lawrenceville,GA) and mixed in equal volumes with 4% (w/v) agarose (TYPE VII, low gelling temperature, Fisher Scientific) and PBS. Sterile glass plates with a 3mm spacer were used to cast this cell-gel solution. The solution was allowed to gel for 20 minutes and eight (8)-mm diameter samples were then cored. Cells were then cultured in these agarose constructs under free swelling conditions in a standard incubator (Steri-Cult Model 3033, Forma Scientific) for two days (denoted henceforth as Day 0). Media was replaced daily.

Compressive Stimulation

To maintain sterility and to prevent media evaporation, dishes containing the constructs were covered with a Teflon FEP film (100Å; DuPont, Circleville, OH) held in place by a delrin ring [2, 162]. These dishes were then placed into each well of the custom-built bioreactor [2]. Constructs were then subjected to dynamic compression profile as described in Experimental Design above [2, 162]. Feeding media was replaced daily.

Fluorescence Analysis

Cyan Fluorescence in the constructs was analyzed qualitatively and quantitatively. Each stimulated and non-stimulated construct was visualized in a fluorescence microscope (Zeiss Axiovert) with a filter set (XF1142, Omega) for cyan fluorescence [2, 162]. Constructs were further visualized using filter sets for green and red fluorescence (XF104-2, Omega and 11002VZ, Chroma) to rule out autofluorescence [2, 162]. TECs were then mechanically homogenized to quantify ECFP fluorescence. Constructs were homogenized using a glass mortar and Teflon pestle. The minced constructs were then placed in a spectrophotometer (Spectra Max M2, Molecular Devices) where ECFP RFUs were quantified using excitation/emission/cutoff wavelengths of 436nm/486nm/475nm, respectively [162].

Determination of Cell Viability

At each time point, cell viability in constructs was determined using an MTT assay (Vybrant MTT Cell Profileration Assay Kit, Invitrogen, Carlsbad, CA). The cell-agarose constructs were first homogenized using a mortar and pestle and incubated in 500µl of 12mM MTT solution for 4 hours. This was followed by additional incubation in 5ml SDS-HCL for 18 hours to completely dissolve the formazan. Formazan absorbance was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, CA) [162, 397]. Viability results were normalized to Day 0 constructs.

Determination of Type II Collagen Content

Type II collagen content was quantified using native Type II collagen detection kit (Chondrex Inc, Catalog #6009, Redmond, WA) [398]. Three constructs from each treatment group and animal were then pooled for such quantification. The details of the assay procedure are described elsewhere [162]. Briefly constructs were lyophilized, their dry weight measured, and subjected to pepsin digestion and elastase digestion following manufacturer's protocols. ELISA was measured according to these protocols[398].

Biomechanical evaluation of the agarose-cell constructs

Constructs for biomechanical evaluation were placed in cryovials and stored at -80ºC Prior to testing each tissue specimen was thawed at room temperature in phosphate buffered saline. Aggregate modulus of the cell-agarose constructs was determined using confined compression testing as previously described [162]. Briefly a 6-mm diameter core was created from the center of each construct and subjected to a constant contact stress of 0.066 MPa until equilibrium displacement was achieved. During testing, specimen height and applied load were continuously measured. Tissue aggregate modulus (HA) was calculated using the formula $HA = (Fo/\pi a2) / (u/h)$ where Fo is applied compressive force, a is specimen radius, h is specimen thickness and u is equilibrium displacement[162].

Statistical Analysis

SPSS software was utilized to perform two way ANOVA with Tukey's HSD post hoc test. to determined the presence of treatment-related differences among values for all response measures. Significance was set at $p < 0.05$.

6.3. Results

Fluorescence Microscopy

Inspection of the TECs showed the presence of homogeneously distributed rounded cells. Cells did not fluoresce ECFP in any of the constructs at day 0 or at day 7. ECFP fluorescent cells were found in all 14, 21 and 28 day constructs (Fig. 6.1). However, no apparent differences in ECFP fluorescence were observed among the S and NS constructs at corresponding time points.

Spectroscopic Analysis

Quantitative results showed that applying a dynamic compressive displacement significantly increased ECFP expression in the 3-D constructs at only later time points. Compared to time-matched, NS controls, compressive stimulation significantly increased RFU values by 1.44 fold and 1.63 fold at 21 and 28 days, respectively ($p<0.05$; Fig. 6.2). There was also a significant increase in RFUs in the S constructs between day 21 and 28, ($p<0.05$; Fig. 6.2).

Type II Collagen Content

Similar to the RFU results by spectroscopic analysis, compressive stimulation increased Type II collagen content in S constructs, but only after 21 and 28 days of stimulation. Type II collagen content was not detectable in day 0 and day 7 samples. Compared to NS controls, S constructs showed 1.72 fold and 1.67 fold increases in Type II collagen content at 21 and 28 days, respectively ($p<0.05$; Fig. 6.3). There was also a significant increase in type II collagen content in S constructs between day 21 and day 28 ($p<0.05$; Fig. 6.3).

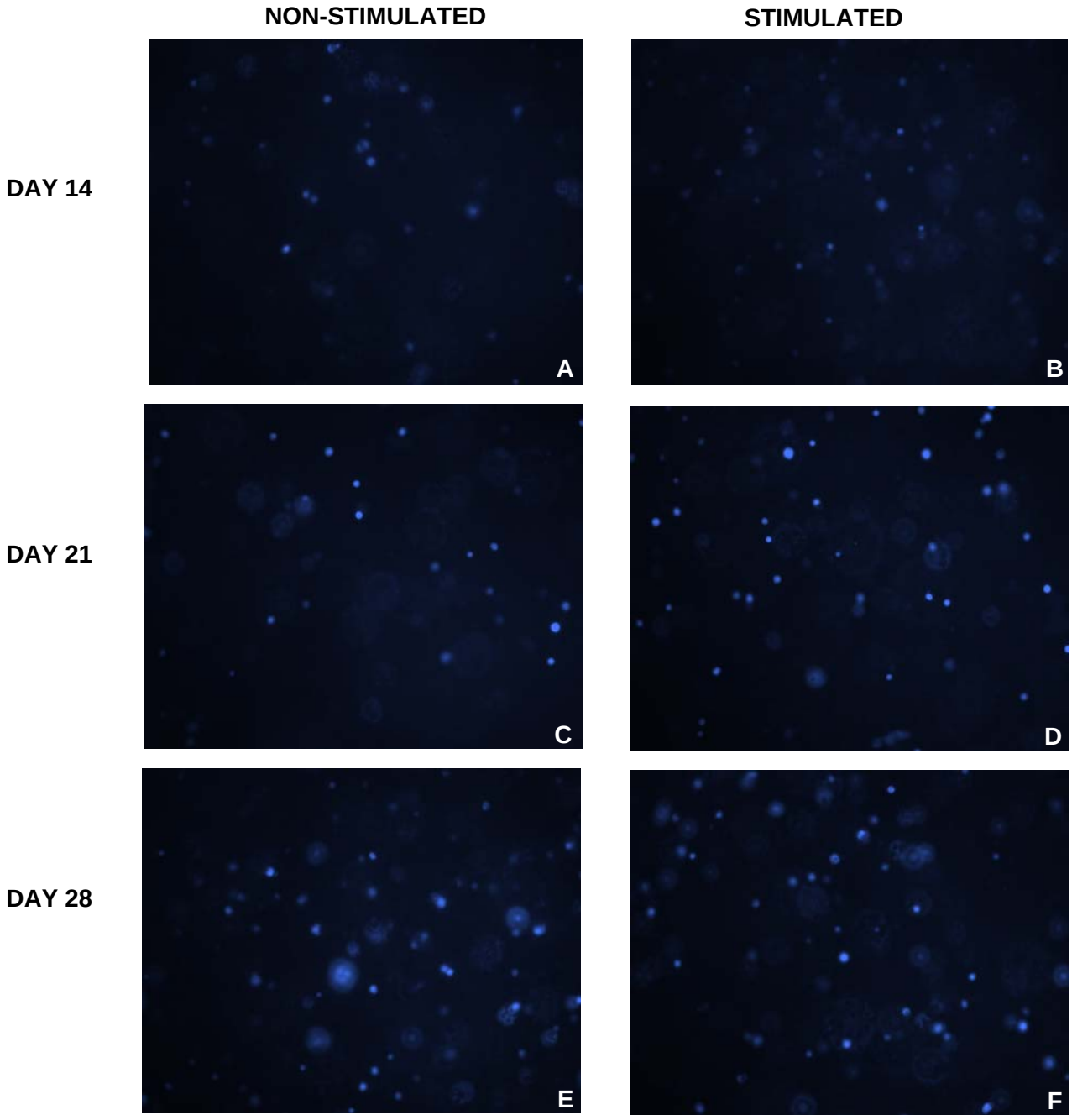

Fig. 6.1. Rounded ECFP-fluorescing cells in non-stimulated (A, C & E) and stimulated (B, D & F) constructs at day 14 (A & B), day 21 (C & D) and day 28 (E & F).

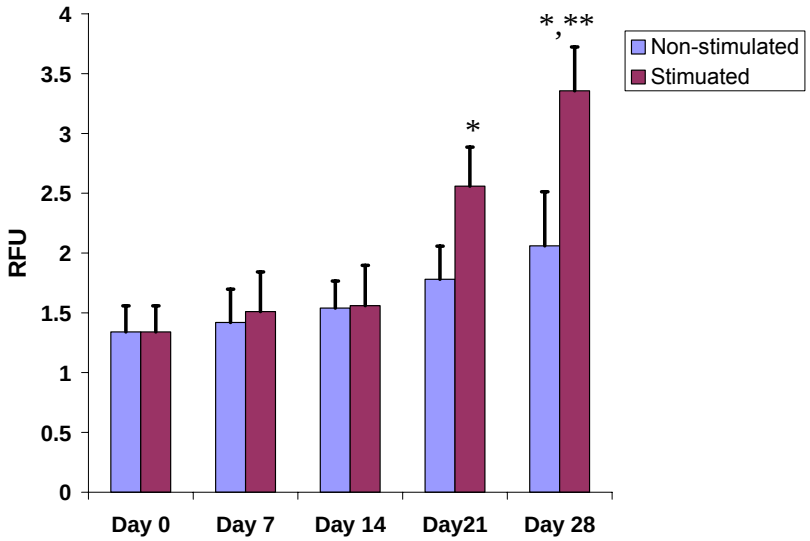

Fig. 6.2. Dynamic stimulation increases ECFP expression by Relative Fluorescence Units (RFU) at day 21 and day 28. * Significant increase compared to non-stimulated control at same time point ($p < 0.05$). ** Significant difference when compared to same treatment at previous time point. Data represented as mean + one standard deviation. N=6 for all groups.

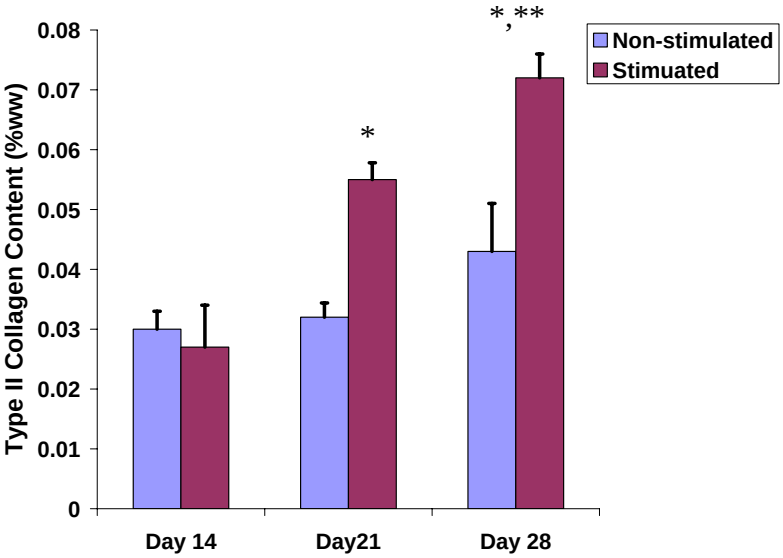

Fig. 6.3. Type II collagen content of constructs increased significantly after 21 and 28 days of dynamic compressive stimulation. * Significant increases compared to non-stimulated controls at same time point ($p < 0.05$). ** Significant increase when compared to same treatment at previous time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups. ww denotes wet weight.

Aggregate Modulus

Compressive stimulation significantly increased the aggregate modulus of the chondrocyte-agarose constructs, but only after 21 and 28 days of stimulation. S constructs showed 1.37 fold and 1.55 fold increases in aggregate modulus compared to NS controls at day 21 and day 28, respectively ($p<0.05$; Fig. 6.4).

Cell Viability

No significant changes occurred in cell viability due to mechanical stimulation or for increasing time in culture ($p>0.05$; Fig. 6.5). Day 28 constructs showed 7% lower cell viability than day 0 constructs.

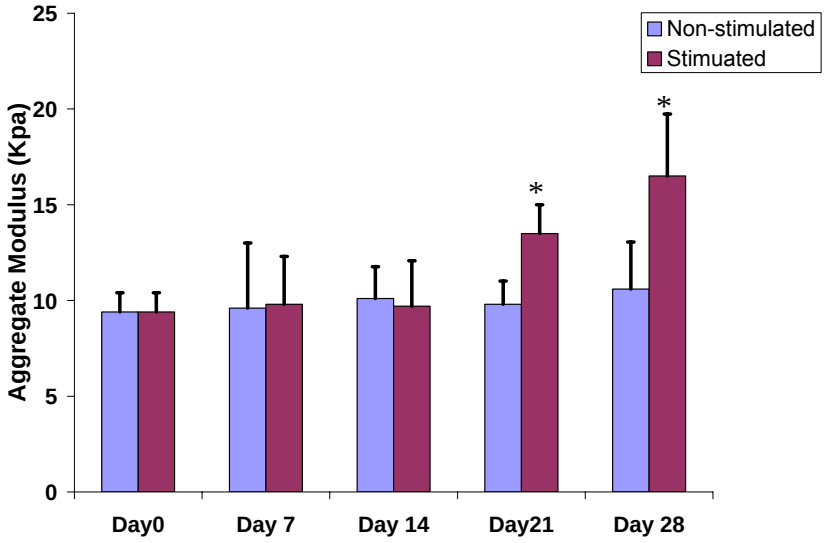

Fig. 6.4. Aggregate modulus of constructs increased significantly only at 21 and 28 days of dynamic stimulation. * Significant increase compared to non-stimulated control at same time point ($p< 0.05$). Data represented as mean + one standard deviation. N=6 for all groups.

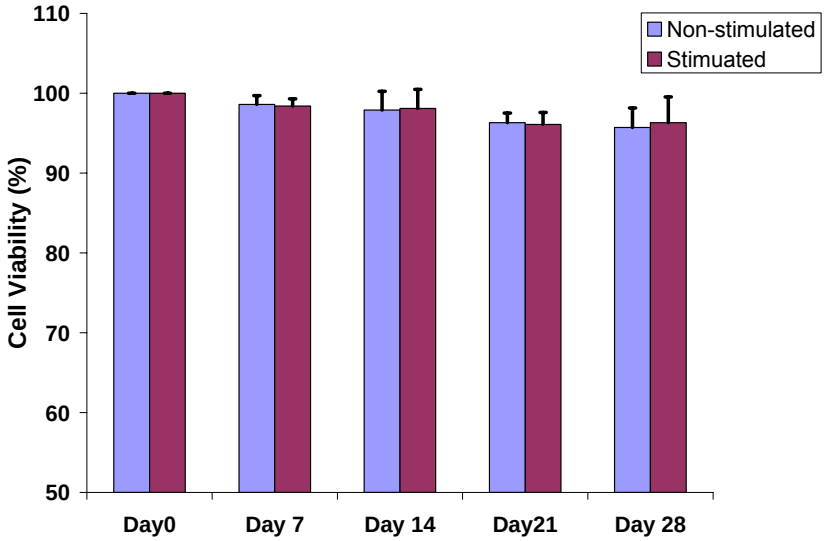

Fig. 6.5. No significant differences were measured in cell viability between stimulated and non-stimulated constructs after 0, 7, 14, 21 and 28 days ($p>0.05$). Cell viability exceeded 95% at all time periods in culture. Data represented as mean + one standard deviation. N=6 for all groups.

6.4. Discussion

Understanding the response of MSCs to physical forces is of critical importance when developing cell-based therapies for cartilage repair. Developing methods that permit rapid visualization of desirable gene expression profiles saves time in identifying the most promising treatment strategies. We chose to develop novel murine transgenic technologies and bioreactors in order to study the effects of compression on gene and protein expression as well as biomechanics for these MSC-agarose scaffolds. Based on our prior work stimulating transgenic chondrocytes in culture, we naturally hypothesized was that dynamic compression applied daily to MSCs in agarose culture would enhances Col2 gene expression, type II collagen content and aggregate modulus of constructs, thus resulting in the formation of stiffer and more functional cartilaginous tissue matrices.

Our quantitative fluorescence results support our first hypothesis that compressive stimulation significantly increases ECFP fluorescence and hence Col2 mRNA expression. The compressive stimulus did increase both measures but unlike our prior study using chondrocytes,

only after longer time points of stimulation. In fact, the cells in our current study only expressed ECFP fluorescence after 14 days of stimulation. These results agree with those found by others examining Col2 gene expression by MSCs [390, 399]. In these studies, MSCs in agarose culture also enhanced Col2 gene expression, but only after 2 weeks in culture. Our results involving dynamic compression are also consistent with prior reports which showed increased Col2 gene expression by stimulating MSCs, but only at time points in culture [203, 389]. It is worth noting, however, that the changes we observed in Col2 gene expression by MSCs in the current study are nearly as rapid as the changes in expression by chondrocytes which show increased Col2 gene expression as early as 2 days in culture [162, 336-339]. The fact that MSCs are slower to respond to biophysical stimulation than fully differentiated articular chondrocytes is not altogether surprising. It has suggested, for example, that the developing pericellular and extracellular matrix may be regulating mechano-stimulation of MSCs undergoing chondrogenesis [400]. Furthermore, cartilage matrix synthesis by articular chondrocytes in dynamically- compressed agarose gels is known to be greater at later time points than earlier time points [162, 401], implying that a well-developed matrix may be required for the transduction of mechanical compression to elicit a cellular biosynthetic response. This is further supported by the fact that the amount of type II collagen in our constructs is greater at later culture periods.

Consistent with our findings for gene expression, our other results support our second and third hypotheses that compressive stimulation significantly increases type II collagen content and aggregate modulus. While both hypotheses were supported, we again found that compressive stimulation only affected protein expression and biomechanics at later time intervals. Similar to the findings for Col2 gene expression, 21 and 28 days of compressive strain were required to increase Type II collagen content (Fig. 6.2). This temporal increase in type II collagen content at later points is similar to the results reported for MSCs [389, 400] and chondrocytes [162]. In a similar fashion, 21 and 28 days of stimulation were required to

positively influence the aggregate modulus of the TECs. Such improvements in aggregate modulus relative to time-matched controls may seem delayed in time, but are still faster than the 42 days required to alter the aggregate modulus of porcine chondrocyte-agarose TECs when subjected to 10% cyclic compressive deformation [400]. When these observations are coupled with our results, it is clear that time in culture and dynamic stimulation can each significantly increase the matrix synthesis and biomechanics of MSC-agarose constructs. However, knowing whether these positive effects can be sustained will require longer-term culture experiments (which are now underway in our lab) and possibly new strategies should gene expression increases decline as we previously found after stimulating chondrocytes [162].

The fact that temporal and stimulation-induced relationships among gene expression, protein expression and biomechanics differ between chondrocytes and MSCs is worth further study. For example, in the chondrocyte-agarose model, the increases we observed in Col2 gene expression at 7, 14, 21 and 28 days only culminated in increased Type II collagen content after 21 and 28 days and increased aggregate modulus only after 28 days [162]. For the MSC-agarose model system, however, the increases we observed in Col2 gene expression were synchronized with the increases in Type II collagen content and aggregate modulus. This suggests differential maturation and structure–function relationships between MSC- and chondrocyte-seeded agarose TECs. Others have also found similar differences between MSCs and chondrocytes [402]. Our system permits these questions to be answered and will be the subject of our ongoing research.

Our study is not without limitations. 1) Autofluorescence of agarose gel fragments may have led to increases in measured RFU values. In this study, we assumed that these changes were constant across groups. 2) Quantification of RFU is done by pooling of all cells and hence we know only the average effect of dynamic compression and not the specific effects of compression on individual cells. Ultimately, more local recordings may be required since a prior study found that gene expression varies in the core vs. the peripheral region of the cell-seeded

hydrogel [389]. 3) Our type II collagen content quantification does not indicate the degree of collagen assembly or alignment. 4) We did not track changes in the production of proteoglycans like aggrecan. Increases in aggrecan content could likely reflect improvements in a construct's aggregate modulus although type II collagen is essential for confining the expansion of GAGs on the aggrecan molecule. In the future we intend to track the production of proteoglycans like aggrecan to more fully understand TEC composition and architecture. 5) These in-vitro studies in culture may not reflect the functionality of TECs once they are implanted at surgery. The murine model represents a continual challenge in this regard due to its small size. Future transgenic studies will need to be performed in larger model systems where surgery can be controlled.

Our results have shown that compressive stimulation and time increase type II collagen gene expression, protein content and aggregate modulus in murine MSCs seeded in agarose gel. We expect that such increases in gene expression will result in rapid synthesis and accumulation of collagen. Future studies will seek to optimize which aspects of the mechanical signal produce the greatest increases in Col2 gene and protein expression as well as aggregate modulus. Such improvements can then be translated to larger animal and tissue repair models where surgery can be even better controlled.

Chapter 7

Influence Of Compressive Loading On Collagen I Gene Expression And Aggregate Modulus In Tissue Engineered Constructs Containing Double Transgenic Fibroblasts Seeded In Agarose

The objectives of this study were to determine how up to 28 days of in-vitro dynamic compression, applied to murine fibroblast-agarose constructs, influences Col1 and Col2 gene expression levels as well as aggregate modulus of the tissue engineered constructs (TEC). Fibroblasts were harvested from the tail tendons of six ten-week old double transgenic mice. These mice carry transgenes that use enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for expression from the Col2a1 and Col1a1 promoters, respectively. Twenty-seven constructs (8mm dia x 3mm thick) per animal were created by seeding MSCs ($10x10^6$ per ml) in agarose gel (2% w/v). Twelve constructs from each animal were stimulated (S constructs) for 7, 14, 21 or 28 days (n=3 each) in a custom bioreactor housed in an electromagnetic system. Another twelve constructs were not stimulated (NS controls) but were exposed to identical culture conditions for the same time periods. The remaining three constructs served as day 0 controls. GFP-T fluorescing cells were present in all constructs. No ECFP expressing cells were seen in constructs. Compared to time-matched, NS controls, 28 days of stimulation significantly decreased GFP-T fluorescence. Compared to time-matched controls, stimulation significantly increased the linear stiffness of constructs at 28 days. This study reveals that compressive stimulation downregulates Col1 gene expression, does not induce Col2 gene expression, but does increase aggregate modulus in compression. Future studies will examine the effects of components of the mechanical signal in culture and address the question of whether long term stimulation would further decrease Col1 gene expression and possibly even lead to detectable Col2 gene expression in culture.

7.1. Introduction

Tendons that wrap around bone have adapted by modulating their structural and biochemical characteristics. Tissues like the rabbit flexor digitorum profundus (FDP) tendon create a fibrocartilaginous (FC) region after birth [13-15]. These contact regions show a basket-weave collagen arrangement [403] and their proteoglycan and GAG contents are two to ten times higher than values in the adjacent normal tendon [404] .Cells in this fibrocartilage rich region also display a more rounded morphology than tenocytes in nearby tendon tissue [405-406].

Many studies suggest that this fibrocartilage (FC) region develops after birth in response to local compressive forces from the opposing bone surface. At birth, the tendon near the bone shows no evidence of fibrocartilage[407].Instead the FC zone forms during early postnatal development, presumably in response to changing physical activity levels [206, 266]. When investigators exposed tendon explants to continuous in-vitro cyclic compression, sulfate incorporation into biglycan and proteoglycan increased up to 1.5-fold and 3- fold, respectively [408]. Similar in-vitro studies demonstrated how compressive loading increased both aggrecan and proteoglycan expression [409-411]. Moreover, surgically translating the tendon to eliminate compressive stresses on the FC-rich contact zones rapidly disrupts the basket-like weave and depletes GAGs [13] and depletion of GAGS [412]. These studies mentioned above demonstrate that the compressive stimuli play a central role in the fibrocartilage formation.

While in-vivo [13, 412] and in-vitro[408-411] studies have been performed on tendons in compression, only a few studies have examined the effects of dynamic compression on fibroblast-seeded scaffolds[206, 413]. Shim et.al applied cyclic hydrostatic pressure to Achilles fibroblasts and observed decreases in Col1 gene expression and upregulation of Col2 and aggrecan gene expression [206]. Elder et.al also applied cyclic hydrostatic pressure to murine embryonic fibroblasts and observed increases in sGAGs [413]. Dynamic compression applied to chondrocyte-seeded scaffolds increases Col2 gene expression [162, 336-339] and applied to

MSC-seeded scaffolds enhances the chondrocytic nature of MSCs [387-391]. However, studies that apply dynamic compression to fibroblasts are needed. Thus the goal of this study was to evaluate the effects of dynamic compression on fibroblast-seeded scaffolds.

Fluorescent transgenic mouse technologies and bioreactors have been created in our lab that permits us to study the effects of compression on constructs containing fibroblasts. In these mice the Col1 gene is linked to Green Fluorescent Protein-Topaz (GFP-T) and the Col2 gene is linked to Enhanced Cyan Fluorescent Protein (ECFP), respectively [162-163]. Thus the cell fluoresce either green or cyan if Col1 or Col2 gene is expressed respectively. Studies from our lab show that MSCs from these mice upregulate Col1 in tension [163] and chondrocytes from these mice upregulate Col2 in compression [162].When gene expression by fluorescence is quantified using a spectrophotometer, the relative fluorescence units (RFUs) and PCR are positively correlated [162-163]. We have also created and validated a bioreactor capable of providing micron precise waveforms to stimulate constructs [2].

Hence the objective of this study was to utilize these technologies to examine the effect of dynamic compression on 3-D murine fibroblast-constructs. By seeding these fibroblasts in scaffolds and placing the constructs in our bioreactor, we hypothesized that compared to non-stimulated constructs at equivalent time points, compressive stimulation would: 1) decrease GFP-T fluorescence, a marker for Col1 gene expression; 2) increase ECFP fluorescence, an indicator of Col2 gene expression; and 3) increase the construct's aggregate modulus in compression.

7.2. Materials and Methods

Experimental Design

Tail tendon fibroblasts were obtained from six (6) ten-week old double transgenic mice. 2% w/v agarose with fibroblasts at a density of 10 x 10^6 cells/ml were created.. A total of twenty-seven (27) cell-agarose constructs (8 mm dia. x 3 mm thick) were created per animal. [162]. Of these twelve (12) of the constructs were stimulated (S) in compression and another twelve (12)

served as non-stimulated (NS) controls at days 7, 14, 21 and 28 (n=3 each). The remaining three (3) constructs served as day 0 NS controls. S constructs all received the same compressive stimulation profile (sinusoidal displacement pattern @ 1Hz to 10% peak strain for one hour followed by 1 hour of rest, repeated three times a day) [162, 366]. The three (3) S and three (3) NS constructs at each time point were assigned as follows: one for ECFP fluorescence in a spectrophotometer, [162-163, 396]one for confined compression to determine aggregate modulus [162, 330], and one for cell viability using an MTT assay [162, 397].

Fibroblast isolation and cultivation

Mice were euthanized by CO_2 according to approved Institutional Animal Care and Use Committee (IACUC) protocols. Tails from these mice were harvested aseptically and the skin removed to expose the parallel tail tendons. Each tendon was dissected free from bone and placed in sterile 100mm culture dishes. Tendons were minced into small pieces and a few ml of FBS was added. After 2 days, 10 ml of media (Advanced DMEM, 10% FBS, pen/strep) was added to each plate. Adherent cells were allowed to grow for 10 to 14 days before first passage. Cells were then trypsinized and replated at 1 x 10^6 cells/100mm dishes and cultured for one additional week. Cells were then repeatedly sub-cultured at a density of 1 x 10^6 cells/100 mm dish until passage four (P4).

Construct Creation

A 4% (w/v) agarose solution was created under sterile conditions and maintained at over 400C in a water bath to prevent gelatin. Fibroblasts at P4 were trypsinized and resuspended in media Advanced DMEM, Invitrogen-Gibco BRL/Life Technologies Inc., Gaithersburg, MD) containing 1% antibiotic/antimycotic and 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville,GA) at a concentration of 20x106 cells/ml. Equal volumes of 4% agarose was the mixed with equal volumes of fibroblast suspension carefully in order to prevent bubbles. This created a final cell-gel solution of 10 x 106 cells/ml in 2% (w/v) agarose. Sterile glass plates with a 3mm spacer were used to cast this cell-gel solution. After 20 minutes of gellation, 8-mm

diameter specimens were cored and transferred to multi-well plates. 5ml of media was added to each well. This fibroblast-fibroblast-agarose tissue engineered constructs (TECs) were then cultured for an additional two days under free swelling conditions in a standard incubator (Steri-Cult Model 3033, Forma Scientific) with media replaced daily. The resulting TECs were henceforth classified as Day 0 constructs.

Compressive Stimulation

Before stimulation, the S constructs were first transferred from the multi-well plates to 35mm diameter glass bottom dishes (Matek Corp) and fed with 2ml of media. The dishes were covered with Teflon FEP film (100Å; DuPont, Circleville, OH). The film is held in place by a custom delrin ring [2, 162]. This film maintained the sterility of constructs and also prevented media evaporation. These dishes were then loaded into our custom bioreactor and subjected to compressive stimulation whose profile is described in the experimental design section [2, 162]. The NS constructs were also cultivated in the same bioreactor but without the compressive stimulation. Feeding media to these constructs was replaced daily.

Fluorescence Analysis

GFP-T and ECFP fluorescence were analyzed qualitatively and quantitatively in the TECs. Each S and NS constructs was washed in PBS to remove the media and visualized in filter sets for GFP-T(XF104-2, Omega) and ECFP(XF1142, Omega) in a fluorescence microscope (Zeiss Axiovert) with a filter set for GFP-T fluorescence and a filter set for ECFP fluorescence [2, 163]. Each TEC was further visualized using a filter set for red fluorescence (11002VZ, Chroma) to rule out autofluorescence. Constructs were homogenized as previously described to extract cells [163]. These homogenates were then pipetted into a black bottom microplate (100µl per well in three wells). RFUs in these were quantified in a spectrophotometer as previously described [2, 163].

Determination of Cell Viability

MTT assay (Vybrant MTT Cell Profileration Assay Kit, Invitrogen, Carlsbad, CA), was performed to determine cell viability. The fibroblast-agarose TECs were homogenized using a mortar and pestle. [162]. The homogenate was incubated for 4 hours in an incubator in 500µl of 12mM MTT solution and then incubated for 18 hours in 5ml SDS-HCL to completely dissolve the formazan. The absorbance of formazan was measured at 570 nm in a microplate spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, CA) [162, 397]. The viability results were normalized to results for day 0 constructs.

Biomechanical evaluation of fibroblast-agarose TECs

Aggregate modulus of the TECs was determined using confined compression testing. At each time point the constructs were placed in cryovials and stored at - 80ºC. On the day of testing tissue specimen was thawed in room temperature phosphate buffered saline (PBS) for 10 minutes. The details of the testing are described elsewhere [162]. Briefly a 6-mm diameter core was created from the center of each construct and subjected to a constant contact stress until equilibrium displacement was achieved. During testing, specimen height and applied load were continuously measured. Tissue aggregate modulus (HA) was calculated using the formula $HA= (Fo/\pi a2) / (u/h)$ where Fo is applied compressive force, a is specimen radius, h is specimen thickness and u is equilibrium displacement[162].

Statistical Analysis

Two way ANOVA with Tukey's HSD post hoc test were performed to determine differences among values for all response measures with significance set at $p < 0.05$. SPSS software was used to perform these tests.

7.3. Results

Qualitatively, we observed homogeneously distributed and rounded cells in all constructs. GFP-T fluorescing cells were present in all constructs (Fig. 7.1) but no GFP-T

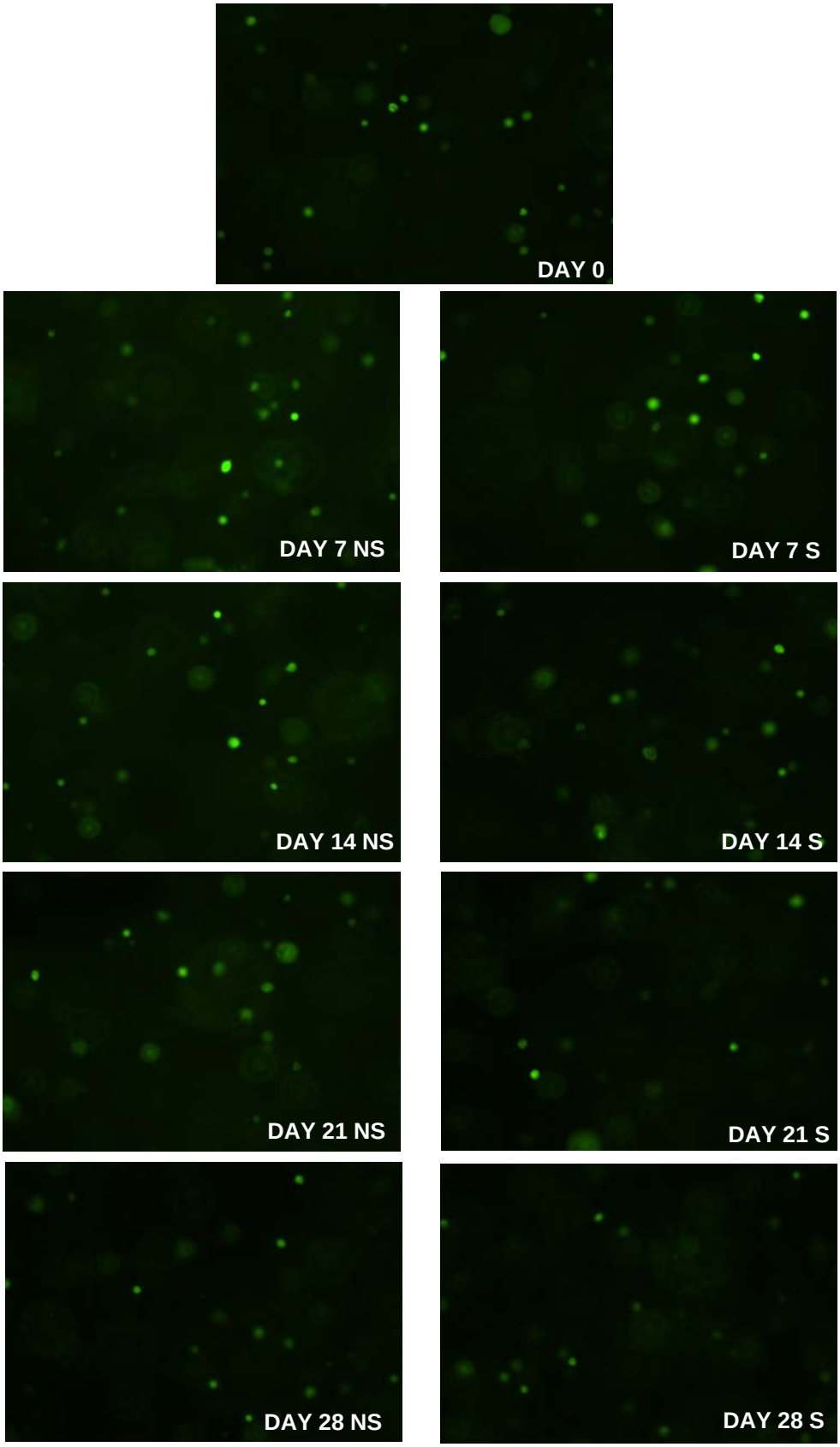

Fig. 7.1. Rounded GFP-T expressing fibroblasts in agarose.

fluorescence was detected using any of the other filter sets. However, no apparent differences could be seen in ECFP fluorescence between the S and S constructs at each of the time points. None of the TECs showed cells displaying ECFP expression.

Quantitative results showed that dynamic compressive strain significantly decreased GFP-T expression in the TECs at 28 days. Compared to time-matched NS controls, the treatment significantly decreased RFU values by 0.56 fold at 28 days ($p<0.05$; Fig. 7.2).

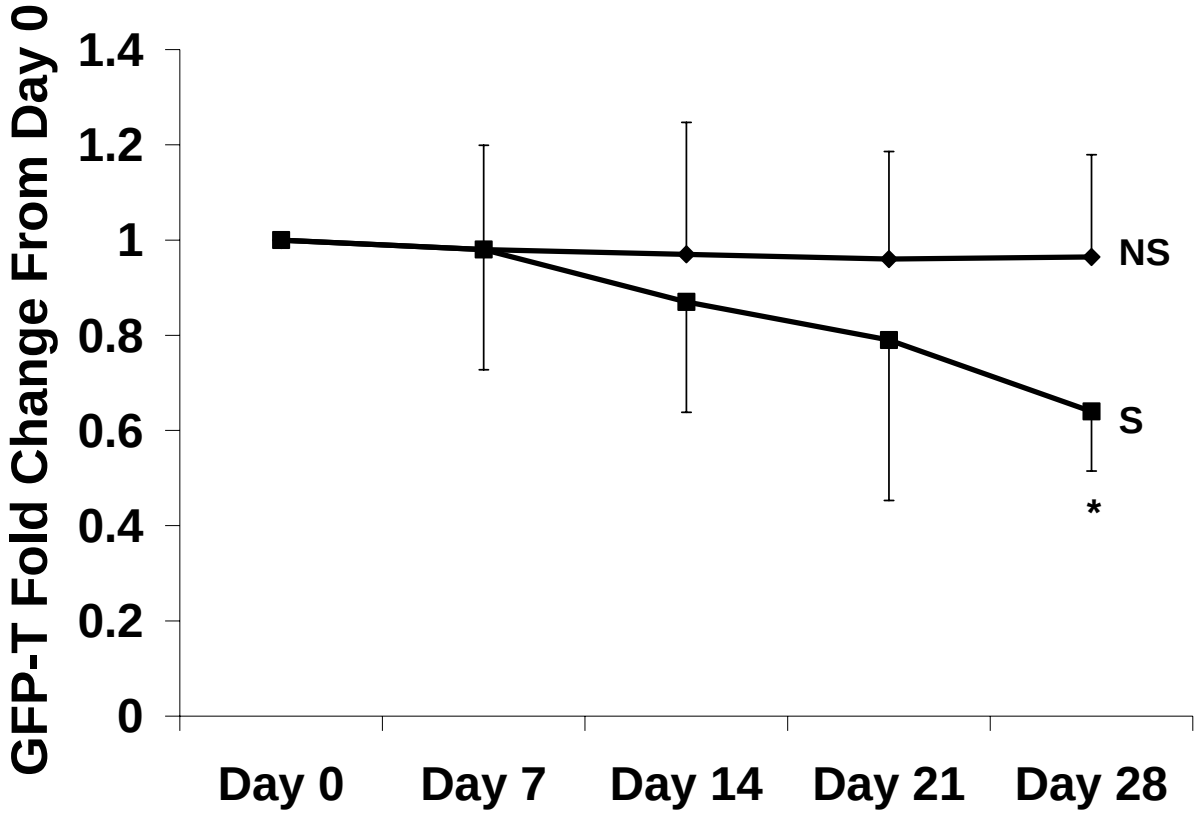

Fig. 7.2. Compressive stimulation decreases GFP-T expression by Relative Fluorescence Units (RFU) day 28. * Significant increase compared to non-stimulated control at same time point ($p< 0.05$). Data represented as mean + one standard deviation. N=6 for all groups. NS- Non-Stimulated, S-Stimulated.

Compressive stimulation increased aggregate modulus of the fibroblast-agarose constructs, but only after 28 days of stimulation. S constructs showed a 1.25 fold increase in aggregate modulus compared to NS controls at day 28 ($p<0.05$; Fig. 7.3).

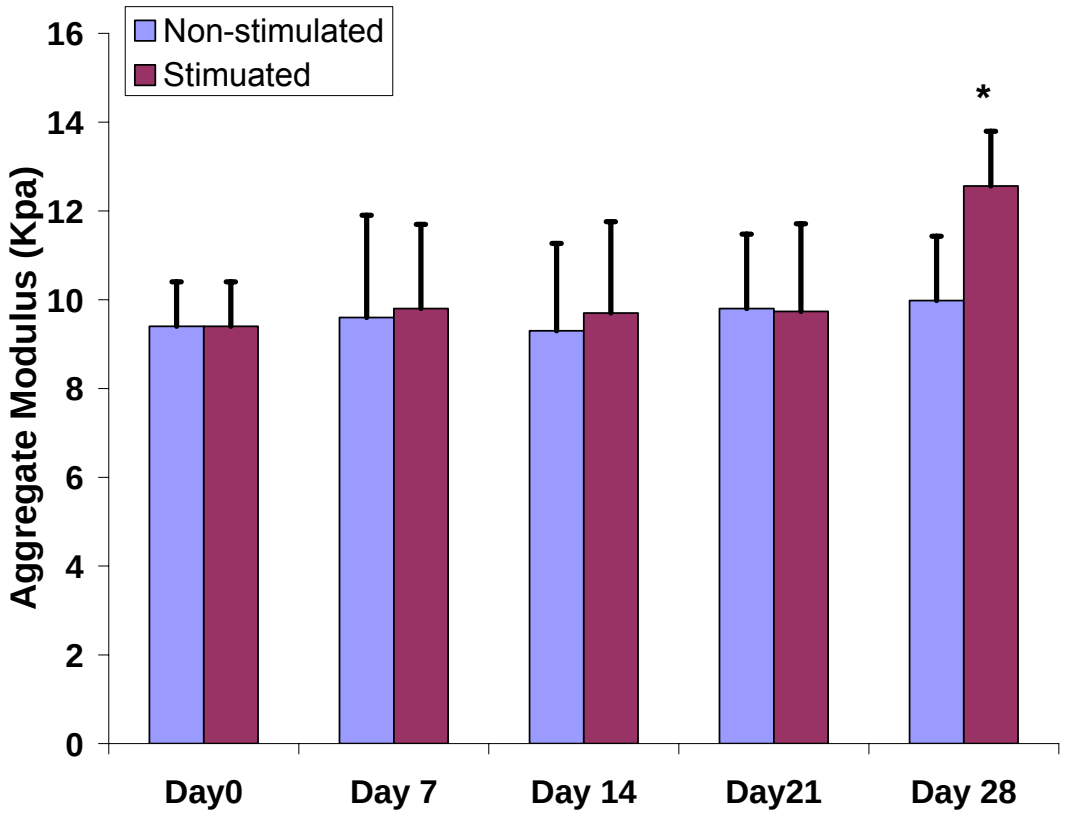

Fig. 7.3. Compressive stimulation increases linear stiffness of constructs 28. * Significant increase compared to non-stimulated control at same time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups.

No significant changes occurred in cell viability due to mechanical stimulation or for increasing time in culture ($p>0.05$; Fig. 7.4). Day 28 constructs showed 8% lower cell viability than day 0 constructs.

7.4. Discussion

The objective of this study was to examine the effects of compression on fibroblast gene expression and resulting biomechanics. In this study, murine tail tendon fibroblasts were cultured, expanded and seeded in 3-D agarose to determine the effect of dynamic compressive strains on Col1/Col2 gene expression and aggregate modulus. Agarose was used as the biomaterial because of its ability to be compressed. The specific loading profile for stimulation was selected because it was previously shown to improve Col2 gene expression, protein, sGAG and mechanical properties of constructs [162, 336-339].

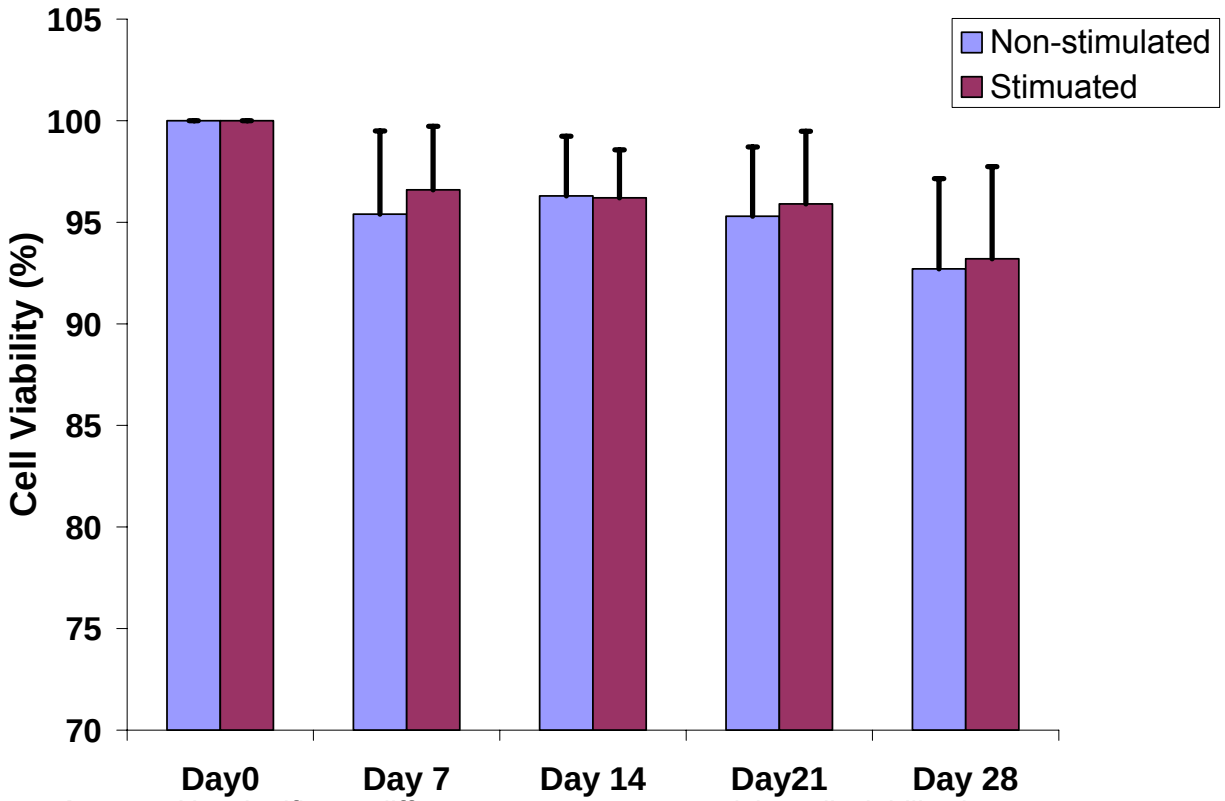

Fig. 7.4. No significant differences were measured in cell viability between stimulated and non-stimulated constructs after 0, 7, 14, 21 and 28 days ($p>0.05$). Cell viability exceeded 92% at all time periods in culture. Data represented as mean + one standard deviation. N=6 for all groups.

Our quantitative fluorescence results support our first hypothesis that compressive stimulation significantly decreases GFP-T fluorescence and hence Col1 mRNA expression but only at later time points. In the current study, tail tendon fibroblasts exposed to dynamic compression downregulated Col1 gene expression. This decrease in Col1 gene expression is consistent with the idea that cyclic tensile strain is the primary stimulus for Collagen type I synthesis [163, 167, 414-421], and with reports that compression downregulates Col1 gene mRNA expression in chondrocytes [422]. Our results are also similar to another study in which fibroblasts from human periodontal ligament were cyclically contracted on a flexible membrane [423]. The cells in that study synthesized less Collagen type I and fibronectin protein and downregulated Col1A1 mRNA compared to non-stretched controls. Similar results were seen when rat Achilles fibroblasts were seeded in pellets and then subjected to cyclic hydrostatic

pressure. The cells in that study showed significantly less Col1 mRNA compared to non-stretched controls[206].

The absence of ECFP expression in any of the TECs indicates that cells were not upregulating the Col2 gene, thus negating our second hypothesis. While a compressive stimulus has been shown to upregulate Col2 gene expression in other cells types [162, 203, 206, 336-339, 389-390, 399, 413, 424-425], in our current experiments none of the constructs showed ECFP-positive cells. Our results directly contradict other reports in the literature, suggesting differences among applied treatments or the cell-scaffolds receiving these compressive signals. For example, when one study subjected rat Achilles fibroblasts in pellets to cyclic hydrostatic pressure, investigators showed increased the expression of Col2 mRNA [206]. It is noteworthy, however, that the mechanical profile we delivered in the current study is identical to the one that resulted in upregulation of Col2 gene expression when applied to murine chondrocyte-agarose scaffolds[162]. Such differences suggest that either fibroblasts are either terminally differentiated and incapable of responding to compressive stimulation or that the signal must be applied for longer periods to elicit the response. It is also plausible that fibroblasts might require a different compressive signal than chondrocytes to upregulate Col2 gene expression. Another reason could be that our fluorescence method is not sensitive enough to detect small changes in Col2 expression and thus might require the use of PCR.

At the same time, our biomechanical results support our third hypothesis that compressive stimulation significantly increases aggregate modulus of constructs. Aggregate modulus of stimulated constructs was significantly higher than non-stimulated controls at day 28. While compression decreased the expression of Col1 gene expression and did not induce Col2 gene expression it is plausible that they also increased the synthesis of proteoglycans and GAGS in our system. Compression has been shown to increases the synthesis of proteoglycans and GAGS[426-427]. Such accumulation of GAGs could have lead to increases in aggregate modulus we observed at day 28.

Our study is not without limitations. 1) We homogenized agarose constructs to record fluorescence. Agarose chunks in the homogenates could have increased the observed RFU Values. We assumed that these increases were constant across groups. 2) The experimental design did not allow us to track the changes in proteoglycans and collagen in these constructs, which could explain the increases in aggregate modulus. In the future we intend to track the production of proteoglycans and collagen. 3) We did not observe any ECFP expression. It is plausible that the cells were exhibiting Col2 expression, but in such low amounts that it could not be detected by our fluorescence methods. In the future we plan to use qRT-PCR techniques along with our fluorescence methods.

We conclude that although compressive stimulation of fibroblast-agarose constructs decreases Col1 gene expression, this stimulus was not sufficient enough or long enough to induce Col2 gene expression. However, the compressive stimulus was substantial enough to increase the aggregate modulus of constructs. In the future we plan to track protein and proteoglycan synthesis in fibroblast-agarose constructs as well as extend the experiment beyond 28 days to determine whether and when Col2 gene expression might be present. We also plan to examine different components of the compressive stimulus to evaluate their effects on Col1/Col2 gene expression in these cells.

Chapter 8

Tensile Stimulation Of Murine Stem Cell-Collagen Sponge Constructs Increases Collagen Type I Gene Expression And Linear Stiffness.

The objectives of this study were to determine how tensile stimulation delivered up to 14 days in culture influenced type I collagen gene expression in stem cells cultured in collagen sponges and to establish if gene expression, measured using a fluorescence method, correlates with an established method, real time quantitative RT-PCR (qRT-PCR). Using a novel model system, mesenchymal stem cells (MSCs) were harvested from six double transgenic mice in which the type I and type II collagen promoters were linked to green fluorescent protein-topaz (GFP-T) and enhanced cyan fluorescent protein (ECFP), respectively. Tissue engineered constructs were created by seeding 0.5 x 106 MSCs onto type I collagen sponge scaffolds in a silicone dish. Constructs were then transferred to a custom pneumatic mechanical stimulation system housed in a standard incubator and stimulated for 5 hours/day in tension for either 7 or 14 days using a repeated profile (2.4% peak strain for 20 seconds at 1 Hz followed by a rest period at 0% strain for 100 seconds). Control specimens were exposed to identical culture conditions but without mechanical stimulation. At three time points (0, 7 and 14 days) constructs were then prepared for evaluation of gene expression using fluorescence analysis and qRT-PCR, and the remaining constructs were failed in tension. Both analytical methods showed that constructs stimulated for 7 and 14 days showed significantly higher collagen type I gene expression compared to non-stimulated controls at the same time interval. Gene expression measured using qRT-PCR and fluorescence analysis were positively correlated (r = 0.9). Linear stiffness of stimulated constructs was significantly higher at both 7 and 14 days compared to non-stimulated controls at the same time intervals. Linear stiffness of the stimulated constructs at day 14 were significantly different from that of day 7. Future studies will vary the mechanical

signal to optimize type I collagen gene expression to improve construct biomechanics and in-vivo tendon repair.

8.1. Introduction

Injuries to soft tissues (tendon, ligament and meniscus) represent almost half of the 33 million musculoskeletal injuries occurring in the US each year [428-433] and often lead to surgery [434-436]. Inadequate healing of these injuries places patients at risk to dysfunction and disability. Tissue engineering [244] is an appealing conceptual alternative when conventional repair techniques (autografts, allografts, xenografts, and prostheses) [177, 281-289]prove unsatisfactory.

Tissue engineered constructs made by seeding mesenchymal stem cells (MSCs) in scaffolds are being used to repair soft tissue defects [51, 148, 150, 248, 290, 434], but these are usually vulnerable early after surgery because they lack the stiffness and strength of native tissue structures [248, 434]. To address this problem, investigators have been applying principles of Functional Tissue Engineering (FTE) [159-160] to use recorded in-vivo tissue forces [157-158] as design parameters for new generations of reparative tissue constructs. Some in FTE [159-160] have also delivered aspects of these in-vivo mechanical signals to precondition constructs while still in culture. Such preconditioning improves the material properties of constructs for soft tissue engineering by increasing the synthesis of extracellular matrix proteins such as collagen [172, 324, 417, 437].

Unfortunately, any mechanical and biological benefits of mechanical stimulation are usually not assessed until the end of mechanical stimulation in culture [161, 248, 438]. Typically, four or more weeks may be required before a destructive test is performed to judge whether a stimulus up-regulates gene expression or increases protein accumulation. This delay hampers the ability of the investigator to quickly optimize the stimulus in culture. A strategy that permits investigators to monitor near real-time gene expression throughout the tissue engineering process could allow them to either modify the stimulus or terminate an experiment leading to an

undesirable outcome. Unfortunately, no such method currently exists for tissue engineers to rapidly assess how mechanical (chemical, etc.) stimuli affect near real-time gene expression.

To address this need, we bred double transgenic mice having type I and type II collagen promoters linked to Green Fluorescent Protein-Topaz (GFP-T) and Enhanced Cyan Fluorescent Proteins (ECFP), respectively. These intra-cellular proteins are expressed when the type I and type II collagen genes are activated, respectively. Such fluorescent proteins have recently served as visual reporters for transgene activity and can be viewed in real time in living tissues [174-178]Investigators have used these promoter-fluorescent protein reporters: 1) to examine early embryonic development, 2) to conduct cell culture studies, and 3) to identify cells within a defined lineage in primary cell culture [174-178] Another value of this technology, when applied to tissue engineering, could be the ability to track how mechanical stimulation affects near real-time collagen gene expression during maturation of constructs in culture.

The first objective of this study was to determine how a controlled mechanical stimulus applied to a stem cell-collagen sponge construct in culture influences the expression of the type I collagen gene as well as linear stiffness. The second objective was to establish if gene expression, measured using a fluorescence method, correlates with an established method, real time quantitative RT-PCR (qRT-PCR). We hypothesized that: 1) mechanical stimulation would increase type I collagen gene expression as well as linear stiffness and 2) the fluorescence data and qRT- PCR data would be positively correlated.

8.2. Materials and Methods

Experimental Design

Bone marrow was harvested from both the femur and tibia of six (6) six-week to eight-week old double transgenic mice as previously described [395, 439]. After isolating and expanding the MSCs to second or third passage using a previously-published protocol[439], fifteen (15) constructs were created from each animal by seeding the cells at a concentration of 0.5×10^6 cells/construct in a type I collagen sponge (P1076, Kensey Nash Corp., Exton, PA).

Hence a total of 90 samples were created using cells from six (6) animals. For each animal, the resulting fifteen (15) constructs were assigned to five (5) treatment groups- Day 0 Non-Stimulated, Day 7 Stimulated, Day 7 Non-Stimulated, Day 14 Stimulated and Day 14 Non-Stimulated (Table 8.1). Thus, three (3) constructs were available per animal from each treatment group. One construct was assigned to evaluate GFP-T fluorescence in a spectrophotometer (measured in Relative Fluorescence Units (RFU)) [329], one construct was assigned to evaluate changes in type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression using real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) [167], and the remaining construct was failed in tension to determine its linear stiffness [171]. Stimulated (S) constructs were stretched between 0% and 2.4% peak strain [158] at 1 Hz for 20 seconds followed by a 100-second rest period. This pattern was repeated for five (5) hours/day. Peak amplitude, frequency and duty cycle were chosen based on studies performed in our lab on the effects of tensile stimulation on rabbit MSCs seeded in collagen scaffolds [167, 171]. The non-stimulated constructs (NS) served as controls. The sample size of six (6) animals was sufficient to detect a 30% treatment effect with an 80% power. Differences were considered significant for $p < 0.05$.

Double transgenic mice

The mouse containing the transgene pOBCol3.6GFPtpz was acquired courtesy of David Rowe, University of Connecticut Health Center. This transgene contains a 3.6 Kb fragment of the rat Col1a1 promoter, enhancer sequence and GFP-T. GFP-T expression in these transgenic mice is evident in skin, tendon and osseous tissues[176].

Plasmid pCol2-ECFP was derived by replacing the β-gal gene with ECFP (Clontech, Palo Alto, CA), in the expression gene containing the mouse type II collagen promoter and enhancer [179] (provided by W. Horton, Northeastern Ohio College of Medicine). This pCol2-ECFP construct was injected into mice blastocysts. Founder mice showed high levels of ECFP expression in cartilaginous tissue.

Treatment Conditions	Constructs/ Treatment Condition/Animal	Response Measures (Assignment Of Constructs)
Day 0 Non-Stimulated	3	Gene expression by qRT-PCR (n=1) Gene expression by RFU (n=1) Linear Stiffness (n=1)
Day 7 Stimulated	3	Gene expression by qRT-PCR (n=1) Gene expression by RFU (n=1) Linear Stiffness (n=1)
Day 7 Non-Stimulated	3	Gene expression by qRT-PCR (n=1) Gene expression by RFU (n=1) Linear Stiffness (n=1)
Day 14 Stimulated	3	Gene expression by qRT-PCR (n=1) Gene expression by RFU (n=1) Linear Stiffness (n=1)
Day 14 Non-Stimulated	3	Gene expression by qRT-PCR (n=1) Gene expression by RFU (n=1) Linear Stiffness (n=1)

Table 8.1. Treatment conditions, response measures, and assignment of constructs.

Mice transgenic for either pOBCol3.6GFPtpz or pCol2-ECFP were then bred to produce double transgenic animals. No apparent phenotypic differences were observed between the double and non-transgenic mice. Both GFP-T and ECFP have a 24- hour half life which indicates that fluorescence decays to half of its original by 24 hours.

Mesenchymal stem cells

Mice were euthanized by CO_2 according to Institutional Animal Care and Use Committee (IACUC) protocols. Their long bones were excised, transected, and placed in adapted centrifuge tubes [395, 439]. Marrow was extracted by centrifugation for one minute at 400g. Extracted cells were plated at 7.5 x 10^6 cells/100 mm dishes and fed media with supplements (MesenCult™ Basal Medium for Mouse Mesenchymal Stem Cells and Mouse Mesenchymal Stem Cell Stimulatory Supplements, Stem Cell Technologies, Vancouver, BC). Cells were allowed to attach for two days, washed with phosphate buffered saline (PBS, Gibco BRL/Life Technologies Inc., Gaithersburg, MD) to remove non-adherent cells, and fed fresh media. Adherent cells were allowed to grow for seven to ten days before first passage. Cells were then trypsinized and replated at 1 x 10^6 cells/100mm dishes and cultured for one additional week.

Cells at P2 and P3 were then sub-cultured at a density of 1 x 10^6 cells/100 mm dish and cultured for another week.

Scaffold and construct preparation

Sterilized collagen type I sponges (94% pore volume; 62 μm mean pore diameter) were provided by Kensey Nash Corporation (Exton, PA). Scaffolds were cut from these sponges such that they fit in the wells of a silicone dish [171]. Before seeding with cells, each scaffold was soaked overnight in PBS and then placed in each well of the dish. MSCs were suspended in media at a concentration of $2x10^6$ cells/ml. 250μl of this cell suspension was pipetted on top of each scaffold. All constructs were placed in an incubator (Steri-Cult Model 3033, Forma Scientific, Marietta, OH; 37ºC, 5% CO_2, 95% relative humidity) for 2 weeks and fed three times weekly with Advanced DMEM, 1% antibiotic/antimycotic, 1% glutamax and 10% FBS.

Mechanical stimulation

After 2 days of incubation, the silicone dishes containing the constructs to be stimulated were placed into a computer controlled five station pneumatic mechanical stimulation system housed within an incubator (Steri-Cult Model 3033, Forma Scientific, Marietta, OH) [171] and stretched using the pattern described in Experimental Design. Dishes were removed from the incubator after either 7 or 14 days and constructs were then prepared for either evaluation of gene expression using fluorescence analysis and qRT-PCR or failure testing in tension.

Fluorescence Microscopy

Each construct was washed in PBS for 1 hour with gentle shaking to remove media and then visualized for GFP-T fluorescence in a fluorescence microscope (Axiovert 25, Carl Zeiss Inc., Göttingen, Germany) equipped with filter sets for visualizing GFP-T and ECFP (XF104-2 and XF114-2, respectively; Omega). To rule out cell auto-fluorescence, each construct was also visualized for rhodamine using a specific filter set (11002VZ, Chroma).

Spectrophotometric Analysis

After imaging, constructs were digested for 40 minutes in 4 ml of 100U/ml type I collagenase (Sigma Chemical, St. Louis, MO) in an incubator with gentle shaking. The resulting collagen fragments were further digested for 20 minutes in 2 ml of trypsin (Invitrogen-Gibco BRL/Life Technologies Inc., Gaithersburg, MD). Digests were then centrifuged at 2000 rpm for 6 minutes and the supernatant was discarded. The remaining pellets were re-suspended in 1 ml of PBS and filtered using a 100μm nylon mesh (BD Falcon, Bedford, MA). These digests were then pipetted into a black bottom microplate (200μl per well in three wells). GFP-T fluorescence in these digests was quantified in Relative Fluorescence Units (RFUs) [329] by reading the microplate in a spectrophotometer (Spectra Max M2, Molecular Devices) using an excitation wavelength of 491 nm and an emission wavelength of 529 nm, with a cut off filter of 519 nm. Pilot studies on GFP-T expressing fibroblasts harvested from the ribs of new born mice did not show any qualitative differences in GFP-T fluorescence when subjected to the above mentioned collagenase, trypsin digestion and filtration steps.

RNA extraction and conventional gene expression analysis

RNA extraction and conventional gene expression analysis were performed according to previously published protocols [167]. Briefly, the constructs were stored in RNAlater (QIAGEN Inc., Valencia, CA) for 2 days at -4^{o}C. The RNAlater was aspirated carefully and the constructs were frozen in liquid nitrogen to prevent RNA degradation. RNA from each construct was extracted using a RNeasy mini kit (QIAGEN Inc., Valencia, CA). Conventional reverse transcriptase (RT) reaction (MuLV reverse transcriptase, Applied Biosystems, Foster City, CA) was performed [167] to create first-strand complementary deoxyribonucleic acid (cDNA). Mouse specific primers were used for type I collagen and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression. The forward and reverse primer sequences and the resultant products are summarized in Table 8.2. Before use in the experiment, all primers were tested under conventional and real-time quantitative RT-PCR conditions to ensure specificity with only

one band by electrophoresis. The conventional PCR of the reverse transcribed RNA was performed according to previously published protocols [167]. The amplified products were verified by 2% agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) and SYBR safe DNA gel stain (Invitrogen Molecular Probes, Eugene, OR).

Gene	Primer sequence	Product size (bp)	Annealing temperature
Collagen I	TGT GTG CGA TGA CGT GCA AT GGG TCC CTC GAC TCC TAC A	132	58ºC
GAPDH	AAT GGT GAA GGT CGG TGT G CCT TCG GGT AGT GGT AGA AG	200	55ºC

Table 8.2. Sequence of primers used for gene expression analysis and product size in base pairs

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR (qRT-PCR) was performed to quantify mRNA levels of the genes according to previously published protocols [167]. qRT-PCR was perfomed by monitoring SYBR Green fluorescent dye (SYBR Green PCR master mix, Applied Biosystems, Foster City, CA) bound to double-strand DNA with a continuous fluorescence detector (DNA Engine Opticon 2 System, MJ Research Incorporated, Waltham, MA). All samples were run in duplicate since differences in cycle number values between samples were less than 0.3 cycles. Standard curves were created for each target gene to quantify gene expression for each cell-sponge construct. The absolute amount of the corresponding gene mRNA in each construct was obtained from the corresponding gene standard curve. Gene expression was normalized by calculating the ratio between type I collagen and GAPDH genes for each sample.

Biomechanical evaluation of the constructs

After 0, 7 or 14 days in culture, constructs for biomechanical analysis were placed in cryovials and frozen at -80^{0}C. On the day of testing, constructs were removed from the freezer

and thawed to room temperature. Small squares of gauze were used to cover the post holes in each end of the constructs. These gauzes provided a surface that would minimize slippage and the premature failure of the specimens in the grips. Constructs were inserted into custom grips in a materials testing system (100R6, TESTRESOURCES, Shakopee, Minnesota) and fixed at a gauge length of 12 mm to minimize Saint Venant's gripping effects on specimen properties[440]. The specimens were then failed under displacement control at a rate of 10%/sec in a PBS bath at room temperature [51]. Linear stiffness was calculated from the linear region of the force-elongation curve generated by the constructs during failure testing.

Statistical analysis

The duplicate gene expression measures and triplicate RFU measures for each cell line from each of the six mice were averaged prior to statistical analysis. Gene expression and mechanical properties of the stimulated vs. non-stimulated stem cell-collagen sponge constructs were compared using a mixed-effects model (SAS proc mixed) with culture time and stimulation as fixed factors and animal as a random factor [334]. All conclusions regarding the significance of mechanical stimulation on gene expression and mechanical properties were made for $p<0.05$.

8.3. Results

Cells did not fluoresce in any of the constructs at day 0. However, GFP-T fluorescent cells were found in all 7 and 14 day constructs. No fluorescence signal was evident when using the ECFP and rhodamine filter sets, indicating the specificity of the GFP-T signal and the lack of detectable levels of type II collagen gene expression, even in the end regions where the construct attaches to the posts. The fluorescing cells appeared elongated in all constructs at both time periods of stimulation (Figs. 8.1A -8.1D). Elongated cells in both the controls (Figs. 8.1A & 8.1C) and the stimulated constructs (Figs. 8.1B & 8.1D) were randomly oriented with respect to the direction of applied tensile strain.

Tensile stimulation significantly increased measured RFU values at both time points. At day 7, RFU values in the S constructs increased by 2.2 fold compared to those for the NS

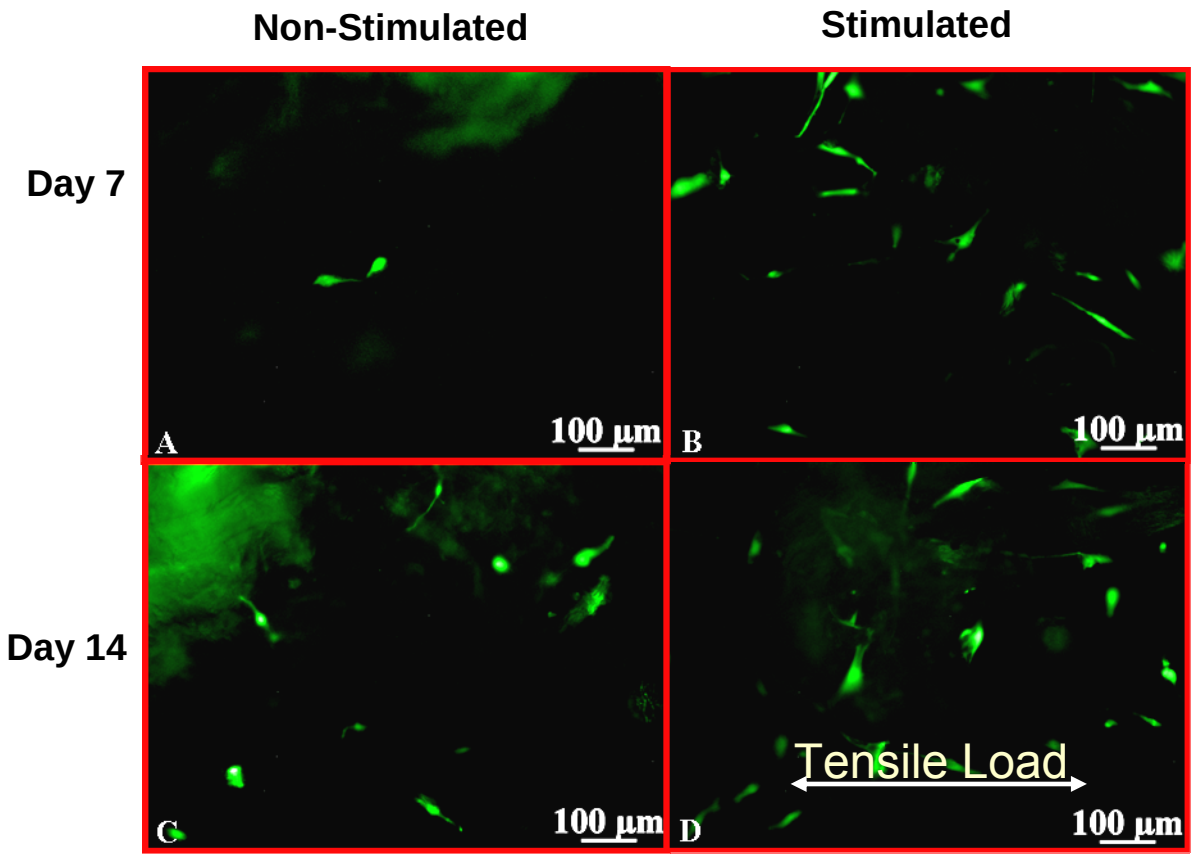

Fig. 8.1. Elongated GFP-T fluorescent cells in A) non-stimulated constructs and B) stimulated constructs at day 7 as well as C) non-stimulated constructs and D) stimulated constructs at day 14. Original magnification x10. Greater fluorescence was observed in the stimulated vs. non-stimulated control constructs at 7 days.

constructs (p=0.0002) (Fig. 8.2). At day 14, RFU values in the S constructs increased by 1.5 fold compared to those for the NS constructs (p=0.01) (Fig. 8.2). RFU values for the S vs. NS construct groups averaged 6.3 ± 0.22 vs. 2.8 ± 0.69 at day 7 and 4.63 ± 0.16 vs. 3.04 ± 0.97 at day 14 (mean ± SD). No difference was seen between NS constructs at day 7 and 14 (p>0.5) (Fig. 8.2). There was a significant decrease in RFUs in S constructs between day 7 and 14 (p=0.0001) (Fig. 8.2).

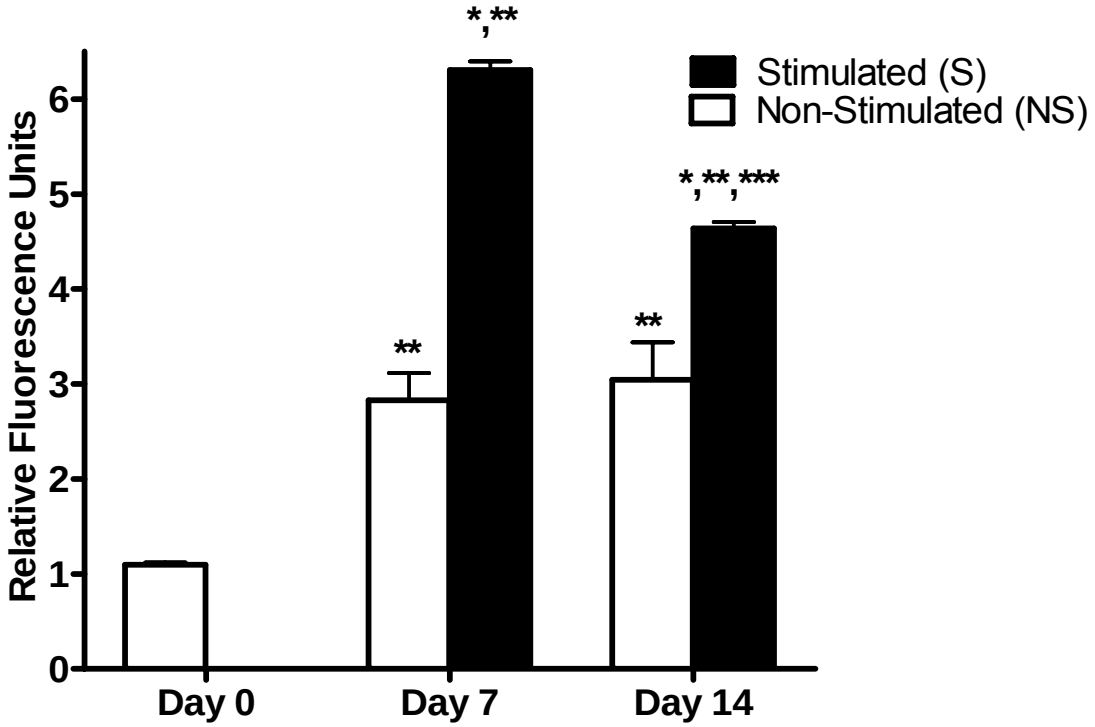

Fig. 8.2. Tensile stimulation increased GFP-T expression by Relative Fluorescence Units (RFU). *Significantly different from non-stimulated controls at same time interval ($p < 0.02$). **Significantly different from day 0 ($p<0.00001$) ***Significantly different from day 7 stimulated constructs ($p<0.0002$). Data represented as mean ± standard deviations. N=6 for all groups.

Seven and fourteen days of in-vitro mechanical stimulation significantly increased type I collagen gene expression in stem cell-collagen sponge constructs measured using qRT-PCR. S constructs showed a 12-fold increase in type I collagen gene expression ($p = 0.001$) relative to NS controls at day 7 and a 5-fold increase at day 14 ($p = 0.01$) (Fig. 8.3). Type I collagen gene expression as measured by qRT-PCR for the S vs. NS construct groups averaged 7.8 ± 2.6 vs. 0.6 ± 0.5 at day 7 and 2.8 ± 1.1 vs. 0.5 ± 0.4 at day 14 (mean ± SD). No difference was seen between NS constructs at day 7 and 14 ($p>0.5$) (Fig. 8.3) but there was a significant decrease in type I collagen gene expression in the S constructs between day 7 and 14 ($p=0.005$) (Fig. 8.3).

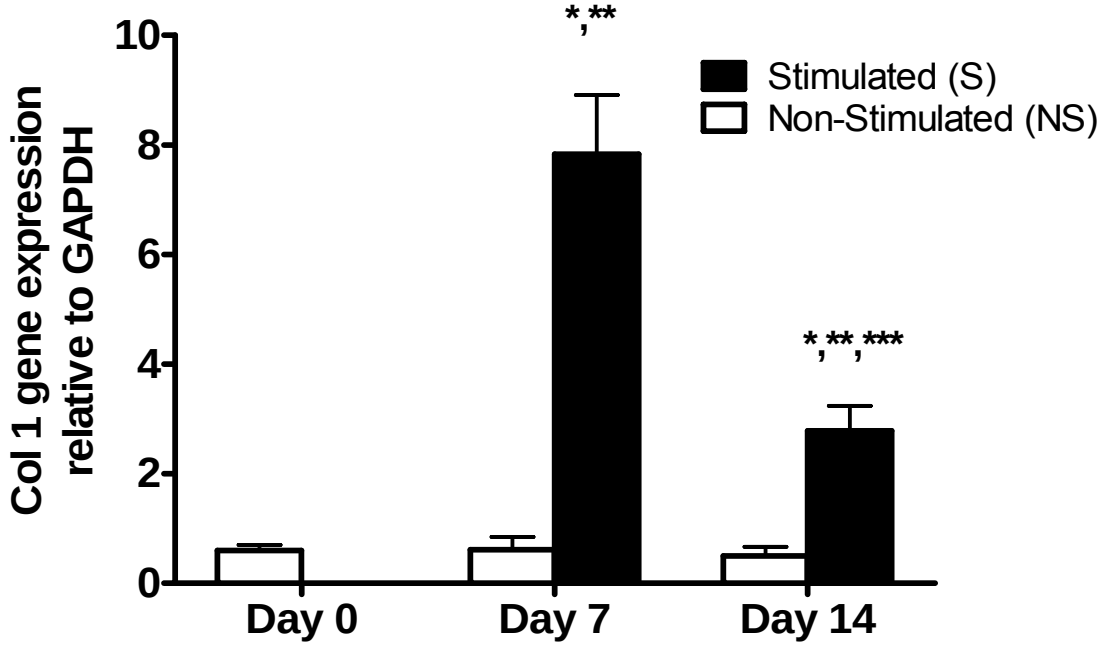

Fig. 8.3. Tensile stimulation increased type I collagen gene expression by qRT-PCR. *Significantly different from non-stimulated controls at same time interval ($p < 0.02$). **Significantly different from day 0 ($p<0.0003$) ***Significantly different from day 7 stimulated constructs ($p<0.006$). Data represented as means ± standard deviations. N=6 for all groups.

Mechanical stimulation did not significantly increase GAPDH gene expression ($p>0.5$) at day 7 or day 14 in culture. GAPDH values for the S vs. NS construct groups averaged 5.8 E-07 ± 1.7 E-07 vs. 5.6 E-07 ± 1.3 E-07 at day 7 and 5.4 E-07 ± 1.4 E-07 vs. 5.3 E-07 ± 1.2 E-07 (mean ± SD). The GAPDH values for day 0 constructs were 5.8 E-07 ± 1.7 E-07. The results from qRT-PCR and fluorescence analysis were positively correlated (Fig. 8.4, $r^2 = 0.79$). The slope of the linear regression curve was 0.56.

Both 7 and 14 days of tensile stimulation increased the construct's linear stiffness compared to NS constructs but had no effect on dimensions ($p>0.05$; Table 8.3). S constructs showed a 2.3-fold increase in linear stiffness ($p = 0.0006$) relative to NS controls at day 7 and a 1.6-fold increase at day 14 ($p = 0.003$) (Fig. 8.5). Linear stiffness for the S vs. NS construct groups averaged 0.035 ± 0.006 N/mm vs. 0.015 ± 0.003 N/mm at day 7 and 0.029 ± 0.004 N/mm vs. 0.0175 ± 0.4 N/mm at day 14 (mean ± SD). No difference was seen between NS

constructs at day 7 and day 14 (p=0.1) (Fig. 8.5). Tensile stiffness of both 7 and 14 day constructs were significantly less than the corresponding stiffness values of day 0 constructs (p=0.005) (Fig. 8.5).

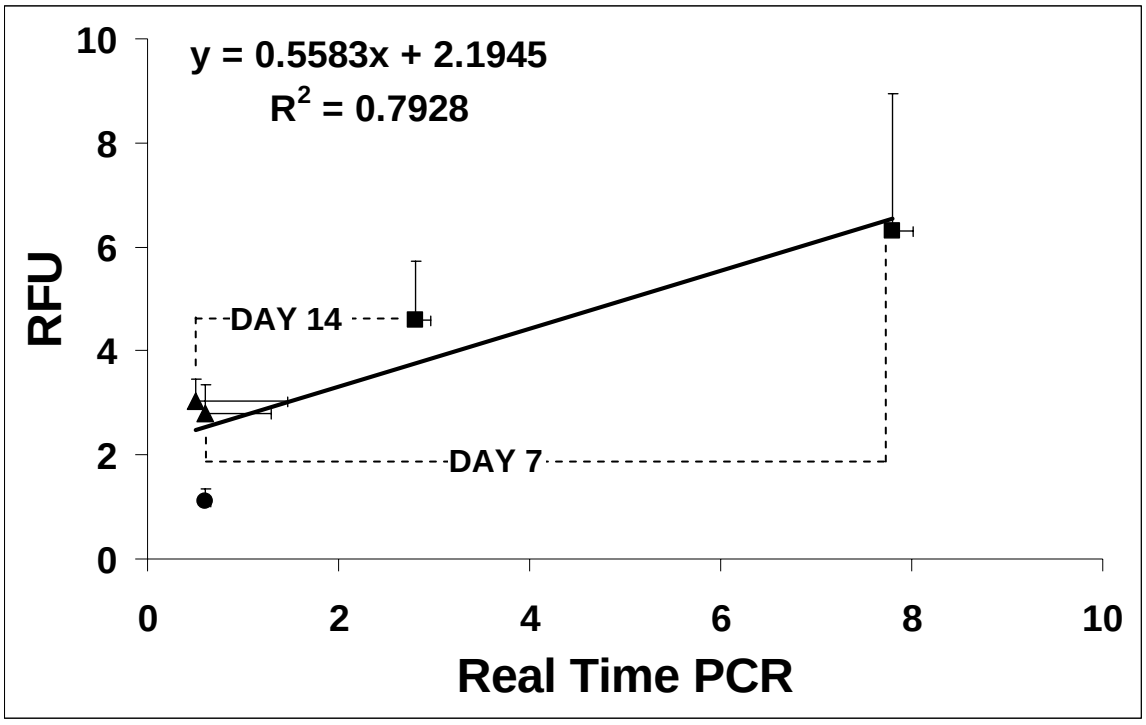

Fig. 8.4. Type I collagen gene expression as measured by relative fluorescence units (RFU) and by qRT-PCR were positively correlated (r^2 = 0.79). Each point represents the average of 6 samples (means ± standard deviations). Circle denotes Day 0 constructs, triangles denote non-stimulated constructs and squares denote stimulated constructs.

Evaluation time points	Length(mm)	Width (mm)	Thickness (mm)
Day 0	23.2 ±0.5	8.5 ±0.7	2.7±0.1
Day 7 Stimulated	23.8 ±0.4	8.3 ±0.5	2.6 ±0.4
Day 7 Non-Stimulated	23.1 ±0.6	8.1 ±0.5	2.5 ±0.5
Day 14 Stimulated	23.6. ±0.3	8.0 ±0.6	2.5 ±0.5
Day 14 Non-Stimulated	23.2 ±0.4	8.1 ±0.9	2.4 ±0.5

Table 8.3. Tensile stimulation did not significantly affect construct dimensions (p>0.05). Shown are means + standard deviations for all three parameters.

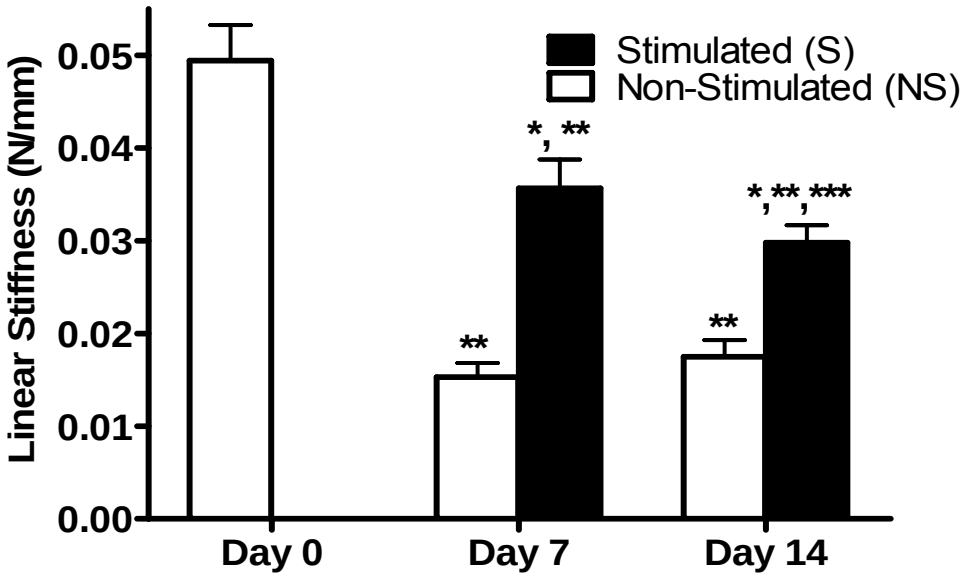

Fig. 8.5. Tensile stimulation increased linear stiffness. *Significantly different from non-stimulated controls at same time interval ($p<0.004$). **Significantly different from day 0($p<0.0005$). ***Significantly different from day 7 stimulated ($p<0.05$). Data represented as means ± standard deviations. N=6 for all groups.

8.4. Discussion

Understanding how to control matrix production and assembly during culture is one of the primary objectives in developing functional tissue engineered replacements for load-bearing connective tissues [159]. Although externally-applied deformations have been shown to strengthen tissue engineered constructs [171-172, 324, 417, 437], long culture times are typically required for cells to synthesize enough matrix to improve resulting mechanical properties that can remotely approach the levels of the native tissue. This slow process could reflect the fact that mechanical stimuli have yet to be optimized for inducing such cellular-based benefits. In particular, we still know little about how various components of the stimulation profile (e.g. strain amplitude, frequency, and duty cycle) affect construct quality and functionality in culture and after surgery. We do know, however, that determining the importance of each component can be a very expensive and time-consuming process. Therefore, a strategy to

evaluate how these factors influence the production of extracellular matrix components like type I (tendon and ligament mid-substance) or type II collagen (soft tissue insertions, articular cartilage, meniscus, intervertebral disc) would appear to be a very beneficial step in this development effort. It was for these reasons that we bred the double transgenic mouse model for the current study. By using this fluorescence technology we could repeatedly track changes in gene expression during rest periods between bouts of mechanical stimulation in culture rather than use destructive microarray or qRT-PCR techniques that could only detect final changes after stimulation. Also this technology provides a unique way to detect near real-time changes in expression in culture by one or both of these genes and to readjust the mechanical signal applied to the evolving construct to obtain the desired spatial and temporal gene expression patterns.

This study utilizing MSCs harvested from these mice was designed to evaluate the effects of effects of uniaxial tension on gene expression and biomechanics in cell-collagen constructs. We hypothesized that: 1) mechanical stimulation would increase type I collagen gene expression as well as linear stiffness and 2) the fluorescence data and qRT- PCR data would be positively correlated. In our study we found that tensile stimulation increased GFP-T fluorescence and type I collagen gene expression compared to controls, thus validating the first part our hypothesis. The increases in both measures (Figs. 8.2 & 8.3) that we observed in stimulated constructs reinforce the importance of stimulating a cell-scaffold construct with a defined mechanical signal in a cell culture system. The fact that the tensile stimulus chosen in this study significantly increased GFP-T fluorescence in the construct's cells without affecting ECFP activity suggests that tensile stimulation did not affect collagen type II gene expression.

Several reasons could explain the increases in GFP-T fluorescence (type I collagen gene expression) we observed after seven and fourteen days of tensile stimulation. Tensile strains are known to trigger the creation of cell-surface stretch receptors and integrins, activating a cascade of genes responsible for the synthesis and secretion of ECM components

[441]. Of course the stimulus we applied may have also produced other cellular changes (cell proliferation, mass transfer rates of nutrients, metabolites and waste materials [173], expression and synthesis of various growth factors and cytokines), some of which are known to be sensitive to mechanical stimulation [396, 398, 442-443]. Several of these factors will be examined in greater detail now that we have demonstrated a significant positive effect of mechanical stimulation on gene expression in this model system.

Our findings are generally consistent with previous reports examining how tensile stimulation affects gene expression of cells and cell-based constructs, although the specific treatments, conditions and response measures vary greatly among studies. Investigators using human fibroblasts [417-418, 444] as well as human mesenchymal[290, 420] and human and bovine stromal cells [173] have demonstrated positive effects of mechanical stimulation on type I collagen gene expression and protein synthesis. However, these studies were conducted either using cells on monolayer [420] or after placing cells in three-dimensional polyurethanes[417] and collagen gels [173, 290]. These differences in local matrix environment can be important to driving cell phenotype and gene expression patterns. The 2.4 % peak strain that we delivered in the current study is far less than the 5% and 10% [417-418, 420, 444] peak strain treatments used by other groups and not as complex as reported biaxial strain patterns[173] While the studies by Park et.al and Noth et.al [290, 420] matched the frequency (1 Hz) delivered in our study and the 14 days for treatment assessment [173, 290] was identical to our time interval, other aspects of these studies differed markedly. In fact, direct comparison of our results to those used by other investigators was difficult given the broad range of frequencies (0.0167 Hz[173], 0.25 Hz[418], and 0.167 Hz [444]) and time assessment periods (from 6 to 24 hrs) [417-418, 420, 444] reported in the literature. Studies will ultimately need to either limit these stimulation conditions across studies or examine the interactive effects of these treatment components if we are to understand how to control and optimize gene and protein expression in tissue engineering.

Our findings are also different from a previous study in the literature [173]. Although Altman and co-workers[173] examined mechanically-induced changes in gene expression using stem cells over the same time interval used in the current study, they used collagen gels rather than sponges and found that tensile stimulation significantly increased type I collagen gene expression at day 14 but not at day 7. Such differences between our two outcomes could be due to different cell sources (bovine vs. murine) or mechanical strain patterns (combined axial and torsional strains vs. uniaxial tensile strains), although other differences may have also contributed. For example, a cell's biosynthetic activity is strongly related to its ability to attach to its collagen scaffold [445-450]. Once accomplished, subsequent collagen synthesis within these three-dimensional collagen lattices is then regulated by transcriptional and post-transcriptional mechanisms mediated by α and β integrins[448]. Similarly, synthetic scaffolds can also provide a conducive environment, as shown by Chastain and co-workers [451] who found increases in α6 and β6 gene expression over a 5-week culture period using mesenchymal stem cells deposited in PLGA scaffolds. Regardless of scaffold type, increases in type I collagen gene expression will clearly require the appropriate signals and microenvironment for effective cell-based therapy.

We also found in this study that collagen type I gene expression measured using real time quantitative RT-PCR and fluorescence analysis were positively correlated (Fig. 8.4) thus validating our second hypothesis. This correlation serves as a validation for using fluorescence as an indicator of average gene expression within tissue engineered constructs. Additional correlations of this type may soon allow investigators to use fluorescence as a reasonable estimate of expression of genes like type II collagen than using more traditional approaches like qRT-PCR. These correlations may also be possible at even finer levels in the tissue engineered constructs, including expression by groups of cells or even individual cells in the future.

We also found that applying both seven (7) and fourteen (14) days of tensile stimulation increased the construct's linear stiffness compared to non-stimulated constructs in culture (Fig.

8.5) validating the last part of our first hypothesis. Other investigators have noted positive effects of stimulating collagen-based constructs on biomechanics as well as gene and protein expression using different cell types (tendon fibroblasts [398] , smooth muscle cells [396] , ligament fibroblasts [444], and MSCs [173, 420] and methods of stimulation [173, 396, 398, 418, 420, 444] . Our results are also similar to another study in our laboratory where mechanical stimulation of rabbit mesenchymal stem cells in collagen sponge constructs not only increased construct stiffness but also produced increases in type I and III collagen mRNA expression using qRT-PCR [167].

Day 7 and day 14 constructs showed significantly lower stiffness compared to day 0 constructs (Fig. 8.5). It has been shown that when chondrocytes [452] and fibroblasts [453] are seeded in collagen based constructs, they produce MMPs which degrade the collagen scaffold. It is plausible that in our study the mouse MSCs also produced MMPs which degraded the collagen scaffold and in turn, decreased its stiffness. Such decreases in stiffness are likely offset by tensile stimulation. In the future, we plan to also monitor MMP production in our stimulated and control constructs.

The stiffness of our constructs is still orders of magnitude less than that of that of native tendons[171] and hence cannot currently replace the entire ruptured tendon. Instead, we have been using these constructs for “biological augmentation” in a load-protected regime to repair central defect injuries to the rabbit patellar tendon. Implanting such stimulated cell-scaffolds in-vivo can speed up the repair process as the cells are already acclimatized in-vitro to some of the deformations that they might experience in-vivo. Further these cells are already synthesizing type I collagen in-vitro and might fill the repair site much faster. We have shown that such compliant constructs produce rabbit patellar tendon repairs whose average stiffness is three orders of magnitude greater than the stiffness of initial constructs at surgery and capable of matching the stiffness of normal patellar tendon up to 150% of peak in-vivo force values **[171]**.

The results obtained from this study have the potential to be translated to higher species such as humans. If we can demonstrate through in-vitro studies that murine cell-based constructs respond to mechanical stimuli (and chemical stimuli) in ways similar to rabbit cell-based constructs by a range of response measures, it is then our hope to be able to apply the results of genetically-based experiments in the mouse to new therapies in larger animals where such tools do not yet exist. The benefits that we formulate and test in lower species and then validate in larger preclinical models can then be translated to improved repair in humans.

Our study has limitations. 1) In this study we recorded non-destructively the average effects of uniaxial tension on GFP-T expression in MSCs. We are currently examining techniques to measure fluorescence without destroying the constructs. 2) We did not quantify the number of fluorescing cells. This prevented us from distinguishing whether the increases in fluorescence were due to increased numbers of fluorescing cells and/or increased production of GFP-T by already fluorescing cells. Determining which of these effects predominates will require more local recordings of gene expression at the individual cell level combined with in-situ hybridization. Fluorescing cells could also be counted in a non-destructive way by applying statistical methods if we had the optical systems that could scan through the entire depth of the opaque constructs. 3) We selected each fluorescent protein to have a 24-hour half life, thus providing near, rather than actual, real-time gene expression. While it would have been ideal to have instantaneous feedback of gene up-regulation much like a conventional transducer, the longer period ensured that we could detect measurable changes in fluorescence before signal dissipation. 4) Cell viability within the constructs was not determined. Diminished cell viability in individual constructs could have adversely affected our RFU results for GFP-T fluorescence and thus increased inter-specimen variability. 5) This method of fluorescence analysis is limited to the specific transgenic species described with two modified fluorescent proteins. 6) We chose to measure only the construct's linear stiffness because we were concerned that the lower aspect ratio of 2:1 (less than the ideal ratio of 3:1 to avoid St. Venant effects) might alter the structure's

failure properties. 7) These constructs can be only used to repair tendons in a load-protected regime. We are currently investigating ways to further improve the stiffness of our constructs.

In conclusion, this study has demonstrated that short-term tensile stimulation increases type I collagen gene expression and linear stiffness of murine stem cell-collagen sponge constructs and that two measures used to track changes in type I collagen gene expression are correlated. Future studies will focus on optimizing the effects of these in-vitro signal components on expression of the type I collagen gene and the degree to which they affect construct and then repair biomechanics. Given that construct stiffness is positively correlated with repair stiffness after implanting rabbit autologous MSC-collagen constructs in patellar tendon defects [171] our efforts to optimize stiffness in culture might also positively impact tendon repair biomechanics and gene expression across multiple species and injury scenarios. We also envision that once optical technologies have been developed to visualize fluorescence in opaque constructs in a bioreactor at magnifications consistent with light microscopy, we and others should be capable of tracking real-time, local changes in gene fluorescence. Ultimately, we hope to speed tendon repair and match the tangent stiffness of normal tendons to levels well above peak in-vivo force levels [171].

Chapter 9

Tensile Strain Downregulates Col2 Gene Expression In Double Transgenic Murine Chondrocytes Seeded In Collagen Sponges

The objectives of this study were to determine how tensile displacements, applied to murine chondrocyte-collagen tissue engineered constructs (TECs), influence Col1 and Col2 gene expression as well as construct linear stiffness. We hypothesized that up to 28 days of tensile stimulation would downregulate Col2 expression, upregulate Col1 expression and increase TEC stiffness. Chondrocytes were harvested from the ribs of six newborn double transgenic mice carrying transgenes with enhanced cyan fluorescent protein (ECFP) and green fluorescent protein (GFP-T) as reporters for expression from the Col2a1 and Col1a1 promoters, respectively. Twenty-seven TECs were created per animal by seeding 0.5×10^6 chondrocytes in type I collagen scaffolds. Twelve of these TECs were stimulated (S constructs) for 7, 14, 21 or 28 days in a pneumatic system (n = 3 each) housed in an incubator and another twelve matching constructs were not stimulated (NS controls) but exposed to identical culture conditions and time intervals. The remaining three TECs per animal served as Day 0 controls. ECFP-fluorescing cells were present in all constructs and time intervals but no GFP-T expressing cells were observed. Compared to time-matched NS controls, 21 and 28 days of stimulation significantly decreased ECFP fluorescence and 28 days of stimulation significantly decreased linear stiffness. This study reveals that tensile stimulation not only downregulates Col2 gene expression but fails to upregulate detectable Col1 gene expression within 4 weeks. The inability of these specialized cells to express the Col1 gene would be consistent with an unaltered TEC stiffness, but the observed *reduction* in stiffness suggests that Col2 expression patterns may also control tensile stiffness even without a perceived change in chondrocyte phenotype. Current studies in the lab are examining type I and type II collagen protein levels in

these TECs and whether longer time intervals and different components of tensile stimulation in culture might result in dedifferentiation of these chondrocytes and corresponding positive effects of Col1 expression on TEC biomechanical stiffness.

9.1. Introduction

Articular cartilage maintains homeostasis as it responds to normal internal stresses produced by dynamic chemical equilibrium and modified by complex external physical forces applied to joint surfaces [454-456]. Although articular cartilage primarily resists compressive external loading, these loads create transverse tensile and shear strains, particularly in the tissue's superficial layer. Efforts to model rapid loading on articular cartilage also reveal the presence of tensile forces acting at the cartilage-bone interfaces as well as in type II collagen fibers near the articular surface [457]. Larger forces associated with traumatic injury can induce cracks or fissures in the articular surface leading to degeneration and long-term arthritis [458-463]. Continued loading of these damaged regions can generate regions of high tensile stress near the crack tips, resulting in tensile strains on neighboring chondrocytes [464]. These altered stress environments can also lead to progressive tissue degradation in adjacent and otherwise normal articular cartilage.

Many forms of mechanical stimulation modulate chondrocyte matrix synthesis both in tissue explants and in 3-D culture environments [166, 366, 465-472]. Static and cyclic mechanical compression and cyclic shear can enhance as well as inhibit extracellular matrix (ECM) synthesis and gene expression by chondrocytes [366, 469-472]. However, relatively few studies have explored how uniaxial or biaxial tensile signals modulate chondrocyte phenotype or matrix synthesis [464, 473-477]. Cyclic tensile strains (10%-20% peak-to-peak) applied to chondrocyte-fibrin gel constructs inhibit proteoglycan synthesis whereas lower peak strains (5%) show no such effect [477]. In another study, tensile strains ranging between 0% and 10% increased cell proliferation but actually decreased matrix synthesis [464]. Tensile strain-induced matrix deformation can also regulate proliferation and differentiation of chondrocytes cultured in

3-D collagen scaffolds [473, 475]. Understanding the role of tension on chondrocyte metabolism is important because tissue engineering approaches to repairing damaged articular cartilage may involve inserting a scaffold with markedly different material properties than the surrounding tissue into a defect site. Under functional loading, regions of the repair or native tissues near this material property mismatch may experience substantial tensile strains[464]. This altered local mechanical environment could in turn affect the development of the repair tissue as well as the maintenance or the continued degradation of the native cartilage [464].

Tensile stimulation has been shown to upregulate collagen I gene expression in other cell types like fibroblasts and MSCs. Groups have shown that tensile stimulation applied to human fibroblasts [416-418], mesenchymal stem cells [419-420] and stromal cells [421] as well as bovine stromal cells [421] increases Col1 gene expression and type I collagen protein synthesis. Similarly, our group has demonstrated that tensile displacements applied to tissue engineered constructs containing either rabbit [167] or murine [163] MSCs upregulate Col1 gene expression. Whether such tensile signals might downregulate Col2 expression and upregulate Col1 expression in chondrocytes is intriguing especially if expression by both genes could be monitored in near real time.

Our laboratory has created novel transgenic technologies to study the effects of mechanical stimulation on gene expression by both undifferentiated and specialized cells. In collaboration with Cincinnati Children's Hospital, we created a double transgenic murine model in which the Col1 and Col2 genes are linked to Green Fluorescent Protein- Topaz (GFP-T) and Enhanced Cyan Fluorescent Protein (ECFP), respectively [2, 163]. Cells from these mice fluoresce either green or cyan if either Col1 gene or Col2 gene is activated respectively. We have shown that after suspending different cell types from these mice in scaffolds, MSCs show increased Col1 gene expression in response to tensile strains [163] and chondrocytes show elevated Col2 gene expression in response to compressive strains [2]. Our group has also

demonstrated that when these fluorescence patterns are quantified in a spectrophotometer, the resulting relative fluorescence units or RFUs are positively correlated with qRT-PCR [2, 163].

Using this double transgenic murine technology, the objective of the current study was to examine how cyclic tensile displacements influence tissue engineered constructs containing chondrocytes. We hypothesized that compared to non-stimulated constructs at equivalent time points, tensile stimulation would: 1) Decrease ECFP fluorescence, a reporter of Col II gene expression; 2) Increase GFP-T fluorescence, a measure of Col1 gene expression; 3) Decrease the construct's linear stiffness. 4) We also hypothesized that non-stimulated constructs would maintain ECFP fluorescence over equivalent time periods.

9.2. Materials and Methods

Experimental Design

Chondrocytes were harvested from the ribs of six (6) newborn double transgenic mice and passaged until P2 [2]. Type I collagen sponges (Kensey Nash Corp., Exton, PA) previously shown to increase Col1 gene expression in murine and rabbit MSCs [163, 167] was used as the scaffold. Chondrocyte-collagen constructs were created by seeding 0.5×10^6 cells/construct. Twenty seven constructs were created per animal. Twelve constructs were assigned to stimulated (S) groups and another twelve constructs were assigned to non-stimulated (NS) controls at days 7, 14, 21 and 28. The remaining three (6) constructs served as day 0 NS controls. Stimulated (S) constructs were elongated between 0% and 2.4% peak strain at 1 Hz for 20 seconds followed by a 100-second rest period. This pattern was repeated for five (5) hours/day. Peak amplitude, frequency and duty cycle were chosen based on studies performed in our lab on the effects of tensile stimulation on murine MSCs [163]. The six (6) S and six (6) NS constructs at each time point were assigned as follows: one for ECFP/GFP-T fluorescence in a spectrophotometer (measured in Relative Fluorescence Units (RFU)) [2, 163, 446], one for cell viability using an MTT assay[478], and one for determining linear stiffness[163].

Chondrocyte isolation and expansion

Newborn mice were euthanized using protocols in accordance with protocols approved by Institutional Animal Care and Use Committee (IACUC) protocols. Chondrocytes were harvested from the rib cages and expanded using previously-published procedures [2, 333, 479]. Chondrocytes were passaged until P2. While ECFP expression was evident, no intracellular GFP-T expression was seen in cultured chondrocytes from P0 through P2.

Scaffold and construct creation

Scaffolds were cut from sponges provided by Kensey Nash Corporation (Exton, PA). The dimensions of the scaffolds are such that they can fit in the wells of silicone dishes. Holes were created in each scaffold to fit over the posts in each well of the dish [163, 171]. Before seeding with cells, each scaffold was soaked in 70% ethanol overnight and rinsed three times in PBS. Chondrocytes were suspended in media at the chosen concentration ($2x10^6$ cells/ml). 250µl of this cell suspension was pipetted on top of each scaffold. All constructs were placed in an incubator (Steri-Cult Model 3033, Forma Scientific, Marietta, OH; 37ºC, 5% CO2, 95% relative humidity) for two days to permit the cells to acclimate to the environment. Constructs were fed three times weekly with Advanced DMEM, 1% antibiotic/antimycotic, 1% glutamax and 10% FBS.

Mechanical stimulation

After letting the cells acclimatize in the collagens sponges for 2 days of incubation (I denoted as Day 0), only the silicone dishes containing the S constructs were stimulated using the pattern described in the Experimental Design. This stimulation was done in a pneumatic mechanical stimulation system housed in a standard incubator (Steri-Cult Model 3033, Forma Scientific, Marietta, OH) [163, 171]. The details of this pneumatic system can be obtained here [2]. Dishes were removed from the incubator at the prescribed intervals and constructs were

then prepared for evaluation of gene expression using fluorescence analysis, failure testing in tension, or cell viability.

Fluorescence Analysis

ECFP and GFP-T fluorescence in constructs were analyzed qualitatively and quantitatively. Each S or NS construct was visualized in a fluorescent microscope (Zeiss Axiovert) with a filter set (XF1142, Omega) for ECFP fluorescence and a filter set (XF104-2, Omega) for GFP-T fluorescence [2, 163]. Further visualization using filter set for red fluorescence (11002VZ, Chroma) ruled out autofluorescence. Constructs were digested as previously described to extract cells [163]. These digests were then pipetted into a black bottom microplate (200µl per well in three wells). ECFP/GFP-T RFUs were quantified in a spectrophotometer as previously described [2, 163].

Determination of Cell Viability

At each time point, cell viability in constructs was determined using an MTT assay (Vybrant MTT Cell Profileration Assay Kit, Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Details of the assay are described elsewhere[478].

Biomechanical evaluation of the cell-collagen constructs

After the specific time periods in culture, constructs for biomechanical analysis were placed in cryovials and frozen at -80^{0}C. On the day of testing, constructs were removed from the freezer and thawed to room temperature. TECs were inserted into custom grips in a materials testing system (100R6, TESTRESOURCES, Shakopee, Minnesota) and then failed under displacement control at a strain rate of 10%/sec [163]. Linear stiffness was calculated from the linear region of the force-elongation curve generated by the constructs during failure testing [163].

Statistical Analysis

The presence of treatment-related differences among values was determined for all response measures using a two-way ANOVA with Tukey's HSD post hoc testing (SPSS

software). Animal was chosen as a random factor, culture time and tensile stimulation as fixed factors, and response measures as dependent variables. Significance was set at $p < 0.05$.

9.3. Results

Fluorescence Microscopy

Fluorescence microscopy using ECFP and GFP-T filter sets revealed ECFP-expressing cells in all constructs (Fig 9.1) but no GFP-expressing cells in any construct. Fluorescence was observed in the red filter set, thus ruling out autofluorescence. ECFP fluorescent cells were found in all constructs at all time points of culture. However, no apparent differences in ECFP fluorescence were observed among non-stimulated and stimulated constructs at the three time points. ECFP expressing cells appeared elongated and were not aligned in the direction of the applied tension.

Spectroscopic Analysis

Quantitative results showed that applying a dynamic tensile displacement significantly decreased ECFP expression in the 3-D constructs, but only at later time points. Compared to time-matched, NS controls, compressive stimulation significantly decreased RFU values by 0.71 fold and 0.56 fold at, 21 and 28 days, respectively ($p<0.05$; Fig. 9.2).

Linear Stiffness

Stiffness of non-stimulated constructs appeared to increase over time but the increases were not significant ($p>0.05$; Fig. 9.3). Tensile stimulation significantly decreased the stiffness of constructs at 28 days in culture. S constructs showed a 0.79 fold decrease in linear stiffness compared to NS controls at day 28 ($p<0.05$; Fig. 9.3).

Cell Viability

No significant changes occurred in cell viability due to mechanical stimulation or for increasing time in culture ($p>0.05$; Fig. 9.4). Day 28 constructs showed 6% lower cell viability than day 0 constructs.

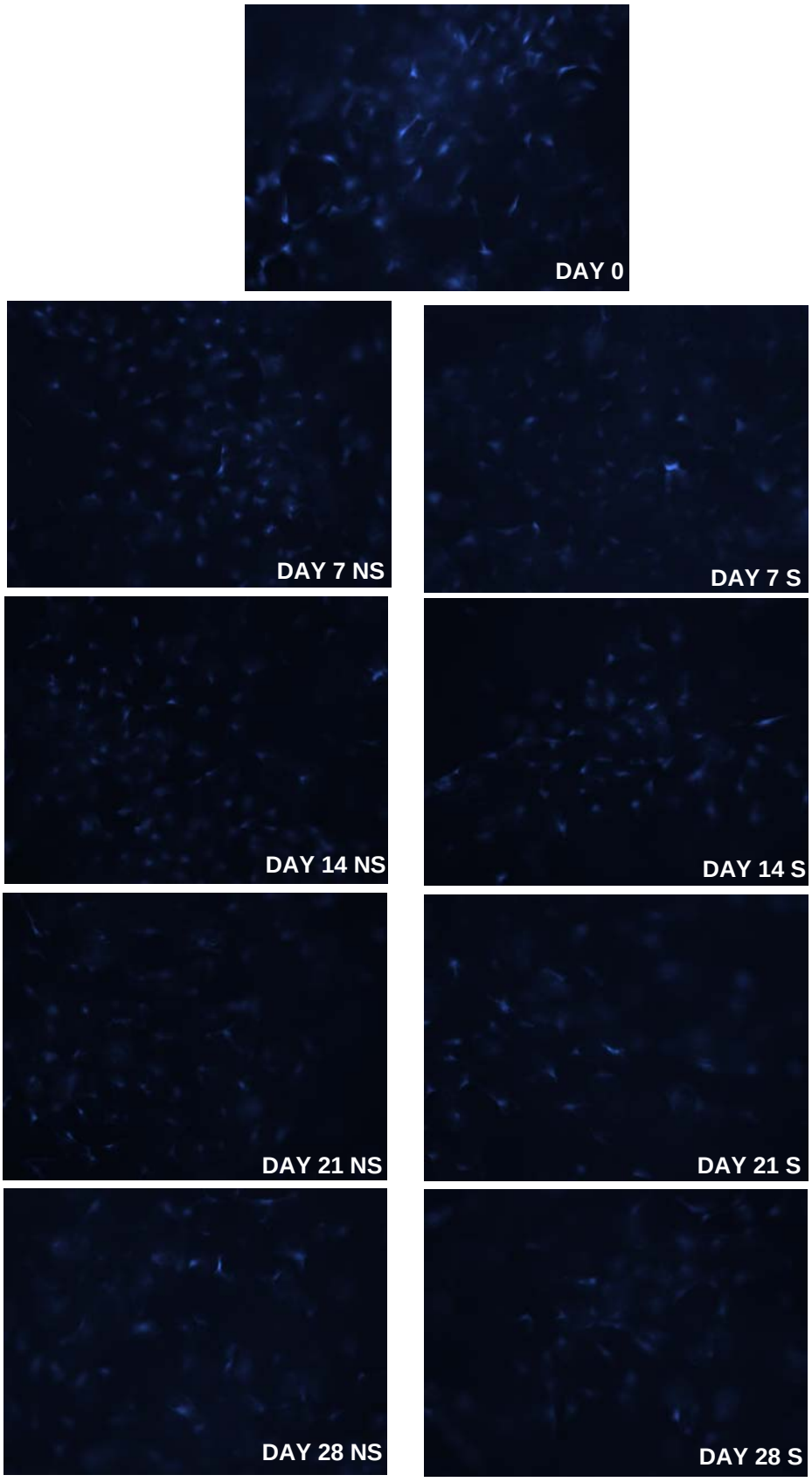

Fig. 9.1. Elongated ECFP expressing chondrocytes in collagen sponges

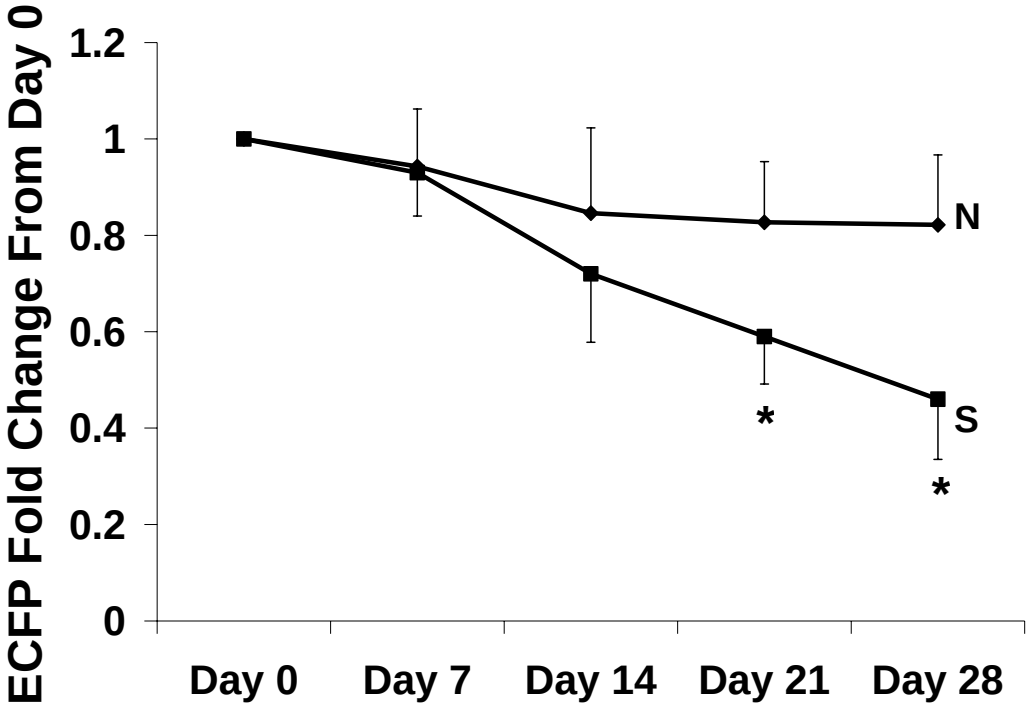

Fig. 9.2. Tensile stimulation decreases ECFP expression by Relative Fluorescence Units (RFU) at day 21 and day 28. * Significant increase compared to non-stimulated control at same time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups. NS- Non-Stimulated, S-Stimulated.

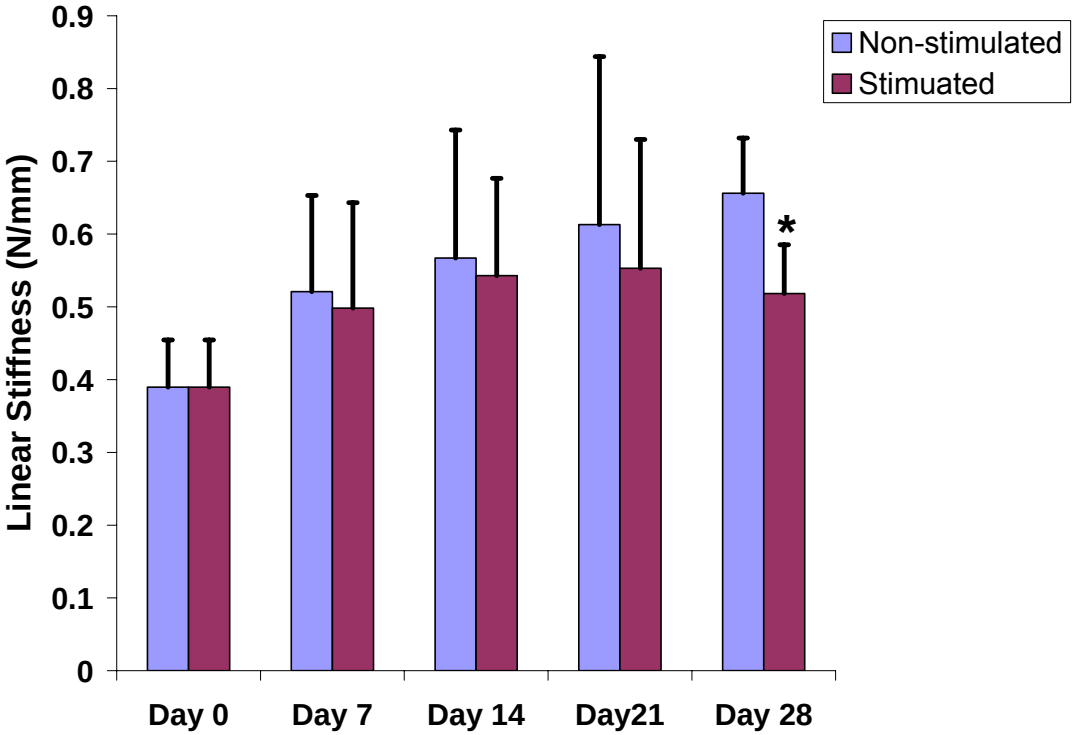

Fig. 9.3. Tensile stimulation decreases linear stiffness of constructs 28. * Significant increase compared to non-stimulated control at same time point ($p < 0.05$). Data represented as mean + one standard deviation. N=6 for all groups.

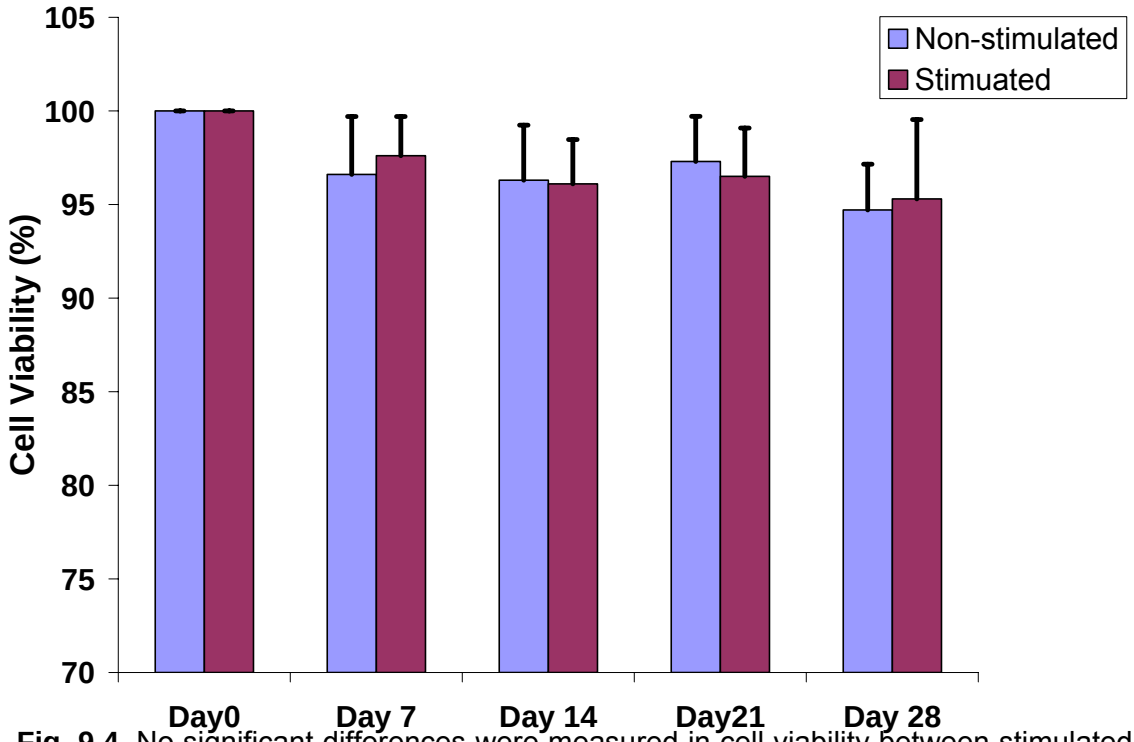

Fig. 9.4. No significant differences were measured in cell viability between stimulated and non-stimulated constructs after 0, 7, 14, 21 and 28 days ($p>0.05$). Cell viability exceeded 94% at all time periods in culture. Data represented as mean + one standard deviation. N=6 for all groups.

9.5. Discussion

Understanding how chondrocytes respond to altered mechanical loading environments offers both in-vitro and in-vivo benefits. Determining how these cells respond to tensile signals can help tissue engineers better understand how to manipulate cartilage constructs in-vitro. These experiments also have the potential to help investigators discover how the post-implantation loading environment further alters cartilage replacement tissues. These goals motivated our group to develop the model, to isolate double transgenic murine chondrocytes, to examine the effects of tension on chondrocyte-collagen scaffolds, and ultimately to determine the plasticity of these cells to mechanical loading. Using this specialized transgenic murine model also allowed us to test our hypotheses that applying tension to chondrocyte-seeded gels would decrease ECFP fluorescence, increase GFP-T fluorescence and thereby increase the construct's linear stiffness.

Our quantitative fluorescence results support our first hypothesis that tensile stimulation significantly decreases ECFP fluorescence in chondrocytes. The fact that tensile stimulation of S constructs produces significant decreases in ECFP RFU values relative to NS controls at 21 and 28 days (Fig. 9.2) indicates that specialized cells do respond to tensile signals in a somewhat plastic manner, albeit with a decline in gene expression. However, these temporal changes are delayed in time compared to the more rapid increase in Col2 gene expression that we and others have observed when chondrocytes are exposed to dynamic compression [2, 336-337]. These changes are also slower than the response of double transgenic murine MSCs to similar patterns of tensile stimulation [163, 167, 416-421].At the same time, it should be noted that the Col2 gene is not exclusively sensitive to mechanical signals in chondrocytes. Genes such as MMPs, CTGF, and runx2/cbfa1 in chondrocytes also respond depending on the mode of stimulation [480]. So while chondrocytes respond to oscillatory tensile stimulation in a fundamentally different manner than they do to compression, monitoring the sensitivity of other structural genes like Col1 are also important.

Our second hypothesis that GFP-T fluorescence, an indicator of Col1 gene expression, would increase in chondrocytes exposed to tensile stimulation was not accepted. While a tensile stimulus upregulates Col1 gene expression in other cells types [163, 167, 416-421], none of the S or NS constructs showed GFP-T positive cells during the course of the experiments. The mechanical profile we delivered to these constructs was identical to the one we used when stimulating murine MSC-collagen sponge scaffolds [163]. However, unlike that study where tensile signals significantly upregulated Col1 gene expression using MSCs, we found no such effect using chondrocytes after 28 days of stimulation. It is possible that chondrocytes require a different mechanical signal to elicit this effect. It is also plausible that longer periods of stimulation are required to show GFP-T expression in these more terminally differentiated chondrocytes or that these levels are below the level of detection using this transgenic technology. Studies are ongoing in our laboratory to test each of these possibilities and to

discover when and if chondrocytes are capable of showing GFP-T expression in response to different tensile stimuli in culture.

Our third hypothesis that tensile displacements applied to chondrocyte-collagen constructs would increase TEC stiffness was also refuted. We were initially surprised that tensile displacements actually decreased linear stiffness of stimulated constructs compared to non-stimulated controls at day 28.Given the fact that tensile stimuli have no effect on Col1 gene expression in these constructs, we might have expected no change rather than a decline in TEC stiffness. Previous studies have shown that compressive stimulation of chondrocyte-seeded collagen constructs increases matrix protein [166, 481-482] and proteoglycan synthesis [426-427] and application of tensile forces produces the opposite effect [483]. Others have also shown that tensile stimulation of chondrocyte-scaffold constructs increases MMP gene expression [164, 480].Thus, it is conceivable that such decreases in protein, proteoglycan synthesis and increase in MMPs could explain the decrease in linear stiffness observed in our constructs. Our future studies will also be monitoring these other gene and protein measures to determine if our chondrocyte-collagen scaffolds demonstrate similar effects.

Our quantitative fluorescence results do support our final hypothesis that Col2 gene expression will not decrease with culture time in non-stimulated constructs. Quantitative ECFP RFU data (Fig. 9.1) shows that there are slight decreases in ECFP RFU in NS constructs over time, but such decreases are not statistically significant. Previous studies have shown that collagen scaffolds have the ability to maintain chondrocytic characteristics [166, 426-427, 481-482, 484-485]. Collagen gels has been shown to provide an environment in which chondrocytes can expand [427] and produce matrix proteins [166, 481-482], proteoglycans [426-427] and maintain Col2 gene expression [166, 485]. Protein synthesis is also enhanced when collagen-chondrocyte constructs are exposed to growth factors [484]. Our results confirm that type I collagen sponges do have the ability to maintain Col2 gene expression in chondrocytes.

Our study is not without limitations. We pooled our cells within a sample in order to detect sufficient fluorescence levels. This pooling means that we only know the average effects and not the specific effects of tension on individual cells. We are currently examining techniques like in situ hybridization to examine more local effects of tension on individual cells. We did not track cell-induced changes in the production of proteins and proteoglycans nor changes in integrin expression. These factors likely play an important role in regulating intracellular gene expression and the ability of cells to sense their near environment. Our future studies will track the synthesis of these constituents and their effect on our outcome measures. We applied only one stimulus pattern to the constructs. We still need to systematically vary components of the mechanical signal and determine their effects on gene expression, protein synthesis and biomechanical properties. Finally, we chose to measure only the construct's linear stiffness. Stiffness was selected because we were concerned that the lower aspect ratio of 2:1 (less than the ideal ratio of 3:1 to avoid St. Venant effects) might alter the structure's failure properties. Future studies will examine how constructs with larger aspect ratios influence our results.

We conclude that tension applied to cell-seeded TECs decreased Col2 gene expression but did not induce increase Col1 gene expression in mouse chondrocytes. Tension also decreased the linear stiffness of constructs. Expanding our studies to examine longer time intervals and different components of tensile stimulation as well as monitoring protein and proteoglycan synthesis should provide a more comprehensive picture of the ability of specialized chondrocytes to undergo dedifferentiation or transdifferentiation in response to more complex mechanical signals. Ultimately, such culture experiments using double transgenic stem and differentiated cells should help investigators in selecting cell sources and scaffold types that more rapidly accelerate the repair of diseased and injured connective tissues.

Chapter 10

Discussion and Conclusions

Chapters 2 through 9 describe a series of studies using a fluorescent promoter transgenic technology in the mouse model to examine Col1 and Col 2 gene expression during natural healing of patellar tendon defect injuries, during growth and development, and following mechanical stimulation of tissue engineered constructs containing primitive or specialized cells. The major findings from these studies are summarized and discussed in this chapter. This chapter also highlights the overarching influence of all of these studies on tendon tissue engineering.

Punch defect healing of murine patellar tendons produce spatial and temporal changes in Col1 gene expression and results in inferior tissues.

Our results from Chapter 2 document the spatial and temporal changes in swelling and disorganization levels, Col1 and Col2 gene expression levels, following punch defect and sham injuries. Both swelling and disorganization decreased with healing time. Col 1 gene expression after injury increased and then decreased in intensity while also becoming less localized. This emphasizes how an entire tissue can be affected by a local punch injury, possibly as a result of the disruption of vascular supply as well as loss of biomechanical tension to the tendon and its tenocytes proximal and distal to the defect site. Our temporal Col1 expression results are in agreement with others in the literature, which shows Col1 expression occurring in healing tendons between 10-24 days [256] [255] [253] [249]. Our Col1 gene expression patterns might indicate which phase of healing is occurring in these tendons and establish timelines for these phases. Very little Col1 gene expression (together with observed swelling and disorganization) at 1 week indicates that the healing tendons are still in the inflammatory phase. High levels of

Col1 expression at 2 weeks suggest that the healing tendons are in the proliferative phase. Decreases in Col1 (together with decreases in swelling and disorganization) at 4 weeks suggests that the tendons may have entered the remodeling phase.

No ECPF expression (Col2 expression) was seen in the entire tendon. This indicates that the Col2 gene is not involved during the repair of tendon mid-substance. This is not surprising as Col2 expression is not seen in the mid-substance of the normal tendons. Since Col2 expression is localized to the insertions, Col2 expression might be a factor in the healing of insertions. The temporal patterns of Col1 expression we observed during mid-substance healing suggests that temporal patterns of Col2 expression might also exist during insertional healing This is supported by preliminary insertional injury studies done in our lab which show elevated Col2 gene expression in the insertions by 2 weeks (not shown). These insertional injuries have to be examined further to determine the complete temporal and spatial expression patterns for Col2 expression there.

Force-elongation and stress-strain curves demonstrate that while not different from each other at 4 and 6 weeks, healing tendons were still significantly compliant and weak compared to normal tendons ($p>0.05$). Poor biomechanics of natural healing was expected as an early study in our lab using rabbit PT window defects showed that natural healing was inferior [257]. What is surprising is that even long term healing (63 days) of murine intrasynovial tendon adhesions produce inferior tissues [256]. These results taken together with our own reinforce that even in the long term, true tendon regeneration is not occurring. Thus, new strategies are required to augment such repair. One promising approach is to determine what happens during normal tissue development as to provide possible stimuli that we might impose during adult healing. This was the motivation for the studies on growth and development.

In summary this repair model and models like it should allow researchers to develop a roadmap for tissue engineering that more closely simulates normal development, potentially leading to regeneration rather than repair of tendon injuries.

The current study was not without limitations. ***1. Our design did not permit us to determine when Col1 and Col2 expression levels first became detectable*** since we only started monitoring the expression of both genes at 7 days. We are currently examining expression after only 3 days. Moreover, once gene expression can be truly monitored in real time, we can answer this question. ***2. The biomechanical results for the healing regions included some adjacent strut tissue due to the extremely small size of the repair area***. Thus, our biomechanical results may have overestimated the true properties of the repair tissues. This inclusion of adjacent struts might also explain why 4 and 6 week healing properties were not different. **3. We recognize that the small size of these tendons increases the difficulty of inducing repeatable injuries in the murine model**. Future studies, particularly those involving tissue engineering, may require using larger animals if genetic markers can be incorporated. ***4. We could not directly explain our biomechanical results based on gene expression patterns.*** The improvements we observed in biomechanical properties between 4 and 6 weeks were quite encouraging, but our experimental design did not include an assessment of either gene expression or tissue structure at 6 weeks post injury. However, based on gene expression results between 2 and 4 weeks combined with our 6-week biomechanical results, we can speculate that gene expression levels likely undergo further decreases toward control levels with corresponding reductions in tissue thickness and more parallel alignment of collagen fibers. ***5. These acute punch defects do not replicate the clinical problems.*** This punch defect model provides a way to biologically and mechanically evaluate the effects of natural healing. Rabbit models which are closer to the clinical conditions are being developed in our lab and should also be extended to the mouse model in the future.

Developing knee structures undergo spatial and temporal changes in Col1 and Col2 gene expressions, BMP-2 expression and TGF-β1 expression.

Results from chapter 3 indicate that there are spatial and temporal changes in ECFP/GFP-T fluorescence patterns in several knee structures (i.e. Col1/Col2 gene expression).

These changes in fluorescence around birth suggest that knee structures undergo regulatory changes which could be controlled by either biologic or physical factors. A recent study of rat supraspinatus entheses indicates that physical factors do not play a significant role in enthesis maturation in the first 14 days postnatally, implying that biologic factors may drive early postnatal development [272]. Thus our Col1/Col2 expression patterns could be a result of differential expression of biological factors, especially BMP-2 and TGF-β1, as these have been shown to have important roles during joint development [486].

Immunohistochemistry did reveal differences in growth factor patterns across age groups. For example, BMP-2 was only evident in the PT mid-substance until P1 and in the tibia at P14. BMP signaling has been suggested to control regional differentiation in tendon. Generally BMP signaling is inhibited in the tendon mid-substance, allowing tendon fiber formation, but is activated at the insertion site, allowing fibrocartilage formation. The appearance of BMP-2 in the tendon mid-substance is contrary to what we expected. It has been suggested that BMP-2 acts a regulator of TGF-β1 signaling which is important in the mid-substance.

The absence of BMP-2 in the tendon mid-substance beyond P1 is also significant suggesting that BMP-2 is likely important only during early development of the insertions and that other members of the BMP family could play important roles during later development.

pSmad1,5,8 expression did not mirror that of BMP-2 expression. Since only the BMP family acts through the pSmad1,5,8 pathway, other members of the BMP family are also active during development. Future studies are necessary to determine which BMPs are expressed at these developmental time points. Once these BMPs have been identified, these should be used to stimulate TECs in culture to see if such stimulation improves not only the in-vitro mechanical properties of TECs but also the resulting in-vivo repair mechanical properties.

Unlike BMP-2, TGFβ1 was seen in the patellar tendons at all ages. TGF-β1 has been shown to have a central role during morphogenesis and tissue formation by influencing the differentiation of connective tissue precursors into tendon forming cells [275]. Thus this

expression of TGF-β1 can help to explain the increases in Col1 gene expression we observed. Also the appearance of TGF-β1 at all time points suggests that this growth factor is likely important not only during early development, but also during later development of the tendon mid-substance. This suggested role of TGF-β1 during development taken together with results in the literature showing improvement in collagen synthesis [276-277] and improvements in healing of tendon mechanics [278] makes this a key factor to be investigated during the development of tissue engineered constructs for tendon repair. In fact a preliminary study in our laboratory demonstrated that adding 10 ng/ml of TGF-β1 significantly increases rabbit MSC-collagen stiffness which was similar to the stiffness produced by optimization of the mechanical stimulus.

pSmad2,3 expression mirrors that of TGF-β1 expression. This suggests that the expressed TGF-β1 acts on the cells in the tendon mid-substance and further emphasizes the importance of TGF-β1 in the formation of tendon mid-substance and possibly delivery of this growth factor locally to TECs.

Improving the integrity of tendon tissue engineered constructs in culture is one of the most important problems facing tendon tissue engineers. Our growth and development results motivate new strategies to solve this problem. For example, we observed that Col1-expressing cells increased fluorescence levels and were already well-aligned with the tendon axis by P3. Such knowledge about organization and gene expression of cells in the developing tendon could be used to try and recreate more useful tissue engineered constructs, e.g. by the application of tensile displacements to improve their integrity.

Recreating tendon zonal attachment sites after injury is another important problem facing tendon tissue engineers [171] [62, 267-269]. Our growth and development results motivate new strategies to solve this problem. Our results showing: 1) appearance of Col2 before Col1 during development, 2) early co-expression of Col1 and Col2 in insertion regions, and 3) differences in expression of BMP-2 vs.TGF-β1, suggest that to recreate insertions during

tendon repair, investigators should monitor and attempt to enhance the co-expression of Col1 and Col2 in the end regions of tendon constructs. Researchers should also strive to regionalize the expression of BMP-2/TGF-β1, if these are lacking in the current set of FTE tendon constructs. Since mechanical signals are known to modulate gene expression, the next set of in vitro studies were conducted to identify appropriate mechanical signals and cell source (primitive vs specialized) necessary to create constructs showing both Col1 and Col2 gene expression.

An important question which arises from these growth and development studies is the correlation of our murine results with findings from larger species, especially humans. It is important to first establish if there are correlations between the mouse and another higher species (for example, rabbit) and then the next higher species (for example, goat) and so on until humans. It is plausible that there are some correlations which can be found by taking into account differences between mice and humans. We might have to deliver different concentrations based on either body mass or tissue size as well as corresponding periods in the life cycle (e.g. early skeletal maturity and in senescence).

In summary, the results from this study illustrate some of the problems that need to be addressed to effectively repair tendon injuries. We need to reconstitute sufficient key regulatory events from the normal spatial and temporal differentiation of the patellar tendon to enhance the maturation of cells in vitro, and thus enhance tendon repair. Our growth and development data begin to suggest biological benchmarks with which to compare tissue engineered constructs and repair. These biological benchmarks complement the mechanical benchmarks our group has been developing over the past decade.

The current studies were not without limitations. 1) ***We only monitored GFP-T and ECFP fluorescence as early indicators for Col1 and Col2 gene expression in sections of the murine knees***. While these fluorescent markers may appear to be subjective measures, we have previously shown positive significant correlations between relative fluorescence units

or RFUs and qRT-PCR for Col1 and Col2, respectively [2, 163, 279]. 2) ***We did not measure actual Type I or Type II collagen protein expression from these sections.*** However, we have shown in culture that Type II collagen protein is present within 7 days after upregulation of Col2 gene expression [2, 163, 279]. 3). ***Although our double transgenic model should allow us to track changes in Col1 and Col2 expression in near real time, currently we must sacrifice animals and section knees to image the fluorescence.*** Once we can identify powerful in vivo optical technologies (e.g. dual photon methods) that we can use to visualize at the cellular level, we should be able to image the entire knee with the required magnification and conduct cost- and time-efficient longitudinal studies that eliminate assignment of animals for gene expression patterns at different ages and healing times.

Cell-culture conditions can be created and maintained in a bioreactor housed in the ELF system. This system delivers precise displacements to compliant TECs.

Results from chapter 4 indicate that the number of number of viable, proliferative cells within the bioreactor was not significantly different from that of cells cultured in the standard incubator. This result confirms our ability to create and maintain cell-culture conditions within the bioreactor.

Results from chapter 4 also indicate that the actuator displacement recorded by the internal LVDT of the electromagnetic system is similar to the displacements recorded by a sonomicrometry system. This confirms our ability to apply precise displacements to compliant TECs.

Several features of our bioreactor emphasize its potential for Functional Tissue Engineering. Our bioreactor combines both the culture and stimulation in a one step process. We can also deliver multiple precise stimulation profiles including in vivo profiles to multiple specimens. We can record loads and displacements during culture thus permitting real-time calculation of stiffness. We can compare these values with those for normal tissues, thus

providing biomechanical feedback for adjusting the stimulation to better "challenge" the constructs and to accelerate their maturation in culture for more rapid delivery at surgery.

The current studies were not without limitations. 1) ***We only monitored cell viability in monolayer***. However, we have previously shown that we can maintain over 90% viability in MSC-agarose and chondrocyte-agarose constructs. 2) ***We only monitored cell viability up to 14 days in culture.*** Important to note is the fact that we have also maintained over 90% cell viability in 3-D constructs up to 28 days in culture.

Positive correlations exist between response measures.

Our results from chapters 5 and 8 and Fig 10.1 suggest that fluorescence and collagen mRNA expression are positively correlated and type II collagen content and aggregate modulus are positively correlated. These correlations serve as a validation for using fluorescence as an indicator of average gene expression within these tissue engineered constructs. These correlations are important as they suggest that RFU measures can be used to indirectly estimate gene expression in constructs and type II collagen content can be used to indirectly estimate aggregate modulus or vice-versa. Such correlations could definitely speed up the tissue engineering process. While it takes about 3-5 hours to determine the RFUs in constructs, it takes about 2-3 days to evaluate mRNA content from cells in these TECs. Also these correlations will reduce the costs associated with performing real time PCR and with using multiple samples.

Although the correlations between these response measures is encouraging, further studies are needed to determine if such correlations are found in other cell types or other model systems (e.g. in the Col2 rabbit). Discovering correlations of this type in larger models like the rabbit may soon allow investigators to use fluorescence as a reasonable surrogate of gene expression as opposed to more traditional approaches like qRT-PCR.

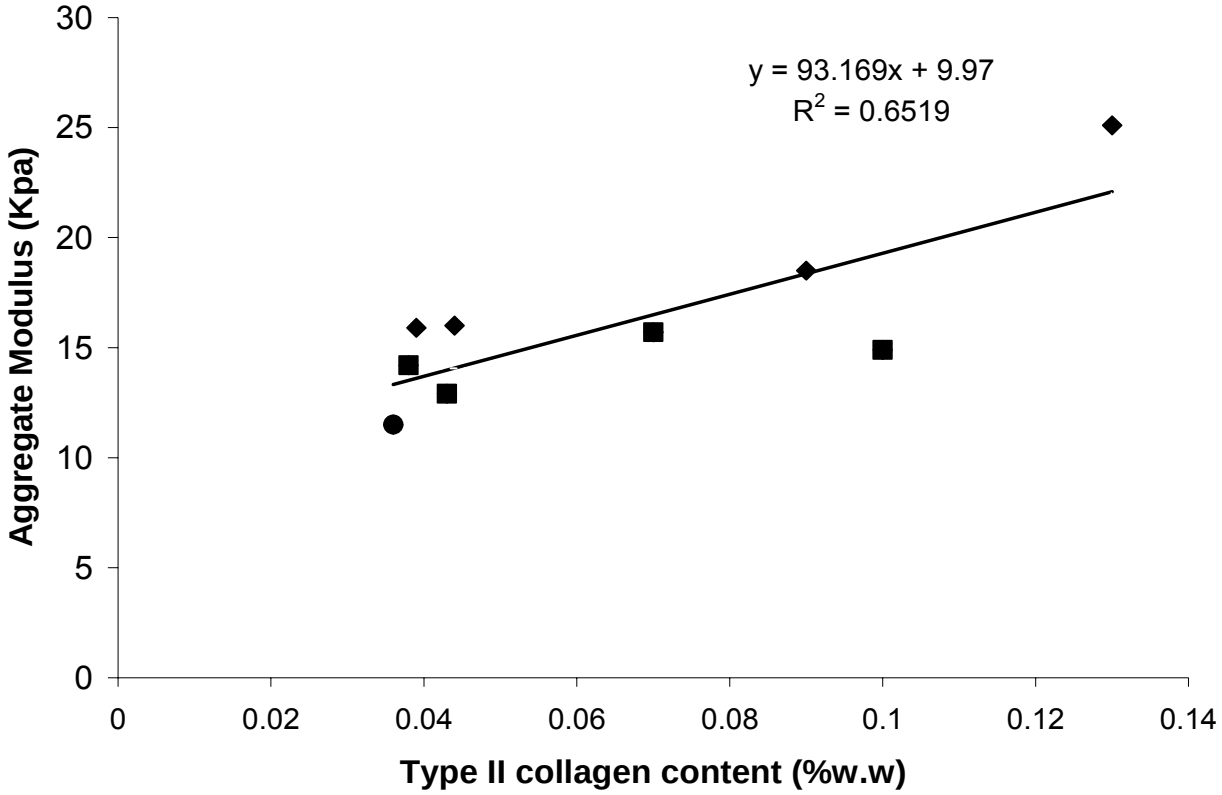

Fig. 10. 1. Positive correlation exists between aggregate modulus and type II collagen content in chondrocyte agarose constructs.

Mechanical stimulation affects gene expression when compared with non-stimulated constructs.

Results from chapters 5 to 9 show the ability of mechanical stimulation to either increase or decrease gene expression compared to non-stimulated controls. Generally, compression increased Col2 gene expression (chapters 5, 6) and decreased Col1 gene expression (chapter 7) while tension increased Col 1 gene expression (chapter 8) and decreased Col2 gene expression (chapter 9).

Our findings for different cell types regarding the effects of compressive and tensile stimulation on collagen gene expression are consistent with other reports in the literature. For example, our Col2 gene expression results showing increases in our chondrocyte-agarose and chondrocyte-MSC systems are similar to results reported in the literature [336] [487] [390, 399] [336-339, 488]. Our Col1 gene expression increases in MSC-collagen constructs are similar to

the results of other studies in the literature [417-418, 444] that have demonstrated positive effects of mechanical stimulation on type I collagen gene expression. Our results are similar to other studies in which fibroblasts exposed to cyclical contraction [423] and hydrostatic pressure [206] expressed significantly lower Col1 mRNA.

Several mechanisms have been put forth by investigators to explain such changes in Col2/Col1 gene expression. Compression has been shown to induce transcriptional activation of SP1 binding sites residing in the proximal region of the col2a1 gene promoter [340], cell/tissue strain, fluid pressurization and flow, electrokinetic phenomena, convective transport, and release of cytokines/growth factors which can trigger the complex chain of events that modulate the production of ECM proteins like Type II collagen [341-342, 489]. Tensile strains are known to trigger the creation of cell-surface stretch receptors and integrins, to activate a cascade of genes responsible for the synthesis and secretion of ECM components [441], to produce cellular changes (cell proliferation, mass transfer rates of nutrients, metabolites and waste materials [173], expression and synthesis of various growth factors and cytokines) [396, 398, 443, 490]. Of particular interest in the above mentioned mechanisms is the expression and synthesis of various growth factors and cytokines. In the future RT-PCR should be performed to determine which cells express which growth factors during mechanical stimulation. Once identified, experiments can then be performed to determine if stimulating these constructs with growth factors during mechanical stimulation further improves their gene expression.

Taking the results of all studies in this dissertation together it becomes evident that when using mechanical stimulation as a treatment, to increase Col1 expression one has to apply tension and to increase Col2 gene expression one has to apply compression. Thus to create constructs with both Col1 and Col2 gene expression one should apply both compressive and tensile signals to constructs. By designing grips which can provide compression to the end of constructs and by utilizing them in our pneumatic system we can create constructs with Col1

expressing cells in the middle and Col2 expressing cells in the ends. Such constructs containing dual Col1 and Col2 gene expression would be necessary for tendon insertions into bone.

Mechanical stimulation affects mechanical properties when compared with non-stimulated constructs.

Results from chapters 5 to 9 show the ability of mechanical stimulation to either increase or decrease the construct's mechanical properties over non-stimulated controls. Both compression and tension increased the aggregate modulus of agarose constructs (chapters 5 to 7) and linear stiffness of collagen constructs (chapter 8), respectively. The only exception was when chondrocytes were subjected to tension (chapter 9). The increase we observed in stimulated constructs reinforces the importance of pre-conditioning a cell-scaffold construct with a defined mechanical signal in a cell culture system as we have shown that in vitro stiffness correlates with in vivo repair stiffness [171]..

The increases in mechanical properties could be attributed to increases in collagen content of our constructs. Results from chapters 4 and 5 confirm that mechanical stimulation increases type II collagen content of the constructs. Other factors could have also played a role. For example, accumulated proteoglycans such as aggrecan in agarose constructs could have led to increases water content and aggregate modulus. Collagen orientation could have improved in our MSC-collagen constructs. We have previously shown that the sub-failure properties are correlated with collagen content[491]. Hence future studies should try to measure proteoglycans by the use of ELISA and collagen orientation by the use of SALS.

The decreases in linear stiffness of chondrocyte-collagen constructs can be explained by the decrease in protein and proteoglycan synthesis [483]. Tensile stimulation has also been shown to increase MMP gene expression [164, 480]. Such decreases in protein and proteoglycan synthesis combined with increases in MMPs could explain the decrease in linear stiffness observed in our constructs. Future studies will need to track MMP and protein production by ELISA.

Compressive mechanical stimulation increases Type II collagen content of stimulated constructs.

Results from chapters 5 and 6 show the ability of compressive mechanical stimulation to increase type II collagen content in agarose constructs. This increase in type II collagen content (Table 10.1) at later points (days 21 and 28) is similar to the results reported for MSCs [389,

	Day 0	Day 7		Day14		Day 21		Day 28	
		NS	S	NS	S	NS	S	NS	S
CA	0.036 (0.004)	0.043 (0.008)	0.039 (0.003)	0.038 (0.006)	0.044 (0.012)	0.07 (0.004)	0.09* (0.005)	0.1 (0.004)	0.13* (0.005)
MA	-	-	-	0.03 (0.003)	0.027 (0.007)	0.03^ (0.002)	0.05*^ (0.003)	0.04^ (0.008)	0.072*^ (0.004)

Table 10.1. Compressive stimulation increased Type II collagen content (%ww) in S constructs. Type II collagen content were higher in CA constructs than MA constructs. * Significant from NS constructs, ^ Significant from CA constructs. N=6.

400] and chondrocytes [488]. For equivalent design and testing conditions, chondrocytes produce inherently greater gene (Table 10.2) and matrix expression than do MSCs (Table 10.1), leading to improved mechanical function. These studies suggest that chondrocytes could be an excellent cell source for regenerating cartilage. However, a major limiting factor using chondrocytes is whether sufficient autologous cells can be harvested, especially in elderly and osteoarthritic patients who exhibit decreased chondrogenic capacity. Hence MSCs are a promising alternative cell source for cartilage repair due to their ease of isolation and expansion and their chondrogenic differentiation potential. These findings suggest that an inherent limitation in matrix formation by MSCs and that culture conditions must be optimized to create constructs whose properties are similar to that of chondrocytes.

	Day 0	Day 7		Day14		Day 21		Day 28	
		NS	S	NS	S	NS	S	NS	S
CA	11.2 (1.2)	18.1 (2.2)	24.3* (2.6)	21.6 (4.3)	32.3* (2.3)	23.2 (3.5)	29.6* (2.1)	18.9 (1.1)	23.7* (3.2)
MA	2.8^ (1.3)	2.9^ (1.7)	3.2^ (1.9)	3.2^ (1.4)	3.3^ (2.0)	3.7^ (1.7)	5.4*^ (1.9)	4.3^ (2.7)	7.1*^ (2.2)

Table 10.2. Compressive stimulation increased ECFP RFU in S constructs. ECFP RFU higher in CA constructs than MA constructs. * Significant from NS constructs, ^ Significant from CA constructs

While my experimental design did not allow me to quantify collagen content in fibroblast-agarose constructs, scaffold material prevented me from quantifying newly synthesized collagen content in MSC-collagen and chondrocyte-collagen constructs. Future studies should try to use 1) radio-labeling techniques to quantify newly synthesized collagen in collagen constructs and 2) ELISA in fibroblast-agarose constructs. Such determination of collagen content is necessary to see if there are correlations between collagen content and biomechanical properties. If such correlation exists then collagen content might become an important predictor for subsequent biomechanics.

Differential maturation and structure-function relationship in chondrocyte-agarose and MSC-agarose systems.

Results from chapters 4 and 5 and Figs 10.1 A &B show temporal and stimulation-induced relationships among gene expression, protein expression and biomechanics. If additional studies could corroborate these findings in the murine model and in other model systems, gene and protein expression patterns might then serve as predictors of biomechanical response, both in vitro and ultimately in vivo.

In the chondrocyte-agarose constructs (chapter 4 and Fig 10.2 A), the earlier increases in Col2 gene expression translated to later increases in Type II collagen content and still later

increases in aggregate modulus [162].. These results are not surprising. While gene expression must obviously precede protein deposition, any biomechanical benefits arise only after the protein is assembled into a more functional matrix.

For the MSC-agarose model system (chapter 5 and Fig 10.2 B), the increases we observed in Col2 gene expression mirrored the increases in Type II collagen content and aggregate modulus suggesting differential maturation and structure–function relationships in MSC- vs. chondrocyte-seeded agarose TECs. Our results are similar to reports in the literature which also show differential maturation and structure-function relationships between chondrocyte- and MSC-seeded constructs [492-493]. It is not surprising that the undifferentiated MSCs do not respond to biophysical stimulation in a similar manner to fully differentiated chondrocytes. Long-term stimulation of MSCs might be needed to create constructs whose characteristics match those of chondrocytes stimulated for a shorter time. It is possible such long-term stimulation might be actually detrimental. One recent study showed that long-term mechanical compression (up to 48 days) produced lower compressive modulus and GAG content than non-stimulated constructs [400].

It is important to note that these studies were done using cells from different animals, hence making straight-forward comparisons more difficult. Future studies should utilize chondrocytes and MSCs from the same animal to eliminate inter-animal effects.

Specialized cells respond more rapidly and to a greater extent than MSCs when stimulated with signals that they normally experience in vivo.

Results from chapters 4 and 5 show that chondrocytes upregulate Col2 gene expression more rapidly than MSCs when cell-seeded TECs are exposed to compression (Fig. 10.3). MSC-laden constructs also possessed properties lower than those of chondrocyte-seeded constructs at equivalent time points (Table 10.1, Table 10.2 and Table 10.3). These findings for gene expression, protein content and biomechanical properties of MSCs vs. chondrocytes are

consistent with observations by others [492, 494-495]. This suggests that methods for inducing MSC-based chondrogenesis have yet to be optimized to produce cells whose functional matrix-forming potential matches that of native chondrocytes. This also suggests that if one's primary aim is to tissue engineer cartilage and sufficient chondrocytes are available, then using chondrocytes will likely be more beneficial than using MSCs.

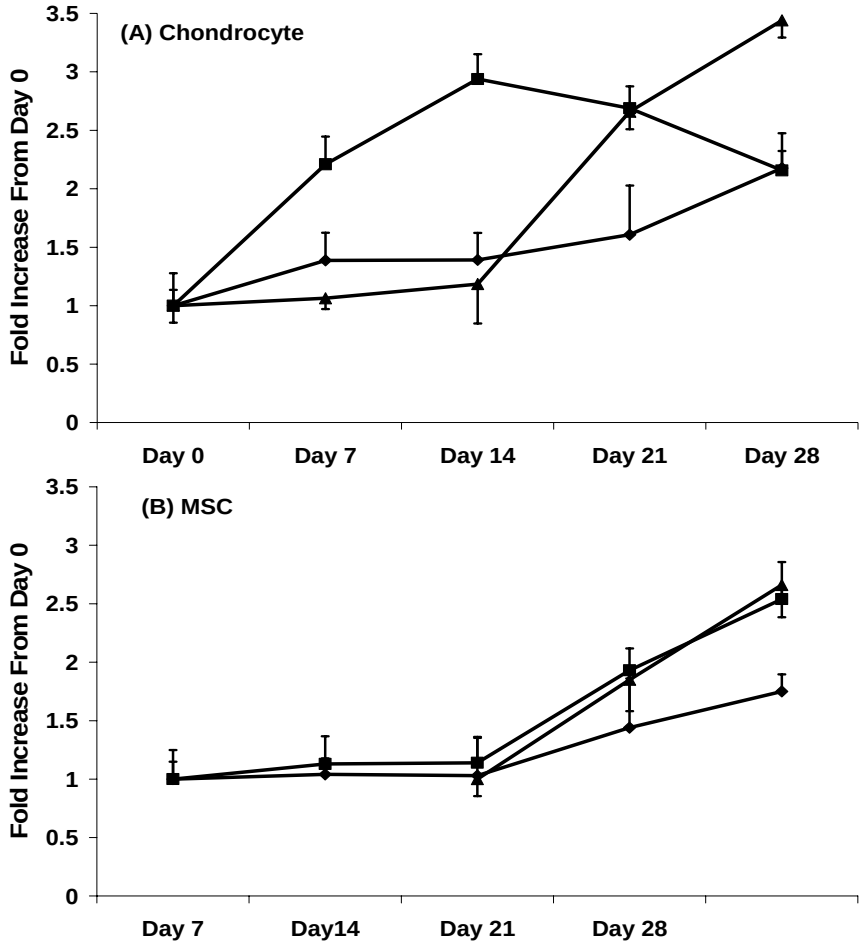

Fig 10.2 Different relationship among RFU, type II collagen content and aggregate modulus between A) Chondrocyte-agarose and B) MSC-agarose systems. Squares denote RFU, triangles denote Type II collagen content and circles denote aggregate modulus

I only performed tensile experiments on MSCs and not on fibroblasts. But preliminary studies performed in our lab (thanks to Nat Dyment and Andrea Lalley) suggest that fibroblasts in TECs show greater Col1 gene expression when exposed to tension than when MSCs in

TECs are placed in tension (Fig 10.4). This also suggests that if one's primary aim is to rapidly tissue engineer tendon mid-substance, then using fibroblasts may be more beneficial than using MSCs.

Comparisons between MSCs vs chondrocytes exposed to compression and MSCs vs fibroblasts exposed to tension lead me to conclude that specialized cells respond more rapidly to signals that they experience normally in vivo than do MSCs. It should be noted that these results were obtained using cells from different animals. To eliminate inter-animal effects, future studies should be performed using cells from the same animal.

	Day 0	**Day 7**		**Day14**		**Day 21**		**Day 28**	
		NS	**S**	**NS**	**S**	**NS**	**S**	**NS**	**S**
CA	11.5 (1.0)	12.9 (3.4)	15.9 (2.5)	14.2 (1.5)	16.0 (2.4)	15.7 (3.1)	18.5 (4.4)	14.9 (2.3)	25.1* (1.5)
MA	9.4 (1.1)	9.6 (3.4)	9.8 (2.5)	10.1 (1.6)	9.7 (2.3)	9.8^ (1.2)	13.5*^ (1.5)	10.6^ (2.4)	16.5*^ (3.2)

Table 10.3. Compressive stimulation increased aggregate modulus (KPa) in S constructs. Aggregate moduli were higher in CA constructs than in MA constructs. * Significantly different from NS constructs, ^ Significantly different from CA constructs

Specialized cells respond more slowly than MSCs when stimulated with signals they do not normally experience in vivo

Our quantitative fluorescence results in chapters 7 and 9 show that stimulation of specialized cells with signals they do not normally experience in vivo decreases Col2 fluorescence in chondrocytes and Col1 expression in fibroblasts but only at later points of stimulation (Fig. 10.5 A&B). The fact that such decreases occur only at later time intervals in culture indicates that specialized cells show modest plasticity. In particular, these temporal changes are delayed in time compared to the more rapid increases in Col2 gene expression that we and others have observed when chondrocytes are exposed to dynamic compression (chapter 5) [2, 336-337]. These changes are also slower than the response of double transgenic murine MSCs to similar patterns of compressive (chapter 6) and tensile stimulation (chapter 8)

[163, 167, 416-421]. These results lead one to conclude that MSCs are generally more plastic than specialized cells (i.e., they respond more rapidly than specialized cells to unconventional mechanical signals in culture).

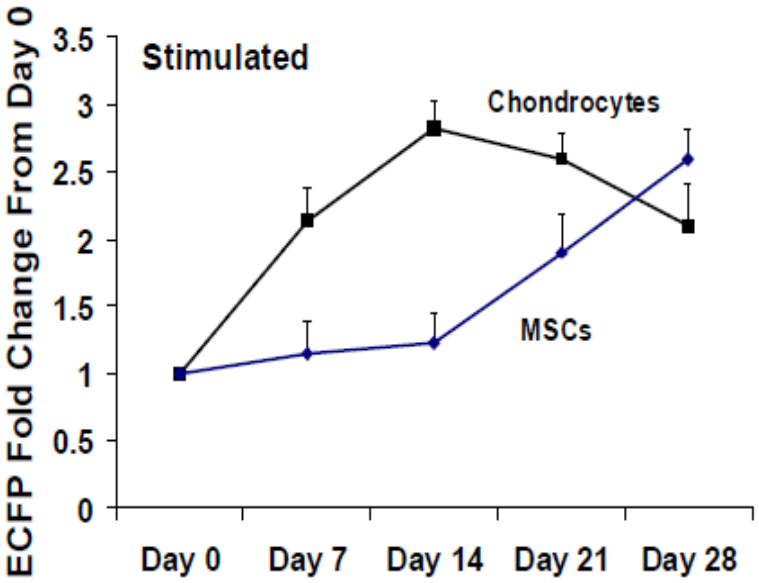

Fig 10.3. Compressive response of chondrocytes vs MSCs in TECs. Chondrocytes show more rapid increases in ECFP expression than MSCs when stimulated in compression.

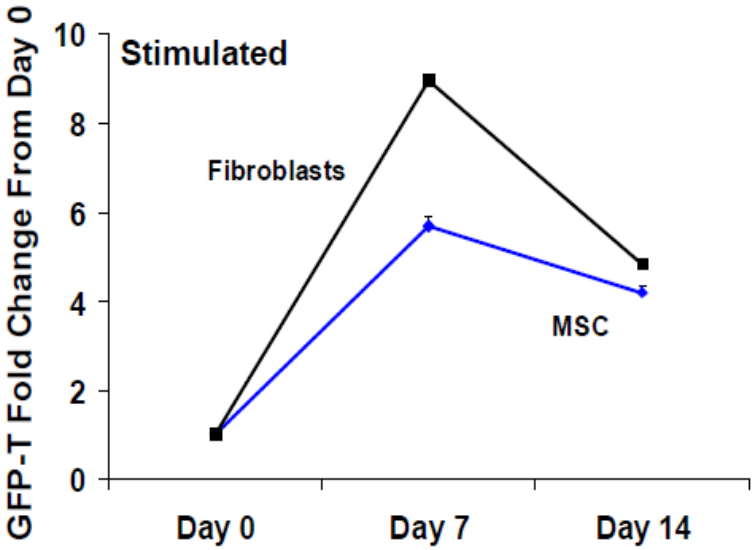

Fig 10.4 Tensile response of Fibroblasts vs MSCs in TECs. Fibroblasts show more rapid increases in GFP-T expression than MSCs when stimulated in tension.

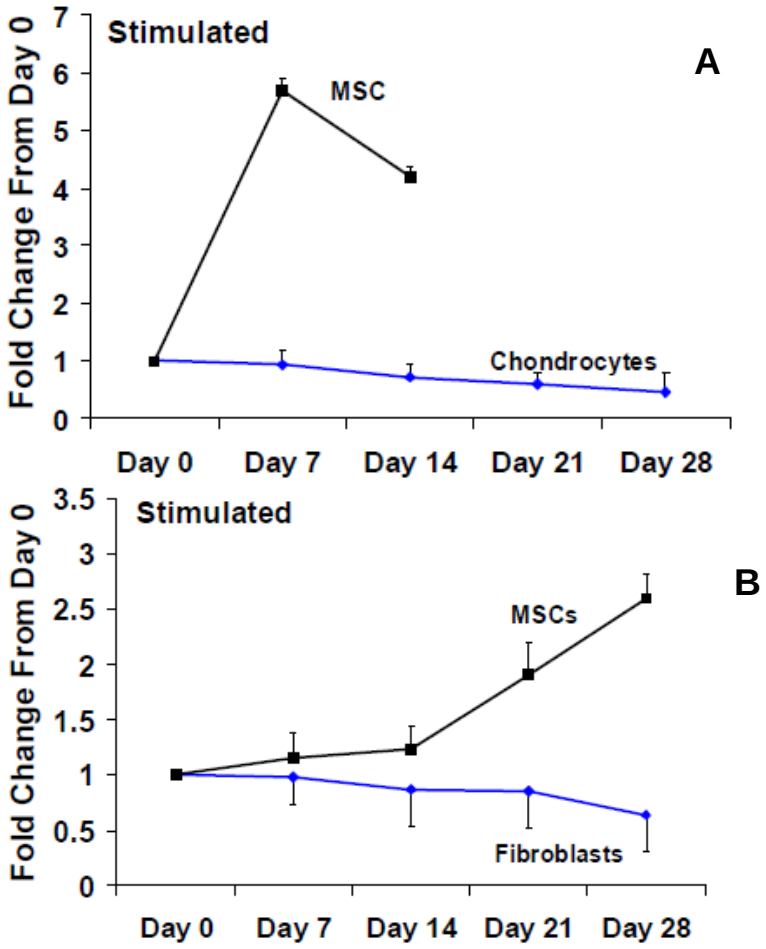

Fig 10.5. A. Chondrocytes respond more slowly than MSCs when stimulated in tension B. Fibroblasts respond more slowly than MSCs when stimulated in compression

MSCs may be a better candidate to create constructs where both Col1 and Col2 gene expressions are desired

Even after 28 days in culture, fibroblasts did not express the Col2 gene (chapter 7) nor did chondrocytes express the Col1 gene (chapter 9). Though the mechanical profile we delivered was the same as the one which upregulated Col1 and Col2 gene expression in MSC-scaffolds (chapters 6 and 8), it might be plausible that the specialized cells require a different mechanical signal and longer time in culture to express the other gene. It is also plausible that these genes were expressed at such low levels that we could not detect the fluorescence. RT-

PCR studies are needed in the future to determine which genes are actually being expressed and their spatial and temporal profiles.

Our results also show that mechanically stimulated TECs containing MSCs can significantly express both Col1 and Col2 after 14 days in culture (chapters 6 and 8) (Fig 10.6). These results have huge implications when one is trying to engineer tissue with both Col1 and Col2 gene expression. Results from our studies suggest that MSCs rather than specialized cells are a suitable cell source if we need to create structures containing both Col1 and Col2 expressing cells.

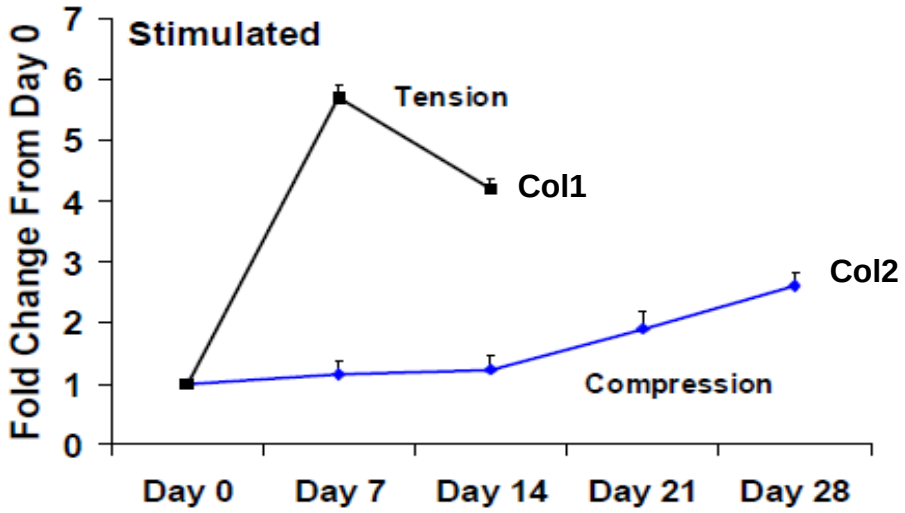

Fig 10.6 Compressive vs tensile response of MSCs in TECs

Our in vitro studies are not without limitations. 1) ***We did not track real-time changes in collagen gene expression in the intact construct***. These structures are currently too thick and opaque to perform such non-destructive imaging. We continue to seek novel imaging technologies to observe such changes in cellular fluorescence that would permit us to perform longitudinal studies on the same constructs over time. 2) ***The fact that we pooled our cells to record fluorescence means that we only know the average effect of dynamic compression and not the specific effects of compression on individual cells***. It has been previously shown that gene expression varies between the core and the annulus region of the hydrogel [389]. In the future, we plan to evaluate gene expression in both regions of the agarose

constructs as well as in the mid and end regions of the collagen constructs. 3) ***Autofluorescence of agarose gel and collagen fragments could increase overall RFU values***. In these studies, we assumed that these changes were constant across groups. 4) ***We did not specifically track changes in the production of proteoglycans like aggrecan in agarose constructs***. Increases in aggrecan content could reflect improvements in a construct's aggregate modulus. In the future we intend to track the production of specific proteoglycans like dermatan sulfate. ***5) We chose to measure only the collagen construct's linear stiffness*** because we were concerned that the lower aspect ratio of 2:1 (less than the ideal ratio of at least 3:1 to avoid St. Venant effects) might alter the structure's failure properties. In the future we are planning to utilize longer constructs to increase the aspect ratio and hence minimize St. Venant effects. ***6) We applied only one stimulus pattern to the constructs***. We still need to systematically vary components of the mechanical signal so as to optimize gene and protein expression as well as construct biomechanics in the shortest possible time interval. We are, however, performing these studies using rabbit MSCs as part of another research project. 7) ***Our plasticity experiments on specialized cells were only performed up to 28 days in culture***. We may need to compressively stimulate our TECs for longer durations to induce Col1 gene expression in chondroyctes as well as Col2 gene expression in fibroblasts.

<u>Technical problems to be overcome in order to perform in vivo studies in the mouse</u>

One of the important findings in the rabbit model is that in-vitro construct stiffness and the in-vivo repair stiffness are positively correlated (302). Such correlations may also exist in the mouse model but in-vivo studies are necessary to establish such correlations. The small size of the mouse not only makes in vivo surgeries a challenge but also makes it difficult to evaluate the effect of implanting tissue engineered constructs to repair defects in the patellar tendon. One of the major problems encountered is securing the tissue engineered constructs in the defect site. While this is achieved with the help of sutures in the rabbit model, such a process will not work in the mouse as the sutures are quite large relative to width and thickness

of the tendon and would create a significant injury to the tissues. Also, simply inserting the constructs in the defect site does not produce a 100% success rate. We conducted preliminary studies in our laboratory where Di-I labeled MSCs were seeded in either KN sponges or ESL-GAG sponges. These TECs were then used to fill the punch defect site in six animals (n=3 for each sponge type). After one week, Di-I labeled cells were seen in the repair sites of only two animals implanted with KN sponges and in none of the repair sites containing the ESL-GAG sponges. These preliminary results strongly emphasize the need to find ways to secure the constructs in the defect site without inducing ancillary effects unrelated to the treatment and to translate our results to larger animals where more reproducible surgery is possible.

Several factors could have influenced the results of the in-vitro plasticity experiments

Three experimental factors could have influenced the results of my in-vitro experiments. These include: 1) animal variability, 2) type of biomaterial, and 3) stimulation profile. I will discuss each of these factors and how they might have affected the outcomes.

Comparing the results of studies using different animals and ages can be difficult. While I was able to control the age of animals from which I harvested fibroblasts and MSCs, I was forced to use newborn rib chondrocytes in my chondrocyte-agarose constructs as the yield of chondrocytes from adult ribs was very low and rather heterogeneous (i.e. they contained fibroblasts). Such differences in age make comparisons of results between MSCs, chondrocytes and fibroblasts difficult. Also I also used cells from different animals in these plasticity experiments once again making comparisons difficult. To minimize such animal effects, future studies should directly compare and contrast the plasticity of cells obtained from the same animal.

Use of different biomaterials in the plasticity experiments made comparisons between studies more challenging. I used agarose for compression studies and collagen for tensile studies. This was because agarose does not resist tension very well and collagen does not resist compression. Use of different biomaterials makes straight-forward comparisons difficult because of the different cell-matrix interactions. It is well known that cell-matrix interactions have a major

influence on the biosynthetic response of cells (466, 489). Use of different biomaterials makes it hard to decouple cell-matrix interaction effects and mechanical stimulation effects while comparing cell compression vs cell tension studies.

In this dissertation I used only one profile for compression and one profile for tension for the in-vitro cell studies. The results of these studies suggest that MSCs are more plastic than specialized cells. The conclusions that I have reached are only valid for the experimental conditions described in the chapters. It is possible that by varying the profiles of mechanical stimulation we might find that specialized cells are more plastic than MSCs and future studies should focus on these issues.

Other factors could have also influenced the results of the plasticity experiments. Observed fluorescence is an indicator of the gene expression of only that promoter to which it is linked. It is important to keep in mind that Col1 and Col2 are under the regulation of many promoters. In our mouse model the topaz and the cyan fluorescent proteins are linked to only one promoter for the Col1 and Col2 genes, respectively. Hence these fluorescent proteins do not indicate the activity of all promoters for Col1 and Col2 genes but only the promoter to which each is linked. These could have played a role in the in-vitro experiments. For example, tensile stimulation could have activated the promoter linked to GFP-T in MSCs, but not the promoter of interest in the case of chondrocytes. This could explain why we did not see GFP-T expressing cells when chondrocytes were stimulated in tension. This could also explain why no ECFP-expressing cells were seen when fibroblasts were exposed to compression.

It is also possible that in chondrocytes exposed to tension, GFP-T was produced but in such low quantities that it was impossible to detect. The same problem could have occurred when fibroblasts were exposed to compressive strains. Hence in the future, real time PCR should be performed in these plasticity experiments of specialized cells to see the levels of Col1 gene expression found in chondrocytes and the levels of Col2 gene expression observed in fibroblasts. We will be initiating these experiments shortly.

Translating results from murine to rabbit models is possible

The main limitation of the mouse model is that we cannot use it to reproducibly test our tissue engineered constructs after surgery. Still this mouse model has its advantages in tissue engineering especially if we take advantage of the genetic tools that are available to perform in vitro studies and then translate the results to larger animal models such as the rabbit where validation of these in vitro studies can be performed in-vivo with ease. Establishing correlations in TEC behavior across these two species might be important if we are to more effectively repair tendon injuries in larger models.

These studies are being performed in our laboratory in order to determine if transgenic and wild type mouse and rabbit cells respond in similar ways to mechanical and chemical signals in culture. For example, tensile stimulation of constructs containing either double transgenic (DT) murine MSCs or rabbit MSCS show similar patterns of increasing Col1 gene expression (307,406). Similarly, compressive stimulation of constructs containing either DT murine chondrocytes or single transgenic rabbit chondrocytes show similar patterns of Col2 gene expression in both species. These results are encouraging and suggest that we can create corresponding animal models and translate our results across species. These comparison studies are ongoing in our laboratory.

To formally link these results across species and unify our mouse and rabbit protocols, we will need to perform similar experiments using cells from both species. Currently differences exist in the way we make and mechanically stimulate mouse and rabbit MSC constructs. These limitations make comparison of results somewhat difficult. Hence following the same protocols for both species becomes important. It should be noted, however, that we may not be able to completely unify these protocols. For example, we already have shown that culturing mouse MSCs requires a special formulation of media unlike that for rabbits. Still, creating conditions as similar as possible across species will make interpretation of our inter-species data easier.

Can chemical stimulation be used to modulate Col1 and Col2 gene expression?

In this dissertation, I chose to mechanically stimualte TECs to modulate Col1 and Col2 gene expression levels in primitive and specialized cells. I could have chosen chemical stimulation (e.g., growth factors) to modulate these genes within TECs. Some of the candidates for chemical stimulation are transforming growth factor beta (TGF-β), basic fibroblast growth factor (b-FGF), insulin-like growth factor (IGF) and bone morphogenetic protein (BMP). TGF- β has been shown to stimulate collagen and glycosaminoglycan synthesis by fibroblasts [276-277]. TGF-β1 induces a dose-dependent increase in the expression of procollagen type I and III mRNA which improves failure load and stiffness in the healing tendons [278]. Investigators have also shown a dose-dependent increase in the number of proliferating cells and the level of expression of type III collagen after in vivo supplementation with b-FGF [496]. This growth factor offers the potential to stimulate the proliferation of fibroblasts in culture and increase the speed of healing of tendon repairs. Another growth factor, insulin-like growth factor (IGF), has been reported to elicit dose-dependent effects on collagen and proteoglycan synthesis in rabbit flexor tendon [497-498]. BMPs have also been shown to increase the expression of Col2 in a variety of cell types. Hence chemical stimulation could conceivably be used in place of or in addtion to mechanical stimulation to modulate Col1 and Col2 genes within TECs.

The important question which naturally arises when comparing these two methods of stimulation is whether chemical stimulation is as effective as mechanical stimulation in altering gene and protein expression as well as biomechanical outcome. With some exceptions, preliminary studies suggest that chemical stimulation may be better than mechanical stimulation when preparing cells for tissue engineering applications. When we exposed DT murine MSC's to BMPs, the cells began to express ECFP by seven days. When these MSC's were mechanically stimulated, however, ECFP expressing cells were only present at fourteen days. Similarly, addition of 10 ng/ml of TGF-β1 to rabbit MSCs led to significantl increases in construct stiffness to levels

produced by the optimized mechanical stimulus [499]. Clearly the effects of chemical stimulation have to be examined further.

But the use of chemical stimulation to modulate genes of interest and to improve the biomechanical properties of constructs has some drawbacks. First and foremost is the cost associated with the use of growth factors as these are are very expensive. We still need to optimize the components of the chemical signals (e.g dosage concentration, frequency and total time of stimulation) and remain concerned about duration of effect and potential adverse reactions should high concentrations of factors be introduced.

Overarching Conclusions and Discussions

Inferior biomechanics of natural healing of patellar tendons even after 6 weeks of healing emphasizes the need for strategies such as FTE to improve and speed the repair process. Normal growth and development data suggests that we should create constructs that mimic both Col1 and Col2 gene expression patterns to recreate zonal insertions. One strategy to create constructs with both Col1 and Col2 gene expressions is to introduce primitive cells such as bone marrow-derived mesenchymal stem cells or MSCs into biological scaffolds and then precondition these MSC-based constructs to produce tendon-like cells in the mid-substance and fibrocartilage-like cells near the attachments. We might accomplish this by using different stimulation methods in the mid-substance vs. the ends. Another possible strategy would be to use specialized cells (fibroblasts or chondroctyes) in different regions of the constructs and then apply mechanical strains to create constructs with tendon-like cells in the mid-substance and fibrocartilage-like cells in the insertions. Examining the plasticity of MSCs vs. chondrocytes and fibroblasts helps us in choosing which of the two strategies mentioned above is more effective. Results from all these studies taken together leads me to conclude the following. Specialized cells (fibroblasts and chondrocytes) respond more rapidly and to a greater extent than MSCs when stimulated with signals that they normally experience in vivo. MSCs respond more rapidly and to a greater extent than specialized cells when constructs containing the latter cells are

stimulated with signals they do not normally experience in vivo. If we want to create a TEC with both Col1 and Col2 expressing cells, then MSCs seem to be a better candidate than using two specialized cell types like chondrocytes and fibroblasts. In total, precursor MSCs are generally more appropriate to use in a tissue application where cells experience multiple types of mechanical stimuli and hence may be more suitable for composite tendon tissue engineering.

The conclusions that I have reached are only valid for the experimental conditions described in the chapters. It is possible that by varying the profiles of mechanical stimulation we might find that specialized cells are more plastic than MSCs. It is also entirely plausible that by changing the biomaterial, we might find that specialized cells are more plastic than MSCs under these new conditions. Further in vitro and in vivo studies must be conducted to answer these questions of plasticity, in-vitro response and in-vivo repair outcome.

Chapter 11

Recommendations

By applying the principles of FTE our group has been successful in using autologous mesenchymal stem cell MSC-collagen constructs to accelerate repair of tendon injuries in the adult rabbit model [51, 150, 257, 500-502]. In spite of early encouraging success, our group faces numerous challenges in tendon tissue engineering. 1) The repair often consists of inferior scar tissue. 2) The zonal insertion of tendon into bone is not restored, making it vulnerable to re-injury. 3) The healing time is too long. 4) Cultured TE constructs can only be used in a load protected regime. 5) The tendon tissue engineering process is iterative with incremental success.

To overcome these challenges we clearly need new strategies. 1) One strategy is to mimic events occurring during growth and development during the tissue culture process. 2) Another strategy is to use either primitive cells and or specialized cells to produce tendon like cells in the mid-substance and fibrocartilage-like cells near the attachments. Our double transgenic mouse system with fluorescent transgenes for Col1 and Col2 becomes very useful as it allows us to investigate the above mentioned strategies quickly and effectively.

Using these transgenic mice, the first part of my dissertation has been focused on understanding Col1 and Col2 gene expression patterns in natural healing and normal development. Using cells from these transgenic mice As well as bioreactors, the second part of my dissertation research has focused on how mechanical signaling of cell-based TECs in culture affects the plasticity of primitive MSCs and specialized chondrocytes and fibroblasts. The results from these studies are presented in chapters 2 through 8. Based on the findings from the studies, this Chapter gives recommendations so that future investigators might improve their understanding of these aspects, a step essential to achieving tendon repair with recreated zonal insertions while decreasing the time post surgery to achieve this desired repair outcome.

This Chapter also describes some of the limitations of this research and ways to overcome these limitations.

11.1. Measurement of Fluorescence in Constructs

11.1.1. Average Effects of Stimulation on Fluorescence

The fact that we pooled our cells to record fluorescence means that we only know the average effect of stimulation. This average effect might mask differences in expression in different regions of the constructs. For example it has been previously shown that gene expression varies in the core and the annulus region of the hydrogel [389]. Future studies should look at the gene expression in the annulus and core regions of compressed hydrogel and also around the posts and mid-regions of the collagen constructs.

This average effect also does not let us determine the specific effects of stimulation on individual cells. Two techniques could be applied to determine the effects on individual cells. One is in-situ hybridization and another one uses laser capture micro dissection (LCM) in conjugation with micro array analysis to quantify local expression. This knowledge about the local gene expression of cells becomes even more powerful if we can couple it with the local stress and strain fields in the neighborhood of these cells. Local stresses and strains in our constructs could be computed using Finite Element Analysis.

11.1.2. Quantification of Fluorescing Cells

We did not quantify the number of fluorescing cells in constructs containing primitive cells. This prevented us from distinguishing whether the increases in fluorescence were due to increased numbers of fluorescing cells and/or increased production of ECFP/GFP-T by already fluorescing cells. Determination of the number of ECFP and GFP-T fluorescing cells becomes even more important when we have constructs with a mixture of ECFP and GFP-T cells. The number of fluorescing cells can be obtained by passing the collagen digests and agarose homogenates through FACS. Future studies should explore the option of utilizing FACS to count the number of fluorescent cells.

11.1.3. Non-destructive Assessment of Constructs

We are currently utilizing non-destructive methods to quantify fluorescence, as these constructs are currently too thick and opaque to perform non-destructive imaging. I strongly recommend that we continue to seek novel imaging technologies to observe such changes in cellular fluorescence that would permit us to perform longitudinal studies on the same constructs over time. This will not only speed up the tissue engineering process, but also reduce cost and the number of animals used in the study.

The other limitation that I faced is to determine when Col1 and Col2 expression levels first became detectable as we only image these constructs at set time points. This limitation can be overcome if we can couple imaging technologies with our existing bioreactor systems.

11.2. Differentiation of Cells in Constructs

One important limitation that our group faces while using rabbit autologous-MSCs is that we cannot determine what if any percentage of MSCs have differentiated into fibroblasts in these constructs. This is because of the lack of cell surface markers in the rabbit model. These surface markers are available in the mouse model. I recommend that the future studies utilize these markers to study the level of differentiation in our cellular constructs as Col1 and Col2 gene expression does not provide information regarding the state of the differentiation of cells. This information is essential to determine if the cells are undergoing differentiation or de-differentiation and therefore their degree of plasticity.

11.3. Experimental Conditions

As mentioned in the discussion chapter three experimental factors could have influenced the results of my in-vitro experiments. These include 1) animal factors, 2) type of biomaterial and 3) stimulation profile. Future studies should be done to either minimize or eliminate these factors. To eliminate animal factors, future studies should compare and contrast plasticity of cells obtained from the same animal. While I was able to control the age of animals from which I harvested fibroblasts and MSCs, I was forced to use newborn rib chondrocytes as the yield of chondrocytes

from adult ribs was very low and heterogeneous. These differences in ages could have influenced the results of the plasticity experiments. It is plausible that chondrocytes from older animals (e.g. 10 week old) might behave differently. It is also plausible that new born fibroblasts and MSCs might behave differently than cells harvested from 10 week old animals. Hence future studies should compare and contrast the response of new born and juvenile cells.

Use of different biomaterials in the plasticity experiments made comparisons between studies more difficult. I strongly suggest that we identify a novel biomaterial that can resist both compression and tension and use that for the plasticity experiments. PLGA seems to be an ideal candidate as investigators have been already been using this material in tendon and ligament repair it can be stimulated in both in tension and compression, it is biocompatible, and it has been shown to enhance cellular attachment, proliferation and matrix production [503-506].

Also I used only one compressive profile and one tensile profile for in-vitro cell studies. The results of these studies suggest that MSCs are more plastic than specialized cells. The conclusions that I have reached are only valid for the experimental conditions described in the chapters. It is possible that by varying the profiles of mechanical stimulation we might find that specialized cells are more plastic than MSCs and future studies should focus on these issues.

11.4. Growth and Development of Knee Structures

We only performed histological studies to map spatial and temporal expression of Col1 and Col2. We did not measure type I and II collagen production. Future studies should try to quantify type I and II collagen proteins and try to correlate these with gene expressions.

Although our double transgenic model should allow us to track changes in Col1 and Col2 expression in near real time, currently we must sacrifice animals and section knees to image the fluorescence. Similar to what I have said in section 10.1.3 we need to identify powerful in-vivo optical technologies that can visualize at the cellular level. With this technology we should be able to image the entire knee with the required magnification and conduct cost- and time-efficient

longitudinal studies that eliminate assignment of animals for gene expression patterns at different ages.

Our growth and development data in the double transgenic (DT) mouse provides an initial, semi-quantitative assay of Col1 and Col2 gene expression during embryonic and early post-natal tendon development. We did not measure type I and II collagen production. Future studies should try to quantify type I and II collagen proteins and try to correlate these with gene expression levels and patterns.

Little information is available regarding the pathways that are required for tendon development, nor its regionalization. We need to identify key agonists/antagonists and signaling pathways in both the tendon mid-substance and insertions. This process can help us to identify the genes truly critical in developing a functional tendon. This will allow us to better understand mechanisms of normal development, which nearly always produce functional tendon, and contrast these with adult natural healing, which most often produce a nonfunctional tendon and cell-based healing which produces a somewhat functional tendon. These studies can be performed by combining LCM with microarray techniques. Future studies should be done to identify these candidate markers and signaling pathways.

11.5. Injury Model in the Mouse Tendon

We could not directly explain our biomechanical results based on gene expression patterns. The improvements we observed in biomechanical properties between 4 and 6 weeks were quite encouraging, but my experimental design did not include an assessment of either gene expression or tissue structure at 6 weeks post injury. Future studies should also perform biomechanics after 1 and 2 weeks of healing and also perform histological studies at 6 weeks of healing.

We only investigated one type of injury i.e. the circular punch defect in the mouse tendon. We tried to create a full length defect in the patellar tendon and did not pursue it further for the following reasons. 1) Our techniques did not produce reproducible injuries. 2) Full length defects

led to rather severe limping of the mouse. 3) It was conveyed to us from Dr. Soslowsky that these defects led to spontaneous rupture. Because of all these reasons, full length defects may not be suitable for the mouse tendon. I suggest that we try half length defects. Such defects can be easily created reproducibly by using a rectangular punch and by modifying the backing which slides under the tendon so as to investigate both soft tissue and insertional healing.

Healing of the mid-substance injury did not induce any ECFP expression which is not surprising. This injury model in my opinion does not utilize the potential of our double transgenic mice, the value of which is in the insertion sites where both Col1 and Col2 is expressed. Hence we should be inducing injuries in the insertions of the tendon. Preliminary studies suggest that Col2 is upregulated during healing in insertions and these might provide clues for recreating zonal insertions. I strongly suggest that we perform healing studies of the insertions in the future.

11.6. Translational of Results from Murine to Rabbit Models

As mentioned in the discussion chapter, establishing correlations in TEC behavior across murine and rabbits might be important if we are to more effectively repair tendon injuries in the animal model. Studies performed in lab show that both transgenic and wild type mouse and rabbit cells behave in the same way. For example, tensile stimulation of MSCs increases Col1 gene expression in both species. Also compressive stimulation of chondrocytes increases Col2 gene expression in both species. These results are encouraging and suggest that we can translate results across species.

We need to perform similar experiments in both species and to make the translational of results easy we need to unify our mouse and rabbits protocols. Currently differences exist in the way we make and stimulate mouse and rabbit MSC constructs and these limitations make comparison of results difficult. Hence following the same protocols for both species become important.

11.7. In-vitro Testing System of Tensile Constructs

Our in-vitro mechanical testing protocol and the testing system itself should also be improved. Gripping issues lead to the low aspect ratio (≈ 2:1) of our constructs resulting in biaxial strains near the grips. It is not surprising, therefore, that failures frequently initiated near the grips due to these elevated stress values. These grip failures likely altered failure mechanisms and thus failure biomechanical properties due to St. Venant's effects [507]. Therefore future studies should explore methods to eliminate these end effects, such as cutting a "dog-bone" shaped test specimen.

The biomechanical properties of the constructs could also have been affected by the friction in the mechanical testing system. Although the magnitude of the friction was kept very low, the error produced by this friction could have been substantial due to very small forces acting on the construct. We have explored the possibilities of using vertical systems and found that these systems have very little friction. Therefore, I recommend that future studies utilize this testing system to test the tensile properties of constructs.

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