AN *IN VITRO* STUDY OF CELLULAR AND MOLECULAR MECHANISMS OF LIGAMENT SCARRING

by

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When ligaments such as the medial collateral ligament (MCL) are injured, they generally heal but form scar tissue, which is composed of a disorganized collagen matrix that is over-produced by fibroblasts. Scar tissue has inferior structural and mechanical properties, which can lead to joint instability. Excessive fibroblast contraction is thought to contribute to tissue scarring. Previous studies have shown that both TGF-β1 and TGF-β3 increase fibroblast contraction and collagen synthesis. However, TGF-β1 enhances scar tissue formation whereas TGF-β3 actually reduces it. In addition, both TGF-β isoforms have been found to increase the expression of α-SMA, which correlates with increased fibroblast contractility. An increase in tension at the wound site has also been found to increase α-SMA protein levels. Therefore these factors are all important in the wound healing process. The overall objective of this thesis research was to investigate cellular and molecular mechanisms that affect scar tissue formation in healing ligaments. Contraction forces, collagen synthesis, and α-SMA protein expression of healing and normal MCL fibroblasts in response to treatment with TGF-β1, TGF-β3, and collagen gel tension were investigated. A novel culture force monitor (CFM) system was used to quantify forces of fibroblast contraction. It was found that healing MCL fibroblasts produced

greater contractile forces and higher levels of collagen synthesis than normal MCL fibroblasts. In addition, treatment with TGF-β1 or TGF-β3 increased contraction forces in healing fibroblasts compared to untreated controls, with TGF-β1 consistently producing greater contraction forces than treatment with TGF-β3. TGF-β1 and TGF-β3 also induced higher levels of α-SMA protein expression compared to untreated fibroblasts. Consistent with the contraction forces, fibroblasts treated with TGF-β1 expressed higher levels of α-SMA protein than those treated with TGF-β3. Further, it was found that when tension in gels embedded with normal MCL fibroblasts was released, expression of α-SMA protein also decreased. Thus, this study showed that healing and normal fibroblasts have differential contractile and collagen synthesis abilities. The results of this study showed that the presence of TGF-β1, TGF-β3, and tension in the matrix should be regulated to improve ligament healing. Decreasing the ratio of TGF-β1 to TGF-β3 in an injured ligament may decrease fibroblast contraction and thus reduce scar formation in healing MCLs. Finally, reducing tension levels in healing ligaments and hence down-regulating α-SMA protein expression may also decrease ligament scarring.

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NOMENCLATURE

α-SMA	Alpha-Smooth Muscle Actin
CFM	Culture Force Monitor
DCFM	Dynamic Culture Force Monitor
DMEM	Dulbecco's Modified Eagle Medium
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FPCG	Fibroblast Populated Collagen Gel
MCL	Medial Collateral Ligament
MPER	Mammalian Protein Extraction Reagent
PIP	Carboxy-terminal peptide
TGF-β	Transforming Growth Factor-β

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1.0 INTRODUCTION

Many sports injuries involve the rupture of the medial collateral ligament (MCL) [1], which plays an essential role in stability of the knee [2, 3]. Although treatment of an injured MCL is most often conservative [4, 5], i.e. non-surgical with a period of immobilization, the ligament tends to heal with formation of scar tissue [3, 6], which can cause inferior structural and mechanical properties [2, 3]. While a normal ligament has fibroblasts that are aligned with collagen fibers along the longitudinal axis of the ligament, the healed ligament has a disorganized and overproduced collagen matrix, which is characteristic of scar tissue [7]. Therefore, in order to better understand the cellular and molecular mechanisms involved in the wound healing process, it is important to first investigate the differences between cells from a healing environment and cells from a normal environment.

In addition to collagen synthesis, excessive contractile forces are thought to contribute to the formation of scar tissue [8, 9]. Growth factor isoforms transforming growth factor (TGF)-β1 and TGF-β3 have been shown to increase cell contraction and collagen synthesis [10-13]. However, in rat skin wounds, exogenous addition of TGF-β3 was found to decrease scar formation, whereas TGF-β1 increased the formation of scar tissue [14]. In addition, TGF-β1 and TGF-β3 both increase the expression of α-smooth muscle actin (SMA), which correlates to an increase in fibroblast contraction [15-18]. Further, tension in the matrix also regulates α-SMA expression,

with increased tension increasing α-SMA expression [19, 20]. Therefore, quantifying the differences between fibroblasts in a healing ligament to those from an uninjured ligament may help to elucidate mechanisms involved in ligament scarring. Regulating the ratio of TGF-β1 to TGF-β3 or altering the tension in a healing matrix may help to decrease scar tissue formation and therefore, improve the function of injured ligaments. Thus, the overall goal of this thesis was to investigate the cellular and molecular mechanisms involved in scar formation in healing ligaments. To this end, we first looked into the possible differences in function, i.e. contraction and collagen synthesis, between healing and normal MCL fibroblasts and then investigated the regulation of α-SMA and cell contraction by TGF-β1, TGF-β3, and gel tension.

This thesis is organized as follows. The next chapter (Chapter 2) is a review of previous studies and literature on the following topics: tissue wound healing, transforming growth factor-β, α-smooth muscle actin, and tension vs. α-SMA. Chapter 3 discusses the specific aims of this thesis and provides a hypothesis from each aim. The subsequent chapters discuss each aim in further detail. Chapter 4 investigates the differences in contraction and collagen synthesis between fibroblasts from a healing MCL and those from a normal, uninjured MCL. In chapter 5, the effects of TGF-β1 and TGF-β3 on fibroblast contraction and α-SMA protein expression of healing fibroblasts were investigated. Chapter 6 focuses on the effect of relaxation of tensioned collagen gels seeded with normal MCL fibroblasts on α-SMA expression. Finally, chapter 7 provides a summary of the findings from this thesis study and discusses future research directions.

2.0 LITERATURE REVIEW

2.1 TISSUE WOUND HEALING

Injuries to the MCL have been shown to heal without surgical intervention [4, 5]. However, the healed ligament has inferior mechanical properties in comparison to uninjured tissue, even at one year post-injury [2, 3, 21]. The altered structural composition of the scar tissue that forms after injury hinders the ligament from functioning as a normal ligament does. Injured MCLs have been shown to have a cross-sectional size almost twice that of the sham control [2, 22] as well as greater laxity in the ligament [2, 3], which leads to an increase in rotation of the knee [4]. Thus, scarring of the injured MCL causes impaired function of the ligament.

Scar tissue is thought to form from excessive collagen production and contraction at the wound site [7, 8, 23]. While some contraction is necessary for wound closure, excessive contraction may contribute to the inferior quality of the healed ligament. Studies have found that, unlike adult wounds, fetal wounds tend to heal without scar formation [8, 24-26]. This differential healing process may be caused by the different levels of growth factors, such as TGF-β1 and TGF-β3, found in fetal wounds as compared to adult wounds [24, 27-29]. TGF-β1 and TGF-β3 have both been found to increase cell contraction and collagen synthesis [10-13]. However, in rat skin wounds, TGF-β3 decreased scar formation, whereas TGF-β1 actually

increased it [14]. Interestingly, both TGF-β1 and TGF-β3 increase α-SMA protein expression in fibroblasts as well [15-18]. Increased contraction of fibroblasts has been attributed to an increase in α-SMA expression [18, 30]. Expression of α-SMA is also increased by increased tension at the wound site [19], such as during wound closure [16]. This increase in α-SMA expression may signify the presence of myofibroblasts in the healing environment, which are specialized cells that are known to produce increased contractile forces in the healing environment [31]. Therefore, regulating the factors that increase α-SMA, such as TGF-β1, TGF-β3, and tension in the matrix, may decrease contraction forces, in the healing environment and help to restore the biochemical and biomechanical properties of an uninjured ligament.

2.2 TRANSFORMING GROWTH FACTOR-β (TGF- β)

In general, there are four distinct stages in the healing process: inflammation, formation of granulation tissue, deposition of new extracellular matrix (ECM), and remodeling. During the inflammatory stage, neutrophils and macrophages invade the wound area and remove cellular debris and pathogens from the injured tissue [32]. Following this stage, platelets and other immunological cells begin to secrete TGF-β [29, 33], which stops the inflammatory response. This growth factor stimulates fibroblasts to produce collagens [10, 13, 33] and induces fibroblast contraction, which are both necessary for wound healing and closure.

As mentioned previously, the transforming growth factor (TGF)-β family has been implicated in the wound healing process [18]. TGF-β has five known isoforms, the active forms of which are disulfide-linked homodimers. Consisting of 390 amino acids, TGF-β1 maps to

human chromosome 19q13 (www.grt.kyushu-u.ac.jp/spad/account/ligand/tgf-beta.html). Activation of TGF-β1 occurs through TGF-β receptors I and II, which are transmembrane serine/threonine kinases [34-36]. Smad proteins 3, 6, and 7 may all be important in the regulation of receptors I and II. Using microarray analysis, it was shown that 95% of the gene targets of TGF-β are dependent on Smad3 [37]. The same study demonstrated that embryonic fibroblasts from mice lacking the expression of Smad3 did not contract with the addition of TGF-β1, while the same cells from mice expressing this protein were contractile [37]. In the presence of TGF-β itself, Smad6 and Smad7 inhibited signals from TGF-β receptors, giving them a regulatory control over production and secretion of this growth factor [36]. Although TGF-β1 is found largely in platelets, it can also be produced by other types of cells, such as activated macrophages, neutrophils, fibroblasts, and keratinocytes [35].

Another isoform of TGF-β is TGF-β3, which was characterized much after TGF-β1 in the late 1980s. The human form of TGF-β3 is a polypeptide that is 410 amino acids in length [38] with a highly conserved C-terminal region [39]. Although there is a lot of sequence homology between TGF-β1 and TGF-β3, two of the major differences are the number of potential N-glycosylation sites and the number of cysteine residues [38, 39]. While TGF-β1 has only three N-glycosylation sites, TGF-β3 has four. In addition, TGF-β3 has a total of five cysteine residues, which is one more than TGF-β1 [38]. These two isoforms are also differentially expressed in tissues in the body. Adult wounds contain greater amounts of TGF-β1 compared to TGF-β3, whereas fetal wounds have a higher ratio of TGF-β3 to TGF-β1 [24, 27-29].

TGF-β1 plays a large role in wound healing. It is secreted by platelets at the injury site and aids in extracellular matrix deposition among other things [34, 36]. Over-expression of TGF-β1 can result in tissue fibrosis, not only in the skin, but also in the liver and kidney [36]. In particular, TGF-β1 increases cellular contraction, as well as collagen synthesis in fibroblasts [11-13], both of which contribute to the formation of scar tissue [7-9]. TGF-β1 increases fibroblast contraction in a dosage-dependent manner, but exceedingly high concentrations induce lower increases in contraction [11].

Furthermore, TGF-β1 has also been found to increase α-SMA expression [15-17, 40], which is a marker of myofibroblasts [17, 19, 30, 41]. This growth factor further promotes fibroblast differentiation by increasing the presence of other structural features of myofibroblasts, such as stress fibers, fibronexus adhesion complexes, and fibronectin fibrils [42]. Interestingly, while TGF-β1 is present in abundance in adult tissues, only small amounts are present in fetal tissue [27-29, 43, 44]. Contrastingly, TGF-β3 is much more abundant in fetal tissues than in adult tissues, which may largely contribute~~s~~ to the differential scar formation that occurs between fetal and adult wounds. TGF-β1 has been shown to increase scar formation in rat skin wounds and anti-TGF-β1 agents have been shown to improve healing by decreasing scar formation [14, 45]. Further, in a rabbit model, scarring was decreased in wounded eyes treated with TGF-β1 antisense oligonucleotides [46]. Therefore, regulation of TGF-β1 has been shown to improve healing in many different wound healing models.

Functionally similar to TGF-β1, TGF-β3 has also been found to affect biological functions of cells, such as cell contraction and collagen synthesis [10, 11, 18, 29, 47]. Interestingly, studies

investigating the potency of TGF-β3 on cell contraction in collagen gels differ in their results. Some studies show that TGF-β3 induces greater contractile ability in AKR-2B fibroblasts, fetal and adult skin fibroblasts, as compared to TGF-β1 [18, 47] while others show that TGF-β3 is less potent than TGF-β1 in adult skin and patellar tendon fibroblasts [11, 12]. TGF-β3 also affects α-SMA expression in both adult and fetal skin fibroblasts [18]. Moulin et al. found that without any growth factor treatment, α-SMA expression was higher in fetal fibroblasts as compared to adult fibroblasts [18]. However, after treatment with TGF-β3, there was a significant increase in the expression of α-SMA in adult skin fibroblasts but not in fetal skin fibroblasts. Further, fetal wounds heal without scar formation [8, 24-26]. In addition, in rat skin wounds, exogenous addition of TGF-β3 or neutralization of TGF-β1 actually decreased the formation of scar tissue [14, 48]. Therefore, treatment with TGF-β3 may increase levels of fibroblast contraction and α-SMA expression to those essential for proper wound healing, without scar formation.

The findings of these studies suggest that TGF-β1 and TGF-β3 are both important in the wound healing process. Both TGF-β1 and TGF-β3 have been found to affect many biological functions of cells, such as cell contraction and α-SMA expression. Although these isoforms have similar functions, they have a differential effect on scar formation in wounded skin. Therefore, to reduce scarring in injured ligaments such as MCLs, TGF-β1 and TGF-β3 may need to be differentially regulated.

2.3 α-SMOOTH MUSCLE ACTIN (α-SMA)

Expression of α-SMA is an important factor in the wound healing process. This protein appears as stress fibers in fibroblasts and often results in increased contractile forces of the cells [31]. α-SMA consists of 19 amino acids and has a molecular weight of 42 kDa [49]. Similar to α-SMA, β-actin is another actin isoform, which is expressed in all eukaryotic cells and has the same molecular weight. However, β-actin expression is generally constant as it is essential for cell functions, such as cell motility and growth [50].

α-SMA is a specific marker of specialized cells called myofibroblasts [17, 19, 30, 41]. Myofibroblasts are hybrid cells with properties of both fibroblasts and smooth muscle cells [8, 30, 31]. Many studies have shown that myofibroblasts are only transiently present in wound sites during wound healing [30, 40, 41, 51], i.e. they disappear once the wound is closed and scar tissue is formed. For example, a previous study showed that myofibroblasts were not present at the beginning of the healing process [30]. However, by day 14, they were abundant and by day 30 had disappeared again. Further, in fetal sheep skin tissue, an excisional wound healed without scar formation when α-SMA positive cells were absent [24]. However, when α-SMA positive cells began to appear, there was an increase in both cell contraction and scar formation.

The expression of α-SMA is regulated by many factors, such as growth factors, i.e. TGF-β, and tension on tissues. Growth factor isoforms TGF-β1 and TGF-β3 have been found to increase protein expression of α-SMA [15-18, 40, 52], as was discussed in the **Transforming Growth Factor-β** section. In addition, it was found that in rats where α-SMA protein expression had

been inhibited by vanadate, skin wounds healed with marked improvement of scarring [53]. There was no evidence of α-SMA at the wound site and the collagen fibers were neatly organized in the healed area, i.e. no scar formation. Thus, the presence of α-SMA may hinder proper healing.

2.4 TENSION VS. α-SMA EXPRESSION

As with α-SMA expression, the presence of tension in healing tissue also has a significant impact on the healing process. It can alter the expression of many proteins in the matrix, which can thereby affect the repair or regeneration of the wound tissue. *In vitro*, tension in a collagen matrix embedded with cells can be produced by anchoring collagen gels at the sides or bottom. This model mimics the wound healing environment much better than free floating collagen gels because wound closure creates tension in the healing matrix. In comparing this stressed model (e.g., anchored collagen gel) to unstressed models (e.g., floating collagen gel), several studies have found differential protein expression. Skin fibroblasts showed higher total protein synthesis, in particular elevated collagen expression, in stressed collagen gels [54]. Tension can be induced in matrices both intracellularly and extracellularly. When cells contract, they move through the intertwined collagen matrix and elongate themselves [55], creating intracellular tension. Extracellular tension can be added to the matrix by applying forces to the matrix. It was found that in matrices with high tension, i.e. collagen coated plastic, α-SMA protein expression of gingival fibroblasts was increased about 9 fold compared to matrices without tension, i.e. free

floating collagen gels [19]. Further, when mechanical tension was induced in a skin wound by splinting the wound, α-SMA protein expression was increased by 420% [20].

Tension in the matrix has also been linked to the differentiation of fibroblasts into myofibroblasts [19, 20, 56]. The increase in α-SMA caused by increased tension may contribute to this differentiation. It has been shown that tension induces α-SMA expression in rat skin wounds [20]. However, when tension was released from rat skin wounds, expression of α-SMA and ED-A fibronectin, another marker for myofibroblasts, decreased [20]. In cardiac fibroblasts, cells with low basal levels of α-SMA showed up to a 2 fold increase in α-SMA protein expression with the application of an extracellular force [57]. Thus, both *in vivo* and *in vitro*, tension was found to affect the level of α-SMA expression and myofibroblast differentiation. Therefore, regulation of tension in the matrix may reduce scar formation in healing tissues such as ligaments.

3.0 SPECIFIC AIMS OF THESIS

Although the MCL tends to heal, it does so with the formation of scar tissue [3, 6]. Because of its inferior mechanical properties, scar tissue in the healing MCL can result in a decrease in the stability of the knee joint due to increased laxity [2, 3]. Therefore, it is necessary to reduce scarring in healing MCLs by regulating those factors that contribute to the formation of scar tissue, including excessive fibroblast contraction and collagen production [7-9]. Differentiation of fibroblasts into myofibroblasts at the wound site may also contribute to inferior healing, by increasing contractile forces and scar formation [53]. Myofibroblasts are known to increase contraction at the wound site [20], and are marked by the presence of α-SMA [17, 19, 30, 41, 52]. TGF-β1 and TGF-β3 have both been found to regulate fibroblast contraction and collagen synthesis, as well as α-SMA expression *in vitro* in fibroblasts. Interestingly, TGF-β3 has been found to decrease scar formation in rat skin wounds, whereas TGF-β1 increases scarring [14]. The application of tension to skin wounds by splinting the wound also increases α-SMA expression [20], which may indicate an increase in the presence of myofibroblasts. Although many studies have investigated the regulation of growth factors and tension in skin wounds, few have investigated their role in ligament healing. Therefore, the overall objective of this study was to investigate the cellular and molecular mechanisms involved in ligament healing that may contribute to scar tissue formation.

3.1 SPECIFIC AIM 1

To determine the differential contraction and collagen synthesis of healing vs. normal rat MCL fibroblasts. While normal fibroblasts simply maintain the matrix, healing fibroblasts need to repair and regenerate the matrix of injured tissue. Fibroblast contraction and collagen synthesis are both necessary to regenerate the matrix in healing ligaments. Contraction and collagen synthesis are up-regulated in keloid skin fibroblasts compared to normal fibroblasts [58, 59]. Therefore, it is likely that healing ligament fibroblasts also produce higher levels of contraction and more collagen than normal fibroblasts.

Hypothesis 1: Healing fibroblasts generate larger contraction forces and produce more collagen type I than normal fibroblasts.

3.2 SPECIFIC AIM 2

To determine the effects of TGF-β1 and TGF-β3 on fibroblast contraction and α-SMA expression of healing fibroblasts. TGF-β1 and TGF-β3 have both been found to increase fibroblast contraction and α-SMA protein expression *in vitro* [11, 12, 15, 18, 42]. However, in skin and tendon fibroblasts, TGF-β1 increases contraction to a greater degree than TGF-β3 [11, 12]. TGF-β1 mRNA levels were also greater in hypertrophic scar fibroblasts as compared to normal dermal fibroblasts [60]. Further, TGF-β3 has been found to reduce scarring in skin wounds, whereas TGF-β1 increases it [14]. Therefore, TGF-β1 and TGF-β3 differentially regulate fibroblast contraction and α-SMA protein expression.

Hypothesis 2a: TGF-β1 and TGF-β3 induce greater contraction forces and greater α-SMA protein expression in healing fibroblasts compared to untreated fibroblasts.

Hypothesis 2b: TGF-β1 induces greater contraction forces and α-SMA protein expression in healing fibroblasts than TGF-β3.

3.3 SPECIFIC AIM 3

To determine the effect of gel tension on α-SMA protein expression in normal rat MCL fibroblasts. Previous studies have shown that fibroblasts in matrices under greater extracellular tension expressed higher α-SMA protein levels [19]. Further, tensioning skin wounds by adding a splint under them induced greater α-SMA protein expression than their released counterparts [20]. Transiently expressed during tissue wound healing [30, 41], α-SMA protein is a marker of myofibroblasts, which ~~and~~ are known to produce increased contractile forces [20]. Therefore, decreasing the level of tension in the matrix may also decrease α-SMA protein expression.

Hypothesis 3: Reducing tension in fibroblast populated collagen gels (FPCGs) decreases α-SMA protein expression.

This thesis first investigated the differences in contraction and collagen synthesis between healing and normal ligament fibroblasts and then looked into the effects of TGF-β1, TGF-β3, and tension on these cells. Both excessive fibroblast contraction and collagen synthesis are major factors in the formation of scar tissue. Tension in the matrix, TGF-β1, and TGF-β3 are known to increase expression of α-SMA, which may contribute to the increase in fibroblast contractility during healing. The findings of this thesis will aid in better understanding the cellular and molecular mechanisms of tissue scarring.

4.0 CONTRACTION AND COLLAGEN SYNTHESIS OF HEALING VS NORMAL RAT MCL FIBROBLASTS

4.1 ABSTRACT

Fibroblasts from a healing ligament must have differential expression from those in an intact ligament, because they must be able to regenerate the wounded matrix around them caused by injury. The purpose of this study was to investigate the differences in contraction forces and collagen synthesis between fibroblasts from a healing ligament and those from an intact/normal ligament, since both excessive contraction and increased collagen synthesis have been implicated in the formation of scar tissue. The formation of scar tissue at the site of injury in a ligament may contribute to the inferior structural and mechanical properties of the injured ligament. When seeded in collagen gels, healing fibroblasts were found to generate 1.6 and 1.7 fold greater contraction forces at 15 and 20 hours, respectively, compared to normal fibroblasts. Further, healing fibroblasts produced 10% more type I collagen than normal fibroblasts. Taken together, these results show that healing fibroblasts have a different phenotype than normal fibroblasts. This study also highlights the importance of using healing fibroblasts to study the cellular and molecular mechanisms of ligament healing.

4.2 INTRODUCTION

The MCL is injured frequently during sports activities [1]. Although it has been found to heal sufficiently with conservative treatment, i.e. without surgery but with immobilization of the knee [4, 5], the healing process results in formation of scar tissue [3, 6]. Excessive collagen production as well as excessive contraction forces exerted by cells at the wound site both contribute to this scar formation [7, 8]. The increased contraction and collagen synthesis cause the matrix at the wound site of an injured MCL to be highly disorganized and the fibroblasts do not align along any one direction [6]. While fibroblasts in the healing process repair and remodel the wounded tissue, those from normal tissue simply maintain the matrix around them. Therefore, healing and normal fibroblasts may exhibit differences in function, such as contraction and collagen synthesis. Identifying the differences between healing and normal fibroblasts responsible for these different functions can provide insight into the cellular and molecular mechanisms of tissue wound healing, and therefore aid in devising new therapeutic strategies to enhance the quality of healing tissues.

Previous studies have shown that cells from a healing environment produce increased amounts of collagen and TGF-β1 [59, 61, 62]. In a study comparing peripheral blood mononuclear cells (PBMCs) from burn patients to those from normal individuals, it was found that the cells from the burn patients produced greater amounts of collagen [62]. Similarly, fibroblasts from hypertrophic scars secreted more type I collagen than normal dermal fibroblasts [60]. Keloid fibroblasts also secreted a significantly larger amount of TGF-β1 compared to

normal skin fibroblasts [61]. Healing fibroblasts need to contract more than normal cells in order to close the wound quickly as well as produce more collagen for the formation of the healed tissue. TGF-β1 has been found to regulate both collagen production as well as contraction forces of fibroblasts [11-13, 63]. Therefore, a greater presence of TGF-β1 at the wound site may largely contribute to the formation of scar tissue.

Although some studies have been done to investigate the biological differences between healing and normal fibroblasts in skin wounds [59-62], few studies have been done to compare these differences in ligaments such as the MCL. These differences are especially important to explore in ligaments in order to restore the original function of the ligament. Thus, this study had two objectives: the first was to determine the functional differences in terms of contraction forces between healing and normal MCL fibroblasts; and the second objective was to determine the difference in collagen synthesis between healing and normal MCL fibroblasts. Studying the differences between normal and healing MCL fibroblasts as this study proposes would allow for the identification of the cellular mechanisms that may contribute to scar tissue formation during the healing process.

4.3 MATERIALS AND METHODS

In the right knee of eight rats, a 2-mm gap injury was created in the MCL (IACUC #0210947). In the contralateral leg, a sham surgery was done, which involved creating a skin incision, gently elevating the MCL, and finally closing the skin incision with sutures. The sham surgery allowed

us to take into account any differences that may have been due to the surgery instead of the injury itself. After 10 days, the rats were sacrificed, and "healing MCLs" from the right knees of the rats and "normal MCLs" from the left knees of the rats were harvested. The granulation tissue area of the injured MCLs was clearly marked by the differing matrix composition; the area was pinker in color and also much thicker than the rest of the ligament. Only this area of the injured MCL and the 2 mm midsection of the sham-operated MCL were excised for culture experiments. The reason for choosing 10 days is that the healing tissue is in the proliferation phase and fibroblasts are abundant [64].

To obtain fibroblasts from the MCLs, each MCL was washed twice with PBS, cut into small pieces in aseptic conditions, and then placed in a small Petri dish with 2 mL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S; Invitrogen). Cells from the MCL tissues grew out onto the Petri dishes and were then sub-cultured. The cells were grown as monolayer cultures in DMEM supplemented with 10% FBS and 1% P/S on plastic tissue culture dishes in an atmosphere of 5% CO_2 and 100% humidity at 37^{o}C. Note that cells from the injured MCLs were considered as "healing" fibroblasts, whereas those from sham-operated MCLs were referred to as "normal" fibroblasts. Also, note that healing and normal fibroblasts of the same passage (either 3 or 4) and from the same rat were compared for each individual experiment in this study. Fibroblasts of low passage numbers were used in order to maintain the difference in phenotype.

Fibroblast contraction was measured using a multi-station culture force monitor (CFM) system that was developed previously in our laboratory [65]. Briefly, semi-conductor strain

gauges were attached to each of four cantilever beams. A power supply provided 4 V of excitation to the strain gauges. A Labview program tracked the voltage of each cantilever beam through a data acquisition card. Each beam was calibrated by adding pieces of wire with known weights and recording the change in voltage. These weights were converted into force measurements and a linear trendline was drawn using MS Excel. The accuracy of the CFM system is 0.1 dynes.

To measure cell contraction forces, fibroblast populated collagen gels (FPCGs) were attached to the CFM system. A collagen solution was made from the combination of 0.1 M NaOH, 10x PBS, and collagen type I (Cohesion Technologies Inc.) in a ratio of 1:1:8. Fibroblasts were trypsinized from Petri dishes, centrifuged, and resuspended in growth medium. For each MCL FPCG, 1.5 x 10^6 cells were incorporated into 3 mL of collagen solution with growth medium. The FPCG solution was pipetted into a silicone dish of dimensions 9 cm x 3 cm x 1 cm with a porous vyon bar at each end and allowed to polymerize for about one hour at 37°C. The FPCG solution attached to the vyon bars as it polymerized. Following incubation, 7 mL of growth medium was added to each silicone dish. The vyon bar at one end was connected to the silicone dish and thus was stationary, while the vyon bar at the other end connected to the cantilever beam using a wire connection. After the FPCGs were attached to the CFM system, contraction forces were monitored for up to 48 hours. Every 10 min, 100 data points were collected in a 10 sec period using the Labview program. These 100 data points were averaged and the average value was used as the force measurement for that time.

To promote collagen synthesis in the FPCGs, ascorbic acid, at a concentration of 0.06 mg/mL, was added to the 7 mL of medium around each FPCG. Samples of medium were collected during the contraction experiments at 20 hours to measure levels of collagen synthesis using the Procollagen Type I C-Peptide EIA Kit (Takara, Japan), which assesses collagen production by a standard sandwich ELISA method for procollagen type I carboxy-terminal peptide (PIP). The detection of collagen synthesis using this method was first reported by Taubman et al., using polyclonal antibodies [66]. Briefly, in a 96-well plate that was pre-coated with a monoclonal antibody, an antibody-peroxidase conjugate solution, which is a secondary monoclonal antibody, was added to the appropriate wells. Either standard or sample was added to each well containing conjugate solution within 5 minutes. After 3 hours of incubation at 37°C, the sample solution was removed, and the wells were washed 4 times with PBS. Next, a Substrate Solution was added to each well, and the plate was incubated at room temperature. After 15 minutes, a Stop Solution of 1 M H_2SO_4 was mixed into each well. The color intensity of the solution in each well was proportional to the amount of PIP in the sample. The absorbance of the samples was read on a plate reader at 450 nm.

To ensure that the differences in contraction forces were not due to differences in cell numbers, an MTT Assay was performed on FPCGs in 6-well plates to assess cell viability. This assay measures the mitochondrial activity of cells to assess cell growth. Cells were mixed with a collagen solution as mentioned previously and plated in 6-well plates at a cell number of 10 x 10^4 cells per well overnight. To each well, 200 µL of MTT (tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/mL) was added and the plate was incubated for 3 hours at 37°C. The plate was then centrifuged at 1900 RPM for 5 min.

The supernatant was extracted, and the insoluble product was dissolved with the addition of 1 mL extraction buffer (15% w/v Lauryl Sulfate (SDS), 44% v/v Dimethyl Formamide (DMF), 41% v/v H_2O). The plate was incubated overnight at 37°C and absorbance of the samples was read at 550 nm. Cell viability was measured at 24 and 48 hours.

An unpaired *t*-test was used to determine whether a statistical difference existed in contraction forces between healing and normal fibroblasts. A paired *t*-test was used to determine whether there was a significant difference in collagen synthesis between healing and normal fibroblasts. In both analyses, the significance level was set at 0.05.

4.4 RESULTS

When grown in tissue culture dishes, healing and normal MCL fibroblasts were seen to have a similar morphology **(Fig 1)**.

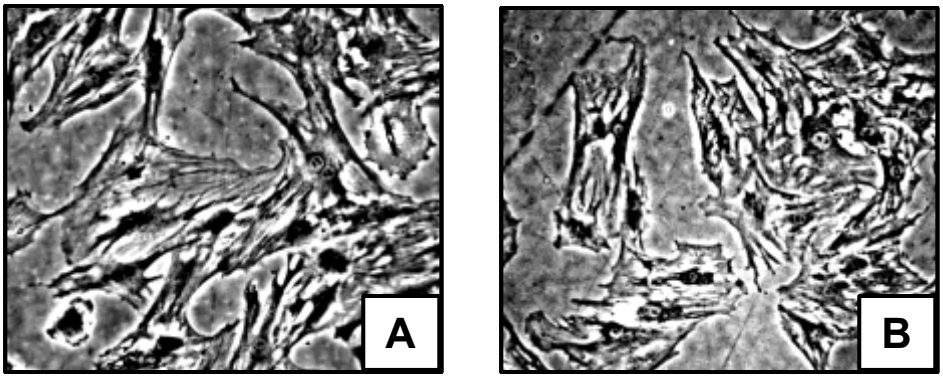


Figure 1 The morphology of healing (**A**) and normal (**B**) fibroblasts was similar. They look similar in culture under the same experimental conditions.

Using the CFM system, healing fibroblasts were found to have larger contraction forces than their normal counterparts. Tracking the forces every 10 min, healing fibroblasts were seen to reach maximum contraction forces around 25 hours **(Fig 2)**. Although it appears that the contraction forces decrease after reaching this maximum force, we believe that this occurs because we do not replace the medium around the FPCGs during the course of the experiment. In comparing contraction forces between healing and normal fibroblasts every hour, it was found that healing fibroblasts produce a significantly greater force of contraction than normal fibroblasts at 15 hours of contraction **(Fig 3)** ($p<0.05$). The differences in contraction forces remain significant as is evident at 20 hours of contraction. Specifically, the healing fibroblasts showed 1.58 and 1.69 times greater contraction forces than their normal counterparts at 15 and 20 hours.

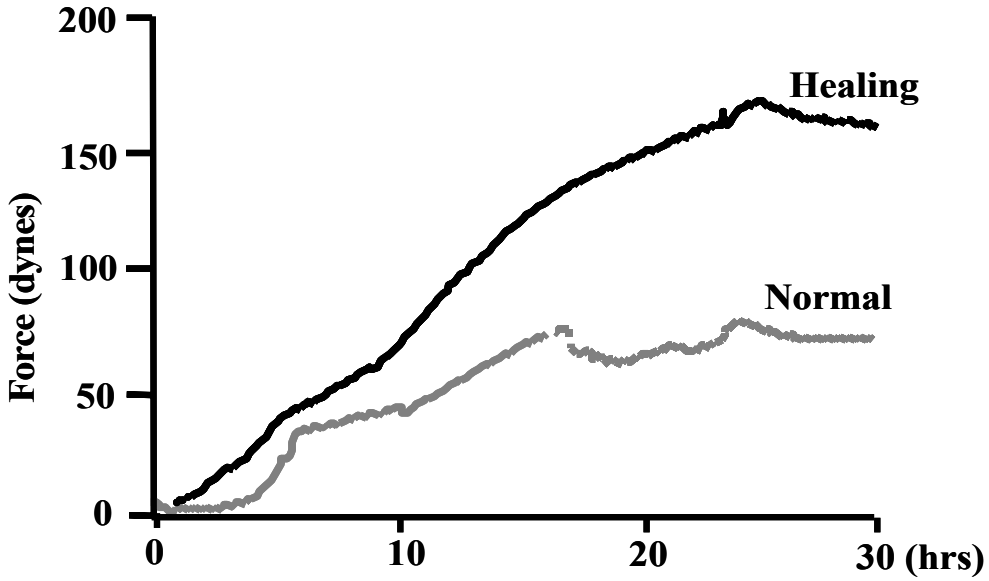


Figure 2 Fibroblast contraction tracked from the CFM system. Healing fibroblasts produced a greater contraction than normal fibroblasts.

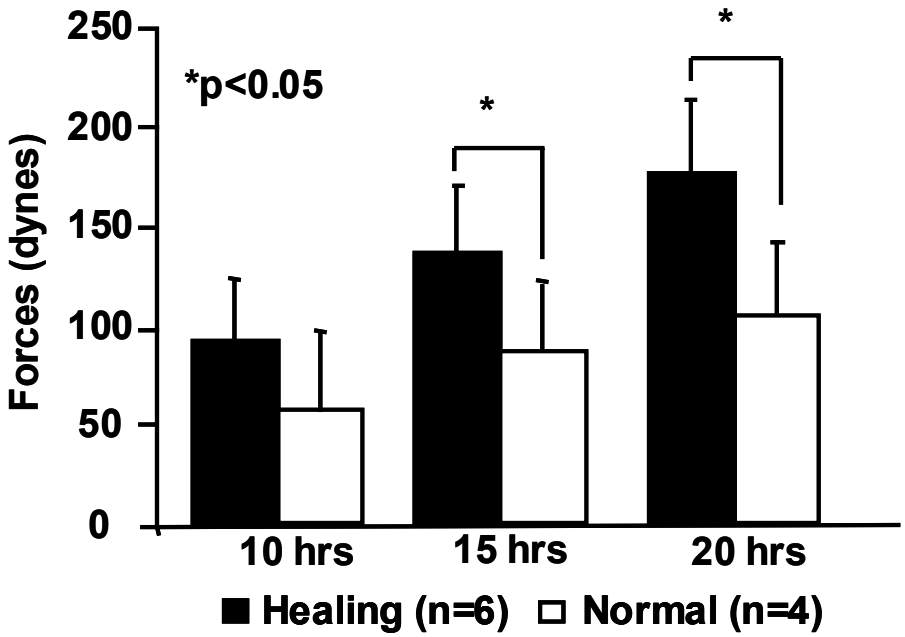


Figure 3 Contraction of healing and normal fibroblasts. Beginning at 15 hours, healing fibroblasts produced a significantly greater contraction than normal fibroblasts. Similar results were obtained at 20 hours.

Cell number was not a contributor to the differences that were seen. The difference in cell numbers between collagen gels with healing cells and those with normal fibroblasts was not significant at 24 hours **(Fig 4)**. Although the difference in healing and normal fibroblast cell numbers did reach significance at 48 hours ($p < 0.05$), we only compared contraction forces at 10, 15, and 20 hours, so this did not affect our findings. Furthermore, the healing fibroblasts consistently produced significantly more collagen than normal fibroblasts in all experiments **(Fig. 5)** ($p < 0.05$). On average, healing fibroblasts produced 10% more collagen than their normal counterparts.

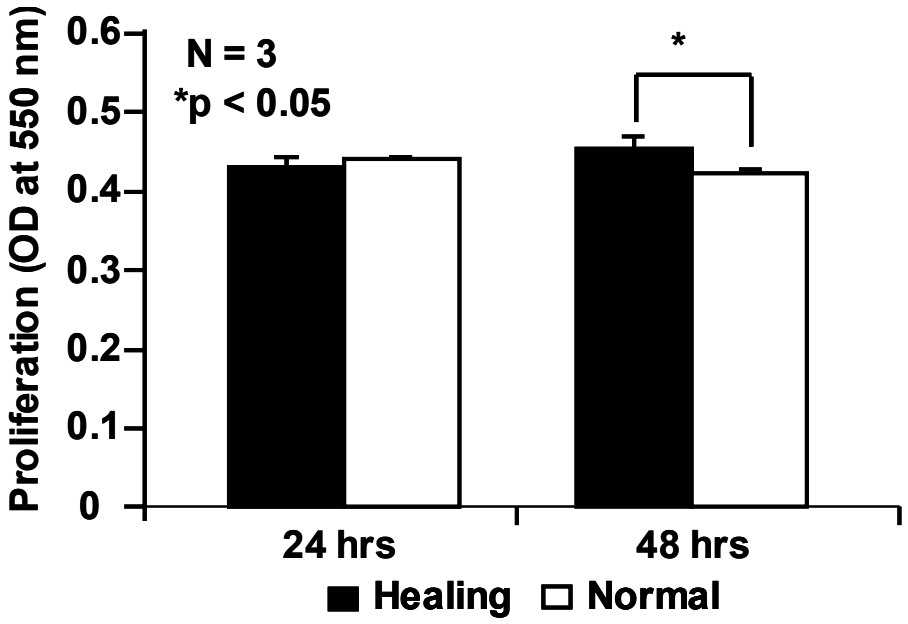


Figure 4 Proliferation of healing and normal fibroblasts in collagen gels. Proliferation of healing fibroblasts was significantly faster than normal fibroblasts at 48 hours but not at 24 hours.

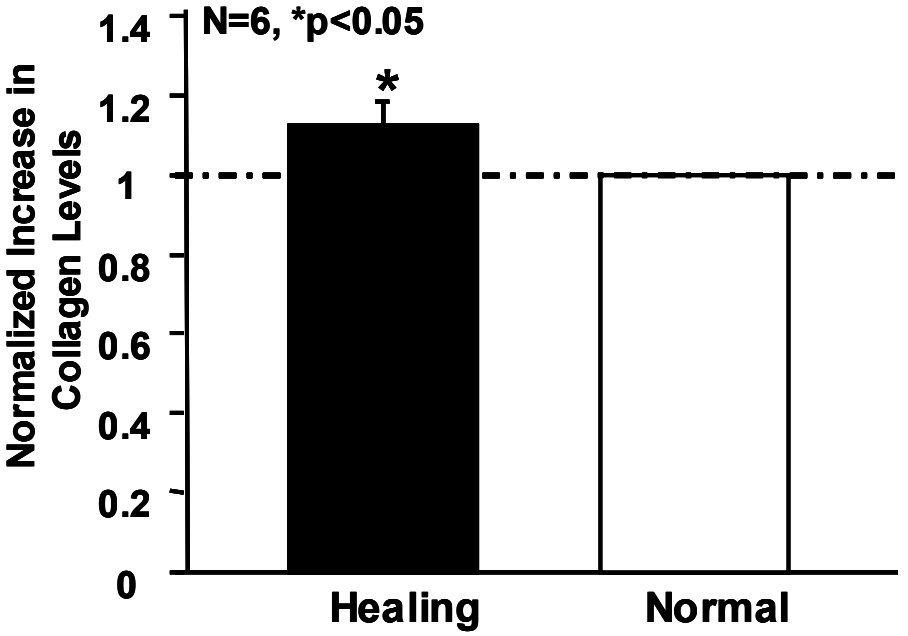


Figure 5 Collagen production by healing and normal fibroblasts. Medium was collected after 20 hours of contraction to assay collagen levels. It was found that healing fibroblasts consistently produced more collagen than normal counterparts.

4.5 DISCUSSION

This study demonstrated that healing fibroblasts generate greater forces of contraction and produce more collagen than their normal counterparts. Healing MCL fibroblasts are surrounded by a different environment than those from a normal ligament and therefore, it would be expected for them to have differential expression. They need to regenerate the matrix around them and pull the wound closed, so they need the ability to have increased collagen synthesis as well as increased contractile forces. The increased contractile forces may also influence the amount of collagen produced since contracting fibroblasts in stressed, anchored collagen gels have been shown to produce more collagen than the more relaxed fibroblasts that are found in unstressed, free floating gels [54].

Differences between healing and normal MCL fibroblasts were revealed not only on a functional level in this study, but also on a protein level. Healing fibroblasts have a different function than normal fibroblasts and therefore, must also express different levels of proteins in order to fulfill this function. Fibroblasts from keloids generate higher levels of contraction [58] and have elevated collagen synthesis than normal fibroblasts [59]. Also, hypertrophic scar fibroblasts secrete more type I collagen than normal dermal fibroblasts [60]. Thus, since this phenomenon occurs in skin following injury, similar events may occur in ligaments following rupture. Fibroblasts contributing to the formation of scar tissue in the MCL must also be more

contractile and produce greater amounts of collagen. It is important to identify these differences in order to focus on the aspects that are most important in improving the wound healing process.

It should be noted that the differences found in this study were not due to changes in cell number. It was found that there was not a significant difference in cell number between healing and normal fibroblasts at 24 hours. Healing fibroblasts produced significantly greater forces of contraction much before 24 hours and reached maximum contraction forces around 25 hours. Further, the medium was sampled to assess collagen type I secretion levels before 24 hours.

There are also a few limitations in this study that are important to note. The fibroblasts used for these experiments were of passages 3 or 4. Although the healing fibroblasts most likely did lose their phenotype a little with each passage, differences between the healing and normal fibroblasts in collagen synthesis and contractile forces were still evident. The differences that were noted would most likely be even greater at lower passage numbers, and probably not as evident with increasing passage numbers. Also note that this study investigated the differences between healing and normal fibroblasts at only one time point after the injury was made, after 10 days. At 5 days post-injury, the ligament may still be in an inflammatory stage and therefore, it may be harder to isolate the fibroblasts from the other cell populations. Further, several weeks post-injury, the healing phase may slow down because not as much remodeling needs to be done. Thus, the differences may not be as pronounced. A future study tracking the changes in levels of collagen synthesis and fibroblast contraction that occur over the course of wound healing in the MCL would be interesting to look into. Further, it would be interesting to look at the differences in other collagen types, such as type III, which is known to be elevated in healing tissue [67].

Thus, this study showed that healing fibroblasts produce greater contractile forces and more collagen than normal fibroblasts, both of which may be potential contributors to the scar tissue that forms following rupture of the MCL. Therefore, regulating these factors may lead to reduced scar formation in healing ligaments such as the MCL.

5.0 THE EFFECT OF TGF-β1 AND TGF-β3 ON HEALING RAT MCL FIBROBLASTS

5.1 ABSTRACT

TGF-β1 and TGF-β3 are important regulators of many functions during wound healing. *In vitro*, they have both been found to increase cellular contraction, which contributes to the formation of scar tissue. TGF-β1 and TGF-β3 have also been found to increase α-SMA expression. Cellular contraction may be regulated by the presence of α-SMA at the wound site. Interestingly, while TGF-β1 increased scarring in a rat skin wound, TGF-β3 reduced it. Using an FPCG model, it was found that TGF-β1 and TGF-β3 both increased fibroblast contraction and α-SMA protein expression of healing MCL fibroblasts, with TGF-β3 acting to a lesser extent. TGF-β1 and TGF-β3 increased fibroblast contraction 3.1 and 2.7 fold over untreated controls. Further, α-SMA protein expression was increased 1.8 and 1.3 fold by TGF-β1 and TGF-β3, respectively, compared to untreated controls. Thus, TGF-β1 and TGF-β3 have differential effects on healing MCL fibroblasts. Differential regulation of these growth factors during ligament healing may improve the quality of a healed ligament.

5.2 INTRODUCTION

Growth factors (e.g. TGF-β and PDGF) have been investigated as potential biological therapies to reduce scar tissue formation [21]. Since expression of TGF-β is increased in healing tissue as compared to normal tissue [61], its regulation may be useful in restoring structural and mechanical properties of injured ligaments. In wounded fetal tissue where scarring does not occur, the inflammatory response is greatly reduced, if not absent [25, 29, 68]. Furthermore, fetal wounds are characterized by a low concentration of TGF-β1 and a high concentration of TGF-β3 [27, 28]. In rat skin wounds, addition of TGF-β3 or neutralization of TGF-β1 was shown to decrease the formation of scar tissue [14]. Also, antisense oligonucleotides to TGF-β1 were found to significantly inhibit the formation of scar tissue in the eye of a mouse [46].

Previous studies have shown that the addition of either TGF-β1 or TGF-β3 to a fibroblast-populated collagen gel (FPCG) increases fibroblast contraction [11, 12], although to different extents. At the same dosages, TGF-β1 increases fibroblast contraction more than TGF-β3. Individually, TGF-β1 and TGF-β3 act in similar dose-dependent manners on fibroblast contraction. They have both also been shown to increase protein expression of α-SMA [15-18, 52]. The increased contraction may be mediated by the increased presence of α-SMA. Electroinjection of an antibody to α-SMA inhibited contraction in gingival and periodontal ligament fibroblasts [69]. Further, as the expression of α-SMA signifies the appearance of myofibroblasts, which are known to generate large contraction forces in healing tissue [15-18].

Interestingly, inhibition of α-SMA expression in rat skin wounds using vanadate resulted in less scar formation [53].

TGF-β1 and TGF-β3 regulate many cell functions, such as cell contraction and collagen synthesis during the wound healing process [10, 11, 18, 29, 47]. Their differential effects on levels of cell contraction may be an important factor in the differential effect they have on the formation of scar tissue. It is also important to investigate the potential causes of their differential contractile ability in order to improve ligament healing. Therefore, the objective of this study was to look into the effects of TGF-β1 and TGF-β3 on cellular contraction and α-SMA expression. Regulation of α-SMA may decrease the level of fibroblast contraction seen at the wound site and thus improve the quality of the healed ligament.

5.3 MATERIALS AND METHODS

Healing fibroblasts were obtained from the MCLs in the right knees of eight Sprague-Dawley rats. A 2-mm gap injury was created in the MCL and allowed to heal for 10 days, after which, the rats were sacrificed and the MCLs were excised and placed in cell culture medium. MCLs were harvested after 10 days because the healing tissue was considered to be in the proliferation phase and at that time fibroblasts are abundant [64]. To obtain fibroblasts, MCLs were individually washed twice with PBS and then minced into small pieces in 60 x 15 mm polystyrene tissue culture dishes. Subsequently, 2 mL of DMEM with 10% FBS and 1% P/S was added to each dish and the dishes were maintained in an atmosphere of 5% CO_2 and 100%

humidity at 37°C. Once fibroblasts reached confluence, they were sub-cultured four times for all experiments.

Contractile forces of healing fibroblasts were measured using the multi-station CFM system described in Chapter 4. The collagen solution was prepared by combining 0.1 M NaOH, 10x PBS, and collagen type I in a ratio of 1:1:8. Fibroblasts from confluent Petri dishes were trypsinized and then centrifuged for 5 min at 1000 rpm. The cell pellet was resuspended in AIM-V medium (Invitrogen), a serum-free medium to a concentration of 50 x 10^4 cells/mL. Each FPCG was created by mixing 3 mL of cell solution and 5 mL of collagen solution and adding this FPCG solution into a silicone dish around two porous vyon bars. The silicone dishes were incubated at 37°C for one hour as the FPCG solution polymerized. Solutions of 1 ng/mL TGF-β1 and 1 ng/mL TGF-β3 were made by adding the appropriate amount of growth factor to AIM-V medium. This dosage of TGF-β was chosen based on previous studies by Campbell et al. [12] as well as unpublished data from our laboratory. After polymerization, 7 mL of AIM-V with TGF-β1, AIM-V with TGF-β3, or growth factor-free AIM-V medium was added to the appropriate FPCGs. Gels were connected to the CFM system and contraction was tracked for 20 hours.

To assay α-SMA protein expression, cells were removed from collagen gels after 20 hours of contraction using a protocol adapted from Vaughan et al. [42]. Gels were washed twice with PBS and then incubated with trypsin for 10 min at 37°C. Subsequently, collagenase (Sigma Aldrich) at a concentration of 3 mg/mL was added to each gel and gels were incubated again for

15 min at 37°C. The reaction was stopped using FBS. Samples were centrifuged for 10 min at 1000 rpm and then the cell pellet was resuspended in 100 μL of mammalian protein extraction reagent (MPER; Pierce) containing protease inhibitors. The lysed cell solution was centrifuged again for 5 min at 8000 rpm. The supernatant was extracted and used to assay α-SMA.

A standard Western blotting technique was used to assay α-SMA expression in the protein samples. A BCA Protein assay (Pierce) was used to determine the total protein concentrations of the cellular extracts. Equal amounts of total protein were run on 10% SDS-polyacrylamide gels (BioRad, Inc) at a constant voltage of 125 V. Next, proteins were transferred to a nitrocellulose membrane using a standard transfer module (BioRad, Inc) at constant amps of 250 mA for 90 min. The membrane was blocked overnight at 4°C, or for 1 hr at room temperature, in a 5% dry milk/ PBS-tween 20 solution. Next, the membrane was probed with a mouse monoclonal anti-α-SMA antibody (Sigma Aldrich) at a dilution of 1:1333 in a 1% dry milk/ PBS-tween 20 solution, followed by a peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc.) at a dilution of 1:3333 in a 1% dry milk/ PBS-tween 20 solution. Bands of α-SMA were detected using an ECL detection kit (Amersham Biosciences) and then transferred to film. Membranes were re-probed for GAPDH to verify equal protein loading in the gels.

Statistical analysis of differences in contraction forces between the experimental groups was done using one-way ANOVA, followed by Bonnferroni's test for multiple comparisons, with a significance level set at 0.05.

5.4 RESULTS

Both TGF-β1 and TGF-β3, at dosages of 1 ng/mL, produced significantly greater contraction forces than untreated controls **(Fig 6)**. TGF-β1 consistently showed greater contractile forces than TGF-β3 in all experiments. Contraction forces were tracked for a period of 20 hours, at which point the forces seemed to level off. The greatest increase in contraction forces was seen between 0 and 10 hours. The shape of the curves from all three treatment groups was similar. Quantitatively, compared to untreated controls, gels treated with TGF-β1 and TGF-β3 showed 3.1 and 2.6 fold greater contraction forces, respectively, at 20 hours **(Fig 7)**. Differences between both treatment groups and the control were significant. Although gels treated with TGF-β1 consistently showed greater contractile forces than gels treated with TGF-β3 in all experiments, the difference did not reach statistical significance.

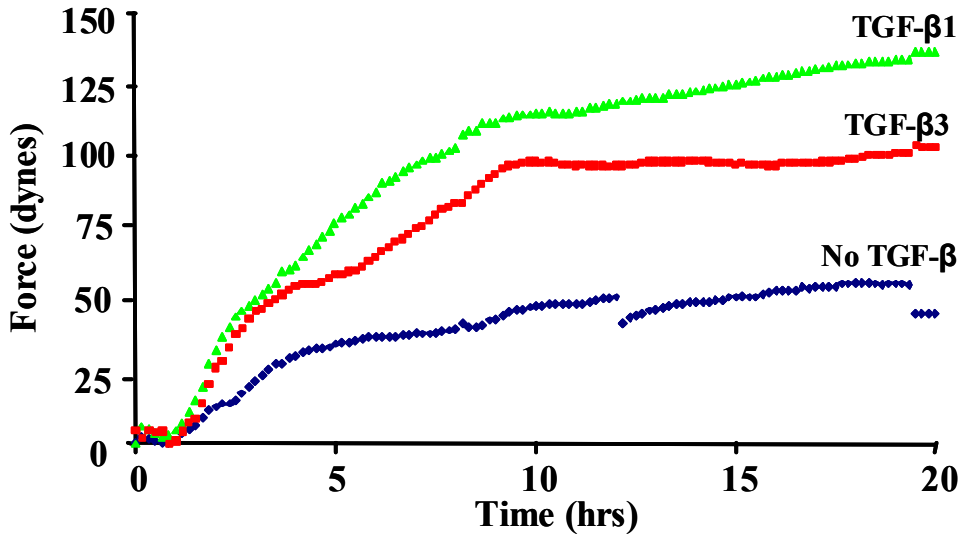


Figure 6 A representative graph of contraction force data tracked from the CFM system. TGF-β1 showed greater contractile forces than both TGF-β3 and the untreated control. The breaks in the No TGF-β curve were probably due to a disturbance in the voltage readings of the CFM system.

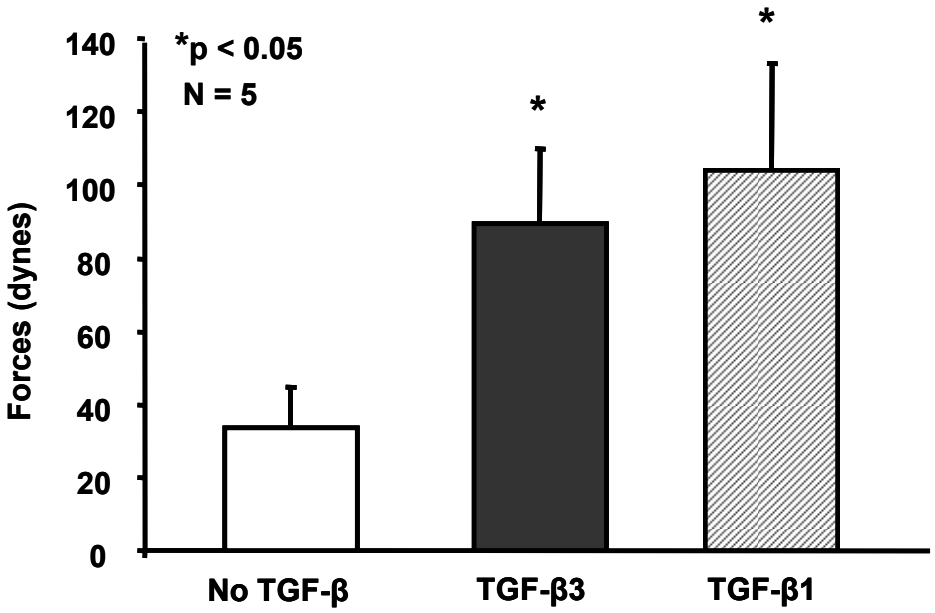


Figure 7 Fibroblast contraction due to TGF-β1 and TGF-β3 treatment. Both TGF-β1 and TGF-β3 significantly induced greater contraction than the untreated fibroblasts at 20 hours after gel contraction. Although TGF-β1 consistently produced greater forces than TGF-β3 in five independent experiments, the difference did not reach statistical significance.

Further, differential α-SMA protein expression was seen between control gels, those treated with TGF-β1, and those treated with TGF-β3. TGF-β1 induced maximal expression of α-SMA among the experimental groups—greater than both TGF-β3 and controls **(Fig 8)**. In comparing the three groups, TGF-β1 and TGF-β3 induced 1.75 and 1.27 fold more α-SMA than controls, respectively **(Fig 9)**. The greatest increase in levels of α-SMA correlated with the greatest increase in forces of contraction seen by treatment with TGF-β1. Similarly, TGF-β3 had greater α-SMA expression and showed greater contraction than untreated controls.

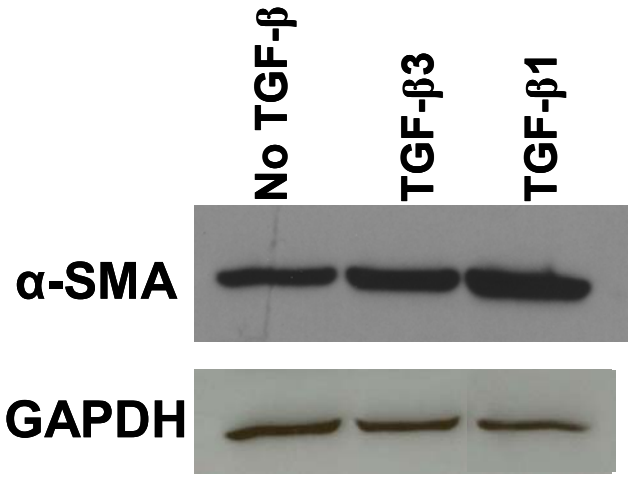


Figure 8 A representative Western blot result of α-SMA protein expression. Fibroblasts treated with TGF-β1 and TGF-β3 expressed higher levels of α-SMA protein than untreated fibroblasts; moreover, TGF-β1 consistently induced higher α-SMA protein expression than TGF-β3 in five separate experiments.

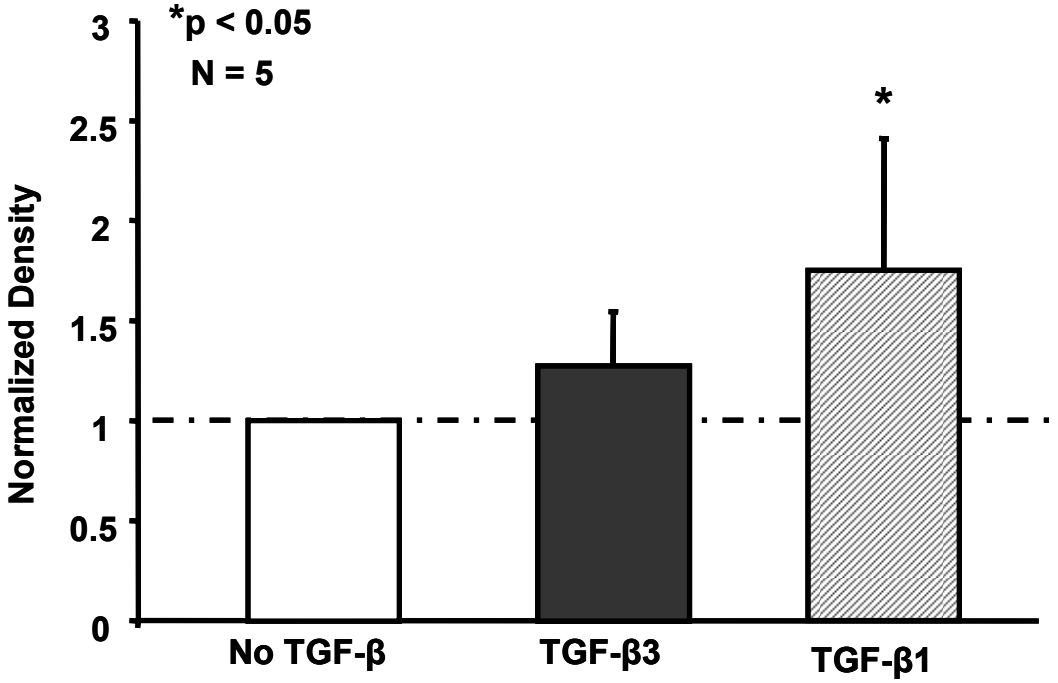


Figure 9 Analysis of Western blot results. TGF-β1 and TGF-β3 consistently produced a higher level of α-SMA than untreated controls. However, only differences between TGF-β1 and untreated controls reached statistical significance. TGF-β1 also consistently expressed more α-SMA than TGF-β3 in all experiments. Data was first normalized to the density of GAPDH bands and then to the density of the untreated controls.

5.5 DISCUSSION

The results of this study showed that TGF-β1 and TGF-β3 produced greater contractile forces than untreated controls. Although previous studies have differed in their reports of whether TGF-β1 or TGF-β3 has a stronger effect [11, 12, 18, 47], this study showed that TGF-β1 produced greater contraction forces than TGF-β3. Further, it was found that TGF-β1 induced more α-SMA than TGF-β3 and controls, which correlates to the contraction data tracked with the CFM system. These results are interesting because although there was a difference in α-SMA

expression between TGF-β1 and TGF-β3 treatment, there was not a large difference in contraction forces between these treatment groups. A possible explanation for this difference is that TGF-β3 causes faster migration of fibroblasts than TGF-β1 [48] and therefore, the "more tractional forces" generated by TGF-β3 treatment are moderately smaller than those "more contractile forces" generated by TGF-β1 treatment.

Previous studies have shown that TGF-β1 and TGF-β3 increase α-SMA expression [15-19], which may indicate differentiation of fibroblasts into myofibroblasts. Myofibroblasts are marked by higher levels of α-SMA expression and have also been shown to cause greater contraction in healing tissue [20]. These specialized cells are found only transiently in the wound site [30]. Thus, this study may indicate that TGF-β1 and TGF-β3 cause differentiation of fibroblasts into myofibroblasts to different extents. The induction of α-SMA expression also increases the contractile forces produced. In rat skin wounds, TGF-β1 was found to increase scar formation, whereas TGF-β3 actually reduced scarring [14]. Thus, the differential contraction forces and α-SMA expression seen in this study may explain this differential healing response. Therefore, α-SMA expression as well as contractile forces needs to be regulated in order to reduce ligament scarring.

A major difference between this study and previous studies is that this study was able to quantify the forces of fibroblast contraction in response to TGF-β treatment using the CFM system. Many previous studies only showed qualitative, visual differences in gel areas. In addition, the FPCGs in this study were under tension and not free-floating, which is closer to the

in vivo situation of ligaments. Another advantage of the CFM system is that it can reveal the pattern of contraction forces of the FPCG.

There are a few limitations in this study. The CFM system is accurate to 0.1 dynes, which allows us to track very small changes in contraction forces. However, this also makes the system extremely sensitive. Therefore, on occasion, there are breaks in the curves from the CFM system, as seen in **Fig 6**. We believe that these are due to jumps in the voltage being sent to each beam, which may come from outside disturbances in the lab environment. However, because the general trends of the curves seem uninterrupted, these breaks do not change our results. Another limitation is that we only investigated one dosage of growth factor. It would be interesting to look into different dosages of TGF-β1 and TGF-β3, as both isoforms have been shown to act in a dosage-dependent manner [11], where the relationship between contraction and dosage may be direct at low dosages but after a maximum effect is reached, the relationship becomes indirect and higher dosages induce smaller increases in contraction. Thus, the differential effect that TGF-β3 and TGF-β1 have on scar formation may be due to the levels of each growth factor present in the wound site. Also, since TGF-β1 and TGF-β3 differentially regulate scar formation in skin wounds and are present together at the wound site, their effect in combination at the appropriate ratios on α-SMA expression and fibroblast contraction should be investigated.

The results of this study suggest that regulating the levels of TGF-β1 and TGF-β3 may be important in improving ligament scarring after injury. TGF-β1 and TGF-β3 were both found to increase fibroblast contraction and α-SMA expression, although to different extents. These are

two factors that have a large influence on the formation of scar tissue. Therefore, down-regulating the ratio of TGF-β1 to TGF-β3 may help to decrease scar formation.

6.0 THE EFFECT OF GEL TENSION ON α-SMA EXPRESSION IN NORMAL RAT MCL FIBROBLASTS

6.1 ABSTRACT

There is an increase in tension in the matrix during wound healing. Previous studies have correlated the presence of tension and α-SMA in the matrix qualitatively by comparing tensioned and completely untensioned gels. The increase in expression of α-SMA at the wound site may signify the differentiation of fibroblasts into myofibroblasts. This study quantitatively altered the amount of tension in collagen gels populated with ligament fibroblasts and then assessed the amount of α-SMA expression. It was found that α-SMA expression and tension in the matrix were directly related: in FPCGs that were relaxed after 5 hours of tension, there was 69% lower α-SMA expression than those subjected to 20 hours of tension. A decrease in tension in the matrix caused a decrease in the expression levels of α-SMA protein. Therefore, decreasing α-SMA expression by easing the amount of tension in the wound healing matrix may help to decrease scarring by reducing myofibroblast differentiation.

6.2 INTRODUCTION

Myofibroblasts are specialized cells that have properties of both fibroblasts and smooth muscle cells [8, 30, 52]. Their differentiation from fibroblasts has been associated with an increase in expression of α-SMA, a marker and contractile component of the cells [8, 23, 41, 70, 71]. It has been hypothesized in several studies that an increase in α-SMA expression also increases contraction of the cells [30, 40, 69]. A previous study showed that electroinjection of an antibody to α-SMA into gingival fibroblasts decreased contraction by 93% [69].

During wound healing, injured tissue is subjected to greater amounts of tension. The process of contraction in tissues that are tensioned is due to the activity of cells trying to move through the collagen matrix. The cells spread and elongate themselves [55], which is contrary to smooth muscle contraction, and then create tractional forces throughout the entangled collagen fibril network [72]. This then creates intracellular tension in the matrix. Extracellular tension is created by anchoring the matrix to a surface. In a free floating gel, the matrix is mechanically relaxed extracellularly. However, in an anchored gel, the matrix remains stressed extracellularly.

A previous *in vitro* study compared tension to α-SMA expression by comparing floating collagen gels to anchored collagen gels, matrices with low vs. high tension, respectively [19]. It was found that after adding 10 ng/mL TGF-β1 to each gel, there was little difference in α-SMA expression in the floating gels. However, there was a 3-fold increase in α-SMA protein expression in the anchored matrices [19]. Further, the deletion of TGF-β1-induced α-SMA by

incubation with an antibody did not increase α-SMA protein levels in cells on any substrate. In addition, when mechanical force acted on cardiac fibroblasts that had low basal levels of α-SMA, expression was increased up to 2 fold [57]. *In vivo*, when tension was increased in a skin wound by splinting it, α-SMA expression was increased compared to untensioned controls [20].

As there is tension in both the skin and the heart, there is also tension present in ligaments. This study looked at the effect of matrix tension on α-SMA expression of normal MCL fibroblasts in collagen gels using a dynamic CFM (DCFM) system, which quantitatively altered tension in a collagen gel matrix. Since α-SMA is related to fibroblast contraction, reducing tension in the matrix may decrease contraction forces in the matrix and thereby reduce ligament scarring.

6.3 MATERIALS AND METHODS

Normal fibroblasts were obtained from MCLs in the left knees of eight Sprague-Dawley rats. MCLs were undermined by a surgeon and then the skin on top of them was sutured. After 10 days, the MCLs were excised and harvested. Ligaments were washed twice with PBS, cut into small pieces in a 60 x 15 mm polystyrene tissue culture dish, and then covered with DMEM containing 10% FBS and 1% P/S. Fibroblasts were maintained in this growth medium at 37ºC, in an atmosphere of 5% CO_2 and 100% humidity, and were sub-cultured to passages of four or less for use in experiments.

Protein expression of α-SMA was measured from FPCGs that were attached to a dynamic culture force monitor (DCFM) system. This system is a modification to the CFM system described in Chapter 4. The DCFM allows for the adjustment of gel tension during the course of the experiment. While one vyon bar still attaches to the cantilever beam, the second vyon bar attaches to a metal block that can be moved manually back and forth to increase or decrease tension in the gel **(Fig 10)**.

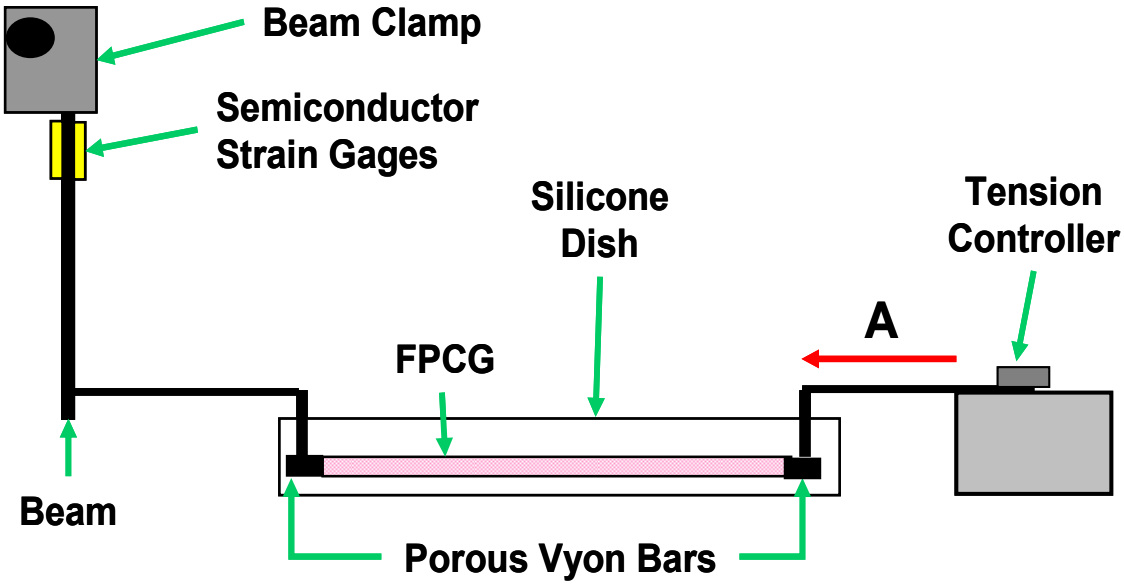


Figure 10 Illustration of dynamic culture force monitor (DCFM) system. The DCFM system can control the amount of tension in the collagen matrix. The FPCG is anchored by a vyon bar on each side. The tension controller attaches to the vyon bar on the right and can be moved in the direction of arrow **A** to reduce tension levels in the collagen matrix.

FPCGs were created using a collagen solution that was made by combining type I collagen, 0.1 M NaOH, and 10x PBS in a ratio of 8:1:1. Normal MCL fibroblasts were trypsinized from Petri dishes and centrifuged for 5 min at 1100 rpm. The cell pellet was resuspended in growth medium at a density of 50 x 10^4 cells/mL. Next, 3 mL of the cell solution was added to 5 mL of

collagen solution for each gel and the mixture was pipetted onto the two vyon bars in individual silicone dishes. After 1 hour of incubation at 37°C, gels were polymerized, 7 mL of growth medium was added to each gel, and FPCGs were attached to the DCFM system. Voltages were tracked using a Labview program as with the CFM system. After 5 hours of contraction, tension was decreased in the relaxed group of gels by moving the vyon bar attached to the tension controller towards the cantilever beam and decreasing the voltage measurements to those at the beginning of the experiment.

Cells were removed from FPCGs after 20 hours of attachment to the DCFM using a protocol adapted from a previous study [42]. FPCGs were washed twice with 1x PBS and then trypsinized and incubated at 37ºC for 10 min. Following, 1 mL of collagenase was added to each gel and the gels were incubated again at 37ºC for 15 min. The reaction was stopped with FBS. The cell solution was centrifuged for 10 min at 1100 rpm. The cell pellet was then resuspended in 100µl of MPER containing protease inhibitors. The lysed cell solution was centrifuged for 5 min at 8000 rpm in a microcentrifuge. The supernatant was extracted and used to assay α-SMA protein expression.

A standard Western blotting technique was used to determine α-SMA protein expression. A BCA protein assay was run on all samples to determine total cellular protein concentration. Equal amounts of total protein were run on 10% Tris-HCl polyacrylamide gels using gel electrophoresis at a constant voltage of 125 V. Protein was transferred to a nitrocellulose membrane using a standard BioRad transfer module at 250 mA for 90 min. The membrane was blocked overnight at 4ºC, or for 1 hour at room temperature, in a 5% dry milk/ PBS-tween 20

solution. A mouse monoclonal anti-α-SMA antibody was used to first probe α-SMA at a dilution of 1:1333 in 1% dry milk/ PBS-tween 20, followed by a peroxidase-conjugated goat anti-mouse antibody at a dilution of 1:3333 in a 1% dry milk/ PBS-tween 20 solution. Bands of α-SMA were exposed using an ECL detection kit and then transferred to film. Membranes were re-probed for GAPDH to ensure equal loading.

A paired *t*-test was used to determine whether a statistical difference existed in α-SMA expression between tensioned and relaxed gels in normal fibroblasts. The significance level was set at 0.05.

6.4 RESULTS

Contraction forces were tracked for 20 hours for all FPCGs **(Fig 11)**. The average difference in contraction forces between tensioned and relaxed gels at 20 hours was consistently about 50 dynes for all five experiments.

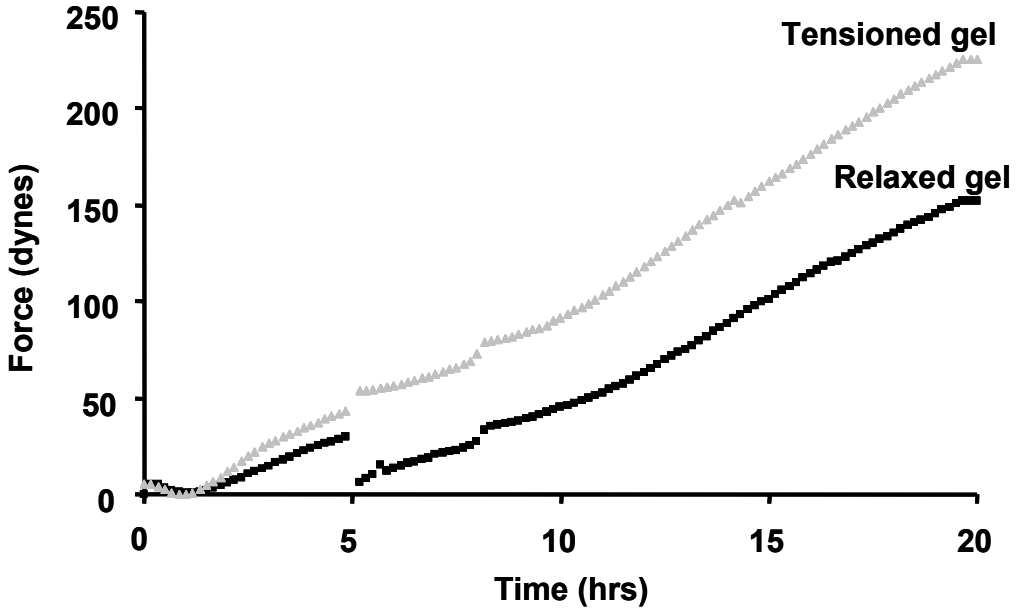


Figure 11 A representative graph of gel contraction from the DCFM. The relaxed gels were released at 5 hours to their starting voltage reading and then allowed to continue contracting. Tensioned gels produced greater maximal contraction forces as compared their relaxed counterparts.

Western blotting showed that α-SMA expression was less in relaxed samples as compared to tensioned samples. Bands of α-SMA were thinner and less dense in the relaxed samples. Analysis of the bands of α-SMA revealed that relaxed gels had 69% less α-SMA expression compared to the control tensioned gels. Therefore, these results suggest that tension and α-SMA expression is correlated: decreasing tension in the matrix also decreased α-SMA expression.

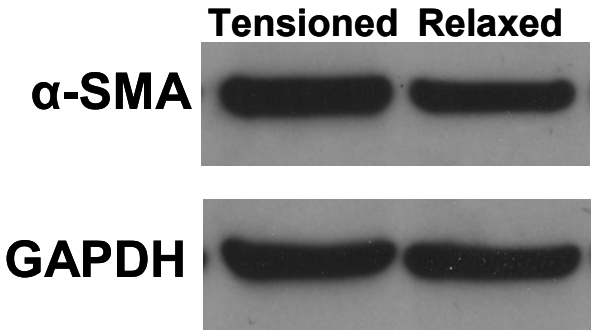


Figure 12 A representative Western blot result of α-SMA protein expression. The bands from tensioned gels were larger than those of the relaxed gels.

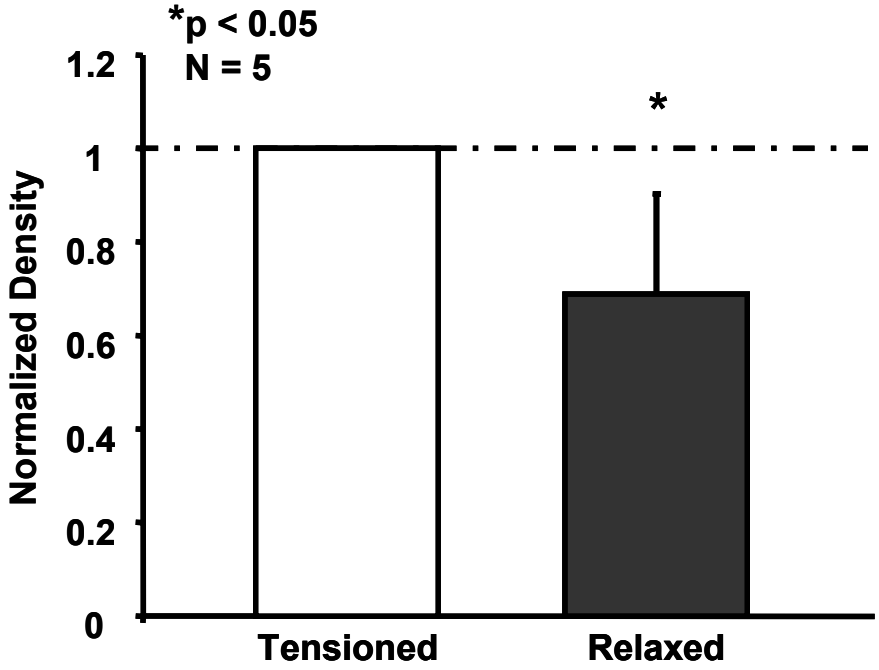


Figure 13 Bar graph of Western blot analysis. The relaxed gels consistently expressed less α-SMA protein than the tensioned gels. On average, α-SMA protein in relaxed gels was 69% of that of tensioned gels.

6.5 DISCUSSION

The results of this study revealed that α-SMA expression decreased with decreased tension. When gels were relaxed at 5 hours of contraction, they showed lower expression of α-SMA expression after 20 hours of contraction compared to gels that were tensioned for 20 hours. The average contraction forces at 20 hours of relaxed gels were about 50 dynes less than tensioned gels. An important factor of this study was that only intracellular tension was regulated. From the contraction curve produced by the DCFM system, it is apparent that after the gel was released, the cells still produced contractile forces and re-created gel tension. Increased expression of α-SMA is linked to the differentiation of fibroblasts into myofibroblasts [17, 19, 30, 41, 52, 70]. Myofibroblasts are transiently present during wound healing [30, 40, 41, 51]. However, their presence has a large impact on the outcome of the healing process, as they have been implicated in the formation of scar tissue [53]. With the lack of α-SMA, the myofibroblast phenotype disappeared and rat skin wounds healed properly without scarring [53]. The presence of α-SMA contributes to the increased contractile ability of myofibroblasts [31]. Excessive contraction forces are an important factor in scar tissue formation [8, 9]. At the wound site, the increase in contraction due to wound closure increases tension in the matrix [16], which may cause differentiation of fibroblasts into myofibroblasts and further enhance the expression of α-SMA.

Note that there are a few limitations in this study. First, the difference in contraction forces between the tensioned and relaxed gels was about 50 dynes. This difference in force is not a

large amount, as there were 1.5 million cells in each gel, which gives a force difference of about 0.33 nN/cell. Therefore, the effect on each cell of the matrix relaxation was very small. Further, this study only released tension in the matrix at one time point in one amount and the gels were allowed to continue to contract and regenerate the tension after this. Future studies should look at a dosage response, where tension is released at different levels and α-SMA expression is determined. This would allow a correlation to be drawn between α-SMA expression and tension levels in the collagen matrix. In addition, this study utilized normal fibroblasts instead of healing fibroblasts. The DCFM device used in this study is novel and so the effect of tension on normal fibroblasts was investigated first. This system can precisely control gel tension and can thus manipulate cell contraction. Releasing the amount of tension in the gel can also change the compliance of the matrix that the cells are in. Now seeing the effect of tension on α-SMA in normal fibroblasts, future studies should investigate the effect on healing fibroblasts.

This study showed that decreasing tension in a collagen matrix decreased α-SMA expression. Therefore, the amount of tension in the wound healing matrix may be an important factor in the formation of scar tissue. In the future, levels of collagen synthesis should be assessed. Previous studies have directly correlated tension and collagen synthesis [54]. Both contraction and collagen synthesis may impact scar formation [7]. Thus, relaxing the tension in the matrix would decrease expression of α-SMA and reduce scarring at the wound site.

7.0 SUMMARY AND FUTURE DIRECTIONS

In summary, this study shows that healing and normal fibroblasts from MCLs exhibit differential abilities of contraction and collagen type I production. When TGF-β1 and TGF-β3 were added to healing fibroblasts seeded in collagen gels, both were found to increase contraction forces compared to untreated controls—but, TGF-β1 consistently produced higher forces than TGF-β3. In addition, TGF-β1 induced greater α-SMA protein expression than both TGF-β3 and untreated controls. Finally, this study showed that normal fibroblasts in relaxed collagen gels had decreased α-SMA protein expression compared to tensioned collagen gels.

The findings of this thesis highlight the importance of using healing fibroblasts instead of normal fibroblasts in studying the cellular mechanisms involved in scar formation during ligament healing, as they are functionally different. Fetal wounds are characterized by an increased presence of TGF-β3 and heal without scar formation, unlike adult wounds. TGF-β1 and TGF-β3 increase contraction and α-SMA expression, which are both important factors in wound healing, to different extents. Thus, regulating the relative expression levels of TGF-β1 and TGF-β3 may result in reduced scarring in healing ligaments. In addition, the effect of tension on α-SMA is an important factor in the healing process. An increase in tension at the wound site may increase differentiation of fibroblasts into myofibroblasts and thus, increase scar formation.

Future studies should look into the effect of TGF-β1 and TGF-β3 at different dosages and in combination on cell contraction, collagen secretion, and α-SMA expression. Previous studies have shown that TGF-β1 and TGF-β3 differentially regulate scar formation in skin wounds and these three factors are all important in scarring. Therefore, the application of these growth factor isoforms in the appropriate ratios may improve ligament healing. Further, the effect of tension on α-SMA expression should be assessed in healing fibroblasts. Tension should be quantitatively altered at different levels so that a correlation between tension levels in the matrix and α-SMA expression can be obtained. Lastly, the application of these factors should be tested *in vivo*, e.g., by injection of TGF-β3 or an antibody to TGF-β1 to the wound site or immobilization of the injured joint to decrease tension at the wound site, to investigate their effect on ligament scar formation in an animal model.

APPENDIX A

EXPERIMENTAL PROTOCOLS

Harvesting Fibroblasts from Ligament Tissue

1. Place ligament in growth medium at animal facility.
2. Wash MCL in cell culture hood twice with growth medium.
3. Mince MCLs into pieces in small Petri dishes.
4. Add 2 mL to each Petri dish. MCLs should not be floating.
5. Incubate at 37°C, 5% CO_2, and 100% humidity.
6. Replace medium every 2-3 days.
7. Subculture fibroblasts once they are confluent in the Petri dishes.

Measuring FPCG Contraction with CFM System

1. Warm medium and trypsin
2. Make collagen gel solution:
    a. For 4 gels (mix in 50 mL conical tube):
        i. 2.2 mL of 10X PBS
        ii. 2.5 mL of 0.1 M NaOH
        iii. 17.6 mL collagen stock solution (Vitrogen)
    b. Mix well, store at 4°C until needed
3. Make Ascorbic Acid solution if necessary
    a. Add 15.50 mg to 20 mL ddH_2O
4. Prepare cells:
    a. Extract media from cell dish
    b. Add 3 mL of trypsin to cells and place in incubator for 3 minutes
    c. Check cells, via light microscope, to ensure that they are unattached
    d. Add 5 mL of growth media (with 10% FBS) to counter the trypsin
    e. Using a pipette, tilt the Petri dish and wash cells to the bottom
    f. Pipette the cells into an appropriate sized conical tube
    g. Centrifuge the cell solution (1000 RPM for 5 minutes)
    h. Set out collagen solution while waiting for the centrifuge
    i. Extract supernatant, leaving the cell pellet and a small amount of solution, try not to loose cells
    j. Add a small amount of the medium (growth medium or AIM-V for TGF-β experiments) that will be used in the experiment and mix well
    k. Count cells with hemacytometer
        i. Clean and dry hemacytometer
        ii. Place cover slip on hemacytometer
        iii. Obtain 10μL of well mixed cell solution and pipette onto hemacytometer
        iv. Count the four corners and find the total cells per mL=$(C_1+C_2+C_3+C_4)x10^4/4$ (cells/mL)
    l. Dilute cells solution to appropriate density (50 x 10^4 cells/ mL)
5. Prepare silicone dishes and gel attachment units to add the FPCG solution
6. Mix:
    a. 13.2 mL cells to the 22 mL collagen solution
7. Pipet 8 mL of mixture onto and between vyon bars for each gel
8. Incubate for 1 hour
    a. While waiting, start CFM setup program on computer
9. Setup up computer and data acquisition system
    a. Turn on power supply and check excitation voltage with volt meter (this will allow strain gages to get to temperature)
    b. Start "Setup" program for CFM
10. Add 7 mL of appropriate medium (growth medium, AIM-V, or AIM-V with TGF-β1 or β3)
11. Label gels

12. Add Ascorbic Acid if testing for collagen synthesis
13. Attach to CFM and provide initial tension (check by seeing ~ 2-3 mV increase)
14. Stop “Setup” program and run CFM “Experiment” program

Preparation of FPCGs in 6-well Plates

1. Warm medium and trypsin
2. Make collagen gel solution:
    a. For 6 wells:
        i. 1.1 mL of 10X PBS
        ii. 1.3 mL of 0.1 M NaOH
        iii. 9.1 mL collagen stock solution (Vitrogen)
    b. Mix well, store at 4°C until needed
3. Prepare cells:
    a. Extract media from cell dish
    b. Add 3 mL of trypsin to cells and place in incubator for 3 minutes
    c. Check cells, via light microscope, to ensure that they are unattached
    d. Add 5 mL of growth media to counter the trypsin
    e. Using a pipette, tilt the Petri dish and wash cells to the bottom
    f. Pipette the cells into an appropriate sized conical tube
    g. Centrifuge the cell solution (1000 RPM for 5 minutes)
    h. Set out collagen solution while waiting for the centrifuge
    i. Extract supernatant, leaving the cell pellet and a small amount of solution, try not to loose cells
    j. Add a small amount of growth medium and mix well
    k. Count cells with hemacytometer
        i. Clean and dry hemacytometer
        ii. Place cover slip on hemacytometer
        iii. Obtain 10μL of well mixed cell solution and pipette onto hemacytometer
        iv. Count the four corners and find the total cells per mL=$(C_1+C_2+C_3+C_4)x10^4/4$ (cells/mL)
    l. Dilute cells solution to appropriate density (12.5 x 10^4 cells/ mL)
4. Prepare silicone dishes and gel attachment units to add the FPCG solution
5. Mix:
    a. 6.9 mL cells to the 11.5 mL collagen solution
6. Pipette 1.4 mL of cell-collagen solution into each well
7. Incubate at 37°C for 1 hour
8. Add 3 mL medium to each well

MTT Assay for Measuring Cell Number in 6-well Plates
(Adapted from: Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods*. 65:55-63)

1. Add 200 µl MTT solution (5 mg/mL) to each FPCG in a 6-well plate
2. Incubate for 3 hours at 37°C with 5% CO_2 and 100% humidity
3. Centrifuge 6-well plate at 1900 rpm for 5 min
4. Remove supernatant
5. Add 2 mL extraction buffer
    a. 15 mL DMF
    b. 14.1 mL ddH_2O
    c. 5 g SDS
6. Incubate overnight
7. Add 200 µl of each sample to a 96-well plate
8. Read plate using microplate reader at 550 nm

Protein Extraction from FPCGs

(Adapted from: Vaughan, M.B., E.W. Howard, and J.J. Tomasek. 2000. Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res*. 257:180-9)

1. Remove growth medium
2. Rinse twice with 1x PBS at room temp for 10 min
3. Incubate with 2 mL 0.25% Trypsin and incubate for 10 min at 37°C
4. Digest with 1 mL 3 mg/mL collagenase (in 130 mM NaCl, 10 mM Ca Acetate, 20 mM Hepes, pH 7.2) and incubate for 15 min at 37°C
5. Add 100 µL of FBS to stop the reaction
6. Centrifuge the samples for 10 min at 1000 rpm
7. Make lysis buffer:
    a. 1 mL M-PER + 15µl of protein cocktail
8. Extract supernatant
9. Resuspend cell pellet in 100 µl lysis buffer
10. Centrifuge sample in microcentrifuge for 5 min at 8000 rpm
11. Collect supernatant
12. Assay for protein using standard BCA Protein Assay (Pierce)

Assaying α-SMA using Western Blot

I. Set up gel module:

1. Make running buffer (100 mL 10 x Tris/Glycine/SDS + 900 mL ddH_2O)
2. Open pre-made gels.
3. Take off white tape at the bottom of the gel.
4. Take out the comb from the top of the gel.
5. Place gel in module and secure it.
6. Add running buffer to the module such that it covers the top of the gel.

II. Prepare samples and running the gel:

1. Turn on dry bath heater.
2. Vortex samples (keep samples on ice). Spin in minispinner.
3. Make loading buffer:
    a. Add a ratio of (10 sample buffer: 1 DTT) to an eppendorf tube (make enough to add 20 μL to each sample).
4. Calculate the volume of each sample that would be required to load 10 μg of protein. Add that volume of sample to its own eppendorf tube (label tubes accordingly).
5. Add DI water to each protein sample to give a final volume of 20 μl.
6. Add 20 μl loading buffer to each sample for a volume ratio of 1 sample: 1 loading.
7. Heat the sample and loading buffer mixture for 4 minutes at 90 to 100ºC (put on ice afterwards).
8. Spin down the sample tubes via centrifuge for 20 to 30 seconds.
9. Add 10 μL of the protein standard to the first well.
10. Add 37 μL of a sample to each well.
11. Attach the electrodes and run at constant voltage of 125 V for 110 minutes (shut down the electrophoresis when the indicating dye nears the bottom of the gel).

III. Transfer of the protein from the gel to the nitrocellulose membrane

1. Make transfer buffer (100 mL 10x Tris/Glycine, 200 mL Methanol, 700 mL ddH_2O)
2. Place transfer module, 2 sponges, 2 pieces of filter paper, and filter paper-nitrocellulose membrane sandwich into a container with transfer buffer.
3. Pour out the running buffer from the electrophoresis module.
4. Remove the gel from the module.
5. Pry the sides of the gel container open.
6. Carefully cut the top, bottom, and edges of the gel.
7. Remove the gel and place it (with protein standards on the right) on top of 2 pieces of filter paper in the transfer module, which is in the transfer buffer. (Gel should be on gray side of module)
8. Make sure all the bubbles are removed.
9. Place the nitrocellulose membrane on top of the gel. (Membrane should be on white side of module)
10. Place another 2 pieces of filter paper on top of the membrane and then add the sponge.
11. Clean the electrophoresis module and fill halfway with transfer buffer.

12. Place the transfer module into the electrophoresis module (gray side should be on the black side of the electrophoresis module, white side should line up with red side of electrophoresis module) and secure it.
13. Add transfer buffer to the top of the module.
14. Place in container with ice.
15. Run at constant amps of 250 mA for 90 minutes.

IV. Binding of the proteins

1. Rinse the membrane with DI water.
2. Place the membrane in 5% fat-free milk/PBS-T solution
    a. Shake overnight in the cold room or for 1 hour at room temperature.
3. Add 15 µL of the primary antibody to 20 mL of 1% fat-free milk/PBS-T.
4. Add this solution to the membrane and shake for 1.5 hours at room temperature.
5. Pour off the milk solution and wash the membrane 3 times for 15 minutes with .1% PBS-T.
6. Add 3 µL of the secondary antibody to 20 mL of 1% fat-free milk/PBS-T (1 2º AB: 5000 1% fat-free milk/PBS-T).
7. Add this solution to the membrane and shake for 1 hour at room temperature
8. Pour off milk solution and wash the membrane 3 times for 15 minutes with .1% PBS-T.

V. Exposure of the membrane

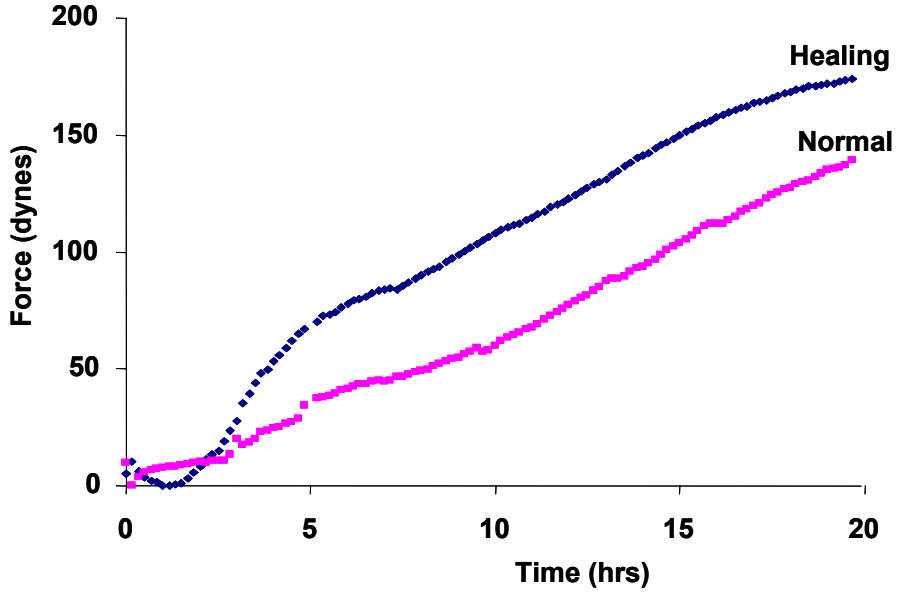
1. Make ECL solution (follow kit instructions):
    a. Add 3 mL of solution A into a 15 ml conical tube.
    b. Add 75 µL of solution B to solution A.
2. Lay out two pieces of saran wrap (bigger than the membrane).
3. Blot dry the membrane and place it on the center of one of the pieces of saran wrap with protein side up (the top left corner should be the cut corner).
4. Add 3 mL of the ECL solution (from 1) to the membrane and spread it across the membrane (ensure that there are no bubbles and that the entire surface is covered).
5. Let sit for 5 minutes.
6. Drain off excess ECL by placing the edge of the membrane onto a piece of filter paper.
7. Place the membrane with the protein side down (top right corner should be the cut corner) on top of the other piece of saran wrap.
8. Wrap the membrane and cut off extreme excess of saran wrap.
9. Place the wrapped membrane into the film tray with the protein side up (top left corner should be the cut corner).
10. Take the membrane, film, exposure tray, and scissors immediately down to the dark room to develop (BST 15th Floor).
11. In the dark room, cut the film to a size somewhat bigger than the membrane.
12. Place the film on top of the wrapped membrane (protein side up) and expose for about ~ 1 min. (time may vary depending on protein concentration, adjust accordingly).

APPENDIX B

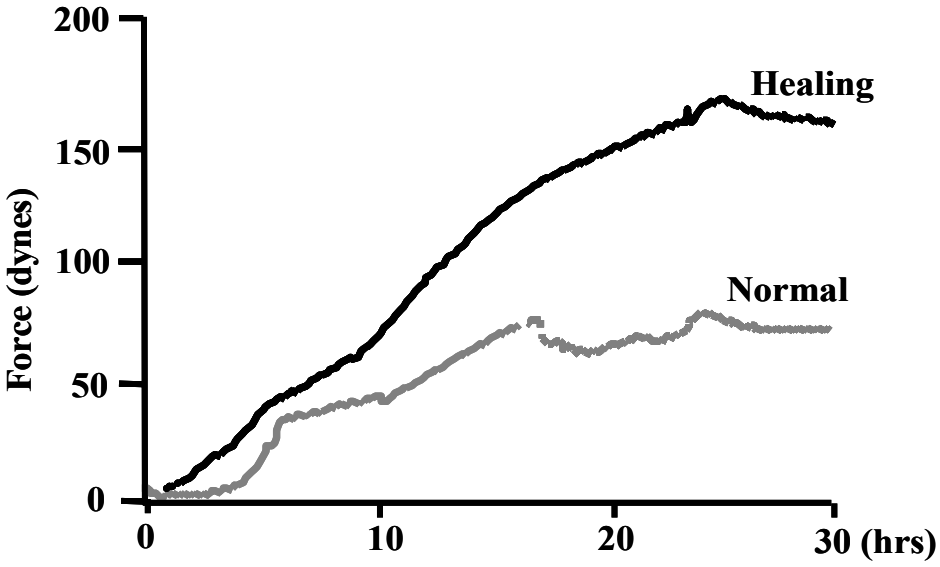
DATA ANALYSIS

Specific Aim #1: The following graphs represent the contraction forces produced by healing and normal fibroblasts in FPCGs connected to the CFM system. Contraction was tracked for 20 to 30 hrs. A total of four experiments were done.

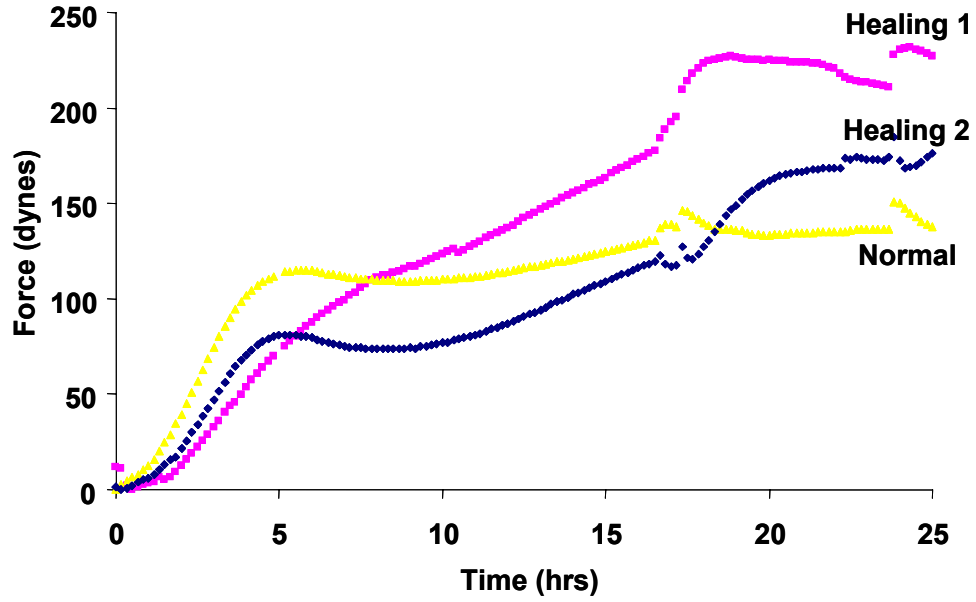
Experiment #1:



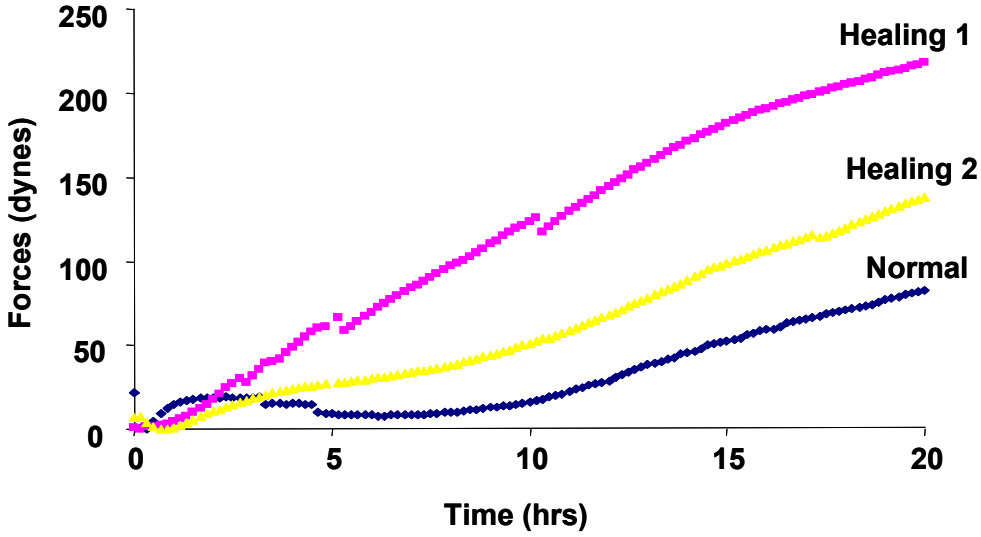
Experiment #2 **(Figure 2 in text)**:



Experiment #3:



Experiment #4:



Analysis of Healing vs. Normal Contraction Data

Healing Fibroblast Contraction Forces (dynes)

Time	Expt #1	Expt #2	Expt #3	Expt #4		
1	0	3.292	3.216	6.185	4.507	1.048
2.00	8.423	7.049	12.095	21.299	17.926	10.58
3.00	27.628	15.186	32.44	47.19	31.969	18.399
4.00	53.42	21.112	53.677	70.811	48.719	24.163
5.00	**67.141**	**30.175**	**111.617**	**80.332**	**61.256**	**26.811**
6.00	77.969	41.380	87.543	79.396	69.767	30.313
7.00	83.952	46.268	99.37	74.849	83.956	33.859
8.00	90	51.786	111.066	73.749	97.002	38.101
9.00	98.899	57.675	116.535	74.104	110.029	44.178
10.00	**107.966**	**64.749**	**123.643**	**76.805**	**123.845**	**51.17**
11.00	114.677	75.106	128.581	80.973	129.35	58.748
12.00	122.541	87.180	137.132	87.054	144.319	67.962
13.00	131.03	97.425	146.767	94.165	158.551	78.266
14.00	141.29	106.256	155.401	102.202	171.237	88.798
15.00	**149.759**	**116.131**	**163.36**	**108.924**	**181.823**	**98.976**
16.00	157.574	123.881	172.87	116.28	190.904	106.724
17.00	163.462	130.899	192.728	116.843	198.101	114.522
18.00	168.31	136.599	223.166	127.132	205.136	119.985
19.00	171.648	141.030	226.223	148.771	211.86	129.885
19.67	**173.923**	**143.830**	**225.106**	**158.628**	**216.078**	**135.6**

Normal Fibroblast Contraction Forces (dynes)

Time	Expt #1	Expt #2	Expt #3	Expt #4
1	7.801	0	12.361	14.681
2.00	10.02	0.668	39.092	18.797
3.00	20.164	1.201	74.418	18.84
4.00	24.470	3.646	102.152	15.373
5.00	**34.031**	**9.896**	**69.52**	**9.448**
6.00	41.470	28.129	114.67	8.262
7.00	44.406	34.45	111.791	8.652
8.00	49.294	36.196	109.782	10.228
9.00	54.958	38.603	109.23	12.906
10.00	**59.901**	**40.909**	**110.254**	**16.196**
11.00	67.281	42.393	111.586	22.537
12.00	77.351	48.287	114.504	28.861
13.00	87.270	53.902	117.42	38.589
14.00	93.575	59.828	120.74	45.795
15.00	**104.039**	**65.358**	**124.706**	**52.655**
16.00	112.026	70.336	128.788	59.428
17.00	119.681	74.184	139.028	65.466
18.00	127.451	64.752	139.753	70.733
19.00	134.814	61.616	135.459	77.134
19.67	**138.914**	**62.683**	**133.604**	**81.259**

Analysis of Time Points

10 hrs

Healing (forces in dynes)						***Average***	***SD***
107.966	64.749	123.643	76.805	123.845	51.17	***91.363***	***31.33195***
Normal (forces in dynes)							
59.901	40.909	110.254	16.196			***56.815***	***39.86709***

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	91.363	56.815
Variance	981.6912	1589.385
Observations	6	4
Pooled Variance	1209.576	
Hypothesized Me	0	
df	8	
t Stat	1.538905	
P(T<=t) one-tail	0.081194	
t Critical one-tail	1.859548	
P(T<=t) two-tail	**0.162389**	
t Critical two-tail	2.306004	

15 hrs

Healing (forces in dynes)						***Average***	***SD***
149.759	116.131	163.36	108.924	181.823	98.976	***136.4955***	***33.27106***
Normal (forces in dynes)							
104.039	65.358	124.706	52.655			***86.690***	***33.46461***

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	136.4955	86.6895
Variance	1106.963	1119.88
Observations	6	4
Pooled Variance	1111.807	
Hypothesized Me	0	
df	8	
t Stat	2.314049	
P(T<=t) one-tail	0.024688	
t Critical one-tail	1.859548	
P(T<=t) two-tail	**0.049376**	
t Critical two-tail	2.306004	

19.67 hrs

Healing (forces in dynes)						***Average***	***SD***
173.923	143.830	225.106	158.628	216.078	135.6	***175.5275***	***37.38843***
Normal (forces in dynes)							
138.914	62.683	133.604	81.259			***104.115***	***37.94548***

t-Test: Two-Sample Assuming Equal Variances

	Variable 1	*Variable 2*
Mean	175.5275	104.115
Variance	1397.895	1439.86
Observations	6	4
Pooled Variance	1413.632	
Hypothesized Me	0	
df	8	
t Stat	2.942468	
P(T<=t) one-tail	0.00932	
t Critical one-tail	1.859548	
P(T<=t) two-tail	**0.018639**	
t Critical two-tail	2.306004	

Collagen Assay Analysis

	(OD at 450 nm)	
	Healing	**Normal**
	0.442	0.357
	0.2115	0.177
	0.336	0.207
	1.125	1.073
	0.859	0.8125
	0.9035	0.8195
Average		***0.574333***

Normalized to Average Normal OD (at 450 nm)

	Healing	**Normal**	**Difference**
	0.769588	0.62159	0.147998
	0.368253	0.308183	0.06007
	0.585026	0.360418	0.224608
	1.958793	1.868253	0.09054
	1.495647	1.414684	0.080963
	1.573128	1.426872	0.146257
Average	***1.125073***	***1***	***0.125073***
SD	***0.636233***	***0.654051***	***0.060407***

t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1.125073	1
Variance	0.404793	0.427783
Observations	6	6
Pearson Correlati	0.995997	
Hypothesized Mea	0	
df	5	
t Stat	5.071641	
P(T<=t) one-tail	0.001931	
t Critical one-tail	2.015048	
P(T<=t) two-tail	**0.003862**	
t Critical two-tail	2.570582	

MTT Assay on Collagen Gels for Proliferation

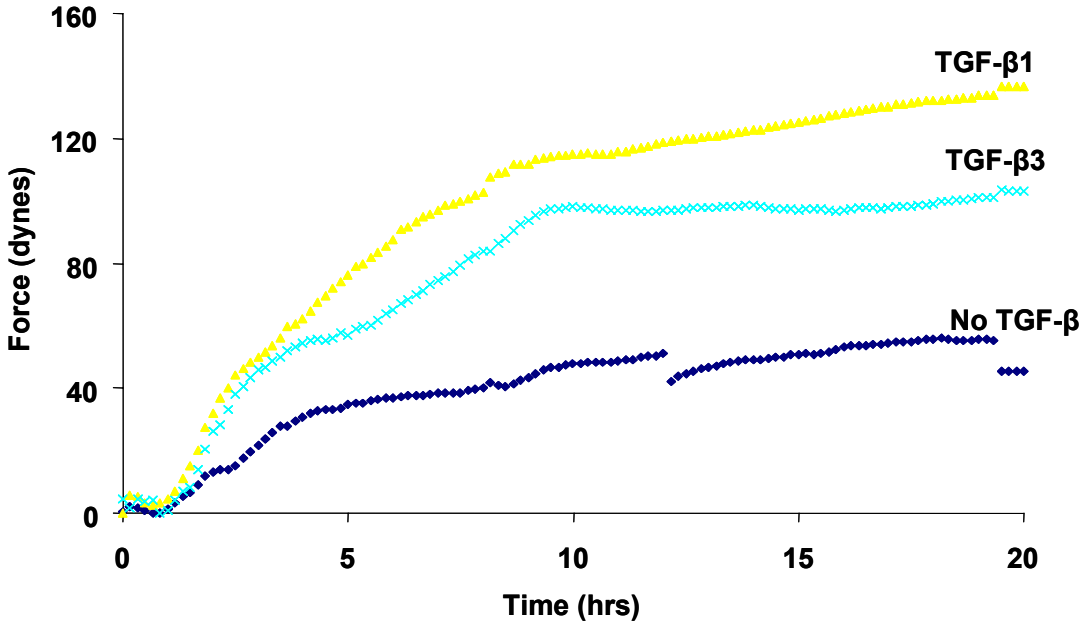
24 hours	**(OD at 550 nm)**	
	Healing	**Normal**
	0.4125	0.454
	0.451	0.4075
	0.4265	0.462
	0.418	0.476
	0.456	0.4075
	0.4685	0.5265
Average	***0.43875***	***0.455583***
Normalized to average Normal OD	***0.963051***	***1***
SD	***0.022814***	***0.044975***

t-Test: Paired Two Sample for Means

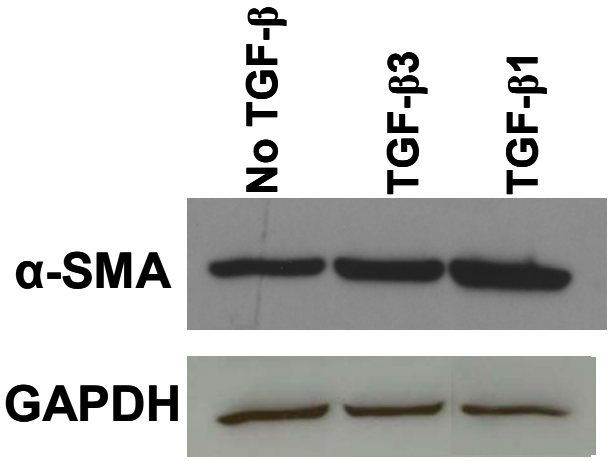
	Variable 1	*Variable 2*
Mean	0.43875	0.455583
Variance	0.00052	0.002023
Observations	6	6
Pearson Correlation	0.044954	
Hypothesized Mean D	0	
df	5	
t Stat	-0.83287	
P(T<=t) one-tail	0.221435	
t Critical one-tail	2.015048	
P(T<=t) two-tail	**0.44287**	
t Critical two-tail	2.570582	

Specific Aim #2: The following graphs represent the contraction forces produced by FPCGs treated with 1 ng/mL TGF-β1, 1 ng/mL TGF-β3, or untreated controls. The bands below each graph are the corresponding results of Western blotting for α-SMA on cells from these FPCGs. A total of five experiments were performed.

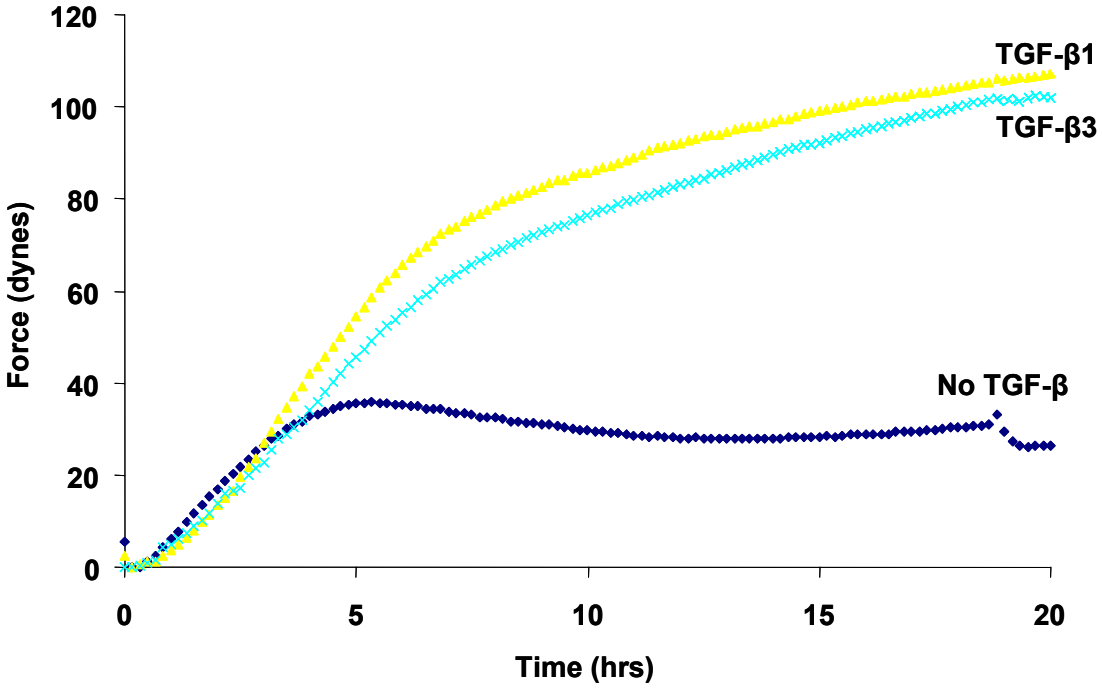
Experiment #1 **(Figure 6 in text)**:

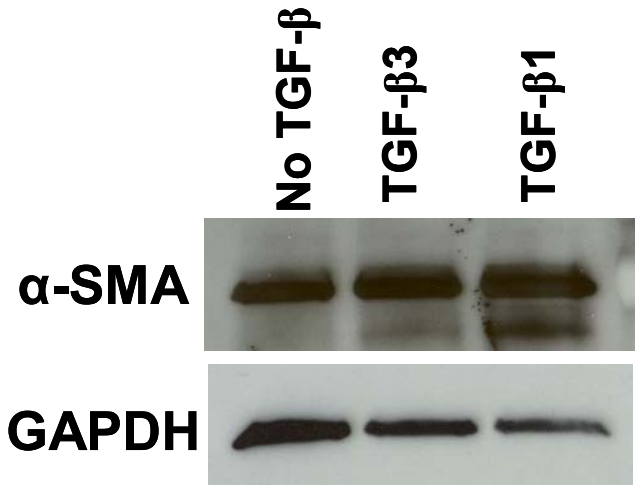


(Figure 8 in text)

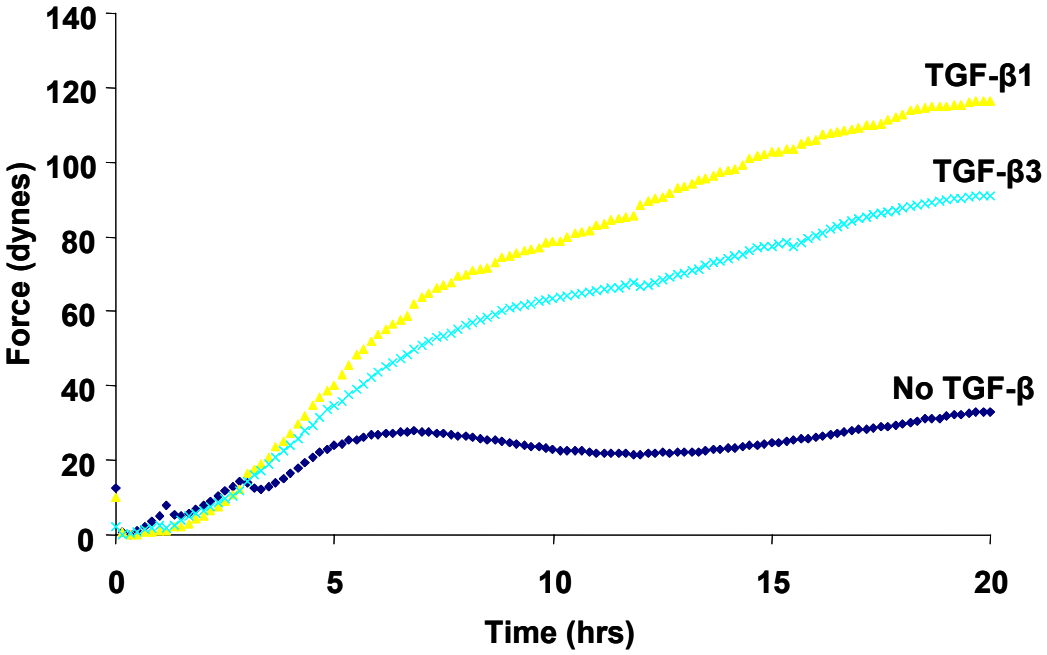


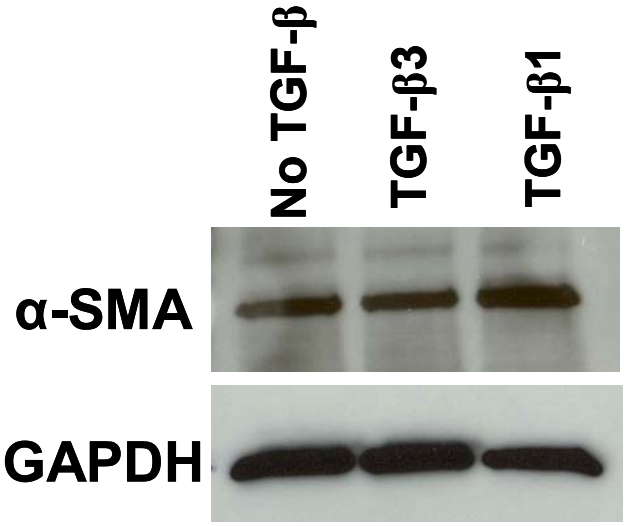
Experiment #2:



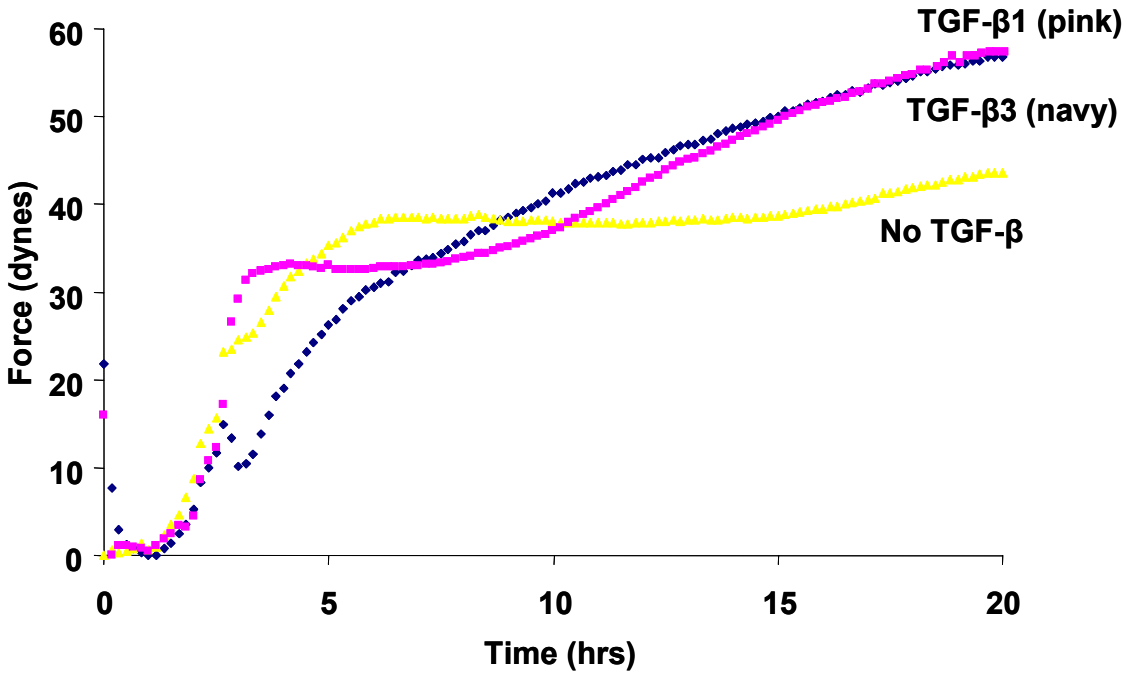


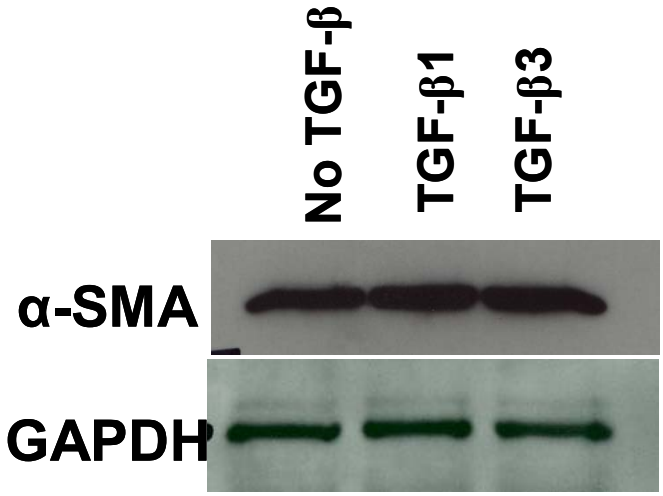
Experiment #3:



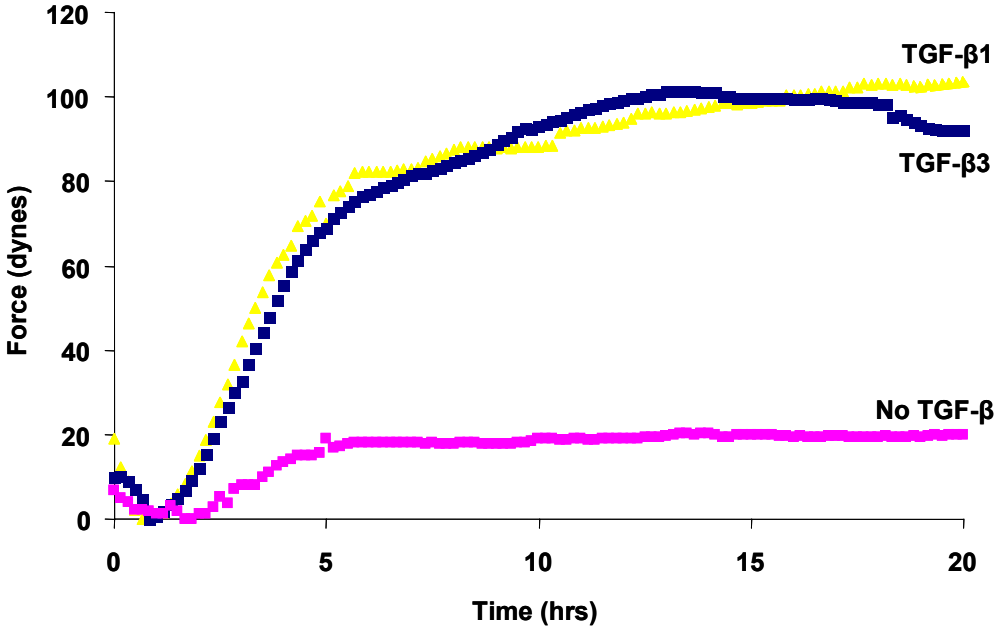


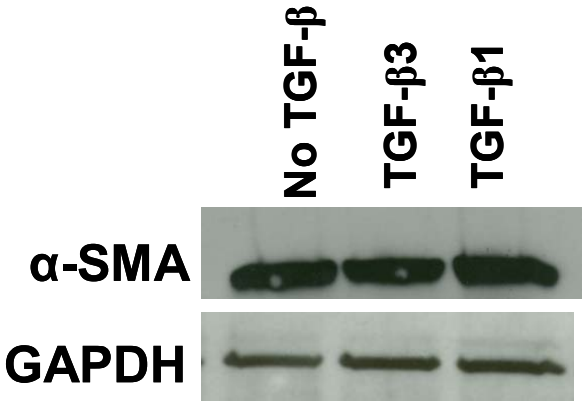
Experiment #4:





Experiment #5:





Contraction Data for TGF-beta

	Contraction Forces (dynes)		
	No TGF-b	**TGF-b3**	**TGF-b1**
1	45.535	103.170	136.605
2	26.38908	106.9959	108.9041
3	33.04903	90.93652	116.3623
4	43.60297	56.68425	56.72601
5	20.00415	87.57687	102.9363
Average	***33.716***	***89.073***	***104.307***
Normalized to average No TGF-b	***1.000***	***2.642***	***3.094***
SD	***10.94976***	***19.8412***	***29.47643***

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	168.5804	33.71608	119.8971
Column 2	5	445.3633	89.07267	393.673
Column 3	5	521.5335	104.3067	868.8601

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between Groups	13799.1	2	6899.551	14.97266	**0.000548**	3.885294
Within Groups	5529.721	12	460.8101			
Total	19328.82	14				

Bonferroni's Test

Treatment groups:

1 = No TGF-β

2 = TGF-β3

3 = TGF-β1

Multiple Comparisons

Dependent Variable: Forces

Bonferroni

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-55.35665*	13.57661	.005	-93.0925	-17.6208
	3.00	-70.59069*	13.57661	.001	-108.3265	-32.8549
2.00	1.00	55.35665*	13.57661	.005	17.6208	93.0925
	3.00	-15.23403	13.57661	.851	-52.9699	22.5018
3.00	1.00	70.59069*	13.57661	.001	32.8549	108.3265
	2.00	15.23403	13.57661	.851	-22.5018	52.9699

*. The mean difference is significant at the .05 level.

Western Blot Analysis for TGF-beta

Density of Western Blot Bands (area x intensity)

	A-SMA				GAPDH		
	No TGF-b	**TGF-b3**	**TGF-b1**		**No TGF-b**	**TGF-b3**	**TGF-b1**
1	2.85547	3.572919	4.97821	1	1.708952	1.492764	1.389714
2	2.866338	3.368201	3.851767	2	3.350838	2.362432	1.698144
3	2.296907	2.399679	3.002839	3	4.157937	4.262572	3.388943
4	0.422083	0.488634	0.5522	4	2.062884	2.070082	1.969812
5	5.80289	5.448611	6.253418	5	1.951973	1.701622	2.156108

	A-SMA				**A-SMA**		
	Normalized Relative to GAPDH				**Normalized Relative to Control**		
	No TGF-b	**TGF-b3**	**TGF-b1**		**No TGF-b**	**TGF-b3**	**TGF-b1**
1	1.67089	2.393492	3.582183	1	1	1.432465	2.143877
2	0.855409	1.425735	2.268222	2	1	1.666728	2.651622
3	0.552415	0.562965	0.886069	3	1	1.019098	1.603992
4	0.204608	0.236046	0.280331	4	1	1.153648	1.370087
5	2.972833	3.20201	2.900326	5	1	1.07709	0.97561
				Average	***1***	***1.269806***	***1.749038***
				SD	***0***	***0.272714***	***0.658291***

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	5	5	1	0
Column 2	5	6.349029	1.269806	0.074373
Column 3	5	8.745188	1.749038	0.433347

ANOVA

rce of Varia	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*
Between G	1.439193	2	0.719596	4.251926	**0.040186**	3.885294
Within Gro	2.030881	12	0.16924			
Total	3.470073	14				

Bonferroni's Test

Treatment groups:

1 = No TGF-β

2 = TGF-β3

3 = TGF-β1

Multiple Comparisons

Dependent Variable: Density

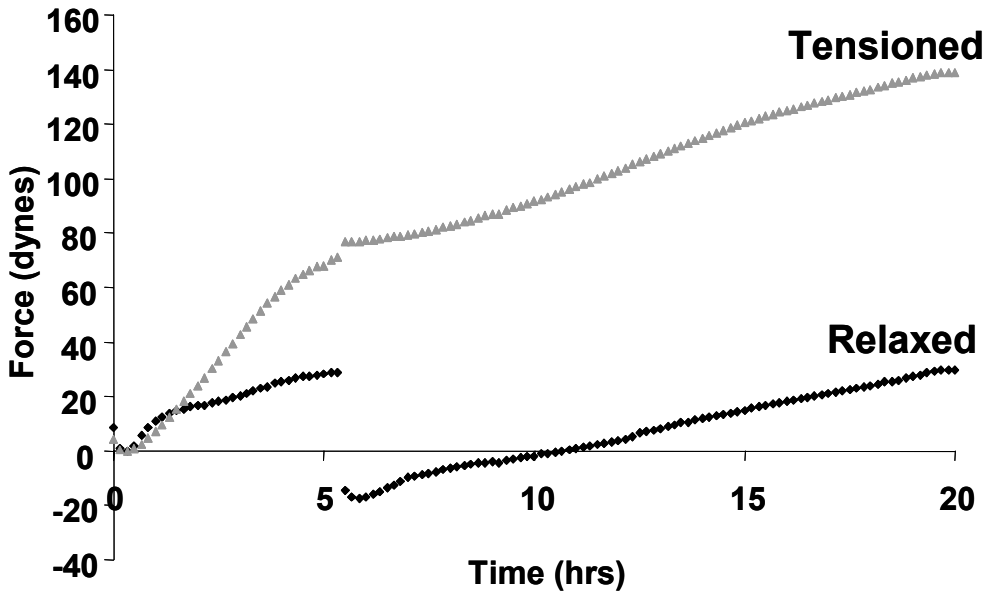
Bonferroni

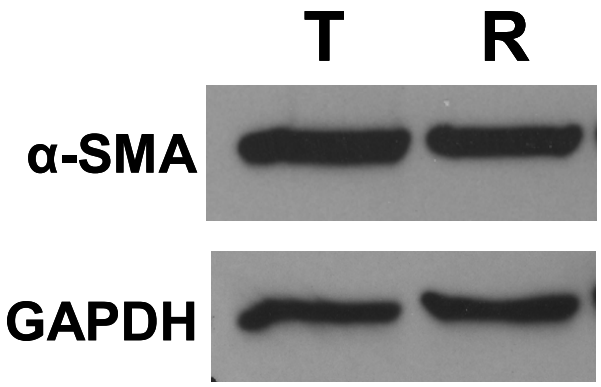
(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.26981	.26018	.961	-.9930	.4534
	3.00	-.74904*	.26018	.042	-1.4722	-.0259
2.00	1.00	.26981	.26018	.961	-.4534	.9930
	3.00	-.47923	.26018	.271	-1.2024	.2439
3.00	1.00	.74904*	.26018	.042	.0259	1.4722
	2.00	.47923	.26018	.271	-.2439	1.2024

*. The mean difference is significant at the .05 level.

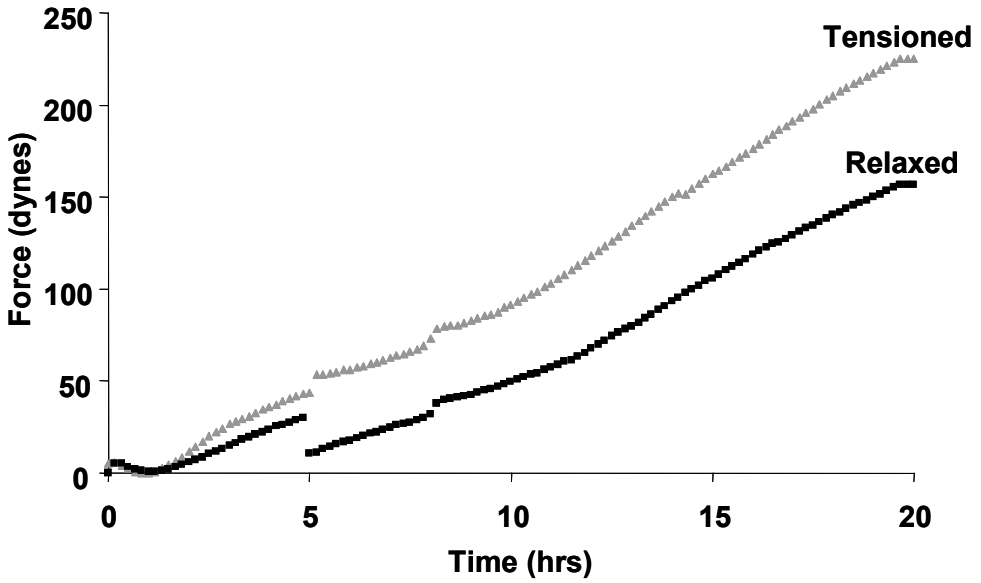
Specific Aim #3: The graphs below represent the contraction forces tracked by the DCFM. At 5 hrs, tension was released in the relaxed gels to approximately the starting levels. The bands below each graph represent the corresponding results from Western blotting for α-SMA from the tensioned and relaxed gels. A total of five experiments were performed.

Experiment #1:

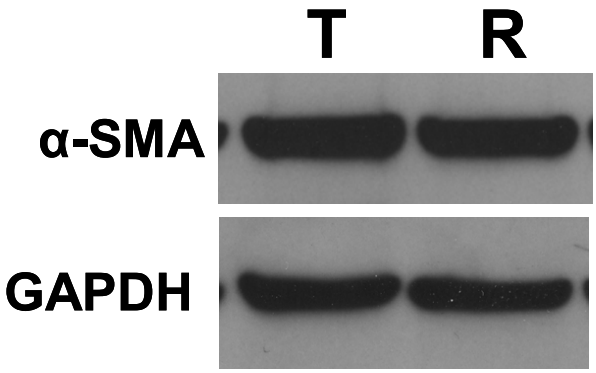




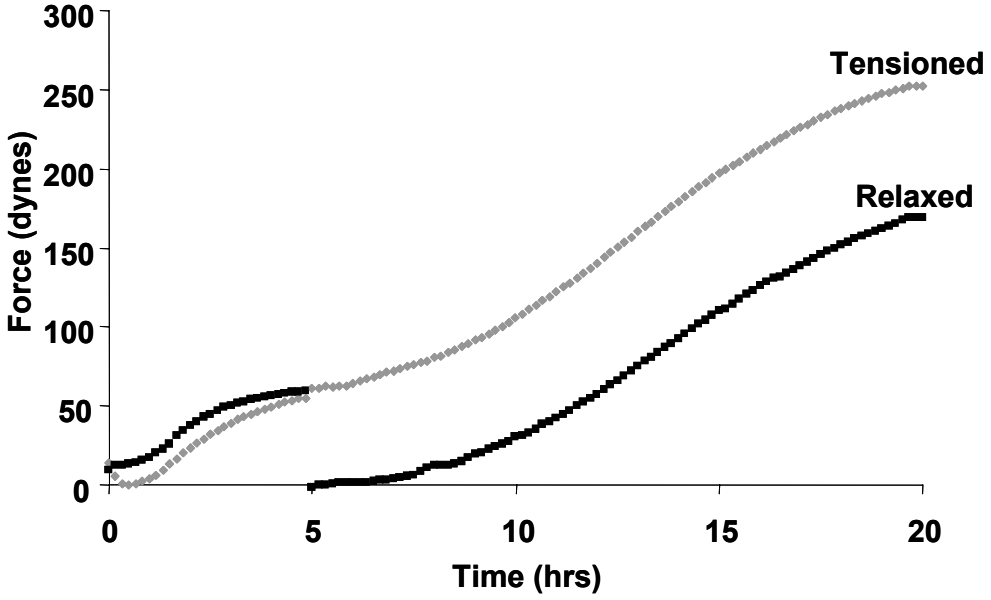
Experiment #2 **(Figure 11 in text)**:

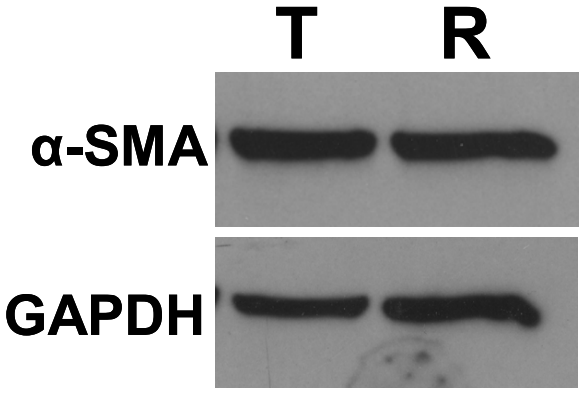


(Figure 12 in text)

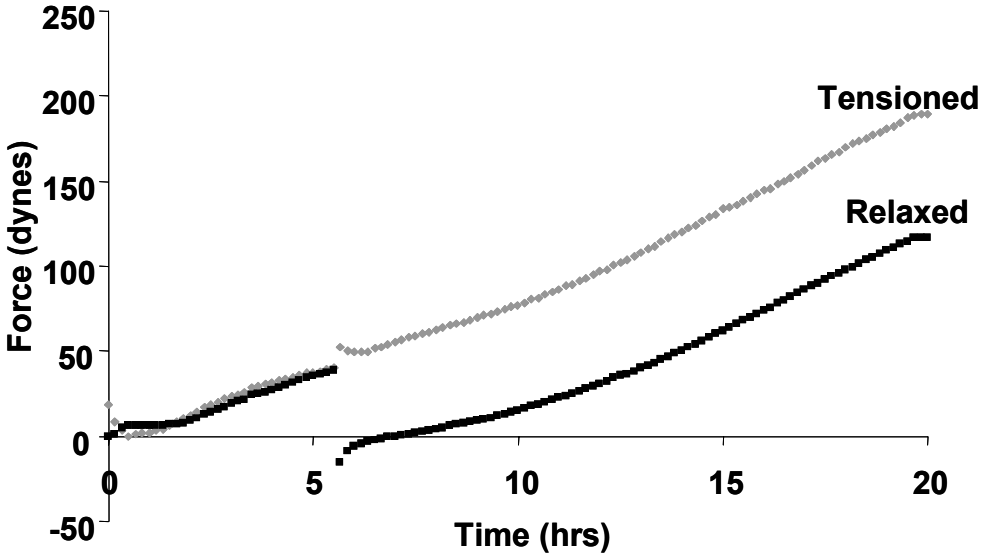


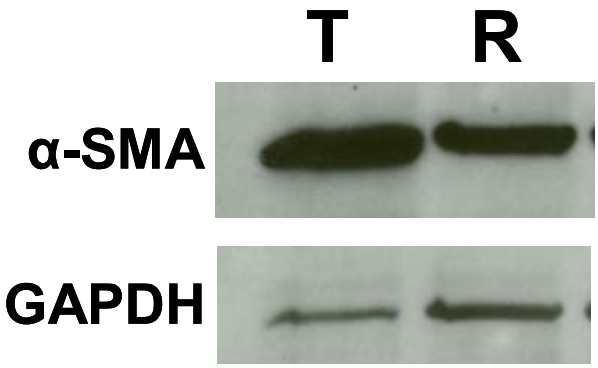
Experiment #3:



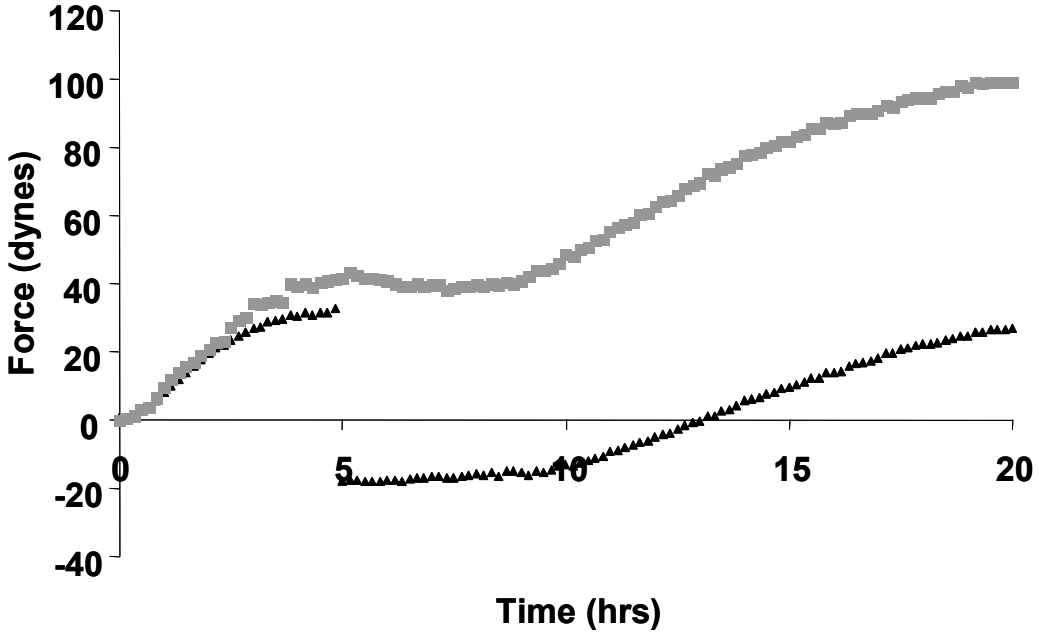


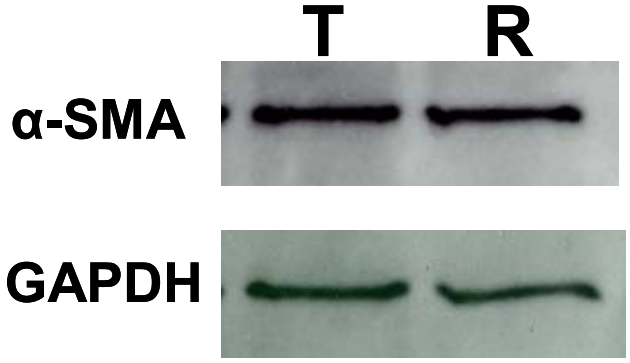
Experiment #4:





Experiment #5:





Contraction Data for Tensioned-Relaxed Experiments

	Contraction Forces (dynes)	
	Tensioned	**Relaxed**
1	138.742	29.645
2	208.574	156.651
3	252.169	168.916
4	189.096	116.495
5	99.317936	26.735
Average	***177.580***	***99.688***
Normalized to average Tensioned forces	***1.000***	***0.561***
SD	***59.77053***	***68.0956***

t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	177.57969	99.68847
Variance	3572.5163	4637.011
Observations	5	5
Pearson Correlation	0.9552889	
Hypothesized Mean Di	0	
df	4	
t Stat	8.3675162	
P(T<=t) one-tail	0.0005578	
t Critical one-tail	2.1318468	
P(T<=t) two-tail	**0.0011156**	
t Critical two-tail	2.7764451	

Western Blot Analysis for Tension-Relax Experiments

Density of Western Blot bands (area x intensity)

	A-SMA	
	Tension	**Relax**
1	0.415601	0.324324
2	0.508583	0.459844
3	0.325651	0.317562
4	5.936804	3.70447
5	2.295991	1.81594

	GAPDH	
	Tension	**Relax**
1	4.697918	4.97569
2	7.428092	7.171498
3	3.121764	4.672022
4	1.412268	2.555504
5	2.627568	2.719313

	A-SMA Normalized Relative to GAPDH	
	Tension	**Relax**
1	0.088465	0.065182
2	0.068467	0.064121
3	0.104316	0.067971
4	4.203737	1.449604
5	0.873808	0.667794

	A-SMA Normalized Relatvie to Control	
	Tension	**Relax**
1	1	0.736808
2	1	0.936519
3	1	0.651586
4	1	0.344837
5	1	0.764234

Average	***1***	***0.686797***
SD	***0***	***0.217391***

t-Test: Paired Two Sample for Means

	Variable 1	*Variable 2*
Mean	1	0.686797
Variance	0	0.047259
Observatio	5	5
Pearson C	#DIV/0!	
Hypothesiz	0	
df	4	
t Stat	3.22159	
P(T<=t) on	0.016116	
t Critical on	2.131847	
P(T<=t) tw	**0.032232**	
t Critical tw	2.776445	

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