GENE EXPRESSION IN RESPONSE TO MECHANICAL LOADING ON THE ANTERIOR CRUCIATE LIGAMENT

by

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Abstract: Many tissues in the body, especially bone, have exhibited adaptive responses to low-magnitude, high frequency mechanical loading. However, the response of ligament to these types of mechanical loads is not well understood. Therefore, the purpose of this study was to identify the mechanisms by which ligaments respond *in vivo* to high-frequency, low-magnitude mechanical loading by identifying (1) if there is a response to this mechanical loading, and (2) what genes are altered in response to this mechanical loading on the ligament. The left ACL of seven rabbits were subjected to *in vivo*, low-magnitude, high-frequency mechanical loading for twenty minutes, in a novel mechanical loading device, the RACL loader. Three rabbits served as external controls and received no loading. Following four hours to allow for genetic response, the ACL's were harvested, and the RNA extracted to determine which genes were altered in expression in response to the loading.

In response to mechanical loading, the loaded ACL had three genes differentially expressed compared to the internal control ACL. None of these three genes had annotations within the rabbit genome. The loaded ACL had 121 genes differentially regulated compared to the external control ACL (including 1 regulating collagen synthesis, and 15 with links to mechanotransductive pathways). This shows that there is a systemic response to mechanical loading in the ligament. Additionally, the genetic results shed light on the possible

mechanotransduction response pathway in ligament. This study provides evidence that ligaments can be adapted through mechanical loading and may be used one day to strengthen a ligament for to reduce injury rates.

Gene Expression in Response to Mechanical Loading on the Anterior Cruciate Ligament

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Chapter I. Introduction

More than 350,000 ACL (anterior cruciate ligament) reconstructions are performed annually in the United States and reconstruction rates are on the rise (Herzog et al., 2017; Nessler et al., 2017). While ACL tears occur in both men and women, women have been found to be three to five times more likely to tear their ACL than men (Lohmander et al., 2004). ACL tears occur in athletes of all ages, especially both collegiate (Rugg et al., 2014) and young athletes, (Wiggins et al., 2017), even more in sports involving pivoting, cutting, jumping and landing such as football, handball, and soccer (Drawer and Fuller, 2002; Lohmander et al., 2007).

ACL tears, for a long time, have been termed "the beginning of the end of the knee" due to life-long post-tear complications, and there is evidence that this is still the case today (DeHaven, 1983). After the first ACL tear, patients are at an increased risk for secondary tears, as one in four young athletes who suffer an ACL tear and return to sport will re-tear that same ACL at some point in their athletic career (Wiggins et al., 2017). Collegiate athletes, with a prior ACL tear, are 8 times more likely to sustain a secondary tear, and spend 50% more time on the disabled list (Rugg et al., 2014). Additionally, more than 50% of people who tear their ACL will develop osteoarthritis in their knee throughout their lifetime associated with pain, functional limitations, and decreased quality of living (Lohmander et al., 2004). Depending on when the tear occurs in a person's lifespan, osteoarthritis can develop at a young age. A study examining cohort of collegiate soccer players, tested 14 years post ACL tear, found 51% of them had developed knee osteoarthritis, while more than 75% of them reported functional limitations in their knee affecting their quality of living (Lohmander et al., 2004). Therefore, prevention of ACL injuries is important.

There have been numerous proposed mechanisms for ACL injury prevention, such as focus on altering movement patterns (Hewett et al., 1999, 1996) , proprioception training (Caraffa et al., 1996) and neuromuscular adaptations (Hewett et al., 2002). Despite focus on ACL injury prevention for the past twenty years, there has not been a reduction of injury rates. In fact, over the past twenty years, ACL injury rates are on the rise (Beck et al., 2017). Investigating a way to strengthen the ACL may be an alternative method to prevent ACL tears.

Tissues in the body have been known to be able to adapt to mechanical loading (Brown et al., 1998; Ingber, 1991). Mechanotransduction is the signaling response to a mechanical load, and while the signaling behind this process is not completely understood, it has been proven in muscle (Gilsanz et al., 2006), tendon (Arampatzis et al., 2007; Arnoczky et al., 2002), and especially in bone (Rubin et al., 2001; Turner et al., 1991; Wolf et al., 2001).

Mechanotransduction in the ligament has been thoroughly studied in the periodontal ligament. Connecting the root of a tooth and the alveolar bone, the periodontal ligament has been shown to produce a variety of chemical responses, such as regulating gene expression and transcription factors, to induce bone and extracellular matrix formation or resorption in response to mechanical loading at the tooth (De Araujo et al., 2007; Garlet et al., 2007; Kanzaki et al., 2002; Kawarizadeh et al., 2005; McCulloch et al., 2000; Rios et al., 2008). However, structurally, the periodontal ligament is not the same as typical ligaments that connect bone to bone in a joint, as its' properties allow it to respond differently to loading compared to typical ligaments. The periodontal ligament is highly vascularized compared to typical ligaments and completely remodels its collagen much faster than a normal ligament (Berkovitz, 1990). This increased rate of remodeling and increased vasculature make it unlike typical ligaments, and questionable that other ligaments would respond to mechanical loading in the same way.

Traditionally, training would occur through a high magnitude load, applied a limited number of times. However, as ligaments are designed to restrict unhealthy joint movement, high magnitude loading of ligaments is likely non-desirable, making low magnitude, high frequency an intriguing possibility. Used on the hind legs of sheep (Rubin et al., 2002), the ulnas of turkeys (Rubin et al., 1996), the tibias of rats (Turner et al., 1991), and the lower extremity of humans (Rubin et al., 2004), these low magnitude, high frequency loads resulted in increased bone mineral density (Rubin et al., 2002, 2004), creation and thickening of trabecular bone (Rubin et al., 2002), increased intracortical pores (Rubin et al., 1996), stiffer and stronger bone in the direction of loading (Rubin et al., 2002), and decreased rate of bone loss (Rubin et al., 2002, 2004).

While bone clearly demonstrates the ability to adapt and strengthen in response to high frequency, low magnitude mechanical loading, it would make sense that ligaments, the connective tissue connecting two bones together, would also exhibit similar adaptive properties. If the bone were to get stronger through loading in the absence of ligament strengthening, it may create an imbalance between the two tissues that may be problematic. It is likely that ligament should respond, through a similar mechanism as bone, with a similar adaptive response.

There is limited research on a typical ligament response to mechanical loading. The exact sensor for ligament mechanotransduction is unknown, but a good means to assess the response to loading is to investigate downstream gene expression. However, the specific genes in ligament that will respond to mechanical loading is relatively unknown (Sarasa-Renedo and Chiquet, 2005). Cyclic loading of human ACL fibroblasts *in vitro* was found to produce increases in collagen I and III mRNA (Kim et al., 2002). However, previous studies have also shown mechanical loading to increase collagen I but not collagen III mRNA in ACL fibroblasts, and

collagen III but not collagen I mRNA in MCL's (Hsieh et al., 2002). These conflicting findings are likely a result of differences in the direction, duration, or frequency of the applied load to the ligament. Nonetheless, this is evidence that ligaments may exhibit mechanotransductive properties. While the majority of this limited work has been done through cyclic testing on cultured fibroblast *in vitro*, mechanical loading on a typical ligament *in vivo* may be a more accurate representation of a true mechanotransductive response.

Purpose

The purpose of this study was to identify (1) if loading a ligament, *in vivo*, will produce a mechanotransductive response, and (2) what genes are altered in their expression level in response to high-frequency, low-magnitude loading.

Significance

The findings of this study will help improve the understanding of ligaments biochemical response to mechanical loading. If ligaments were able to be strengthened through mechanical loading, it may be possible to reduce the risk of ACL tears, however, the response of ligament to these types of mechanical loads is not well understood. Many tissues in the body exhibit an adaptive response to low magnitude, high frequency loading, and it is likely that ligaments would respond similarly. This study will help to identify the mechanisms by which ligaments respond to mechanical loading by identifying (1) if there is a response to this mechanical loading, and (2) what genes are altered in response to mechanical loading on the ligament.

Delimitations

1. Skeletally mature New Zealand White rabbits will be used as participants for this study
2. All rabbits used will be female rabbits
3. All rabbits will have healthy knee complexes

Operational Definitions

Mechanotransduction: The process by which cells sense respond to mechanical stimuli by converting them to biochemical signals that elicit specific cellular responses.

Chapter II. Review of the Literature

Introduction

The purpose of this study was to identify (1) if loading a ligament, *in vivo*, will produce a mechanotransductive response, and (2) what genes are altered in their expression level in response to high-frequency, low-magnitude loading. This review of literature will explore the following concepts: the prevalence and complications associated with ACL tears, the chemical response to mechanical loading that occurs in tissues throughout the body, and how ligaments respond to this mechanical loading.

ACL Tears: Prevalence and Complications

Based on data from 2002-2014, more than 350,000 ACL (anterior cruciate ligament) reconstructions are performed annually in the United States (Herzog et al., 2017; Nessler et al., 2017). A retrospective study analyzing health databases in this 12-year span found the rate of ACL reconstruction to have rose by 22% per 100,000 person-years (Herzog et al., 2017). While the rate of ACL reconstruction rose in both males and females during this time, the rate of female ACL reconstruction rose nearly three times as much compared to males, consistent with the fact that young women have a nearly three to five times higher risk of ACL injury than males (Herzog et al., 2017; Lohmander et al., 2007). This also holds true when controlling for the effect of gender participation. The rate of ACL injury may even be higher than the rate of ACL reconstruction rate, as not all ACL injuries are treated with a reconstruction. These ACL injury rates are increasing in both collegiate (Rugg et al., 2014) and young athletes (Wiggins et al., 2017). The risk of ACL injury is present during any sport, but is increased in sports involving pivoting, jumping, landing and cutting, such as football, handball and soccer (Drawer and Fuller,

2002; Lohmander et al., 2007; Nessler et al., 2017). Soccer, with its combined jumping, landing, pivoting and contact from slide tackles, often occurring simultaneously, increases the risk of ACL tears anywhere from 100 to 1000% (Drawer and Fuller, 2002; Lohmander et al., 2007).

Athletes who tear their ACL are at an increased risk for a secondary tear. Nearly 20% of people will suffer re-injury within two years following reconstruction (Holm et al., 2012), and only 60% of people with ACL reconstructions will return to normal or close to normal knee function (Eriksson et al., 2001). One in four young athletes who suffer an ACL tear and return to a high risk sport will re-tear that ACL at some point during their athletic career, often early in the return to play period (Wiggins et al., 2017). These young athletes who have suffered ACL tears and go on to play collegiate level sports are 8 times more likely to suffer a second tear, and when it does occur, they spend 50% more time on the disabled list, medically unable to play (Rugg et al., 2014).

ACL tears, for a long time, have been termed “the beginning of the end of the knee” (DeHaven, 1983). Along with the many complications following an ACL tear including reconstruction surgery, immobilization, and rehabilitation, ACL tears are also associated with post traumatic OA (osteoarthritis) in the years following the tear. Due to great variability in reported results, an official percentage of people developing OA 10 to 20 years following ACL tears has been reported anywhere from 10-90%, (Frobell et al., 2015; Gillquist and Messner, 1999; Holm et al., 2012; Lohmander and Roos, 1994; Ruano et al., 2017) but a metanalysis of 127 publications reviewing ACL tears and the development of OA has found an overall mean of more than 50% (Lohmander et al., 2004). This means that 10 to 20 years following an ACL tear, at least one of every two patients will develop OA. Additionally, it was recently found that 26% of patients will develop OA within five years of injury (Frobell et al., 2015; Ruano et al., 2017).

No matter the reconstruction or rehabilitation technique used, a vast majority of the patients with ACL tears will develop osteoarthritis and knee problems during middle age (Von Porat et al., 2004). Despite surgical techniques to reconstruct the ACL, often from either a semitendinosus graft, gracillis graft, or bone patellar tendon bone graft, there is no evidence that any type of ACL reconstruction improves chances of not getting OA (Eriksson et al., 2001; Myklebust and Bahr, 2005; Von Porat et al., 2004). Osteoarthritis can develop before middle age too. With the majority of acute ACL tears occurring in in patients younger than 30 years old, a large majority of these individuals develop early-onset OA, often affecting athletes during their playing career (Lohmander et al., 2004; Von Porat et al., 2004).

A high prevalence of soccer players, years after ACL injury, have reported knee OA, pain, and functional limitations. In 103 female soccer players tested, 82% had radiographic changes to their knee and 51% of the women exhibiting OA. In addition, 75% of these women reported having significant symptoms affects their knee related quality of life (Lohmander et al., 2004). In a similar cohort of male soccer players 14 years after ACL tears, similar consequences on the knee were found, in addition to 80% reporting reduced activity level (Von Porat et al., 2004). In these athletes who suffered ACL tears at a young age, the development of OA not only starts at an early age but will continue to progress throughout their lifespan. OA development following ACL tears is strongly correlated with decreased quality of living, functional limitations, and significant pain (Lohmander et al., 2004). Patients with OA report feeling handicapped during functional and social activities, in their relationships, socioeconomically, in their emotional wellbeing, and as a representation of their body image (Carr, 1999).

The high incidence of ACL injuries and the lifelong complications following the injuries make prevention of ACL injuries important. In the past, there have been numerous proposed

mechanisms for ACL injury prevention, such as focus on altering movement patterns (Hewett et al., 1996), proprioception training (Caraffa et al., 1996) and neuromuscular adaptations (Hewett et al., 2002). Despite focus on ACL injury prevention for the past twenty years, there has not been a reduction of injury rates. In fact, over the past twenty years, ACL injury rates are on the rise (Beck et al., 2017). Investigating a way to strengthen the ACL may be an alternative method to prevent ACL tears.

Mechanotransduction in Muscle & Tendon

Tissues in the body have an ability to adapt based on the types of mechanical loads that are applied to it (Brown et al., 1998; Ingber, 1997, 1991). This process, called mechanotransduction, involves the conversion of mechanical signals to a chemical response that triggers a cellular response in the tissue. While not fully understood, there is evidence that when mechanical signals are applied to the tissues of cells, integrins, the actin cytoskeleton, stretch-sensitive ion channels and signaling molecules all help convert this mechanical load to a chemical signal and response (Alenghat and Ingber, 2002; Lavagnino et al., 2007; Liedert et al., 2006).

Integrins, transmembrane receptors that link actin associated proteins to the ECM (extracellular matrix) and to adhesion receptors on the surface of other cells, are a key mediator of mechanotransduction by altering the number, location or strength of adhesive contacts that can transmit physical forces between neighboring cells (Beckerle and Yeh, 1990; Ingber, 1991; Springer, 1990). Actin cytoskeletons, the load bearing architectural structure of the cell, also contributes to mechanotransduction with its scaffolding which holds the cell together (Alenghat and Ingber, 2002). Stretch sensitive ion channels located in the membrane also contribute to mechanotransduction by regulating the molecules that can pass through the membrane ion

channel (Ingber, 1991). Lastly, mechanotransduction occurs with deformation of the cytoskeleton initiating a cascade of signaling molecules that lead to a variety of chemical responses, such as altered gene, growth factor or proteoglycan expression, and re-organization or synthesis of collagen matrix (Arampatzis et al., 2007; Lavagnino et al., 2007; Lavagnino and Arnoczky, 2005; Wang et al., 2007).

Muscle is one tissue that has been shown to exhibit mechanotransductive properties. Young women who underwent low magnitude, high frequency full body vibrations for two minutes a day for twelve months increased muscle mass in the lower extremity and up into the axial skeleton as well (Gilsanz et al., 2006). These mechanical vibrations, induced from standing on a vibrating platform, mechanically loaded the lower extremity muscles, leading to increased muscle mass as a result of the mechanotransductive property of muscle.

Since tendons connect bone to muscle, it is logical that tendon also exhibits mechanotransductive properties. In response to 14 weeks of high loading strain induced through exercise in the Achilles tendon, there was a decrease in strain at a given tendon force, an increase in tendon-aponeurosis stiffness, tendon elastic modulus and Achilles tendon hypertrophy, compared to low loading exercise strain (Arampatzis et al., 2007). At a genetic level, in response to *in vitro* cyclic uniaxial loading of the human and dog patellar tendon, fibroblast proliferation and SAPKs levels increased (stress-activated protein kinases), as well as gene and protein expression of type I collagen and TGF-β1 (Arnoczky et al., 2002; Yang et al., 2004). Additionally, tensile loading of the rat tail tendon caused re-organization of the cytoskeleton fibers in the direction of loading, in addition to increased anabolic gene expression of collagen I and α1 collagen. However, in the absence of tensile loading, there was a loss of organization in the cytoskeletal fibers, and an increase in catabolic gene expression for collagen I, interstitial

collagenase, as well as inhibition of the previously expressed anabolic collagen I gene, α1 collagen (Lavagnino and Arnoczky, 2005). Tendon and muscle tissue display mechanotransductive properties.

Mechanotransduction in Bone

Bone has also been shown to exhibit a strong mechanotransductive response to loading. Wolff's law, the principle that a bone grows in response to the loading placed on it, has become widely accepted, if not scientifically proven (Bertram and Swartz, 1991; Turner et al., 1991). Patterns and periods of loading or de-loading on bones throughout the skeletal system has been shown to lead to either bone formation or bone resorption, respectively. Bone cells, osteoblasts and osteocytes, act as sensors of deformation in the bone tissue that signal an anabolic or catabolic chemical response (Lau et al., 2010; Turner et al., 1991). Through a fluid filled network called lacunae, osteocytes are able to connect to each other, and communicate in response to loading at both the transcriptional and protein levels to produce soluble factors that inhibit osteoclast (bone resorption cell) formation such as COX-2 mRNA (Lau et al., 2010).

The sensitivity of bone to mechanical stimuli has been demonstrated in clinically based studies investigating bone's site-specific formation response to exercise and bone's resorption in response to space flight or extended bed rest (Rubin et al., 2001). This response has been analyzed, using modeling techniques and experimental procedures, and is dependent on, but not limited to strain magnitude, strain distribution, strain frequency, strain gradient, strain rate, number of load cycles and strain history (Rubin et al., 1996, 2001).

Direction of loading on the bone has also been shown to have a strong impact the mechanotransductive response bone. Turkey ulnas were subject to low-magnitude cyclic compression and torsion loading in compression for four weeks. The ulnas subjected to

compression loading produced an increased number of intracortical pores, and a decrease in number of pores lost to resorption compared to turkey ulnas loaded in shear or not loaded at all (Rubin et al., 1996). Overall, this showed that bone tissue is sensitive to not only to low magnitude loading but can also readily distinguish between distinct directions of strain.

Similarly, hind limbs of sheep were subject to very-low magnitude, high frequency vibrations for one year and were found to increase bone mineral content and trabecular number, while decrease trabecular space, thus increasing trabecular density in their femurs (Rubin et al., 2002). Bone quality in the hind legs of these sheep were increased both by creation of new trabeculae, and the thickening of existing trabeculae. Additionally, significant increases in stiffness and strength in the longitudinal direction of the bone were observed, indicating again that the adaption occurred primarily in the weight bearing direction. Low magnitude mechanical loading also assisted in the healing process of broken bone by increasing the rate of callus formation. In response to induced bone fractures in the feet of sheep, their hind legs were subjected to low-magnitude, high frequency vibrations. These vibrations induced a mechanical load on the broken foot and resulted in a slightly enhanced callus formation at the break site (Wolf et al., 2001).

With promising results using low magnitude strain loading, a similar protocol was applied to a human population. After one year of brief periods standing on a vibration platform, especially in highly compliant individuals, participants demonstrated an increased bone mineral density in their lower extremity, with reduced rates of bone loss throughout the body (Rubin et al., 2004). Therefore, low magnitude strains can be beneficial not only to increase bone mineral density, but to slow the rate of bone loss and resorption as well.

The effect of loading frequency on the response to loading in bone has also been investigated. Bone formation has been shown to be positively correlated with loading frequency, based on bending loading applied to the tibia of rats (Turner et al., 1991). Bone formation rate increased almost four-fold from a loading frequency of 0.5 Hz to 2.0 Hz. At even greater frequencies, turkey ulnas loaded at 20 Hz produced around 40% more bony growth compared to a loading frequency of 1 Hz (Rubin and McLeod, 1994). Similarly, when rat ulnas were loaded at higher frequencies, strain related bone formation was enhanced at higher loading frequencies (Hsieh and Turner, 2001). These results show that loading frequency, and specifically greater frequencies allow for a greater mechanotransductive response of the bone, at the same loading magnitude.

While increased loading frequencies on bone often produce an increased osteogenic response to loading, there seems to be a frequency threshold that must be passed in order to induce a loading response. Turner et al, (1991), found no increase in bone formation rate when tibias of rats were loaded at frequencies of under 0.5 Hz. There also seems to be a threshold for magnitude of loading. While low magnitude loads are correlated with increased bone formation, there does appear to be a magnitude threshold where any strain below this threshold of loading will result in no effect of loading on bone formation. For applied bending strains on rat tibias that were below 1050 microstrain, equivalent to 40N or 0.1% strain, there was no evidence of increased bone formation (Turner et al., 1994). Additionally, the load magnitude threshold can be decreased, by increasing loading frequency (Hsieh and Turner, 2001). Therefore, it can be concluded that some combination of a low magnitude, high frequency mechanical loading would be optimal to produce a mechanotransductive response.

Mechanotransduction in Ligaments

While bone clearly displays a mechanotransductive response and the ability to strengthen in response to loading, it would make sense that ligaments, the connective tissue attaching bone to bone would also exhibit similar mechanotransductive properties. If bones have an anabolic response to loading, an absence of a ligament response would create an uneven modulus balance between the two tissues. A stronger bone with the same ligament would place the ligament at an increased risk for failure. Ligaments should respond with a similar adaptation mechanism to loading as bones, in order to be able to withstand similar forces that a stronger bone would withstand.

The response of the periodontal ligament, a ligament that attaches the root of a tooth to the alveolar bone, to mechanical loading has been thoroughly studied. Mechanical loading is applied to the tooth and transferred to the periodontal ligament, where periodontal cells react to this load by regulating the formation or resorption of bone (Lekic and Mcculloch, 1996). For example, in response to compression loading on a tooth, osteoclasts are formed to assist in bone or ECM formation (Kanzaki et al., 2002). Similar to the mechanotransduction in other tissues, the direction, frequency, duration and size of the loading forces determine the magnitude of the response (McCulloch et al., 2000). Without the periodontal ligament, forces applied to the teeth would produce a very limited bone remodeling response, indicating that the loss of the periodontal ligament would terminate mechanotransduction signaling at the tooth (McCulloch et al., 2000).

Mechanical loads to the periodontal ligament also produce a variety of chemical responses such as regulating gene expression of biological mediators such as interleukin-6, interleukin-1β, MMPs (matrix metalloproteinases) and TIMPs (tissue inhibitor of metalloproteinases), COX-2 (cyclooxygenase), alkaline phosphatase activity, type I collagen and

osteocalcin (McCulloch et al., 2000). These biological mediators can lead to bone and extracellular matrix formation, often through a mechanical feedback regulatory mechanism. Mechanical loading in rat periodontal ligament showed the importance of the periostin in maintaining structural integrity of the extracellular matrix (Rios et al., 2008). TGF-B was found to regulate periostin, which through cascade effects regulated collagen 1 fibrosis. This was confirmed, *in vitro*, in human periodontal ligament, and TGF-B was up-regulated in response to cyclic loading (Meikle, 2007), and on the tension and compression side of cycling bending (Garlet et al., 2007).

Collagen can also be synthesized via the ERK1 and ERK2 pathway (Kawarizadeh et al., 2005). Cyclic loading *in vivo* on rat periodontal ligaments found this mechanical stimulus to up-regulate Runx2 (runt-related transcription factor 2) via the ERK pathway. Runx2 stimulates the transcription of osteoblastic markers such as osteocalcin, type 1 collagen, alkaline phosphatase, all of which are pivotal transcription factors of bone and extracellular matrix formation.

RANKL (receptor activator nuclear factor kB ligament), a potent osteoclastogenic factor in the periodontal ligament, can be stimulated by static compressive forces (De Araujo et al., 2007; Kanzaki et al., 2002). This up-regulation occurred through a cascade of transcription factors, starting with the up regulation of Cox2, promoting an up-regulation of PGE_2 (prostaglandin E_2), finally resulting in up-regulation of RANKL, causing a breakdown and remodeling of bone and extracellular matrix. RANKL can also be down regulated by IL-10, a transcription factor that inhibits bone resorption (Garlet et al., 2007). IL-10, an anti-inflammatory cytokine, also up-regulates TIMP's to inhibit MMP's, which play a role in degradation and remodeling of the extracellular matrix. Therefore, IL-10, has an anabolic effect, and through a

cascade of chemical responses can inhibit extracellular matrix and bone decomposition and remodeling (Garlet et al., 2007).

The periodontal ligament has much in common with the connective tissues elsewhere in the body (Berkovitz, 1990). It is made up of primarily collagen, and the cells are mainly fibroblasts. The majority of the collagen in the periodontal ligament is type I collagen, which also forms the major proteins of skin, bone and tendon in our body, but type III collagen also contributes to periodontal ligament structure (Berkovitz, 1990). The periodontal ligament also displays a zig-zag like appearance, known as "crimp" which is seen in other connective tissues around the body. (Berkovitz, 1990). The extracellular matrix of the periodontal ligament also includes collagen V and VI, tenascin, fibronectin, undulin, proteoglycans, and chondroitin sulfate, all things included in normal ligament extracellular matrix (McCulloch et al., 2000).

However, the periodontal ligament is not the same as a typical ligament that connects bones and protects a typical joint. First, the periodontal ligament is not made up of single strands of straight collagen fibers, but rather consists of a complex meshwork, that causes loading to occur unevenly throughout the tissue (McCulloch et al., 2000). Additionally, normal collagen fibrils are known to increase in diameter with age, but periodontal ligament fibril diameters have been found to remain relatively constant throughout the lifespan (Luder et al., 1988). This thinner ligament may result in modified mechanotransductive properties. The most important difference is the rate of remodeling of the periodontal ligament compared to normal ligaments. Due to the high vasculature in the periodontal ligament, the rate of turnover is much faster, remodeling both type I and III collagen completely every 2 to 45 days (Berkovitz, 1990). Normal ligaments in the body have little to no vasculature, meaning diffusion of nutrients in and out of the cells is more difficult, leading to a slower rate of remodeling. For these reasons, it is unlikely

that the mechanotransductive properties of the periodontal ligament can be assumed in normal ligaments.

There is limited research on a normal ligament's response to mechanical loading. The mechanisms by which connective tissues, especially ligaments, convert mechanical signals into a specific response in extracellular matrix gene expression patterns are poorly understood (Sarasa-Renedo and Chiquet, 2005). Additionally, in the majority of this limited research, the loading is done on cultured fibroblasts *in vitro*, rather than *in vivo*, which may affect the response to the mechanical load as it is hard to fully simulate the cellular environment *in vitro*. Human ACL fibroblasts, loaded *in vitro* cyclically for 24 hours expressed increases in collagen I and III (Kim et al., 2002). Additionally, in the presence of a TGF-B antibody, expression of collagen I and III did not increase showing that TGF-B is a mediator of collagen I and III.

The ACL and the MCL (medial collateral ligament) have been shown to exhibit differing responses to loading. Type III collagen mRNA increased in MCL fibroblasts, but not in ACL fibroblasts, in response to cyclic strains (Hsieh et al., 2000). Contrarily, cyclic strains produced increased collagen I mRNA in ACL fibroblasts that were not present in MCL fibroblasts. However, it has also been shown that mRNA expression of type III collagen was higher than that of type I collagen in response to loading of ACL fibroblasts (Kim et al., 2002). These conflicting findings are likely a difference in the direction, duration, elongation rate and frequency of the load applied to the fibroblast. The difference in response between the ACL and MCL to loading is likely correlated with their differing healing capabilities. This difference in healing could also be a result of differing TGF-B1 expression in the two ligaments, as TGF-B1 is one of the most important regulators in the healing and remodeling process of the ligament, through its mediator of collagen synthesis (Kim et al., 2002). Incisions to rabbit MCL's *in vitro* produced an increased

expression of many growth factors, including TGF-B1 (Sciore et al., 1998). There is some belief that a ligament would respond to mechanical loading with a similar mechanism as it responds to healing (Menetrey et al., 1999).

Previous research has attempted to mechanically load ACL ligaments *in vitro*, but in a culture that is more representative of true *in vivo* conditions. A bone-ACL-bone complex, was used to test a constant load of 5 N on an intact rat bone ligament (Hsieh et al., 2002). Type I collagen mRNA was found to increase in the ligament after one hour of constant load, but then to decrease after two hours of constant load. While this is a step in the right direction to testing *in vivo*, the loading still occurs *in vitro*, outside of the organism's body. Additionally, a constant load was applied in this experiment, rather than the low-magnitude high-frequency oscillating loads that produced promising mechanotransductive results in bone. In general, while not fully conclusive nor exhaustive, there is evidence that certain genes and growth factors are potential stimulators of ligament cell proliferation and protein synthesis that are responsive to low-magnitude, high-frequency loading (Menetrey et al., 1999; Woo et al., 1998).

Summary

Not only are ACL tears common, but the side effects from a tear such as reduced return to sport, increased risk for a second ACL injury, and increased risk of osteoarthritis make ACL tears a risk for many athletes. A stronger ligament may reduce the risk of tears. Many tissues in the body, especially bone, have exhibited anabolic responses to low-magnitude, high frequency mechanical loading. However, the response of ligament to these types of mechanical loads is not well understood. Therefore, we aimed to identify if loading a ligament *in vivo* would produce a mechanotransductive response. Identifying a mechanism to load a ligament, and the gene

regulation in response to the mechanical load, may eventually allow for strengthening of ligaments, to reduce the occurrence of ACL tears.

Chapter III. Methods

Participant Information

Ten, female, New Zealand White rabbits were used for this study (3.79 ± 0.34 kg, mean body mass ± SD). This breed of rabbit was chosen based on the similarity of its knee joint complex to humans (Menetrey et al., 1999). For example, rabbits have been used in studies as a model for knee osteoarthritis development (Turhan et al., 2019) and for studies examining ACL reconstruction (Chai et al., 2018). This allows us to make better predictions about the extrapolations of this research to human population. All rabbits were fully mature retired female breeders (1.997 ± 0.329 years, mean age ± SD). The animals were housed locally at the Brody School of Medicine Animal Resource Center in compliance with the guidelines and procedures detailed in the Guide for the Care and Use of Laboratory Animals (U.S. Department of Health and Human Services). All procedures, including but not limited to: housing, anesthesia, euthanasia, and experimental procedures were approved by the Institutional Animal Care & Use Committee (IACUC)(Appendix I). To comply with the IACUC rules on acclimation, the animals were housed for at least five days after arrival before experimentation began. Since the rabbits were anesthetized while in the device, acclimation to the testing device was not necessary.

Seven of the ten rabbits were randomly assigned to receive experimental loading. Based on the assumption that there were no pre-existing differences between the ACL's of the right and left leg, due to constraints with adjusting the loading device, the left leg of each of the seven rabbits were loaded, while the right leg served as a control leg. Additionally, to control for the effects of being in the device, the remaining three rabbits served as controls. These three rabbits were positioned in the device for twenty minutes but underwent no loading.

Instruments and Equipment

A custom-made device, The RACL Loader (Rabbit Anterior Cruciate Ligament Loader) was developed to mechanically load ACL's at specified loading magnitudes and frequencies. This device allowed a rabbit to be positioned in the device, with the lower leg secured in a specific position to cyclically load the ACL *in vivo*. To our knowledge, this is the only device of its kind.

A servo-step motor, attached to a pulley system which moves a slider back and forth on a track, is controlled using a USB connection to a computer, and an ACE-SDE GUI, Version 228 (Arcus Technology, California)(Figure 1). This slider is connected to another slider with a spring and produces an oscillating load when it moves back and forth on the track (Figure 2). The second slider pulls a cast which wraps the rabbit's tibia. A 100% cotton cord connects the cast to the second slider, as this cotton cord has low strain, to ensure loading is not lost in the stretch of the cord. When the first slider moves forward, the spring, when fully compressed, moves the second slider forward. Then, as the first slider changes directions and moves backwards on the track, the spring elongates as the momentum from the second slider is still in the forward direction. As the spring recoils, and changes the second sliders momentum direction, a tension load is applied to the cast, pulling on the ACL. To ensure proper tension, an adjustable carabiner is used to adjust the starting tension of the cord, to account for the needed pre-load on the ACL (Figure 3). To measure the load being applied to the ACL, a tension/compression load cell, sampling at 1000 Hz, (MLP-75, Transducer Techniques) is attached to the second slider to measure the tension load pulling on the cord (Figure 3). The load data is monitored using NI SignalExpress 2013 software (National Instruments, Texas).

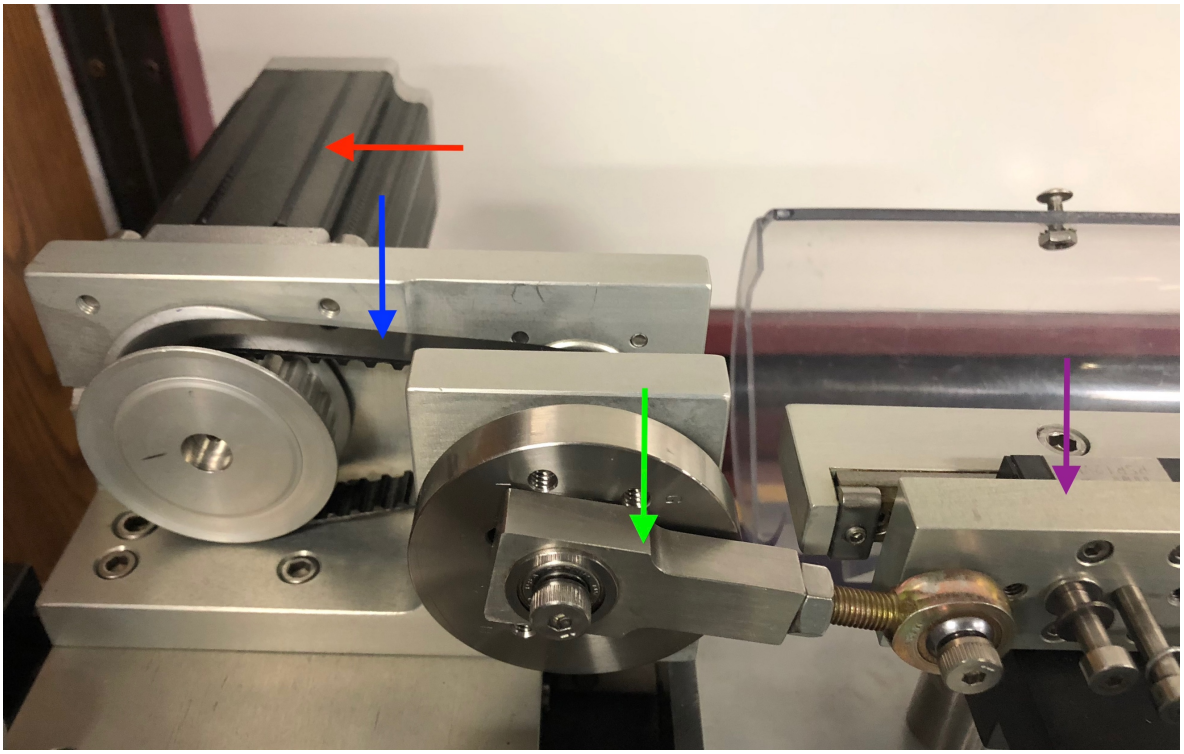

Figure 1: The servo-step motor (red arrow), spins a pulley system (blue arrow), which moves a crank arm (green arrow) connected to a slider on a track (purple arrow).

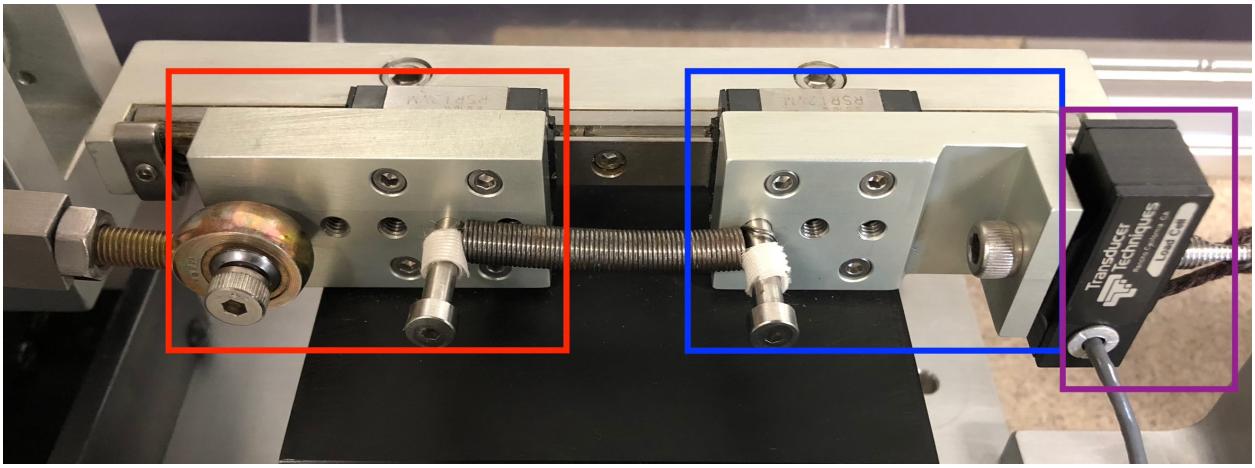

Figure 2: The oscillating first slider (red box) moves the second slider (blue box), by the spring pushing/pulling on the second slider. The tensile load is measured by the load cell (purple box).

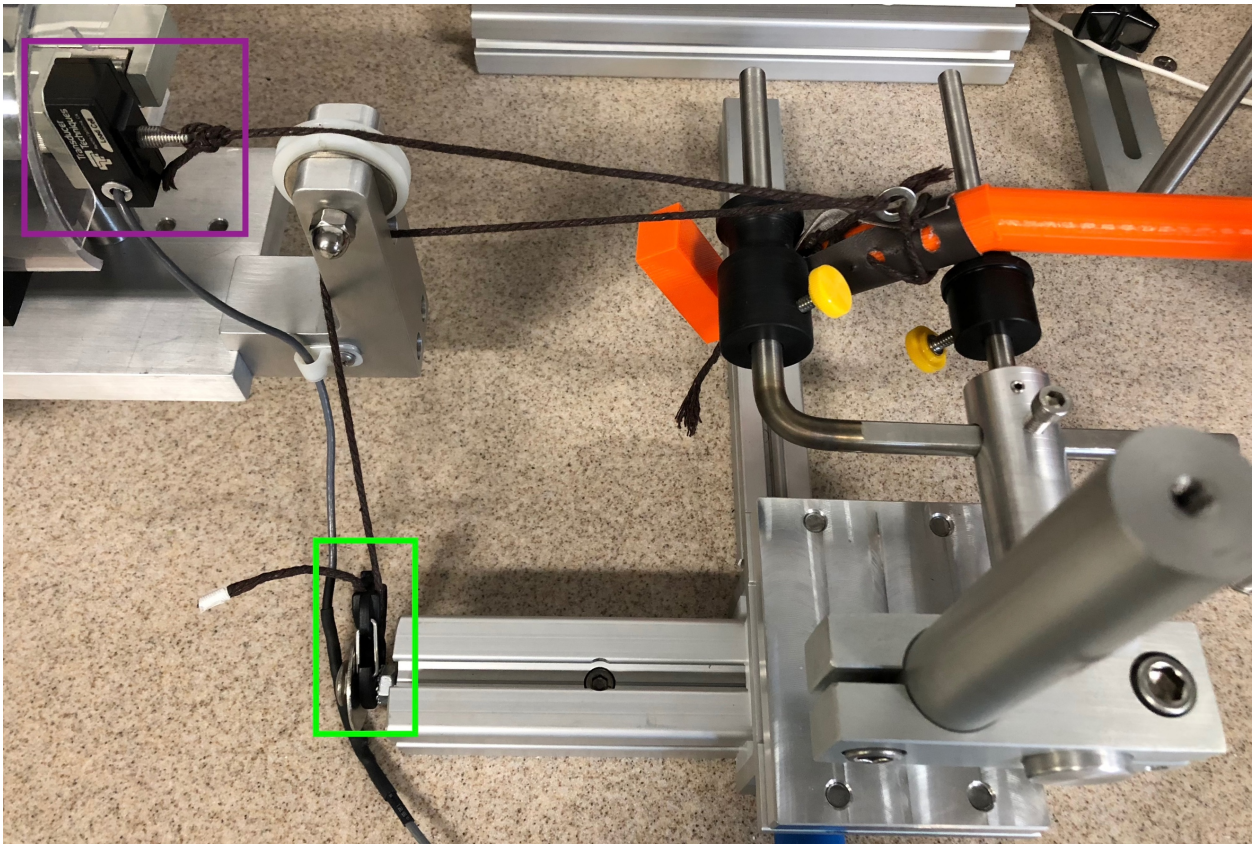

Figure 3: The load cell (purple box) monitors the load that is applied to the ACL, by pulling on the cast. The starting tension of the cord is adjusted using the locking carabiner (green box), to ensure the ACL is loaded at the proper loads.

During the loading, the rabbit was positioned comfortably in the holder, which was adjusted based on the size of the rabbit (Figure 4). With the rabbit in the holder, the left leg was extended straight at the hip, with the lower leg positioned in the loader similar to the 3D printed model leg; with the knee in front of the top bar, and the ankle below the second bar (Figure 5). A soft, non-stretching, leather cast around the shank of the rabbit, tightened via cinching similar to the tightening of a corset, was fit snuggly to ensure all tension on the cast translated to tension on the shank. A cast was used, opposed to a cord around the back of the shank, to ensure even loading and prevent laceration.

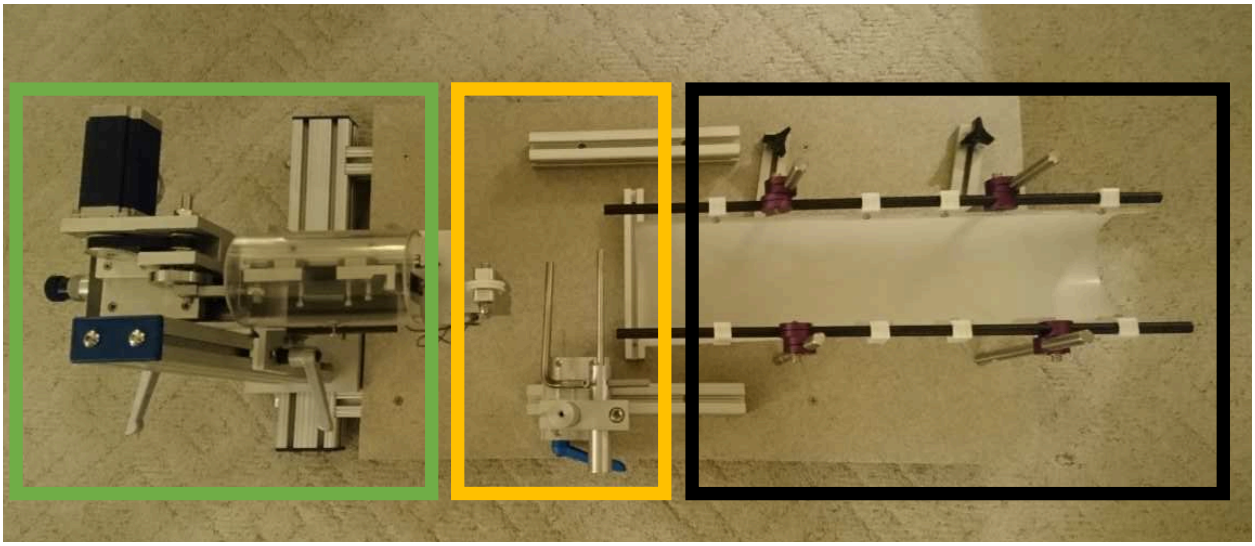

Figure 4: Due to restrictions from the Institutional Animal Care & Use Committee, images with the rabbit in the device are not able to be published. An overview of the RACL Loader is shown, with the motor and spring highlighted in green, the shank attachment highlighted in yellow, and the holder to secure the rabbit highlighted in black.

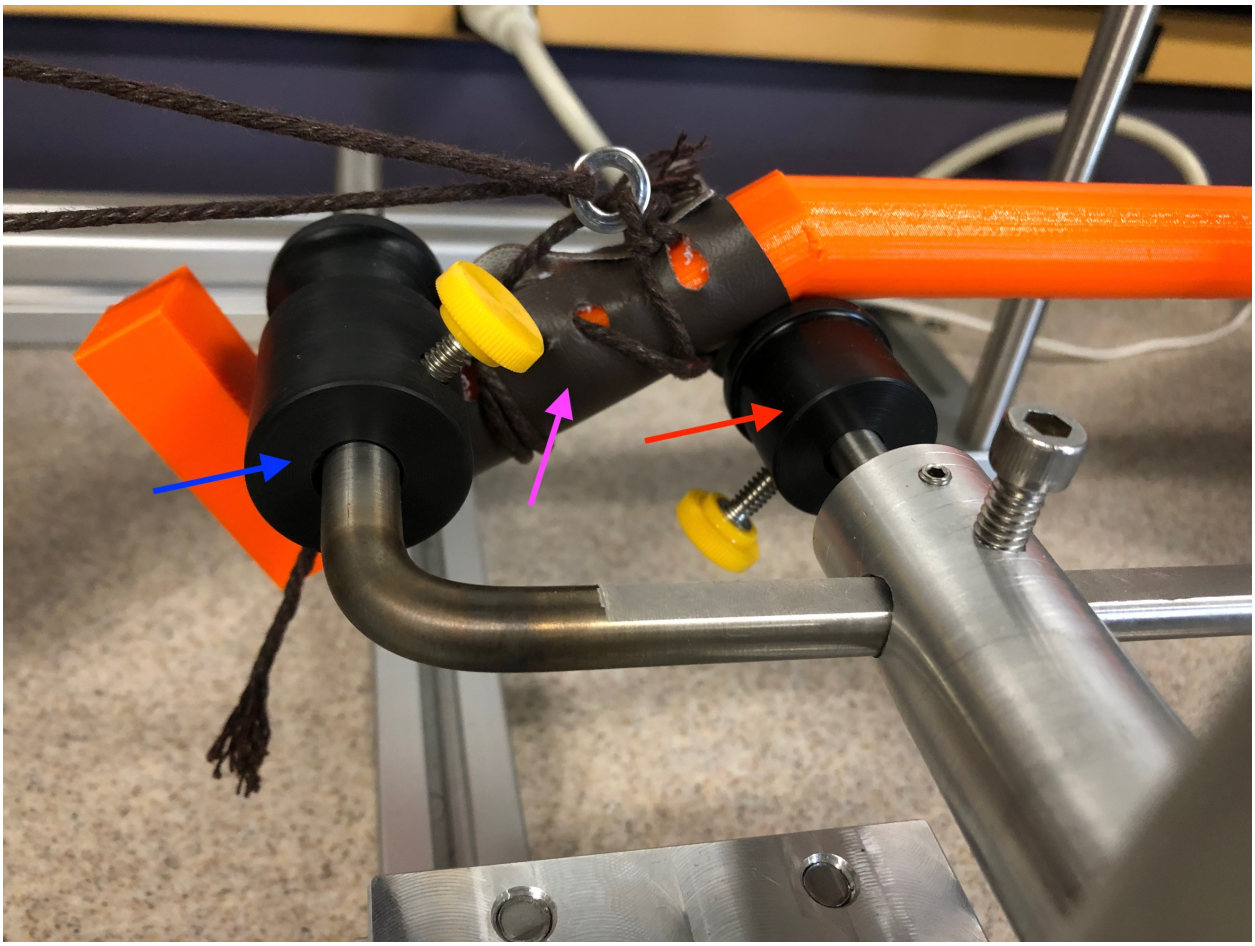

Figure 5: To hold the rabbit left leg in place, the left knee of the rabbit was placed over the first bar (red arrow), while the ankle was secured under the second bar (blue arrow). The leather cinch cast (purple arrow), was tightly secured around the tibia of the rabbit.

Protocol

Prior to loading, the rabbit was anesthetized with isoflurane administered through inhalation. Under anesthesia, 1.5 ml of blood was collected from the right ear prior to loading for analysis of potential circulating factors. Following the blood draw the rabbit was placed in the RACL loader, with the left leg extended and shank cinched in the cast. Care was taken to ensure the left knee was positioned at around 30° knee flexion, and that the cord pulled on the cast between 35°- 40° with respect to the shank. For twenty minutes, the ACL of the rabbit was loaded at an oscillating low-magnitude load (~2.0 - 12N), at a high-frequency (~15 Hz), based on previous similar protocols, which loaded the hind legs of sheep for 20 minutes/day, 5 days/week for a year (Rubin et al., 2002). While this current protocol only produces a single session of 20 minutes of loading, it is designed not to produce a structural response, but rather to induce a genetic response, which should be present after a single bout of loading. Following the loading protocol, anesthesia was reduced allowing the rabbit to return to full consciousness.

During the loading protocol, the rabbit vitals were monitored by a Department of Comparative Medicine (DCM) staff member. Rabbits body temperature was maintained through a DCM approved heating pad, while blood pressure, heart rate, temperature and oxygen levels were monitored by DCM staff.

Based on the time course for collagen genes hypothesized to be affected by loading to be fully expressed, rabbits were sacrificed four hours following the end of loading (Majima et al., 2000; Raab-Cullen et al., 1994; Sullivan et al., 2009; Yang et al., 2004). Genetic response of these collagen genes, such as TGF-ß, had increased gene expression levels up to 4 hours post

loading, but not beyond that, and therefore rabbits were sacrificed 4 hours post loading. During these 4 hours, rabbits returned to their pen and were monitored. After four hours, the rabbits were first sedated with 35-50 mg/kg of ketamine and 3-5 mg/kg of xylazine delivered intramuscularly. A second blood draw of 1.5 ml was then performed under sedation from the ear. Euthanasia was performed with an overdose of 100 mg/kg of pentobarbital sodium delivered into the heart. Following euthanasia, the ACL's from both legs were harvested and the RNA extracted.

Loading Parameters: Frequency & Magnitude

Previous research has found high-frequency, low-magnitude loading to initiate an osteogenic response in bone (Rubin et al., 2002, 1996, 2004; Rubin and McLeod, 1994; Turner et al., 1991). A high-frequency of 30 Hz, designed to simulate vibration has been demonstrated in previous studies to produce a mechanotransductive response (Rubin et al., 2002). This frequency is still far below levels that would jeopardize the structural properties of tissues, at the proper low-magnitude loads. To simulate this vibration frequency, and to account for the limitations of our ligament loading device, 15 Hz was chosen as the frequency for this protocol.

Due to the difference in modulus and yield strain between bone and ligament, the ACL could not be loaded at the same low-magnitude load as bone had been in previous experiments. In the Rubin studies, the magnitude of strain on the bone was approximately 5% to 10% of the peak strain amplitude we experience in normal everyday activities (Rubin and McLeod, 1994). During these experiments, the low magnitude load was approximately 150 με, which is 5-10% of the strain experienced during walking (1000 με) or vigorous activity (3000 με). Since ligaments can stretch 4-5% during the extremes of functional loading (Rubin et al., 2001), applying the principle of loading at strains of 5-10% of normal activity leads us to loading strains of 0.25-

0.50%. Since the loading will occur *in vivo* where strain cannot accurately be measured, this strain must be converted to a mechanical load using a stress-strain curve. Based on previous experiments, as seen in figure 6, a strain of 0.50% equates to approximately 1-2 MPa (Danto and Woo, 1993; Woo et al., 1992). Based on the cross-sectional areas of the rabbit ACL's measured during these experiments, 1-2 MPA then converts to approximately 7 Newtons (Equation 1).

Equation 1: $2\ MPA\ \times\ 3.5\ mm^2\ =7\ Newtons$

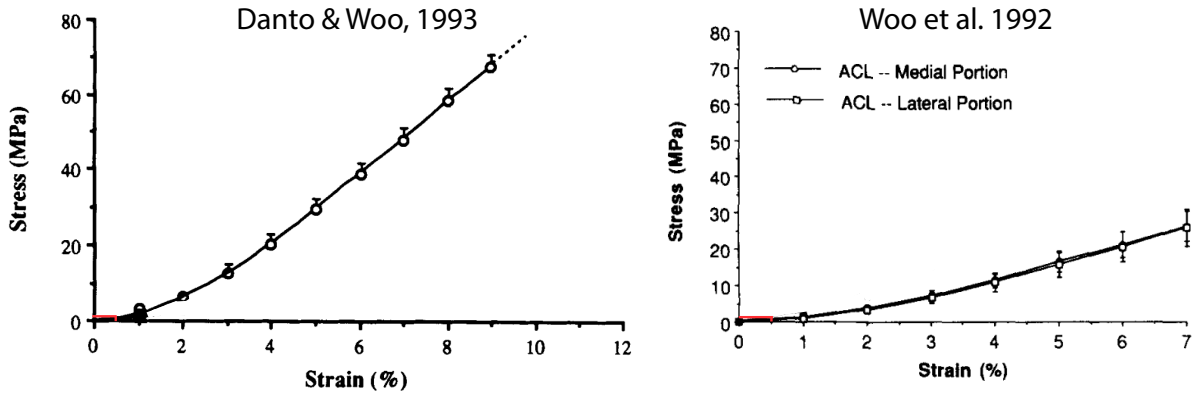

Figure 6: Adapted stress-strain curves from rabbit ACL tensile testing from Woo *et al.* 1992 and Danto & Woo, 1993. Both studies were taken into consideration due to parameter differences in the tensile testing, such as speed, causing different moduli between the studies. A faster tensile testing strain rate was chosen to more accurately represent the faster strains in this study as a result of mechanical loading. A strain of 0.5% equated to 1-2 MPA, as shown in red.

The proper low-magnitude load was also verified using yield strain for ligaments. In previous low-magnitude loads, sheep were loaded at 1/1000 of failure strain of bone (Rubin et al., 2002). With ligaments failure strain at 10-12%, a load 1/1000 of the failure strain equates to a strain of at least 0.01% (Danto and Woo, 1993; Woo et al., 1992). While this strain percentage is less than calculated using normal activity strains previously, loading at a load this low may not account for the toe-region of a ligament's modulus due to creep. A preload is often applied during ligament loading to account for the creep of the ligament. This initial pre-load can vary based on species, often 2.5 N in humans (Lyon et al., 1989; Woo et al., 1991) and 0.5 N in

rabbits (Danto and Woo, 1993; Woo et al., 1990, 1992, 1987). Therefore, we can safely assume any loading below 0.5 N is unlikely to properly load the ACL. To account for this, the ACL will start in at least 0.5 N of tension, to ensure the loading takes place outside of the toe region of the ligament.

However, it is unlikely that 100% of the load applied will be loaded in the ACL, due to the complex architecture of ligaments and tendons in the knee. It has been reported that in the knee position that the loading will occur in, the ACL resists 86% of the applied anterior translation of the tibia, with other ligaments accounting for less than 3% (Escamilla et al., 2012). To account for this, the ligament was loaded so that 86% of the load applied was at least 7.0 N, requiring loading of 8.2 N.

Cyclic loading can affect the tensile properties of the ACL if it is loaded at greater magnitudes. It is essential that the cyclic loading in this protocol is performed at a low magnitude, to induce stress, but not cause damaging structural changes in the ligament. Tested on rabbit ACL's, cyclic loading done at 30% or below of ultimate tensile strength had no effect on the load-to-failure (Sekiguchi et al., 1998). The ultimate tensile strength of a rabbit ACL varies based on the strain rate, hormone levels, pre-conditioning of strain, and flexion angle of the knee during testing, but has been reported in a range from 250 N – 505 N (Panjabi and Courtney, 2001; Slauterbeck et al., 1999). Therefore, even assuming the lower end of the ultimate tensile strength, 30% of 250 N is 75 N. Since 8.2 N is well below 30% of the ultimate tensile strength, 75 N, this chosen load should have no damaging effect on the ligament.

To account for constraints imposed by the design of the device, and the stiffness and displacement of the chosen spring, it was not possible to load at a range from exactly 0.5 N to 8.2 N. It was important to find a range that started the low end of the range above 0.5 N, out of the

toe region, while the upper end of the range included 8.2 N, but stayed below 30% of the ultimate tensile strength, 75 N, to ensure we are not damaging the ligament. Therefore, the ACL was loaded at an oscillating magnitude between on average 2.0 N and 12 N (Figure 7).

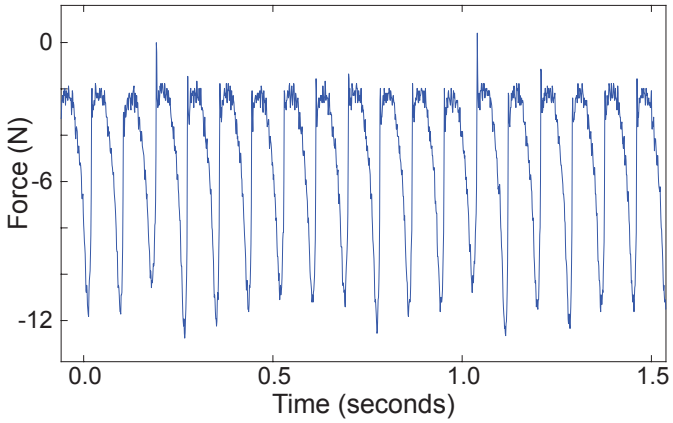

Figure 7: A 1.5 second snapshot of the low-magnitude, oscillating between on average, 2.0 N – 12 N, high-frequency, 15 Hz, load that was applied to the ACL of a rabbit for twenty minutes.

Loading Parameters: Positioning

Throughout the range of motion of the knee, the ACL is loaded differently, and has varying failure and yield strains (Escamilla et al., 2012; Woo et al., 1987). To maximize the amount of load that is translated to the ACL, the knee was fixed at 30º knee flexion, as in this knee position, the ACL carries the most load in weight bearing and non-weight bearing exercises (Escamilla et al., 2012; Li et al., 2005; Woo et al., 1991). Frontal plane and longitudinal plane adjustments to the femur-ACL-tibia-complex (FATC) were not considered as their effect on the loading of the ACL is limited compared to the sagittal plane (Lyon et al., 1989).

During previous loading studies, significant increases in stiffness and strength in the longitudinal direction of the bone were observed, indicating that the adaption occurred primarily in the weight bearing direction (Rubin et al., 2002). Therefore, it is important that the ACL loading occurs in the proper orientation of the FATC. Additionally, the percentage of load placed on the ACL is more affected by the direction of loading, rather than the knee flexion angle (Lyon

et al., 1989; Woo et al., 1987). To ensure the loading was translated to the ACL, loading occurred along the anatomical axis of the ACL, rather than the long axis of the tibia. Based on imaging techniques, at 30º knee flexion, the angle of insertion of the ACL is 52º with respect to the tibial plateau (Li et al., 2005). Therefore, loading occurred along the anatomical axis of the ACL at 38º with respect to the tibia when fixed at 30º knee flexion (Figure 8).

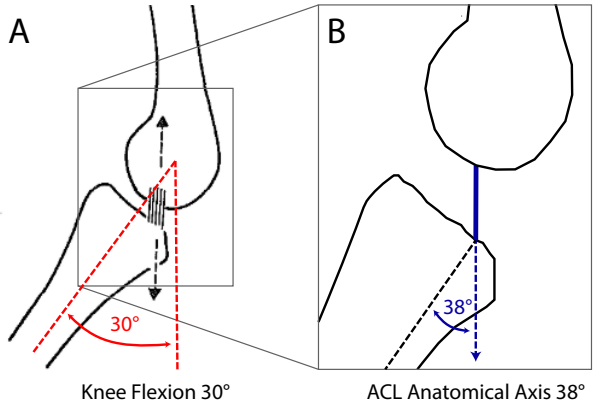

Figure 8: Loading occurred (A) with the knee fixed at 30º flexion, as measured in red, and (B) along the estimated anatomical axis of 38º with respect to the tibia, measured in blue, determined by the elevation angle of ACL. Tensile loading occurred in the direction of the blue arrow.

ACL Harvesting

After four hours following the loading protocol, to allow adequate time for genetic response, the ACL tissue was harvested from both legs in both the experimental and control rabbits. Following euthanasia, the rabbit's legs were skinned, and the hamstrings disinserted on their distal attachment on the posterior tibia. Next, the proximal attachments of the plantar flexors were disinserted to expose the posterior knee. On the anterior side of the knee, the patellar tendon attachment on the anterior tibia was cut, and the quadriceps pulled proximally. The patellar fat pad was grasped with forceps and removed. Any remaining soft tissue on the medial and lateral aspect of the knee was then carefully removed, including the MCL and LCL, leaving the ACL and PCL remaining. The ACL was gently grasped with the forceps, while the

ACL and PCL were cut form their distal attachment on the tibial plateau, taking care to extract as much tissue as possible by cutting right along the tibia. Unattached from the tibia, the PCL was then cut away from the femur, leaving only the ACL secured with forceps, which was then cut from the femoral notch. Upon extraction of the ACL tissue, it was snap-frozen in dry ice, and stored at -80 ºC until RNA sequencing was performed.

RNA Extraction

RNA was extracted per the protocol outlined in Qiagen's RNeasy Plus kit (Qiagen Inc, Maryland) and the assistance of East Carolina University Genomics Core Facility and Department of Biology. Since the time from tissue extraction to homogenization and storage can affect RNA quality, care was taken to ensure that the tissue was immediately frozen at -80 ºC and stayed frozen up until the exact moment of RNA extraction (Grinstein et al., 2018).

While Qiagen suggests the maximum amount of starting tissue to be less than 0.03 g, due to the low cellular density in ligament tissue, the full extracted ACL tissue was used. To extract the RNA, the ACL tissue was first added to a solution of 600 µl of Buffer RLT Plus and 6 µl of β-mercaptoethanol, and immediately disrupted and homogenized with a tissue homogenizer (Pro Scientific Inc, Oxford, CT). Based on previous research investigating the homogenization times that produce the greatest quality RNA yield in mouse tendon, the lysate was homogenized for 180 seconds (Grinstein et al., 2018). Following centrifuging the lysate for 3 minutes at maximum speed (16.9 x g), the supernatant was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at ≥ 8000 x g. The column was then discarded, and the flow through kept. This step ensured all insoluble material and DNA had been removed. 600 µl of 70% ethanol was mixed with the flow through by pipetting and added to an RNeasy spin column placed in a 2 ml collection tube and centrifuged for 15 seconds at ≥ 8000 x

g. The flow through was discarded. 700 µl of Buffer RW1 was added to the spin column and centrifuged for 15 seconds at ≥ 8000 x g, then 500 µl of Buffer RPE and centrifuged for 15 seconds at ≥ 8000 x g, and finally 500 µl of Buffer RPE and centrifuged for 2 minutes at ≥ 8000 x g; all done to wash the spin column membrane. After each centrifuge, the flow through was discarded. To eliminate any possible carryover of Buffer RPE or residual flow through, the RNeasy spin column was then placed in a new 2 ml collection tube and centrifuged at full speed (16.9 x g) for 1 minute. The RNeasy spin column was then placed in a new 1.5 ml collection tube, and 50 µl of RNase-free water was added to the spin column and centrifuged for 1 minute at ≥ 8000 x g to eluate the RNA.

Data Processing & Bioinformatics

The RNA was then stored at -80 ºC and sent to Novogene for Eukaryotic RNA-Seq Quantification analysis (sequencing strategy: PE150) with a coverage depth of 20 million reads per sample. The paired end raw nuecleobase sequencing data for each tissue sample, with sequences of reads and corresponding base quality, was analyzed with the assistance of the genomics core facility at East Carolina University. If the RIN for the ACL tissue was insufficient, the sample underwent a low-input library test for sequencing.

Using *FastP* (Chen et al., 2018), the adapters were trimmed, and the raw data was filtered at a Phred quality score of two. A Phred quality score of two was chosen to ensure the diversity of the sample was not lost (Quinn and McManus, 2015). *FastQC* (Andrews, 2010, http://www.bioinformatics.babraham.ac.uk/projects/fastqc) was then used to compare pre and post filtered data.

A subsample of 10 million reads from a sample in each of the 4 experimental conditions (control rabbit: left and right leg, and experimental rabbit: left, loaded and right, unloaded leg)

were sampled using *SeqTK* (Li, 2012, https://github.com/lh3/seqtk). A *Trinity* assembly to generate a transcriptome was run with the combined forward and reversed reads of the subsampled reads (Grabherr et al., 2013). To annotate the transcriptome, a *Diamond Blast* of the generated transcriptome against the predicted transcripts downloaded from the Ensambl rabbit genome was performed (Buchfink et al., 2014).

Kallisto was then used to quantify the abundances of transcripts and genes in the RNA-sequencing data of each sample (Bray et al., 2015). A *Kallisto* index was first built from the transcriptome, the samples were pseudoaligned, and then bootstrapped 100 times to quantify abundances of the transcripts in each sample by estimated counts and transcripts per million. The *Kallisto* output was then paired with an auxiliary experimental design table, and the proper gene names from the annotated transcriptome to construct a *Sleuth* object (Pimentel et al., 2017). With the *Sleuth* object, estimates of the parameters for the *Sleuth* full and reduced model were created, by smoothing the raw *Kallisto* abundance estimates for each sample using a linear, full, model with a parameter that represents the experimental condition. Differential analysis tests were performed by fitting a second, reduced, model that presumes abundances are equal in the two conditions, and identifying which transcripts fit better with the experimental, full, model, compared to the reduced model.

As the rabbit genome is not fully annotated, *Better Bunny* was used for augmented annotation and orthologue analysis on the differentially expressed rabbit gene ensemble ids (Craig et al., 2012). *Better Bunny* gets its gene description, protein identifies, gene ontology from Ensembl's *BioMart,* and the National Center for Biotechnology Information database. This application allows for a more comprehensive analysis through the use of orthologous relationships, which is important, as the rabbit genome is not fully annotated. It has been

previously reported that the rabbit genome is nearly 75% analogous with the mouse genome (Evsikov, 2019), and the human genome is nearly 99% annotated. Therefore, the mouse and human genome were used as orthologues if the matching identity threshold was greater than 75%. To better group genes by function, gene ontology enrichment analysis was performed using *Enrichr* (Chen et al., 2013; Kuleshov et al., 2016).

Statistics

To test differential expression, a *Sleuth Wald* test was run between the loaded ACL and the internal control ACL, and the loaded ACL and the external control ACL (Figure 9). The Benjamini-Hochberg q-value, at $q \leq 0.05$, was used to adjust the p-value to control for false discovery rate and adjust for multiple test correction. A gene was termed differentially expressed if the q-value ≤ 0.05. B, the beta value, which is the log fold change between conditions was also calculated. The log fold change is important to understand the effect size of the change in gene expression. While log2 fold change is often used to detect biological changes, since this was an exploratory analysis, using an RNA-sequence technique to determine all the genes that were altered in genetic expression in response to loading, the log fold change of log2 was not used as a cut off factor. Too strict of log fold change threshold may eliminate genes of interest from being identified. For one gene, compared in two conditions, condition A and condition B, a positive beta value indicates expression of the gene in condition A is greater than in condition B, while a negative beta value indicates expression of the gene in condition B is greater than in condition A. The magnitude of the beta value indicates the strengthen of the effect size, with greater integers indicating greater effect sizes, and decimal values indicating weaker effect sizes. Gene expression was measured by count distributions, which were calculated as the normalized proportion of genes associated with the number of counts in each gene.

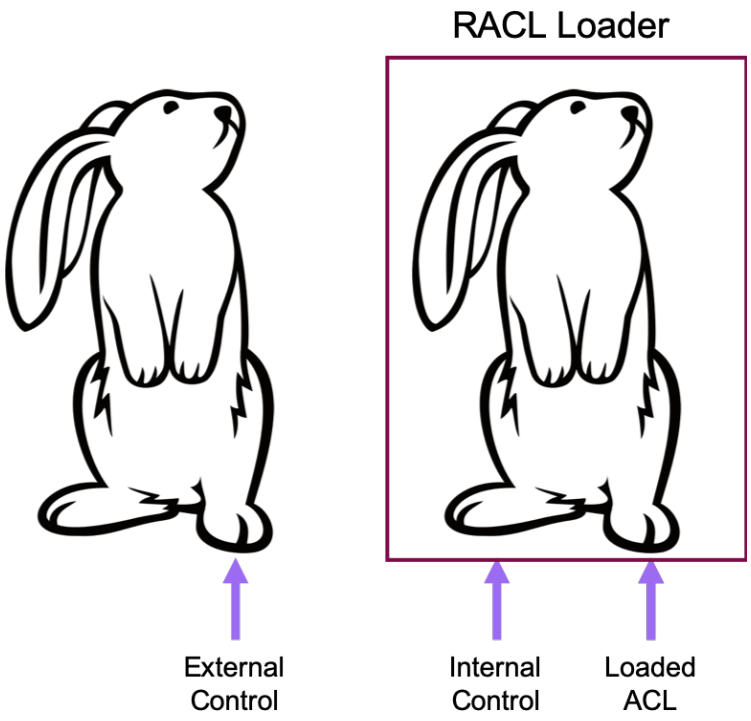

Figure 9: To test differential expression, the genetic response was compared between the loaded ACL and the internal control ACL, as well as another comparison between the loaded ACL and the external control. This figure helps to identify the tissues from which legs were compared. The rabbit on the right, outlined with a box, was the rabbit that received the mechanical loading.

Chapter IV. Results

While the weight of extracted ACL tissue varied from 0.0214 g to 0.1371 g based on the size of the rabbit and the success of the ACL extraction, the average ACL tissue weight was 0.0424 g. After RNA extractions, RNA concentrations also varied from 0.584 ng/µl to 62 ng/µl, with a mean ± standard deviation of 15.1ng/µl ± 14.1 ng/µl. Tested by Novogene, the RIN ranged from 4.10 to 7.70, with a mean ± S.D. of 6.24 ± 1.13 (Table 1). Nine of the twenty samples, due to insufficient total amount of RNA, required a low-input library test. A low-input library test can be used to sequence tissues with insufficient total amount of RNA at a risk. This low-input library was successful in sequencing the samples, as all twenty samples were sequenced.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Right	5.1*	5.6*	4.6*	7.4	5.8	7.5	7.3	6.8	7.1	6.7*
Left	5.5*	5.5*	4.1*	7.7	5.8	5.8*	7.3	7.3*	7.2	4.6

Table 1 – RIN's (RNA integrity number) for the twenty ACL samples that were collected. Samples denoted with a star underwent a low-input library sequence. With the addition of the low-input library test, all twenty samples were successfully sequenced.

Across the twenty ACL tissue samples, there were 45.397 ± 4.535 million mapped reads per sample (mean ± S.D.), with a unique mapping rate of 67.995% ± 12.028. Following quality filtering in *FastP,* there were 45.365 ± 4.522 million mapped reads per sample. All per base sequence quality was above 20 and the per sample quality score spike was all above a mean sequence Phred quality score of 2. Additionally, the per base N content was low and the per base sequence content indicated no biases. These results indicated the sequencing data was filtered properly in *FastP*. Following the *Trinity* assembly of the transcriptome, *Busco* post-assembly QC analysis indicated a very good assembly.

The *Sleuth Wald* test for differential expression between the loaded ACL and the internal control (unloaded control leg in the experimental rabbit) produced three genes that were altered in expression (Table 2). Of these three genes, none of them had annotations within the rabbit genome, the generated transcriptome, or orthologues in the mouse or human genome.

Testing for differential expression between the loaded ACL in the experimental rabbit and the external control (ipsilateral leg on the control rabbit), there were 121 genes that had altered expression (Tables 3-10). Of these 121 genes, 60 genes had annotations within the rabbit genome, the generated transcriptome, or orthologues in the mouse or human genome. When grouping these 60 genes based on function, 1 gene (TNXB) was involved in the synthesis and organization of collagen, four genes (EIF4E, FABP6, IRAK4, and PLCL1) were involved in the regulation of the cytokine interleukin which has downstream effects on collagen synthesis and tissue generation, 15 genes (ABHD2, FAM20C, JUP, KNG1, LIPC, LOC103351662, OR2B2, OR8S1, PSEN1, RALBP1, SLC35G1, SYNPR, TAS2R14, UPK1B and VN1) were involved in mechanotransduction signaling pathways, 2 genes (KPNA7 and VTI1B) were involved in protein transport, 5 genes (DNPH1, LOC100358248, MEST, RAD51C, and SLC27A6) were involved in metabolic processes, and 3 genes (CCDC88A, DFNB31 and TMOD3) were involved in actin cytoskeleton regulation.

Non-Annontated Genes From Loaded ACL & Internal Control			
Rabbit Ensembl Gene ID	P-value	Q-Value	B
ENSOCUG00000018321	2.37E-08	1.31E-03	3.4474
ENSOCUG00000011996	2.63E-07	7.27E-03	4.3385
ENSOCUG00000043566	1.32E-06	2.43E-02	3.8888

Table 2: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, and B (estimates of log fold changes between conditions) for genes that were differentially expressed between the loaded ACL and the internal control ACL. Each of the three genes were non-annotated.

Actin Cytoskeletal Genes						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000003620	CCDC88A	0.0000483	0.026895	-0.7385	coiled-coil domain containing 88A	regulation of actin cytoskeleton organization; TOR signaling; membrane organization;
ENSOCUG00000004645	DFNB31	0.0000101	0.00910301	1.9391	deafness, autosomal recessive 31	actin filament
ENSOCUG00000024437	Tmod3 (Mouse)	0.000000611	0.00117368	-2.0587	tropomodulin 3	actin cytoskeleton organization

Table 3: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of the actin cytoskeletal relating genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Collagen, Interleukins and Tissue Repair Genes						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000007056	TNXB	3.16E-113	1.76E-108	7.38036	tenascin XB	extracellular matrix organization; cell-matrix adhesion; collagen fibril organization; extracellular fibril organization; elastic fiber assembly; collagen metabolic process
ENSOCUG00000001456	EIF4E	1.38E-29	1.54E-25	5.72874	eukaryotic translation initiation factor 4E	Akt/mTOR pathway
ENSOCUG00000005435	FABP6	0.0000164	0.01346358	3.68086	fatty acid binding protein 6, ileal	interluekin cytokine regulation
ENSOCUG00000004103	IRAK4	0.0000946	0.04335622	-0.4732	interleukin-1 receptor-associated kinase 4	signal transduction; cytokine-mediated signaling pathway;
ENSOCUG00000013978	PLCL1	0.0000378	0.02287314	3.13213	phospholipase C-like 1	signal transduction; intracellular signal transduction; signal transducer activity;

Table 4: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of the collagen, interleukins and tissue repair genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Meatbolic Genes						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000002269	RAD51C	0.00000111	0.00188165	2.28188	RAD51 paralog C	DNA metabolic process;
ENSOCUG00000021203	DNPH1	0.0000915	0.04324041	4.72232	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	metabolic process
ENSOCUG00000026015	LOC10035824	0.00000332	0.00402137	3.17679	liver carboxylesterase 2-like	metabolic process
ENSOCUG00000006752	MEST	7.74E-48	1.44E-43	7.04972	mesoderm specific transcript	metabolic process
ENSOCUG00000017136	SLC27A6	0.0000761	0.03752844	-0.8121	solute carrier family 27 (fatty acid transporter), member 6	metabolic process; long-chain fatty acid metabolic process;

Table 5: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of the metabolic genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Protein Transport Genes						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000001792	KPNA7	2.01E-24	1.40E-20	6.16082	karyopherin alpha 7 (importin alpha 8)	transport; protein import into nucleus; protein transport
ENSOCUG00000000586	VTI1B	0.0000655	0.03350369	1.06407	vesicle transport through interaction with t-SNAREs 1B	vesicle-mediated transport; intracellular protein transport; regulation of protein localization to plasma membrane

Table 6: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of the protein transport relating genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Mechanotransduction Pathway Genes						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000013527	ABHD2	0.000104098	0.04641836	3.17467	abhydrolase domain containing 2	response to wounding
ENSOCUG00000010891	FAM20C	0.00000925	0.00901201	0.6543	family with sequence similarity 20, member C	skeletal system development; regulation of fibroblast growth factor receptor signaling pathway; positive regulation of osteoblast differentiation; osteoclast
ENSOCUG00000012063	JUP	0.00000126	0.00207107	-0.5958	junction plakoglobin	signal transduction; establishment of protein localization to plasma membrane; detection of mechanical stimulus; signal transducer activity;
ENSOCUG00000005003	KNG1	0.000000288	0.00064208	0.94991	kininogen 1	negative regulation of cell adhesion; extracellular space; extracellular exosome;
ENSOCUG00000001647	LIPC	3.15E-08	0.0000976	3.89853	lipase, hepatic	extracellular region; extracellular space
ENSOCUG00000029566	LOC10335166	0.00000235	0.0032768	6.41608	membrane-spanning 4-domains subfamily A member 12-like	integral component of membrane
ENSOCUG00000013027	OR2B2	0.00000032	0.00068636	0.87215	olfactory receptor, family 2, subfamily B, member 2	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; integral component of membrane;
ENSOCUG00000004100	OR8S1	0.0000461	0.025974	4.5969	olfactory receptor, family 8, subfamily S, member 1	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; integral component of membrane;
ENSOCUG00000014545	PSEN1	0.0000233	0.01698012	-1.3293	presenilin 1	intracellular signal transduction; Notch signaling pathway; Notch receptor processing;
ENSOCUG00000010172	RALBP1	8.68E-08	0.00024179	4.03047	ralA binding protein 1	signal transduction; small GTPase mediated signal transduction;
ENSOCUG00000004261	SLC35G1	0.0000844	0.04022388	0.50667	solute carrier family 35, member G1	calcium ion export from cell;
ENSOCUG00000012918	SYNPR	0.0000131	0.01108085	1.10668	synaptoporin	transporter activity; integral component of membrane;
ENSOCUG00000022270	TAS2R14	0.00000977	0.00907422	3.77897	taste receptor, type 2, member 14	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; integral component of membrane
ENSOCUG00000008294	UPK1B	0.0000784	0.03830676	3.24825	uroplakin 1B	integral component of membrane
ENSOCUG00000002109	VNN1	0.0000303	0.01940978	1.03334	vanin 1	inflammatory response; negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway

Table 7: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of the mechanotransduction pathway relating genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Differentially Expressed Genes Loaded ACL & External Control						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000020258	5S_rRNA	0.00000624	0.00644452	4.42335	5S ribosomal RNA	no relevant function known
ENSOCUG00000010426	AGFG1	0.0000027	0.00349897	1.05574	ArfGAP with FG repeats 1	no relevant function known
ENSOCUG00000027113	ARL14EPL	0.00000383	0.00435766	3.76404	ADP-ribosylation factor-like 14 effector protein-like	no relevant function known
ENSOCUG00000001523	C12H6orf165	0.00000352	0.0040845	1.3413	Chromosome 12 open reading frame, human C6orf165 (C12H6orf165), transcript variant X1, mRNA	no relevant function known
ENSOCUG00000002767	CEP162	0.0000259	0.01781172	0.89045	centrosomal protein 162kDa	protein binding
ENSOCUG00000007045	CLP1	0.000000167	0.00040487	4.6398	cleavage and polyadenylation factor I subunit 1	no relevant function known
ENSOCUG00000003017	ERICH3	0.0000206	0.01572647	3.37587	glutamate-rich 3	
ENSOCUG00000006154	GINS4	0.00004	0.02400119	-0.4991	GINS complex subunit 4 (Sld5 homolog)	protein binding
ENSOCUG00000022627	HNRNPA1 (Mouse Orthologue)	0.00000183	0.00277791	3.60961	heterogeneous nuclear ribonucleoprotein A1	no relevant function known
ENSOCUG00000023956	HOXD9	0.0000093	0.00901201	-0.872	homeobox D9	regulation of transcription, DNA-templated;

Table 8: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1.

Differentially Expressed Genes Loaded ACL & External Control (continuted)						
Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	Selected Cellular, Biological or Molecular Function
ENSOCUG00000013591	ITLN1 (Mouse Orthologue)	4.25E-14	2.16E-10	4.72554	intelectin 1 (galactofuranose binding)	no relevant function known
ENSOCUG00000011556	KCTD18	1.20E-10	4.44E-07	5.9514	potassium channel tetramerization domain containing 18	protein homooligomerization
ENSOCUG00000013931	LEPROTL1	0.00000164	0.00261609	3.44487	leptin receptor overlapping	no relevant function known
ENSOCUG00000005200	LOC10000948	0.0000026	0.00344981	-0.5738	lipophilin CS (LOC100009480), mRNA	no relevant function known
ENSOCUG00000029693	LOC1003427C	0.00000224	0.00319832	-0.5716	IQ domain-containing protein F5-	protein binding
ENSOCUG00000023695	LOC10034283	0.0000933	0.0433475	1.11918	40S ribosomal protein S3a	structural constituent of ribosome
ENSOCUG00000016686	LOC10035615	0.000079	0.03830676	2.73601	melanoma-associated antigen	no relevant function known
ENSOCUG00000016139	MMAA	0.0000244	0.01720254	-0.5477	methylmalonic aciduria (cobalamin deficiency) cblA type	no relevant function known
ENSOCUG00000008571	MYOM1	0.0000232	0.01698012	3.2322	myomesin 1	protein binding; protein homodimerization activity; identical protein binding
ENSOCUG00000028329	SNORD22	2.31E-09	0.00000756	3.9617	Small nucleolar RNA SNORD22	no relevant function known
ENSOCUG00000014042	SNRPB	0.000000737	0.00136899	3.15642	Small nuclear ribonucleoprotein-associated protein	nucleus; ribonucleoprotein complex
ENSOCUG00000014000	TAF7	0.0000293	0.01920299	-1.4862	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 55kDa	intracellular estrogen receptor signaling pathway; intracellular estrogen receptor signaling pathway;
ENSOCUG00000011199	TIMM8A	1.48E-15	8.24E-12	1.87016	translocase of inner mitochondrial membrane 8 homolog A (yeast)	transport; protein transport; mitochondrial intermembrane space
ENSOCUG00000007954	TPBG	0.00000277	0.00351321	1.53749	trophoblast	positive regulation of synapse
ENSOCUG00000008740	TTI2	0.000107519	0.04756335	3.87513	TELO2 interacting	cytoplasm; centrosome;
ENSOCUG00000019556	U6	0.0000693	0.03511449	0.87307	U6 spliceosomal RNA	no relevant function known
ENSOCUG00000026098	U6	0.0000349	0.02169553	3.26519	U6 spliceosomal RNA	no relevant function known
ENSOCUG00000011756	ZNF358	0.000059	0.03131653	1.72304	zinc finger protein 358	nucleic acid binding; metal ion binding
ENSOCUG00000010370	ZNF605	0.0000026	0.00344981	4.67631	zinc finger protein 605	nucleic acid binding; metal ion binding
ENSOCUG00000005172	ZSCAN30	0.0000285	0.01916284	-6.1404	zinc finger and SCAN domain containing 30	regulation of transcription, DNA-templated; transcription, DNA-templated

Table 9: Gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the selected cellular, biological or molecular function of genes that were differentially expressed between the loaded ACL and the external control ACL. Full cellular, biological and molecular functions for each gene can be found in Appendix 1. This table is a continuation of Table 8.

Non-Annontated Genes							
Rabbit Ensembl Gene ID	P-value	Q-Value	B		Rabbit Ensembl Gene ID	P-value	Q-Value	B
ENSOCUG00000012435	6.73E-66	1.87E-61	6.7657		ENSOCUG00000019190	3.56E-05	0.021806825	1.0477
ENSOCUG00000038847	1.47E-28	1.37E-24	2.3767		ENSOCUG00000011519	4.09E-05	0.024249081	1.6720
ENSOCUG00000018263	2.29E-27	1.82E-23	5.8355		ENSOCUG00000009267	4.25E-05	0.024820369	1.3602
ENSOCUG00000045943	1.16E-22	7.19E-19	5.7612		ENSOCUG00000049897	4.27E-05	0.024820369	1.2684
ENSOCUG00000045723	1.40E-13	6.48E-10	4.4779		ENSOCUG00000008084	4.48E-05	0.025761956	3.1251
ENSOCUG00000011483	1.80E-13	7.73E-10	5.5156		ENSOCUG00000043099	4.56E-05	0.025958068	3.3638
ENSOCUG00000041090	1.92E-09	6.70E-06	4.6008		ENSOCUG00000004584	5.01E-05	0.027574676	4.1039
ENSOCUG00000024326	5.91E-08	0.00017339	3.7485		ENSOCUG00000037648	5.05E-05	0.027574676	3.4492
ENSOCUG00000030701.	1.31E-07	0.00034854	1.6556		ENSOCUG00000034170	5.50E-05	0.029578915	2.7809
ENSOCUG00000043159	1.47E-07	0.00037182	3.9642		ENSOCUG00000037812	5.52E-05	0.029578915	0.7643
ENSOCUG00000009137	2.35E-07	0.00054652	0.9246		ENSOCUG00000042575	6.07E-05	0.031721245	-0.5022
ENSOCUG00000032163	3.71E-07	0.00076609	4.7090		ENSOCUG00000037812	6.09E-05	0.031721245	4.3720
ENSOCUG00000008290	5.36E-07	0.0010678	1.0880		ENSOCUG00000048680	6.45E-05	0.033280566	-0.9989
ENSOCUG00000011050	7.64E-07	0.0013713	3.6433		ENSOCUG00000002262	7.50E-05	0.03731423	5.3667
ENSOCUG00000007169	7.87E-07	0.0013713	2.0961		ENSOCUG00000049084	8.35E-05	0.040137978	2.8928
ENSOCUG00000012277	1.84E-06	0.00277791	3.5317		ENSOCUG00000046523	9.32E-05	0.043347504	-1.3443
ENSOCUG00000046389	2.98E-06	0.00369263	1.5966		ENSOCUG00000044032	9.49E-05	0.043356224	3.5007
ENSOCUG00000034854	3.40E-06	0.00403229	4.4181		ENSOCUG00000007645	0.000112119	0.049208077	6.3503
ENSOCUG00000001189	4.91E-06	0.00525949	3.4952		NA	1.82E-12	7.26E-09	5.5055
ENSOCUG00000038178	9.38E-06	0.00901201	1.1527		NA	2.17E-06	0.003177485	3.0568
ENSOCUG00000027349	9.75E-06	0.00907422	0.9653		NA	4.34E-06	0.004840741	1.0603
ENSOCUG00000036216	1.12E-05	0.00989231	1.7438		NA	4.57E-06	0.004991919	1.9179
ENSOCUG00000037543	1.29E-05	0.01108085	1.1910		NA	6.01E-06	0.006317655	1.1135
ENSOCUG00000034653	1.67E-05	0.01352935	3.0927		NA	1.01E-05	0.009103014	4.0491
ENSOCUG00000046010	1.89E-05	0.01483059	3.6075		NA	1.33E-05	0.011080845	3.1029
ENSOCUG00000011483	2.03E-05	0.01570456	4.5874		NA	2.42E-05	0.017202541	0.9798
ENSOCUG00000000458	2.17E-05	0.01637785	3.7650		NA	2.93E-05	0.019202986	-1.0361
ENSOCUG00000049948	2.35E-05	0.01698012	-0.6036		NA	3.50E-05	0.021695529	2.7821
ENSOCUG00000032529	2.57E-05	0.01781172	1.2351		NA	9.73E-05	0.044109304	-2.3985
ENSOCUG00000009983	2.74E-05	0.01861573	1.3580		NA	9.94E-05	0.044664446	0.7898
ENSOCUG00000029326	3.48E-05	0.02169553	-0.5475					

Table 10: Gene ID's, p-values, adjusted q-values to correct for false discovery rate and B (estimates of log fold changes between conditions) for genes that were differentially expressed between the loaded ACL and the external control ACL. These rabbit Ensembl gene ID's were not annotated to any gene.

Chapter V. Discussion

The purpose of this study was to identify the mechanisms by which ligaments respond *in vivo* to high-frequency, low-magnitude mechanical loading by identifying (1) if there is a response to this mechanical loading, and (2) what genes are altered in response to this mechanical loading on the ligament. This chapter is divided into the following sections: 1. Discussion of Results, 2. Research Significance, 3. Limitations and Future Directions, and 4. Conclusions.

Discussion of Results

Loaded ACL and Internal Control

The gene expression between the left, loaded ACL and the internal control (right, unloaded, control leg in the experimental rabbit) was nearly similar. There were only 3 genes between these two tissue samples that were differentially expressed. When comparing the density plots, it is evident that most genes in the loaded ACL and internal control had a similar distribution counts based on the smoothed empirical densities (Figure 10A). Additionally, we can see the similarity between these two samples based on their QQ-plot (Figure 11A). In the QQ-plot, the quantiles of all gene expression from the loaded ACL and plotted against the quantiles of all gene expression from the internal control. Since this plot forms a nearly perfect straight line, it can be concluded that the two samples share very similar gene distribution, with the exception of the three genes represented by the red dots.

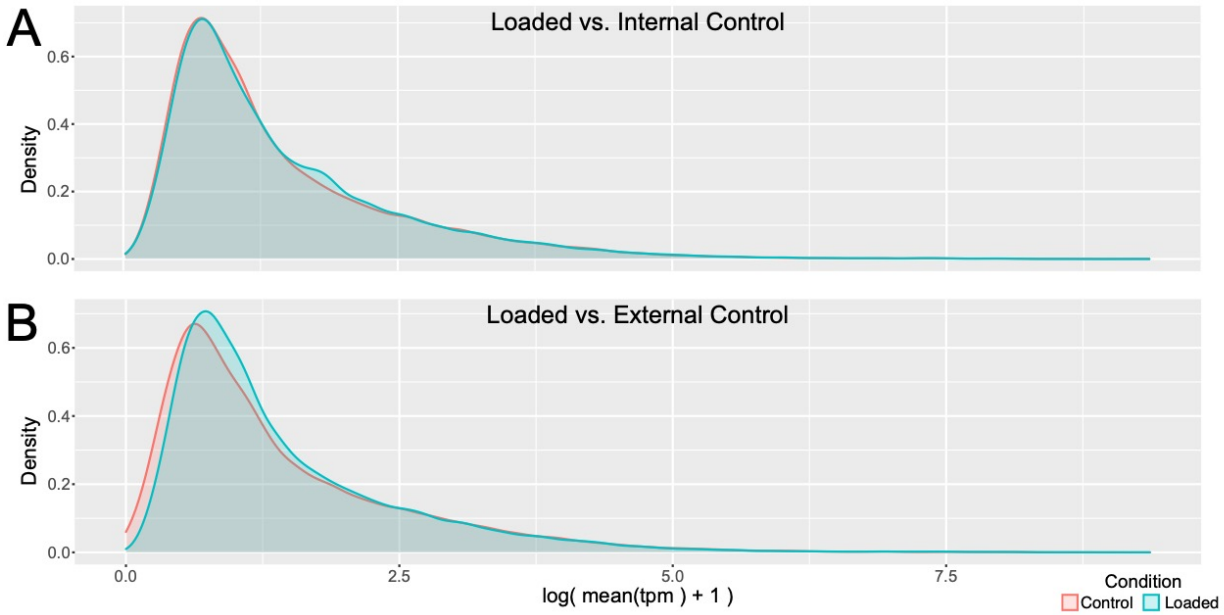

Figure 10: The conditional density plot of the loaded vs. internal control (Figure 10A), and the loaded vs. external control (Figure 10B). The loaded ACL (blue), and control ACL (orange), are more similar, in terms of transcript density per million, in the loaded vs. internal control plot, than in the loaded vs. external control plot.

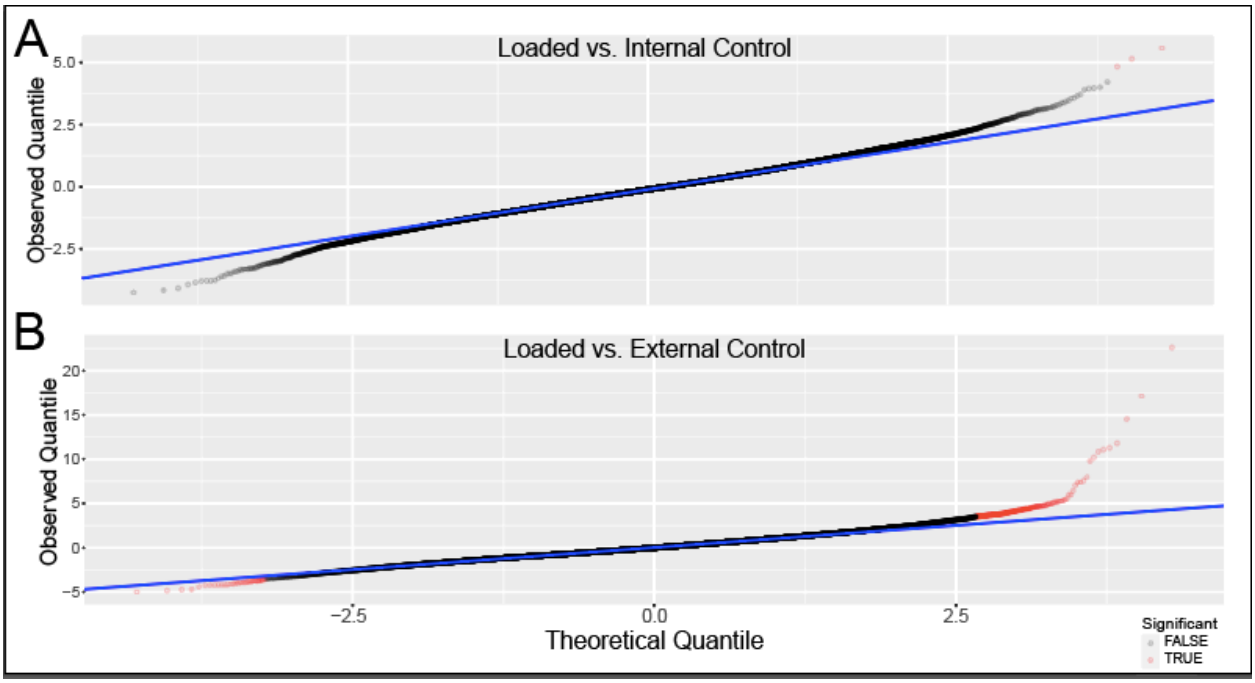

Figure 11: The theoretical quantile plot of the loaded vs. internal control (Figure 11A), and the loaded vs. external control (Figure 11B). The dots in red represent the genes that were differentially expressed between the two compared tissues. The straight blue line represents biological replicates, where each gene is expressed the same in each sample.

These three genes, represented by the rabbit Ensembl gene id's ENSOCUG00000018321, ENSOCUG00000011996, and ENSOCUG00000043566, all have not been annotated in the rabbit genome. Additionally, none of these genes had orthologues within the mouse or human genome. Based on genetic expression, these two tissues samples, from the loaded ACL and the internal control ACL, had genetic expression close to identical. More differences would have likely been detected with larger sample sizes. While it is possible that this result means the acute mechanical loading on the ACL had little effect on-genetic expression, it is also likely that all the effects of the mechanical loading are systemic, and affected both the loaded ACL, and the unloaded ACL.

To further explore the difference between the loaded ACL and the internal control, a differential exon usage test was run as a secondary analysis. This was run to investigate changes in exon expression across conditions. It is important to investigate differential exon usage, as gene expression may be equal across conditions, but certain exons within the gene may be up/down regulated. A DeNovo contig assembly was built in *Trinity* (Grabherr et al., 2013), and the contigs clustered using *Corset* (Davidson and Oshlack, 2014) with *Salmon* alignment (Patro et al., 2017). *Lace* was then used to assemble these outputs into a superTranscript (Davidson et al., 2017). The reads were then mapped to the superTranscriptome using *Star* (Dobin et al., 2016). To count how many reads per block, *Subread's* featureCount package was used (Liao et al., 2014). *DiffSplice* was used to compare the differential transcript usage between the loaded ACL and the internal control ACL (Hu et al., 2013). Finally, *BLAT* was used to align the cluster sequence to their predicted position on the rabbit genome, and the estimated corresponding gene (Kent, 2002; Kent et al., 2002).

The results of the differential exon usage test demonstrated that there were seven clusters that were significantly different between the loaded ACL and the internal control, at a q-value <= 0.05. Of these seven clusters, 5 were related to protein coding genes, and two were unable to be matched to a representative gene. There were two clusters related to ribosomal activity, most closely associated with the EEFIA1 and RPL32 genes, and one cluster, related to the FAM89B, that regulated DNA and RNA metabolism. Two other clusters related to scaffolding proteins and integrins, the SHANK1 and ITGAV gene, respectively, were altered as well. SHANK1 is known to play a role in the connecting neurotransmitter receptors, ion channels, and other membrane proteins to the actin cytoskeleton and G-protein couples signaling pathways (GO:0030159). These clusters are of interest in their relation to mechanotransduction, as integrins are transmembrane receptors that link actin associated proteins to the ECM and to adhesion receptors on the surface of other cells (Beckerle and Yeh, 1990). These integrins are a key mediator of mechanotransduction by altering the number, location and strength of adhesive contacts that can transmit physical forces between neighboring cells (Ingber, 1997; Springer, 1990). However, it must be reminded that the SHANK1 and ITGAV genes were not altered in their expression. Rather, it was the clusters of exons associated with these genes that were altered, which is still greatly significant. Future research should continue to investigate the role these genes/clusters and integrins play in mechanotransduction.

Another secondary analysis was performed using a more liberal *Sleuth Wald* test for differential expression between the loaded ACL and the internal control ACL. Rather than a q-value to correct for false discovery rate, a p-value ≤ 0.0005 was used. However, a log fold effect size threshold of $\beta \geq |2|$ was used, to ensure a significant difference between the expression of the two ACL samples. This allowed for a more liberal approach, to investigate any genes that may

have been missed in our initial, stricter, statistical tests. In all, there was an additional 16 genes that passed this more conservative test. Of these 16, 10 were annotated within the rabbit genome. There were two genes relating to metabolic processes (ALDH2, SPAM1 and TTR), one to membrane transport (SLCO6A1), one to protein binding (NOLC1), and one related to actin cytoskeleton regulation (SHROOM4). Additionally, STK39 and DAPK2, both genes responsible for intracellular signal transduction were altered in expression. Lastly, ABHD2, a gene responsible for response to cell wounding, likely related to mechanotransductive pathways, was also altered in expression. This gene was also the only gene altered in expression in this comparison and the comparison between the loaded ACL and the external control ACL.

Loaded ACL and External Control

Based on the results of comparing the loaded ACL to the external control (the same left leg in an unloaded rabbit) it appears that the mechanotransductive response to the acute mechanical loading may be systemic. The count distribution based on density plots between the loaded ACL and the external control (Figure 10B) are qualitatively less similar than the density plot between the loaded ACL and the internal control (Figure 10A), confirming again that the loaded ACL and external control had more genes differentially expressed than the loaded ACL and internal control. This shows that the loaded ACL and the internal control are more similar than the loaded ACL and the external control. This is supported by the QQ-plot (Figure 11B), as there are many more genes that differ in expression and deviate from the straight line between the loaded ACL and the external control. Of these 121 genes that differ in expression between the loaded ACL and the external control, 57 were annotated from the rabbit genome, while 3 were annotated with the mouse orthologue, leading to 60 annotated genes that were differentially expressed.

Gene Expression Relating to Collagen, Interleukins and Tissue Repair

Of these 121 genes differentially expressed, the gene differentially expressed the greatest between the loaded ACL and the external control was tenascin XB (TNXB). The effect size of 7.38 and q-value of 1.76^{-108}, indicate significant expression difference of TNXB between the two tissue samples. TNXB was upregulated in the loaded ACL over 7 times the amount compared to the external control (Figure 12). TNXB is a gene responsible for formation and organization of type I collagen (Chiquet et al., 2009; Minamitani et al., 2004). Additionally, it is known that type VI collagen is often associated with collagen fibers forming an interconnecting meshwork is regulated by TNXB (Keene et al., 1988; Minamitani et al., 2004). This interconnecting meshwork, along with an increases in collagen gel stiffness which reinforces the space between collagen fibrils (Margaron et al., 2010), may provide additional stiffness and strength to the ligament, which may lead to a ligament more durable to failure. Overall, it can be concluded that TNXB is critical to mediate collagen fibrillogenesis (Margaron et al., 2010).

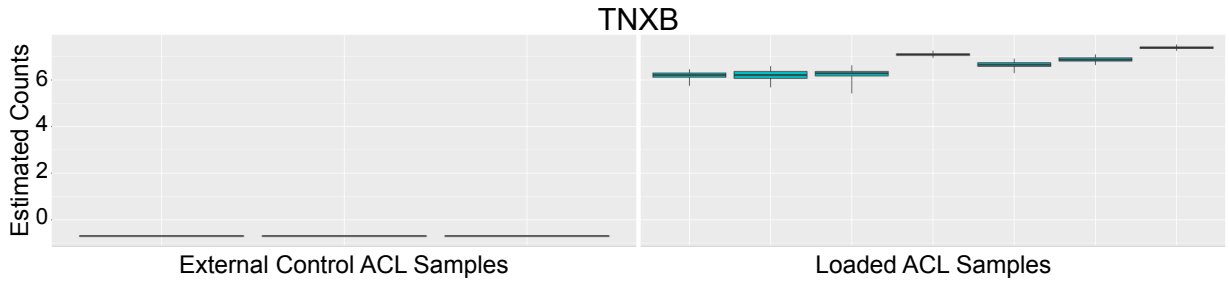

Figure 12: Gene expression of TNXB between external control ACL samples (left), and the loaded ACL samples (right). The external control ACL samples all had zero expression of TNXB.

This *in vivo* mechanical loading of a ligament produced increased expression of genes regulating collagen formation. TNXB being the greatest differently expressed gene between the loaded ACL and the external control is strong evidence that the mechanical loading produced a genetic response which increased collagen synthesis and organization. To our knowledge, this is

the first study to show that *in vivo* mechanical loading of a ligament results in increased genetic expression of genes related to collagen formation, such as tenascin. While tenascin proteins are known to modulate the adhesion of cells on the other extracellular matrix (ECM) components (Minamitani et al., 2004), it is likely that this pathway contributes to the mechanotransductive response of the ligament to the *in vivo* mechanical loading as well.

A group of four genes, all related to the interleukin cytokines and tissue generation, were also altered in their genetic expression in response to low-magnitude, high-frequency loading in the ligament. The interleukin cytokine family has a direct relationship to ECM regulation and fibroblast proliferation. This group of genes, EIF4E, FABP6, IRAK4, and PLCL1, are all related to interleukin or tissue generation and therefore have a direct relationship to collagen regulation in the loaded ligament.

How mechanical stretch causes an increase in protein synthesis is not well understood, but a potential protein synthesis regulator is the translation initiation factor EIF4E (Sadoshima and Izumo, 1997). In this study, EIF4E had increased expression in the loaded ACL compared to the external control ACL, with a significant effect size of 5.72 (Figure 13). EIF4E's response to mechanical loading has been studied in skeletal and cardiac muscle. In skeletal muscle, EIF4E is involved in the Akt/mTOR pathway, and is an important contributor to the initiation of protein synthesis during mechanical loading in skeletal muscle, as it must be present for initiation of protein synthesis and translation to occur (Spangenburg, 2009). The coiled-coil domain containing 88A gene (CCDC88A), a member of the Girdin gene family involved in the cytoskeleton remodeling pathway, was also altered in gene expression and also has relations to mTOR signaling (GO:0031929). Additionally, EIF4E phosphorylation is a mechanism by which increased cardiac load is coupled to accelerated rates of protein synthesis (Wada et al., 1996). As

EIF4E is involved in regulating protein synthesis in skeletal and cardiac muscle, it makes sense that it may also regulate protein synthesis in ligament in response to mechanical loading. More interesting, EIF4E's involvement in the mTOR pathway has been shown necessary to control IL-10 and COX-2 mRNA translation and subsequent production (William et al., 2018). This is significant, as IL-10 is an anti-inflammatory cytokine linked with the ERK1/2 pathway which regulates collagen synthesis (Kawarizadeh et al., 2005), and COX-2 which is known to inhibit bone osteoclasts (Lau et al., 2010). Both are known to respond to mechanical loading with altered gene expression. This is strong evidence that genetic expression of EIF4E in the presence of ligament mechanical loading mediates both IL-10 and COX-2, affecting collagen synthesis.

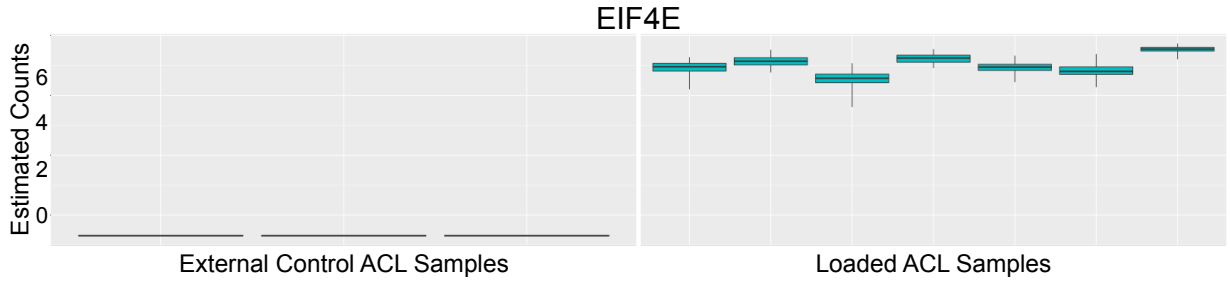

Figure 13: Gene expression of EIF4E between external control ACL samples (left), and the loaded ACL samples (right). The external control ACL samples all had zero expression of EIF4E.

IRAK4, interleukin-1 receptor associated kinase-4, was down regulated in the loaded ACL compared to the external control ACL, with an effect size of -0.4732(Figure 14). Inhibition of IRAK4 has been shown as a protective measure against cell injury *in vivo* (Leaf et al., 2017). This cell injury is likely related to a mechanotransductive response, as IRAK4 has been shown to play a role in the model of fibrogenesis and inflammatory and growth factor signaling. It has also been shown that TGF-β inflammatory and profibrotic responses require IRAK4 (Leaf et al., 2017), further evidence of IRAK4 affecting collagen synthesis.

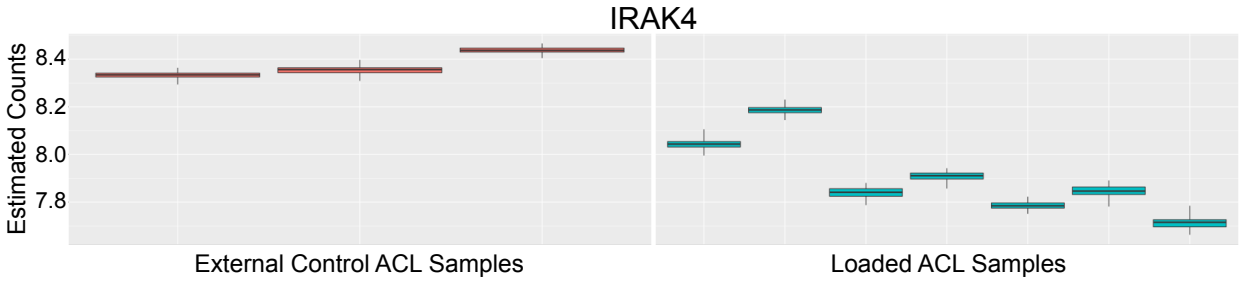

Figure 14: Gene expression of IRAK4 between external control ACL samples (left), and the loaded ACL samples (right).

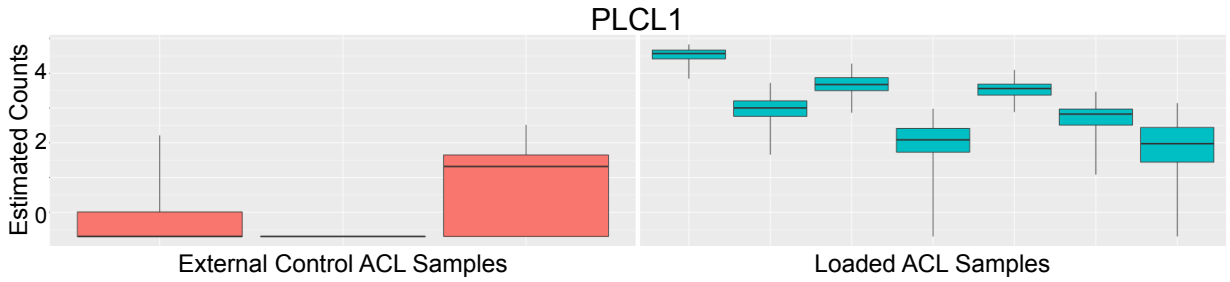

Figure 15: Gene expression of PLCL1 between external control ACL samples (left), and the loaded ACL samples (right).

In addition to these genes that are closely associated with collagen synthesis and interleukins, PLCL1 was also up regulated in the loaded ACL compared to the external control ACL, with an effect size of 3.1321 (Figure 15). PLCL1 is a gene responsible for intracellular signal transduction (Gene Ontology Annotation: GO:0035556). It's possible influence in the mechanotransduction signaling pathway is intriguing, as it is linked to regulating mechanical sensing in bone (Liu et al., 2008; Riddle et al., 2006). More intriguing though is PLCL1's relationship to PLCL, a polymer matrix nanocomposite biomaterial known for improving bone formation *in vivo* and *in vitro* (Won et al., 2014). A substance with rubberlike elasticity, PLCL's scaffolding has been used to mature and develop cartilaginous tissue. These biocompatibility results show PLCL provides an environment where cartilage tissue growth is enhanced and facilitated (Jung et al., 2008). Thus, it has been used in application of many tissue engineering matrices (Won et al., 2014). While PLCL and PLCL1 are not exactly similar, as PLCL1

microspheres cannot be considered effective for cell loading in tissue engineering due to their irregular and small-sized pores (Jin et al., 2014), the relationship between the two cannot be ignored. It is possible that as a result of this *in vivo*, low-magnitude, high-frequency loading, the PLCL1 gene may play a role similar to its relative, PLCL, and help to develop and mature cartilage tissue. As the ends of ligament are composed of fibrocartilage, PLCL may influence the attachment of the ligament to the bone.

Mechanotransductive Genetic Response

While there is limited research on a typical ligament response to mechanical loading, and the exact sensor for ligament mechanotransduction is not well understood, there are a group of 14 genes that were differentially expressed between the loaded ACL and the external control ACL that are hypothesized to have mechanotransductive effects. Seven of these genes, LOC103351662, OR2B2, OR8S1, SLC35G1, SYNPR, TAS2R14 and UPK1B, are likely to have mechanotransductive effects, based on their function within the cell membrane. FAM20C, KNG1, LIPC and VNN1 all have functions within the extracellular space. JUP and RALPB1 may provide additional insight into the ligament sensor for mechanotransduction, as these genes are responsible for signal transduction, and the detection of mechanical stimulus. Lastly, PSEN1, involved in notch signaling pathways, may play a pivotal role in helping to better understand mechanotransduction in the ligament.

A common feature of all connective tissues is that they consist mainly of ECM, within which the connective tissue cells, ligament fibroblasts in this case, are sparsely distributed (Chiquet et al., 2009). Mechanotransduction is hypothesized to be mediated by stretch-sensitive ion channels, signaling molecules, cytoskeleton and integrins, all of which are located in the ECM or within the plasma membrane (Ingber, 1997). Four genes, FAM20C, KNG1, LIPC, and

VNN1, are involved in extracellular space functions, and all had altered expression as a result of the high-frequency, low-magnitude loading. These genes involved in extracellular function may also play a role in mechanotransductive responses. FAM20C, for example is one of the main protein kinases for the extracellular proteins, generating the majority of the extracellular phosphoproteome (Tagliabracci et al., 2016). Additionally, FAM20C plays a role in cell adhesion, which may contribute to ligament mechanotransductive responses. While it is not well understood these genes direct link to mechanotransduction in the ligament, their function within the extracellular space and altered regulation in response to mechanical loading connects them as possible mediators of ligament mechanotransduction.

Additionally, seven genes, LOC1033351662, OR2B2, OR8S1, SLC35G1, SYNPR, TAS2R14, and RALBP1, all with functions within the plasma membrane of the cell, had altered expression as a result of the high-frequency, low-magnitude loading. Again, this link to the plasma membrane makes these genes possible mediators in the ligament mechanotransduction process. For example, RALBP1 is required for cell adhesion signaling, which produces a cascade of signaling in the membrane mediating cell spreading and migration (Goldfinger et al., 2006). RALBP1 has also been linked to signal transduction, converting a stimulus to a downstream cellular process (Gene Ontology Annotation: 0007165). Additionally, SLC35G1 is an important negative regular of plasma membrane calcium-transporting ATPases preventing calcium efflux from the cell (Krapivinsky et al., 2011), and SYNPR is an intrinsic membrane protein of small synaptic vesicles, helping with transporter activity (Gene Ontology Annotation: 0008021). Overall, these genes involved in plasma membrane function, as well as the ones involved in extracellular space and signal transduction, are all likely mediators of ligament mechanotransduction.

Another gene that may provide insight on mechanotransduction in the ligament is junction plakoglobin (JUP). Plakoglobin regulates both desmosomal adhesion as well as cell stiffness and motility (Huang et al., 2008). More importantly, in response to tensional force, plakoglobin is accumulated on the cellular membrane on the site of force application reinforcing the desmosomes (Michaelson and Huang, 2012; Ohashi et al., 2017). Plakoglobin plays an important role in mechanoresponses, as they help cells to migrate in the direction opposite to the pulling force (Ohashi et al., 2017). Exactly how this increase in mechanical force is initially sensed by plakoglobin remains unclear (Leckband and de Rooij, 2014). While plakoglobin was expressed less in the loaded ACL compared to the external control ACL (effect size = -0.5958) (Figure 16), diminished plakoglobin is found in the myocardium of most patients with ARVC (arrhythmogenic right ventricular cardiomyopathy), affecting proteins that join cells together in the heart (Michaelson and Huang, 2012). This diminished plakoglobin expression has been attempted to be explained by the hypotheses that plakoglobin instead localizes to the nucleus and engages in the Wnt-signaling pathway, a pathway responsible for signal transduction through cell-cell surface receptors (Michaelson and Huang, 2012). Future research should be done on the mechanotransductive effect of plakoglobin in the ligament.

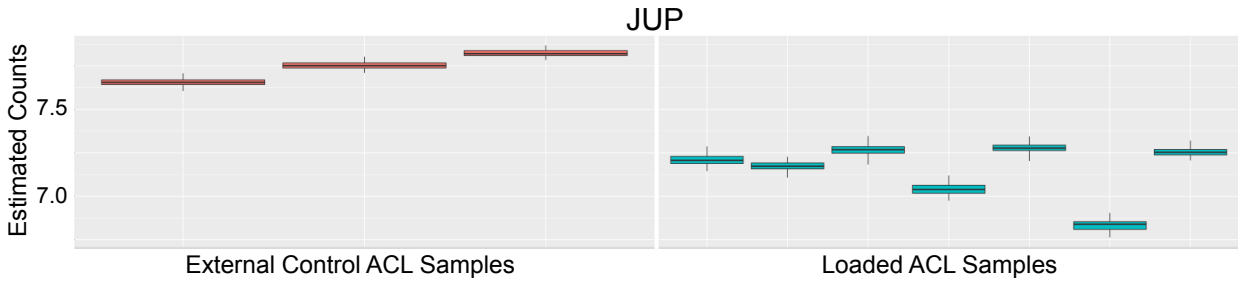

Figure 16: Gene expression of JUP between external control ACL samples (left), and the loaded ACL samples (right).

Presenilin1 (PSEN1), a mediator of Notch signaling, expression was down-regulated as a result of low-magnitude, high frequency mechanical loading on the ligament, with an effect size of -1.3293 (Figure 17). This is significant for better understanding the process of mechanotransduction in the ligament. Notch signaling involves the interaction of receptors and ligands expressed by two neighboring cell (Favarolo and López, 2018). The receiving cell expresses the transmembrane receptor Notch, while the sending cell presents the corresponding transmembrane ligand. PSEN1 is a gene required in the Notch signaling pathway between neighboring cells (Favarolo and López, 2018; Zanotti and Canalis, 2016). Notch1 and Notch2 genes are linked to the differentiation and function of cells of the osteoblastic (bone formation) and osteoclastic (bone resorption) lineages, and play a critical role in osteoblastogenesis and osteoclastogenesis (Zanotti and Canalis, 2016). As we know osteoblastogenesis and osteoclastogenesis can be initiated by high-frequency, low-magnitude loads in bone (Rubin et al., 2002, 2004, 2001), this study now links Notch signaling as a possible regulator of mechanotransduction in the ligament as well.

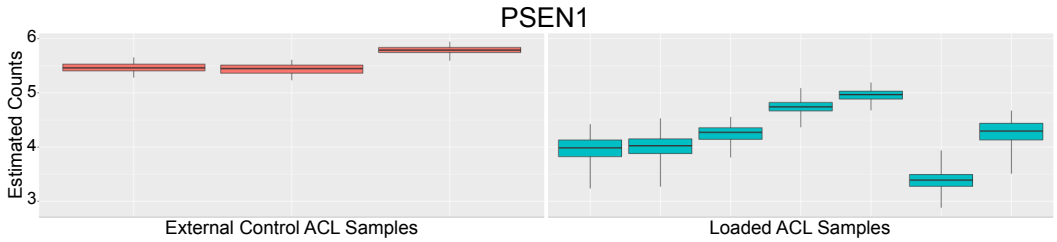

Figure 17: Gene expression of PSEN1 between external control ACL samples (left), and the loaded ACL samples (right).

Systemic Effects of Mechanical Loading

It was found that the gene expression as a result of mechanical loading between the internal control ACL and loaded ACL was more similar than the gene expression was between the external control ACL and the loaded ACL. Only 3 genes between the loaded ACL and the

internal control ACL were differentially expressed. More interestingly, the loaded ACL and external control ACL had 121 genes that were differentially expressed. The comparison that the external control resulted in more differentially expressed genes compared to the comparison with the internal control was surprising. It is also likely that this difference indicates a systemic effect of this mechanical loading. In the rabbit that underwent mechanical loading, the effects of the loading seemed to be systemic, as both ACL's had equal genetic expression. While it is impossible to know if these systemic effects would have resulted in physical changes to the ligament on either leg, or even equally, the systemic effect of mechanical loading in this experiment should be explored.

To further test these apparent systemic effects of mechanical loading, the gene expression between the loaded ACL and the other, right, external control ACL were tested. The results added support to the new, systemic effects, hypothesis. The loaded ACL and other, right, external control ACL produced similar results in terms of gene expression, and magnitude of genetic difference between the two tissues as the original test between the loaded ACL and the original external control ACL. Additionally, if the loaded ACL and internal control were similar in terms of gene expression, it would be hypothesized that the external control ACL and the internal control ACL would also be similar. In general, this was found to be true. The internal control ACL and the external control ACL produced similar results in terms of altered gene expression as the comparison between the loaded ACL and the external control ACL. This further confirms the systemic effect of acute loading.

This systemic effect of mechanical loading may be explained by the hypothesis that in regulating bone mass, mechanical loading may be more of an influence, rather than the control (Lanyon et al., 2009). There may be more to regulating bone mass than just mechanical loading

at the site of interest, as systemic effects may also play a role. One example of this is the development of osteoarthritis, the breakdown of bone, which is believed to be influenced by systemic metabolic factors (Visser et al., 2015). If bone catabolic activity is systemically regulated, it would make sense that bone anabolic activity is also regulated systemically. This was demonstrated by Sample *et al.* 2010. Subjecting the right ulna of rats to *in vivo* cyclic axial loading resulted in increased bone formation in the right ulna, and the left ulna, right humerus and both femurs. The left ulna, right humerus and both femurs were not loaded, but experienced increased bone formation as a result of loading in the right ulna. Additionally, there is strong support for the bilateral/systemic response of muscle (Munn et al., 2005; Slater-Hammel, 1950). It has been demonstrated that exercise in a muscle on one limb will cause contralateral effects on the muscle on the opposite limb. Relevant clinically, this concept can be used to strengthen a muscle while the contralateral limb may be immobilized. This study adds to the support of a systemic response to mechanical loading.

While there is evidence the primary effects of loading are local and not systemic (Cardoso et al., 2009; Kennedy et al., 2012; McBride and Silva, 2012), there is evidence that systemic effects can happen. There is a growing body of evidence that the central nervous system has important regulatory effects on skeletal metabolism (Elefteriou et al., 2005; Sample et al., 2010; Togari and Arai, 2008; Yadav et al., 2008). It is proposed that systemic effects on the skeleton from mechanical loading are mediated via a neuronal crosstalk mechanism between limbs. The sensory innervation of the skeleton exhibits plasticity in response to bone loading, which influences the functional adaptation of the skeleton (Sample et al., 2010). Currently, there is no evidence of the systemic effects of mechanical loading in ligaments. However, this experiment, based off bone's response to loading, demonstrated similar results in ligament. It is

possible that the systemic effects of mechanical loading in bone, also translate to systemic effects in ligaments. Future work should look to investigate the systemic response of protein synthesis as well. While the genetic response in this experiment was systemic, it does not guarantee that the protein synthesis will also be systemic.

Significance

To our knowledge, this is the first study to show that *in vivo* mechanical loading of a ligament results in increased genetic expression of genes related to collagen formation, such as tenascin, and EIF4E, and others relating to tissue repair PLCL1. The increased expression of these specific genes shows that the mechanical loading to the ACL, results in genetic expressions that will likely result in ligament adaptation. Additionally, this study provides more clarity to the mechanotransduction pathway in ligament, a process that is poorly understood in all tissues. Through a combination of genetic responses from PSEN1, JUP, and genes that regulate the ECM and membrane space, these genes are playing a role in the signal transduction response of the cells.

The development of the novel RACL loader is significant to better understanding the response of a ligament to *in vivo*, low-magnitude, high-frequency loading. This device, to our knowledge, is the first of its kind to be able to apply the types of high-frequency loads directly to a ligament *in vivo*. Prior studies often rely on *in vitro* loading to better understand the ligament response, but genetic pathways, especially systemic effects are unable to be quantified *in vitro*. The results of this study that ligament loading may have systemic effects makes it even more important to continue to investigate this response with *in vivo* loading rather than *in vitro* loading.

Most studies investigating gene response to mechanical loading only test a subset of genes that are hypothesized to be affected. This study took a more encompassing approach, through RNA-Seq quantification analysis. This allows a better understanding of the whole genetic response, rather than only a specific subset of genes. This study found genes that had altered expression that had not yet been annotated. In initial exploratory research such as this experiment, it is important to do a broader, encompassing approach such as RNA-seq, as it identifies important genes that are altered. This is beneficial for future research that can now focus on these specific subsets of genes and to identify novel mRNA's that might represent new peptides or genes previously unidentified.

The potential systemic response to mechanical loading is a significant development of this research. If the response truly is systemic in nature, it is possible that there are certain blood markers that may indicate a genetic response. Rather than harvesting of the ligaments to test the effectiveness of the ligament loading, a simple blood test could possibly be used. This is especially important in a potential future long-term ligament training. Especially in humans, it would be ideal to perform a blood test to understand the effectiveness of the ligament training, and then be able to adjust certain loading parameters, such as frequency, magnitude, and duration based on a blood test.

The finding that *in vivo* mechanical loading of a ligament leads to the increased expression of TNXB may be significant in dealing with clinical disorders such as Ehlers-Danlos Syndrome. Ehlers-Danlos Syndrome is characterized by connective tissue defects including disorganization of fibrillar networks, a reduced collagen deposition, and modifications in the mechanical properties of these tissues (Margaron et al., 2010). It is most often associated with tenascin deficiency, as mice deficient in TNXB had fibroblasts that failed to deposit collagen in

an *in vitro* culture (Schalkwijk et al., 2001). An increased expression of TNXB, achieved through *in vivo* mechanical loading on the ligament, may be beneficial in better understanding treatments of Ehler-Dnalos Syndrome, including the possible systemic effects this loading may have throughout the body.

Limitations and Future Directions

The present study had several limitations including: 1) only one set of loading parameters was tested, 2) the amount of load applied to the ACL was not measured 3) the harvested ACL tissue was not snap-frozen in liquid nitrogen and 4) all rabbits tested were females.

In this study, a low-magnitude, ~2-12N, and high-frequency, ~15 Hz, cyclical load was applied to the ACL. While it is known that low-magnitude, high-frequency loading is beneficial, future research should investigate loading at differing low-magnitudes, and different high-frequencies. Rubin *et al.* 2002 loaded the hind legs of sheep at 30 Hz, while this study only loaded the ACL at 15 Hz. While taking the precautions to ensure this increase in frequency will not cause damage to the ligament, it is possible that there is a greater mechanotransductive benefit at a higher frequency than 15 Hz. Future research should investigate optimal frequencies and magnitudes of loading a ligament to produce the greatest mechanotransductive response.

Additionally, in this study, the rabbits ACL was harvested four hours after loading. This time was based on the time course for collagen genes to be expressed in response to mechanical loading (Majima et al., 2000; Raab-Cullen et al., 1994; Sullivan et al., 2009; Yang et al., 2004). While genes that effect collagen expression, such as TNXB, and EIF4E, were altered in their gene expression, it is possible that there was not enough time following loading for these pathways to express collagen genes. Additionally, it is possible that earlier timepoints may be

needed to track the development of the proposed systemic signal via cytokines, exocrines, or endocytosis. Future studies should investigate the optimal time for genetic response in collagen genes based on the optimal magnitudes and frequencies of *in vivo* mechanical loading on a ligament.

It is also plausible that the duration of loading, 20 minutes, was insufficient to cause changes in the expression of collagen genes. In order to see stronger mechanotransductive responses of the ligament, it may be required to load at a duration greater than 20 minutes, or more than once. Future studies should investigate the optimal loading duration, and the effects of an acute load, versus a chronic loading program.

Another limitation in this study was that we were unable to measure the amount of load translated to the ACL. While the load cell measured the force being applied to the tibia-femur-ACL complex, it is unknown how much of this load was actually translated to the ACL. The amount of ACL strain based on knee flexion angle and angle of pull was used to approximate the amount of load translated to the ACL based on specific magnitudes of loading, but exact amounts would require a bone pin strain gauge attached to the femur and tibia. This would be an invasive process that would likely affect the mechanotransductive response and mechanical loading itself. Future studies should look to control for any tibial rotations during loading. While tibial rotations were ignored in this study, as we were focused on loading the ACL in its main loading plane, the sagittal plane, it is possible that not controlling for tibial rotations would reduce the amount of load translated to the ACL.

In the current study, due to lab constraints, following the harvesting of the ACL tissue, it was placed in a vial and placed in a cooler with dry ice. While the ACL tissue qualitatively appeared to be frozen upon storing the tissues in a -80°, it is likely that these tissues were not

snap-frozen. This may have contributed to the low RIN's for nine out of twenty of our ACL samples. Future studies should use liquid nitrogen to snap-freeze the tissues immediately following extraction.

Lastly, all rabbits used in this study were female. It has been shown that estrogen levels can alter the failure load of the rabbit ACL (Slauterbeck et al., 1999). The failure load for rabbits treated with estrogen for thirty days was over 50 N weaker when subjected to failure loading. This difference in failure loading as a result of estrogen levels may mean genders would respond differently to mechanical loading. Females may need require loading at a lower frequency and magnitude than males to produce similar, non-damaging, mechanotransductive effects. Future studies should further investigate this potential gender difference in response to loading.

While this study has promising results in terms of mechanotransductive responses of ligament to mechanical loading, this study is the first of many steps. It is now known that the ligament will respond to mechanical loading. However, future studies must work to characterize optimal loading magnitude, frequency and duration, as well as optimal time for genetic response to occur. It is also important to understand if this increase in genetic response actually translates to a stronger ligament complex. Future studies should also consider tensile testing the ligaments following loading. Once the optimal parameters are known, the effects of an acute loading, versus a chronic loading program should be investigated. Understanding the proper chronic loading program, whether daily or a certain number of times a week, will be important to get to the long-term goal of translating this type of loading to humans, as a preventative measure against ACL, and possibly other ligament, tears.

Conclusions

In conclusion, the purpose of this study was to identify (1) if loading a ligament, *in vivo,* will produce a mechanotransductive response, and (2) what genes were altered in their expression level in response to high-frequency, low-magnitude loading. Rabbit ACL tissue differentially expressed key collagen and mechanotransductive pathway genes in response to low-magnitude, high-frequency mechanical loading on the ACL, indicating that there is a ligament response to loading. While this response was systemic at the time point and loads tested, it better identified what genes respond to mechanical loading, and may play a role in mechanotransduction in the ligament. This study is a key first step in understanding if this low-magnitude, high-frequency mechanical loading on a ligament can be used in the future to reduce ligament injury rates.

Bibliography

Alenghat, F.J., Ingber, D.E., 2002. Mechanotransduction: All Signals Point to Cytoskeleton, Matrix, and Integrins. Sci. Signal.

Arampatzis, A., Karamanidis, K., Albracht, K., 2007. Adaptational responses of the human Achilles tendon by modulation of the applied cyclic strain magnitude. J. Exp. Biol. 210, 2743–2753.

Arnoczky, S.P., Tian, T., Lavagnino, M., Gardner, K., Schuler, P., Morse, P., 2002. Activation of stress-activated protein kinases (SAPK) in tendon cells following cyclic strain: The effects of strain frequency, strain magnitude, and cytosolic calcium. J. Orthop. Res. 20, 947–952.

Beck, N.A., Lawrence, J.T.R., Nordin, J.D., DeFor, T.A., Tompkins, M., 2017. ACL Tears in School-Aged Children and Adolescents Over 20 Years. Pediatrics 139.

Beckerle, M.C., Yeh, R.K., 1990. Talin: Role at sites of cell-substratum adhesion. Cell Motil.cytoskelet. 16, 7–13.

Berkovitz, B., 1990. An update on the innervation of the periodontal ligament. Eur. J. Orthod. 12, 91–100.

Bertram, J., Swartz, S., 1991. the 'Law of Bone Transformation': a Case of Crying Wolff? Biol. Rev. 66, 245–273.

Bray, N., Pimentel, H., Melsted, P., Pachter, L., 2015. Near-optimal RNA-Seq quantification.

Brown, R.A., Prajapati, R., McGrouther, D.A., Yannas, I. V., Eastwood, M., 1998. Tensional homeostasis in dermal fibroblasts: Mechanical responses to mechanical loading in three-dimensional substrates. J. Cell. Physiol. 175, 323–332.

Buchfink, B., Xie, C., Huson, D.H., 2014. Fast and sensitive protein alignment using DIAMOND. Nat. Methods 12, 59–60. https://doi.org/10.1038/nmeth.3176

Caraffa, A., Cerulli, G., Projetti, M., Aisa, G., Rizzo, A., 1996. Prevention of anterior cruciate ligament injuries in soccer: A prospective controlled study of proprioceptive training. Knee Surgery, Sport. Traumatol. Arthrosc. 4, 19–21.

Cardoso, L., Herman, B.C., Verborgt, O., Laudier, D., Majeska, R.J., Schaffler, M.B., 2009. Osteocyte Apoptosis Controls Activation of Intracortical Resorption in Response to Bone Fatigue. J. Bone Miner. Res. 24, 597–605. https://doi.org/10.1359/jbmr.081210

Carr, A.J., 1999. Beyond disability: Measuring the social and personal consequences of osteoarthritis. Osteoarthr. Cartil. 7, 230–238.

Chai, F., Wan, F., Jiang, J., Chen, S., 2018. Micro-scale assessment of the postoperative effect of anterior cruciate ligament reconstruction preclinical study using a 7.1T micro-magnetic resonance imaging. Exp. Ther. Med. 214–220. https://doi.org/10.3892/etm.2018.6080

Chen, E.Y., Tan, C.M., Kou, Y., Duan, Q., Wang, Z., Meirelles, G. V., Clark, N.R., Ma'ayan, A., 2013. Enrichr: Interactive and collaborative HTML5 gene list enrichment analysis tool. BMC Bioinformatics 14. https://doi.org/10.1186/1471-2105-14-128

Chen, S., Zhou, Y., Chen, Y., Gu, J., 2018. Fastp: An ultra-fast all-in-one FASTQ preprocessor. Bioinformatics 34, i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Chiquet, M., Gelman, L., Lutz, R., Maier, S., 2009. From mechanotransduction to extracellular matrix gene expression in fibroblasts. Biochim. Biophys. Acta - Mol. Cell Res. 1793, 911–920. https://doi.org/10.1016/j.bbamcr.2009.01.012

Craig, D.B., Kannan, S., Dombkowski, A.A., 2012. Augmented annotation and orthologue analysis for Oryctolagus cuniculus: Better Bunny. BMC Bioinformatics 13. https://doi.org/10.1186/1471-2105-13-84

Danto, M.I., Woo, S.L., 1993. The mechanical properties of skeletally mature rabbit anterior

cruciate ligament and patellar tendon over a range of strain rates. J. Orthop. Res. 11, 58–67.

Davidson, N.M., Hawkins, A.D.K., Oshlack, A., 2017. SuperTranscripts: A data driven reference for analysis and visualisation of transcriptomes. Genome Biol. 18, 1–10. https://doi.org/10.1186/s13059-017-1284-1

Davidson, N.M., Oshlack, A., 2014. Corset: Enabling differential gene expression analysis for de novo assembled transcriptomes. Genome Biol. 15, 1–14. https://doi.org/10.1186/s13059-014-0410-6

De Araujo, R.M.S., Oba, Y., Moriyama, K., 2007. Identification of genes related to mechanical stress in human periodontal ligament cells using microarray analysis. J. Periodontal Res. 42, 15–22.

DeHaven, K.E., 1983. Arthroscopy in the diagnosis and management of the anterior cruciate ligament deficient knee. Clin. Orthop. Relat. Res. 52–56.

Dobin, A., Gingeras, T.R., Spring, C., Flores, R., Sampson, J., Knight, R., Chia, N., Technologies, H.S., 2016. Mapping RNA-seq with STAR. Curr Protoc Bioinforma. 51, 586–597. https://doi.org/10.1002/0471250953.bi1114s51.Mapping

Drawer, S., Fuller, C.W., 2002. Evaluating the level of injury in English professional 446–452.

Elefteriou, F., Ahn, J.D., Takeda, S., Starbuck, M., Yang, X., Liu, X., Kondo, H., Richards, W.G., Bannon, T.W., Noda, M., Clement, K., Valsse, C., Karsenty, G., 2005. Leptin regulation of bone resorption by the sympathetic nervous system and CART. Nature 434, 514–520. https://doi.org/10.1038/nature03398

Eriksson, K., Anderberg, P., Hamberg, P., Olerud, P., Wredmark, T., 2001. There are differences in early morbidity after ACL reconstruction when comparing patellar tendon and semitendinosus tendon graft: A prospective randomized study of 107 patients. Scand. J.

Med. Sci. Sport. 11, 170–177.

Escamilla, R.F., Macleod, T.D., Wilk, K.E., Paulos, L., Andrews, J.R., 2012. ACL Strain and Tensile Forces for Weight Bearing and Non—Weight-Bearing Exercises After ACL Reconstruction: A Guide to Exercise Selection. J. Orthop. Sport. Phys. Ther. 42, 208–220. https://doi.org/10.2519/jospt.2012.3768

Evsikov, A. V, 2019. Transcriptomics as precision medicine to classify in vivo models of dietary-induced atherosclerosis at cellular and molecular levels. https://doi.org/10.20944/preprints201809.0082.v1

Favarolo, M.B., López, S.L., 2018. Notch signaling in the division of germ layers in bilaterian embryos. Mech. Dev. 154, 122–144. https://doi.org/10.1016/j.mod.2018.06.005

Frobell, R.B., Roos, H.P., Roos, E.M., Roemer, F.W., Ranstam, J., Lohmander, L.S., 2015. Treatment for acute anterior cruciate ligament tear: Five year outcome of randomised trial. Br. J. Sports Med. 49, 700. https://doi.org/10.1136/bjsports-2014-f232rep

Garlet, T.P., Coelho, U., Silva, J.S., Garlet, G.P., 2007. Cytokine expression pattern in compression and tension sides of the periodontal ligament during orthodontic tooth movement in humans. Eur. J. Oral Sci. 115, 355–362.

Gillquist, J., Messner, K., 1999. Anterior cruciate ligament reconstruction and the long term incidence of gonarthrosis. Sport. Med. 27, 143–156.

Gilsanz, V., Wren, T.A.L., Sanchez, M., Dorey, F., Judex, S., Rubin, C., 2006. Low-level, high-frequency mechanical signals enhance musculoskeletal development of young women with low BMD. J. Bone Miner. Res. 21, 1464–1474.

Goldfinger, L.E., Ptak, C., Jeffery, E.D., Shabanowitz, J., Hunt, D.F., Ginsberg, M.H., 2006. RLIP76 (RalBP1) is an R-Ras effector that mediates adhesion-dependent Rac activation and

cell migration. J. Cell Biol. 174, 877–888. https://doi.org/10.1083/jcb.200603111

Grabherr, M.G.., Brian J. Haas, Moran Yassour Joshua Z. Levin, Dawn A. Thompson, Ido Amit, Xian Adiconis, Lin Fan, Raktima Raychowdhury, Qiandong Zeng, Zehua Chen, Evan Mauceli, Nir Hacohen, Andreas Gnirke, Nicholas Rhind, Federica di Palma, Bruce W., N., Friedman, and A.R., 2013. Trinity: reconstructing a full-length transcriptome without a genome from RNA-Seq data. Nat. Biotechnol. 29, 644–652. https://doi.org/10.1038/nbt.1883.Trinity

Grinstein, M., Dingwall, H., Shah, R., Capellini, T., Galloway, J., 2018. A robust method for RNA extraction and purification from a single adult mouse tendon. PeerJ 1–17.

Herzog, M.M., Marshall, S.W., Lund, J.L., Pate, V., Mack, C.D., Spang, J.T., 2017. Incidence of Anterior Cruciate Ligament Reconstruction Among Adolescent Females in the United States, 2002 Through 2014. JAMA Pediatr. 171, 808.

Hewett, T.E., Lindenfeld, T.N., Riccobene, J. V, Noyes, F.R., 1999. The Effect of Neuromuscular Training on the Incidence of Knee Injury in Female Athletes A Prospective Study 27, 699–706.

Hewett, T.E., Paterno, M. V, Myer, G.D., 2002. Strategies for Enhancing Proprioception and Neuromuscular Control of the Knee. Clin. Orthop. Relat. Res. 76–94.

Hewett, T.E., Stroupe, A.L., Nance, T.A., Noyes, F.R., 1996. Plyometric Training in Female Athletes Decreased Impact Forces and Increased Hamstring Torques. Am. J. Sports Med. 24, 765–773.

Holm, I., Oiestad, B.E., Risberg, M.A., Gunderson, R., Aune, A.K., 2012. No differences in prevalence of osteoarthritis or function after open versus endoscopic technique for anterior cruciate ligament reconstruction: 12-Year follow-up report of a randomized controlled trial.

Am. J. Sports Med. 40, 2492–2498.

Hsieh, A.H., Sah, R.L., Paul Sung, K.L., 2002. Biomechanical regulation of type I collagen gene expression in ACLs in organ culture. J. Orthop. Res. 20, 325–31.

Hsieh, A.H., Tsai, C.M., Ma, Q.J., Lin, T., Banes, A.J., Villarreal, F.J., Akeson, W.H., Sung, K.L., 2000. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. J. Orthop. Res. 18, 220–7.

Hsieh, Y.F., Turner, C.H., 2001. Effects of loading frequency on mechanically induced bone formation. J. Bone Miner. Res. 16, 918–924.

Hu, Y., Huang, Y., Du, Y., Orellana, C.F., Singh, D., Johnson, A.R., Monroy, A., Kuan, P.F., Hammond, S.M., Makowski, L., Randell, S.H., Chiang, D.Y., Hayes, D.N., Jones, C., Liu, Y., Prins, J.F., Liu, J., 2013. DiffSplice: The genome-wide detection of differential splicing events with RNA-seq. Nucleic Acids Res. 41, 1–18. https://doi.org/10.1093/nar/gks1026

Huang, H., Asimaki, A., Lo, D., McKenna, W., Saffitz, J.E., 2008. Disparate effects of different mutations in plakoglobin on cell mechanical behavior. Cell Motil. Cytoskeleton 65, 964–978. https://doi.org/10.1002/cm.20319

Ingber, D.E., 1997. Tensegrity: the Architectural Basis of Cellular Mechanotransduction. Annu. Rev. Physiol. 59, 575–599.

Ingber, D.E., 1991. Integrins As Mechanochemical. Cell Biol. 3, 841–848.

Jin, G.Z., Park, J.H., Seo, S.J., Kim, H.W., 2014. Dynamic cell culture on porous biopolymer microcarriers in a spinner flask for bone tissue engineering: A feasibility study. Biotechnol. Lett. 36, 1539–1548. https://doi.org/10.1007/s10529-014-1513-6

Jung, Y., Kim, Soo Hyun, You, H.J., Kim, Sang Heon, Ha Kim, Y., Min, B.G., 2008. Application of an elastic biodegradable poly(L-lactide-co-ε- caprolactone) scaffold for

cartilage tissue regeneration. J. Biomater. Sci. Polym. Ed. 19, 1073–1085. https://doi.org/10.1163/156856208784909336

Kanzaki, H., Chiba, M., Shimizu, Y., Mitani, H., 2002. Periodontal Ligament Cells Under Mechanical Stress Induce Osteoclastogenesis by Receptor Activator of Nuclear Factor κB Ligand Up-Regulation via Prostaglandin E2 Synthesis. J. Bone Miner. Res. 17, 210–220.

Kawarizadeh, A., Bourauel, C., Götz, W., Jäger, A., 2005. Early Responses of Periodontal Ligament Cells to Mechanical Stimulus in vivo. J. Dent. Res. 84, 902–906.

Keene, D.R., Engvall, E., Glanville, R.W., 1988. Ultrastructure of type VI collagen in human skin and cartilage suggests an anchoring function for this filamentous network. J. Cell Biol. 107, 1995–2006. https://doi.org/10.1083/jcb.107.5.1995

Kennedy, O.D., Herman, B.C., Laudier, D.M., Majeska, R.J., Sun, H.B., Schaffler, M.B., 2012. Activation of resorption in fatigue-loaded bone involves both apoptosis and active pro-osteoclastogenic signaling by distinct osteocyte populations. Bone 50, 1115–1122. https://doi.org/10.1016/j.bone.2012.01.025

Kent, W.J., 2002. BLAT---The BLAST-Like Alignment Tool. Genome Res. 12, 656–664. https://doi.org/10.1101/gr.229202

Kent, W.J., Sugnet, C.W., Furey, T.S., Roskin, K.M., Pringle, T.H., Zahler, A.M., Haussler, a. D., 2002. The Human Genome Browser at UCSC. Genome Res. 12, 996–1006. https://doi.org/10.1101/gr.229102

Kim, S.-G., Akaike, T., Sasagaw, T., Atomi, Y., Kurosawa, H., 2002. Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. Cell Struct. Funct. 27, 139–144.

Krapivinsky, G., Krapivinsky, L., Stotz, S.C., Manasian, Y., Clapham, D.E., 2011. POST,

partner of stromal interaction molecule 1 (STIM1), targets STIM1 to multiple transporters. Proc. Natl. Acad. Sci. U. S. A. 108, 19234–19239. https://doi.org/10.1073/pnas.1117231108

Kuleshov, M. V., Jones, M.R., Rouillard, A.D., Fernandez, N.F., Duan, Q., Wang, Z., Koplev, S., Jenkins, S.L., Jagodnik, K.M., Lachmann, A., McDermott, M.G., Monteiro, C.D., Gundersen, G.W., Ma'ayan, A., 2016. Enrichr: a comprehensive gene set enrichment analysis web server 2016 update. Nucleic Acids Res. 44, W90–W97. https://doi.org/10.1093/nar/gkw377

Lanyon, L.E., Sugiyama, T., Price, J.S., 2009. Regulation of bone mass: Local control or systemic influence or both? IBMS Bonekey 6, 218–226. https://doi.org/10.1138/20090382

Lau, E., Al-Dujaili, S., Guenther, A., Liu, D., Wang, L., You, L., 2010. Effect of low-magnitude, high-frequency vibration on osteocytes in the regulation of osteoclasts. Bone 46, 1508–1515.

Lavagnino, M., Arnoczky, S.P., 2005. In vitro alterations in cytoskeletal tensional homeostasis control gene expression in tendon cells. J. Orthop. Res. 23, 1211–1218.

Lavagnino, M., Arnoczky, S.P., Kepich, E., Caballero, O., Haut, R.C., 2007. A finite element model predicts the mechanotransduction response of tendon cells to cyclic tensile loading. Biomech. Model. Mechanobiol. 7, 405–416.

Leaf, I.A., Nakagawa, S., Johnson, B.G., Cha, J.J., Mittelsteadt, K., Guckian, K.M., Gomez, I.G., Altemeier, W.A., Duffield, J.S., 2017. Pericyte MyD88 and IRAK4 control inflammatory and fibrotic responses to tissue injury. J. Clin. Invest. 127, 321–334. https://doi.org/10.1172/JCI87532

Leckband, D.E., de Rooij, J., 2014. Cadherin Adhesion and Mechanotransduction. Annu. Rev. Cell Dev. Biol. 30, 291–315. https://doi.org/10.1146/annurev-cellbio-100913-013212

Lekic, P., Mcculloch, C.A.G., 1996. Periodontal ligament cell populations: The central role of fibroblasts in creating a unique tissue. Anat. Rec. 245, 327–341.

Li, G., DeFrate, L.E., Rubash, H.E., Gill, T.J., 2005. In vivo kinematics of the ACL during weight-bearing knee flexion. J. Orthop. Res. 23, 340–344. https://doi.org/10.1016/j.orthres.2004.08.006

Liao, Y., Smyth, G.K., Shi, W., 2014. FeatureCounts: An efficient general purpose program for assigning sequence reads to genomic features. Bioinformatics 30, 923–930. https://doi.org/10.1093/bioinformatics/btt656

Liedert, A., Kaspar, D., Blakytny, R., Claes, L., Ignatius, A., 2006. Signal transduction pathways involved in mechanotransduction in bone cells. Biochem. Biophys. Res. Commun. 349, 1–5.

Liu, Y.Z., Wilson, S.G., Wang, L., Liu, X.G., Guo, Y.F., Li, J., Yan, H., Deloukas, P., Soranzo, N., Chinnapen-Horsley, U., Cervino, A., Williams, F.M., Xiong, D.H., Zhang, Y.P., Jin, T.B., Levy, S., Papasian, C.J., Drees, B.M., Hamilton, J.J., Recker, R.R., Spector, T.D., Deng, H.W., 2008. Identification of PLCL1 gene for hip bone size variation in females in a genome-wide association study. PLoS One 3. https://doi.org/10.1371/journal.pone.0003160

Lohmander, L.S., Englund, P.M., Ludvig, L.D., Roos, E.M., 2007. The Long-term Consequence of Anterior Cruciate Ligament and Meniscus Injuries. Clin. Sport. Med. Updat. 35, 1756–1769.

Lohmander, L.S., Östenberg, A., Englund, M., Roos, H., 2004. High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. Arthritis Rheum. 50, 3145–3152.

Lohmander, L.S., Roos, H., 1994. Knee ligament injury, surgery and osteoarthrosis: Truth or

consequences? Acta Orthop. 65, 605–609.

Luder, H.U., Zimmerli, I., Schroeder, H.E., 1988. Do collagen fibrils of the periodontal ligament shrink with age? J. Periodontal Res. 23, 46–52.

Lyon, R.M., Woo, S.L.-Y., Hollis, J.M., Marcin, J.P., Lee, E.B., 1989. A New Device to Measure the Structural Properties of the Femur-Anterior Cruciate Ligament-Tibia Complex. J. Biomech. Eng. 111, 350. https://doi.org/10.1115/1.3168390

Majima, T., Marchuk, L.L., Shrive, N.G., Frank, C.B., Hart, D.A., 2000. In-vitro cyclic tensile loading of an immobilized and mobilized ligament autograft selectively inhibits mRNA levels for collagenase (MMP-1). J. Orthop. Sci. 5, 503–510. https://doi.org/10.1007/s007760070030

Margaron, Y., Bostan, L., Exposito, J.Y., Malbouyres, M., Trunfio-Sfarghiu, A.M., Berthier, Y., Lethias, C., 2010. Tenascin-X increases the stiffness of collagen gels without affecting fibrillogenesis. Biophys. Chem. 147, 87–91. https://doi.org/10.1016/j.bpc.2009.12.011

McBride, S.H., Silva, M.J., 2012. Adaptive and injury response of bone to mechanical loading. Bonekey Rep. 1, 192. https://doi.org/10.1038/bonekey.2012.192

McCulloch, C.A.G., Lekic, P., McKee, M.D., 2000. Role of physical forces in regulating the form and function of the periodontal ligament. Periodontol. 2000 24, 56–72.

Meikle, M.C., 2007. Osteogenic Gene Expression by Human Periodontal Ligament Cells under Cyclic Tension 1212–1216.

Menetrey, J., Kasemkijwattana, C., Day, C.S., Bosch, P., Fu, F.H., Moreland, M.S., Huard, J., 1999. Direct, fibroblast and myoblast mediated gene transfer to the anterior cruciate ligament. Tissue Eng. 5, 435–42.

Michaelson, J.E., Huang, H., 2012. Cell-cell junctional proteins in cardiovascular

mechanotransduction. Ann. Biomed. Eng. 40, 568–577. https://doi.org/10.1007/s10439-011-0439-6

Minamitani, T., Ikuta, T., Saito, Y., Takebe, G., Sato, M., Sawa, H., Nishimura, T., Nakamura, F., Takahashi, K., Ariga, H., Matsumoto, K.I., 2004. Modulation of collagen fibrillogenesis by tenascin-X and type VI collagen. Exp. Cell Res. 298, 305–315. https://doi.org/10.1016/j.yexcr.2004.04.030

Munn, J., Herbert, R.D., Hancock, M.J., Gandevia, S.C., 2005. Training with unilateral resistance exercise increases contralateral strength. J. Appl. Physiol. 99, 1880–1884. https://doi.org/10.1152/japplphysiol.00559.2005

Myklebust, G., Bahr, R., 2005. Return to play guidelines after anterior cruciate ligament surgery. Br J Sport. Med 10, 127–131.

Nessler, T., Denney, L., Sampley, J., 2017. ACL Injury Prevention: What Does Research Tell Us? Curr. Rev. Musculoskelet. Med. 10, 281–288.

Ohashi, K., Fujiwara, S., Mizuno, K., 2017. Roles of the cytoskeleton, cell adhesion and rho signalling in mechanosensing and mechanotransduction. J. Biochem. 161, 245–254. https://doi.org/10.1093/jb/mvw082

Panjabi, M.M., Courtney, T.W., 2001. High-speed subfailure stretch of rabbit anterior cruciate ligament: Changes in elastic, failure and viscoelastic characteristics. Clin. Biomech. 16, 334–340. https://doi.org/10.1016/S0268-0033(01)00007-9

Patro, R., Duggal, G., Love, M.I., Irizarry, R.A., Kingsford, C., Biology, C., 2017. Salmon: Fast and Bias-Aware Quantification of Transcript Expression Using Dual-Phase Inference 14, 417–419. https://doi.org/10.1038/nmeth.4197.Salmon

Pimentel, H., Bray, N.L., Puente, S., Melsted, P., Pachter, L., 2017. Differential analysis of

RNA-seq incorporating quantification uncertainty. Nat. Methods 14, 687–690. https://doi.org/10.1038/nmeth.4324

Quinn, E., McManus, R., 2015. Celiac Disease Quality Control and Analysis of NGS RNA Sequencing Data. Celiac Dis. 217.

Raab-Cullen, D.M., Thiede, M.A., Petersen, D.N., Kimmel, D.B., Recker, R.R., 1994. Mechanical Loading Stimulates Rapid Changes in Periosteal Gene Expression. Calcif. Tissue Int. 55, 473–478.

Riddle, R.C., Taylor, A.F., Genetos, D.C., Donahue, H.J., 2006. MAP kinase and calcium signaling mediate fluid flow-induced human mesenchymal stem cell proliferation. Am. J. Physiol. - Cell Physiol. 290, 776–784. https://doi.org/10.1152/ajpcell.00082.2005

Rios, H.F., Ma, D., Xie, Y., Giannobile, W.V., Bonewald, L.F., Conway, S.J., Feng, J.Q., 2008. Periostin Is Essential for the Integrity and Function of the Periodontal Ligament During Occlusal Loading in Mice. J. Periodontol. 79, 1480–1490.

Ruano, J.S., Sitler, M.R., Driban, J.B., 2017. Prevalence of radiographic knee osteoarthritis after anterior cruciate ligament reconstruction, with or without meniscectomy: An evidence-based practice article. J. Athl. Train. 52, 606–609. https://doi.org/10.4085/1062-6050-51.2.14

Rubin, C., Gross, T., Qin, Y.-X., Fritton, S., Guilak, F., McLeod, K., Brook, S., 1996. Differentiation of the Bone-Tissue Remodeling Response to Axial and Torsional Loading in the Turkey Ulna.

Rubin, C., McLeod, K., 1994. Promotion of Bony Ingrowth by Frequency-Specific, Low-Amplitude Mechanical Strain. Clin. Orthop. Relat. Res. 165–174.

Rubin, C., Turner, A.S., Müller, R., Mittra, E., McLeod, K., Lin, W., Qin, Y.-X., 2002. Quantity

and Quality of Trabecular Bone in the Femur Are Enhanced by a Strongly Anabolic, Noninvasive Mechanical Intervention. J. Bone Miner. Res. 17, 349–357. https://doi.org/10.1359/jbmr.2002.17.2.349

Rubin, C.T., Recker, R., Cullen, D., Ryaby, J., McCabe, J., McLeod, K., 2004. Prevention of postmenopausal bone loss by a low-magnitude, high-frequency mechanical stimuli: A clinical trial assessing compliance, efficacy, and safety. J. Bone Miner. Res. 19, 343–351.

Rubin, C.T., Sommerfeldt, D.W., Judex, S., Qin, Y., 2001. Inhibition of osteopenia by low magnitude, high-frequency mechanical stimuli 6, 848–858.

Rugg, C.M., Wang, D., Sulzicki, P., Hame, S.L., 2014. Effects of prior knee surgery on subsequent injury, imaging, and surgery in NCAA collegiate athletes. Am. J. Sports Med. 42, 959–964.

Sadoshima, J., Izumo, S., 1997. the Cellular and Molecular Response of Cardiac Myocytes To Mechanical Stress. Annu. Rev. Physiol. 59, 551–571. https://doi.org/10.1146/annurev.physiol.59.1.551

Sample, S.J., Collins, R.J., Wilson, A.P., Racette, M.A., Behan, M., Markel, M.D., Kalscheur, V.L., Hao, Z., Muir, P., 2010. Systemic effects of ulna loading in male rats during functional adaptation. J. Bone Miner. Res. 25, 2016–2028. https://doi.org/10.1002/jbmr.101

Sarasa-Renedo, A., Chiquet, M., 2005. Mechanical signals regulating extracellular matrix gene expression in fibroblasts. Scand. J. Med. Sci. Sport. 15, 223–230.

Schalkwijk, J., Zweers, M., Steijlen, P., Dean, W., Taylor, G., VanVlijmen, I., VanHaren, B., Miller, W., Bristow, J., 2001. A Recessive Form of Ehlers-Danlos Syndrome Caused by Tenascin-X Deficiency. N. Engl. J. Med. 345.

Sciore, P., Boykiw, R., Hart, D.A., 1998. Semiquantitative Reverse Transcription-Polymerase

Chain Reaction Analysis 429–437.

Sekiguchi, H., Post, W.R., Han, J.S., Ryu, J., Kish, V., 1998. The effects of cyclic loading on tensile properties of a rabbit femur-anterior cruciate ligament-tibia complex (FATC). Knee 5, 215–220. https://doi.org/10.1016/S0968-0160(97)10011-4

Slater-Hammel, A.T., 1950. Bilateral effects of muscle activity. Res. Q. Am. Assoc. Heal. Phys. Educ. Recreat. 21, 203–209. https://doi.org/10.1080/10671188.1950.10624851

Slauterbeck, J., Clevenger, C., Lundberg, W., Burchfield, D.M., 1999. Estrogen level alters the failure load of the rabbit anterior cruciate ligament. J. Orthop. Res. 17, 405–408. https://doi.org/10.1002/jor.1100170316

Spangenburg, E.E., 2009. Changes in muscle mass with mechanical load: Possible cellular mechanisms. Appl. Physiol. Nutr. Metab. 34, 328–335. https://doi.org/10.1139/H09-010

Springer, T.A., 1990. The Sensation and Regulation of Interactions with the Extracellular environment: The Cell Biology of Lymphocyte Adhesion Receptors. Annu. Rev. Physiol. 6, 359–402.

Sullivan, B.E., Carroll, C.C., Jemiolo, B., Trappe, S.W., Magnusson, S.P., Døssing, S., Kjaer, M., Trappe, T.A., 2009. Effect of acute resistance exercise and sex on human patellar tendon structural and regulatory mRNA expression. J. Appl. Physiol. 106, 468–475. https://doi.org/10.1152/japplphysiol.91341.2008

Tagliabracci, V.S., Wiley, S.E., Guo, X., Kinch, L.N., Durrant, E., Wen, J., Xiao, J., Cui, J., Nguyen, K.B., Engel, J.L., Joshua, J., Grishin, N., Pinna, L.A., Pagliarini, D.J., Dixon, J.E., Diego, S., Jolla, L., Chase, C., Diego, S., Jolla, L., Diego, S., Jolla, L., 2016. A single kinase generates the majority of the secreted phosphoproteome. Cell 161, 1619–1632. https://doi.org/10.1016/j.cell.2015.05.028.A

Togari, A., Arai, M., 2008. Pharmacological topics of bone metabolism: The physiological function of the sympathetic nervous system in modulating bone resorption. J. Pharmacol. Sci. 106, 542–546. https://doi.org/10.1254/jphs.FM0070227

Turhan, Y., Arican, M., Karaduman, Z., Turhal, O., Gamsizkan, M., Aydin, D., Kilic, B., Ozakan, K., 2019. Chondroprotective effect of Nigella sativa oil in the early stages of osteoarthritis an experimental study in rabbits. J. Musculoskelet. Nueronal Interact. 19, 362–369.

Turner, C.H., Akhter, M.P., Raab, D.M., Kimmel, D.B., Recker, R.R., Forwood, M.R., Otter, M.W., 1991. Mechanotransduction in bone: do bone cells act as sensors of fluid flow? Bone 12, 73–79.

Turner, C.H., Forwood, M.R., Rho, J.., Yoshikawa, T., 1994. Mechanical loading thresholds for lamellar and woven bone formation. J. Bone Miner. Res. 9, 87–97.

Visser, A.W., De Mutsert, R., Le Cessie, S., Den Heijer, M., Rosendaal, F.R., Kloppenburg, M., Rabelink, T.J., Smit, J.W.A., Jukema, J.W., De Roos, A., Hiemstra, P.S., Huizinga, T.W.J., Pijl, H., De Koning, E.J.P., Assendelft, W.J.J., Reitsma, P.H., Van Dijk, K.W., De Vries, A.P.J., Lamb, H.J., Jazet, I.M., Dekkers, O.M., Biermasz, N.R., Blom, J.W., Rensen, P.C.N., Cobbaert, C.M., Dekker, J.M., Penninx, B.W., 2015. The relative contribution of mechanical stress and systemic processes in different types of osteoarthritis: The NEO study. Ann. Rheum. Dis. 74, 1842–1847. https://doi.org/10.1136/annrheumdis-2013-205012

Von Porat, A., Roos, E.M., Roos, H., 2004. High prevalence of osteoarthritis 14 years after an anterior cruciate ligament tear in male soccer players: A study of radiographic and patient relevant outcomes. Ann. Rheum. Dis. 63, 269–273.

Wada, H., Ivester, C.T., Carabello, B.A., Cooper, G., McDermott, P.J., 1996. Translational

Initiation Factor eIF-4E. J. Biol. Chem. 271, 8359–8364. https://doi.org/10.1074/jbc.271.14.8359

Wang, J.H.C., Thampatty, B.P., Lin, J.S., Im, H.J., 2007. Mechanoregulation of gene expression in fibroblasts. Gene 391, 1–15.

Wiggins, A., Grandhi, R., Schneider, D., Stanfield, D., Webster, K., Myer, G., 2017. Risk of Secondary Injury in Younger Athletes After Anterior Cruciate Ligament Reconstruction 44, 1861–1876.

William, M., Leroux, L.-P., Chaparro, V., Lorent, J., Graber, T.E., M'Boutchou, M.-N., Charpentier, T., Fabié, A., Dozois, C.M., Stäger, S., van Kempen, L.C., Alain, T., Larsson, O., Jaramillo, M., 2018. eIF4E-Binding Proteins 1 and 2 Limit Macrophage Anti-Inflammatory Responses through Translational Repression of IL-10 and Cyclooxygenase-2. J. Immunol. 200, 4102–4116. https://doi.org/10.4049/jimmunol.1701670

Wolf, S., Augat, P., Eckert-Hübner, K., Laule, A., Krischak, G.D., Claes, L.E., 2001. Effects of high-frequency, low-magnitude mechanical stimulus on bone healing. Clin. Orthop. Relat. Res. 192–198.

Won, J.E., El-Fiqi, A., Jegal, S.H., Han, C.M., Lee, E.J., Knowles, J.C., Kim, H.W., 2014. Gelatin-apatite bone mimetic co-precipitates incorporated within biopolymer matrix to improve mechanical and biological properties useful for hard tissue repair. J. Biomater. Appl. 28, 1213–1225. https://doi.org/10.1177/0885328213502100

Woo, S., Hollis, J.M., Adams, D.J., Lyon, R.M., Takai, S., 1991. Tensile properties of the human femur- anterior cruciate ligament-tibia complex The effects of. Am. J. Sports Med. 19, 217–225.

Woo, S., Peterson, R.H., Ohland, K.J., Sites, T.J., Danto, M.I., 1990. The effects of strain rate on

the properties of the medial collateral ligament in skeletally immature and mature rabbits: A biomechanical and histological study. J. Orthop. Res. 8, 712–721. https://doi.org/10.1002/jor.1100080513

Woo, S.L.-Y.Y., Suh, J.-K., Parsons, I.M., Wang, J.-H., Wantanabe, N., 1998. Biologic Intervention in Ligament Healing Effect of Growth Factors. Sports Med. Arthrosc. 6, 74–82.

Woo, S.L., Newton, P.O., MacKenna, D. a, Lyon, R.M., 1992. A comparative evaluation of the mechanical properties of the rabbit medial collateral and anterior cruciate ligaments. J. Biomech. 25, 377–386.

Woo, S.L.Y., Hollis, J.M., Roux, R.D., Gomez, M.A., Inoue, M., Kleiner, J.B., Akeson, W.H., 1987. Effects of knee flexion on the structural properties of the rabbit femur-anterior cruciate ligament-tibia complex (FATC). J. Biomech. 20, 557–563. https://doi.org/10.1016/0021-9290(87)90277-6

Yadav, V.K., Ryu, J.H., Suda, N., Tanaka, K.F., Gingrich, J.A., Schütz, G., Glorieux, F.H., Chiang, C.Y., Zajac, J.D., Insogna, K.L., Mann, J.J., Hen, R., Ducy, P., Karsenty, G., 2008. Lrp5 Controls Bone Formation by Inhibiting Serotonin Synthesis in the Duodenum. Cell 135, 825–837. https://doi.org/10.1016/j.cell.2008.09.059

Yang, G., Crawford, R.C., Wang, J.H.C., 2004. Proliferation and collagen production of human patellar tendon fibroblasts in response to cyclic uniaxial stretching in serum-free conditions. J. Biomech. 37, 1543–1550.

Zanotti, S., Canalis, E., 2016. Notch signaling and the skeleton. Endocr. Rev. 37, 223–253. https://doi.org/10.1210/er.2016-1002

Appendix I

The following tables represent gene ID's, p-values, adjusted q-values to correct for false discovery rate, B (estimates of log fold changes between conditions), and the cellular, biological and molecular function of genes that were differentially expressed between the loaded ACL and the external control ACL and are annotated to the genome. The tables are in the following order:

Actin Cytoskeletal Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000003620	CCDC88A	0.0000483	0.026895	-0.738474	coiled-coil domain containing 88A	regulation of actin cytoskeleton organization; cell migration; regulation of cell proliferation; regulation of protein phosphorylation; activation of protein kinase B activity; regulation of neuron projection development; TOR signaling; regulation of DNA replication; membrane organization; lamellipodium assembly	cytoplasmic vesicle; lamellipodium; membrane; plasma membrane; cytosol; Golgi apparatus; endoplasmic reticulum	actin binding; protein binding; microtubule binding; protein kinase B binding; protein homodimerization activity; phosphatidylinositol binding
ENSOCUG00000004645	DFNB31	0.0000101	0.009103	1.9391015	deafness, autosomal recessive 31	positive regulation of gene expression; establishment of protein localization; inner ear receptor stereocilium organization; cerebellar Purkinje cell layer formation; paranodal junction maintenance	cilium; photoreceptor connecting cilium ; actin filament; ciliary basal body; photoreceptor inner segment; stereocilium; periciliary membrane compartment; stereocilium bundle tip; stereocilium bundle; stereocilia ankle link complex; stereocilia ankle link; USH2 complex	protein binding; protein homodimerization activity; protein heterodimerization activity
ENSOCUG00000024437	Tmod3 (Mouse)	6.11E-07	0.0011737	-2.058725	tropomodulin 3	erythrocyte development; pointed-end actin filament capping; actin cytoskeleton organization		tropomyosin binding

Collagen, Interleukins and Tissue Repair Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000007056	TNXB	3.16E-113	1.76E-108	7.380359	tenascin XB	defense response; single organismal cell-cell adhesion; extracellular matrix organization; cell-matrix adhesion; collagen fibril organization; extracellular fibril organization; triglyceride metabolic process; lipid metabolic process; fatty acid metabolic process; regulation of JUN kinase activity; elastic fiber assembly; collagen metabolic process	extracellular region; proteinaceous extracellular matrix; fibrillar collagen trimer; extracellular exosome; intracellular; extracellular space; extracellular matrix	protein binding; heparin binding; collagen binding
ENSOCUG00000001456	EIF4E	1.38E-29	1.54E-25	5.728741	eukaryotic translation initiation factor 4E	translational initiation; negative regulation of neuron differentiation; behavioral fear response; negative regulation of translation; stem cell maintenance; positive regulation of mitotic cell cycle; regulation of translation; G1/S transition of mitotic cell cycle	cytoplasm; chromatoid body; eukaryotic translation initiation factor 4F complex; mRNA cap binding complex; RISC complex; cytoplasmic stress granule; extracellular exosome; cytoplasmic mRNA processing body; perinuclear region of cytoplasm	translation initiation factor activity; RNA binding; protein binding; eukaryotic initiation factor 4G binding; repressing transcription factor binding; enzyme binding; poly(A) RNA binding
ENSOCUG00000005435	FABP6	0.0000164	0.0134636	3.680858	fatty acid binding protein 6, ileal	transport		transporter activity; lipid binding
ENSOCUG00000004103	IRAK4	0.0000946	0.0433562	-0.47318	interleukin-1 receptor-associated kinase 4	signal transduction; protein phosphorylation; cytokine-mediated signaling pathway; cytokine production; positive regulation of I-kappaB kinase/NF-kappaB signaling; neutrophil mediated immunity; neutrophil migration		nucleotide binding; magnesium ion binding; transferase activity, transferring phosphorus-containing groups; ATP binding; protein tyrosine kinase activity; protein kinase activity; protein serine/threonine kinase activity; protein binding; interleukin-1 receptor binding
ENSOCUG00000013978	PLCL1	0.0000378	0.0228731	3.132131	phospholipase C-like 1	signal transduction; regulation of synaptic transmission, GABAergic; lipid catabolic process; lipid metabolic process; intracellular signal transduction; gamma-aminobutyric acid signaling pathway; positive regulation of receptor binding; regulation of peptidyl-serine phosphorylation	plasma membrane	hydrolase activity; signal transducer activity; inositol 1,4,5 trisphosphate binding; GABA receptor binding; phosphatidylinositol phospholipase C activity; phosphoric diester hydrolase activity; protein binding

Meatbolic Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000002269	RAD51C	0.00000111	0.0018817	2.281878	RAD51 paralog C	DNA metabolic process; DNA repair; positive regulation of G2/M transition of mitotic cell cycle; double-strand break repair via homologous recombination; DNA recombination	Rad51C-XRCC3 complex; Holliday junction resolvase complex; Rad51B-Rad51C-Rad51D-XRCC2 complex; nucleus; mitochondrion; cytoplasm; replication fork; perinuclear region of cytoplasm	nucleotide binding; DNA binding; ATP binding; DNA-dependent ATPase activity; crossover junction endodeoxyribonuclease activity; four-way junction DNA binding; protein binding
ENSOCUG00000021203	DNPH1	0.0000915	0.0432404	4.722322	2'-deoxynucleoside 5'-phosphate N-hydrolase 1	metabolic process; nucleotide metabolic process; deoxyribonucleoside monophosphate catabolic process; nucleoside metabolic process; epithelial cell differentiation	nucleus; cytoplasm; extracellular exosome; nucleoplasm	hydrolase activity, acting on glycosyl bonds; hydrolase activity; deoxyribonucleoside 5'-monophosphate N-glycosidase activity; nucleoside deoxyribosyltransferase activity; hydrolase activity, hydrolyzing N-glycosyl compounds; protein homodimerization activity
ENSOCUG00000026015	LOC10035	0.00000332	0.0040214	3.176789	liver carboxylesterase 2-like	metabolic process		hydrolase activity
ENSOCUG00000006752	MEST	7.74E-48	1.44E-43	7.049721	mesoderm specific transcript	metabolic process; response to retinoic acid; regulation of lipid storage	endoplasmic reticulum; extracellular exosome	catalytic activity
ENSOCUG00000017136	SLC27A6	0.0000761	0.0375284	-0.81206	solute carrier family 27 (fatty acid transporter), member 6	metabolic process; long-chain fatty acid metabolic process; fatty acid transport	integral component of membrane	catalytic activity; very long-chain fatty acid-CoA ligase activity; long-chain fatty acid-CoA ligase activity

Protein Transport Genes

Rabbit Ensembl Gene ID	Rabbit Gene Name	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000001792	KPNA7	2.01E-24	1.40E-20	6.160818	karyopherin alpha 7 (importin alpha 8)	transport; protein import into nucleus; protein transport	nucleus; cytoplasm	binding; protein transporter activity; protein binding
ENSOCUG00000000586	VTI1B	0.0000655	0.0335037	1.064065	vesicle transport through interaction with t-SNAREs 1B	vesicle-mediated transport; intracellular protein transport; regulation of protein localization to plasma membrane	membrane; late endosome membrane; lysosomal membrane; vesicle; recycling endosome; cytoplasm; intracellular membrane-bounded organelle; Golgi apparatus; perinuclear region of cytoplasm	protein binding; chloride channel inhibitor activity; SNARE binding

Mechanotransduction Pathway Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000013527	ABHD2	0.0001041	0.0464184	3.174673	abhydrolase domain containing 2	negative regulation of cell migration; response to wounding		
ENSOCUG00000010891	FAM20C	0.00000925	0.009012	0.654298	family with sequence similarity 20, member C	skeletal system development; regulation of fibroblast growth factor receptor signaling pathway; positive regulation of bone mineralization; positive regulation of osteoblast differentiation; enamel mineralization; odontoblast differentiation; osteoclast maturation; regulation of phosphorus metabolic process; dentinogenesis; biomineral tissue development; protein	extracellular exosome; extracellular space; Golgi apparatus	calcium ion binding; manganese ion binding; protein binding; protein serine/threonine kinase activity
ENSOCUG00000012063	JUP	0.00000126	0.0020711	-0.59581	junction plakoglobin	signal transduction; adherens junction assembly; cell adhesion; skin development; cellular response to indole-3-methanol; bundle of His cell-Purkinje myocyte adhesion involved in cell communication; regulation of ventricular cardiac muscle cell action potential; desmosome assembly; positive regulation of sequence-specific DNA binding transcription factor	cytosol; membrane; Z disc; intermediate filament; intercalated disc; protein-DNA complex; gamma-catenin-TCF7L2 complex; catenin complex; extracellular exosome; desmosome; focal adhesion; cell-cell junction; cell-cell adherens junction; nucleus; cytoplasm; plasma membrane	signal transducer activity; binding; protein binding; cell adhesion molecule binding; alpha-catenin binding; protein phosphatase binding; cadherin binding; transcription coactivator activity
ENSOCUG00000005003	KNG1	2.88E-07	0.0006421	0.94991	kininogen 1	negative regulation of endopeptidase activity; negative regulation of proteolysis; negative regulation of blood coagulation; positive regulation of cytosolic calcium ion concentration; negative regulation of cell adhesion	extracellular exosome; blood microparticle; extracellular space; cytosol	cysteine-type endopeptidase inhibitor activity; protein binding; receptor binding
ENSOCUG00000001647	LIPC	3.15E-08	0.0000976	3.898534	lipase, hepatic	lipid metabolic process; cholesterol metabolic process; cholesterol transport; high-density lipoprotein particle remodeling; low-density lipoprotein particle remodeling; triglyceride catabolic process; very-low-density lipoprotein particle remodeling; cholesterol homeostasis; triglyceride homeostasis; fatty acid biosynthetic process	extracellular region; extracellular space	hydrolase activity; triglyceride lipase activity; carboxylic ester hydrolase activity; protein binding; lipase activity

Mechanotransduction Pathway Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000029566	LOC103351	0.00000235	0.0032768	6.41608	membrane-spanning 4-domains subfamily A member 12-like		integral component of membrane	
ENSOCUG00000013027	OR2B2	0.00000032	0.0006864	0.872147	olfactory receptor, family 2, subfamily B, member 2	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; detection of chemical stimulus involved in sensory perception of smell; sensory perception of smell	plasma membrane; integral component of membrane; membrane	G-protein coupled receptor activity; signal transducer activity; olfactory receptor activity
ENSOCUG00000004100	OR8S1	0.0000461	0.025974	4.596902	olfactory receptor, family 8, subfamily S, member 1	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; detection of chemical stimulus involved in sensory perception of smell; sensory perception of smell	plasma membrane; integral component of membrane; membrane	G-protein coupled receptor activity; signal transducer activity; olfactory receptor activity

Mechanotransduction Pathway Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000014545	PSEN1	0.0000233	0.0169801	-1.32927	presenilin 1	proteolysis; intracellular signal transduction; Notch signaling pathway; proteasomal ubiquitin-dependent protein catabolic process; post-embryonic development; neuron migration; protein phosphorylation; cellular response to DNA damage stimulus; positive regulation of apoptotic process; response to oxidative stress; transcription from RNA polymerase II promoter; autophagosome assembly; autophagy; learning or memory; neuron differentiation; somitogenesis; thymus development; protein transport; T cell activation involved in immune response; blood vessel development; heart development; neuron development; cellular calcium ion homeostasis; forebrain development; T cell receptor signaling pathway; regulation of synaptic transmission, glutamatergic; protein binding; synaptic plasticity; brain development; cerebral cortex development; protein glycosylation; neuron apoptotic process; activation of MAPKK activity; memory; Notch receptor processing; protein kinase activity; skeletal system morphogenesis; hematopoietic progenitor cell differentiation; epithelial cell proliferation; cerebral cortex cell migration; axonogenesis; cell fate specification; mitochondrial transport; neurogenesis; dorsal/ventral neural tube patterning; embryonic limb morphogenesis; protein phosphorylation; beta-amyloid metabolic process; protein kinase activity; heart looping; MAP kinase activity; brain apoptotic signaling pathway; L-glutamate transport; receptor recycling; protein maturation; locomotion; amyloid precursor protein catabolic process; coagulation; myeloid leukocyte differentiation; epidermal growth factor-activated receptor activity; cellular protein metabolic process; skin morphogenesis; myeloid dendritic cell differentiation; resting membrane potential; choline transport; smooth endoplasmic reticulum calcium ion homeostasis; synaptic vesicle targeting; Cajal-Retzius cell	endoplasmic reticulum membrane; endoplasmic reticulum; integral component of membrane; membrane; Golgi apparatus; Golgi membrane; nucleus; cytoplasmic vesicle; growth cone; dendritic shaft; intracellular; axon; neuronal cell body; dendrite; ciliary rootlet; membrane-bounded organelle; rough endoplasmic reticulum; nuclear outer membrane; nuclear membrane; kinetochore; membrane raft; aggresome; smooth endoplasmic reticulum; gamma-secretase complex; mitochondrion; plasma membrane; centrosome; integral component of plasma membrane	hydrolase activity; aspartic-type endopeptidase activity; peptidase activity; protein binding; cadherin binding; PDZ domain binding; calcium channel activity; endopeptidase activity; beta-catenin binding

Mechanotransduction Pathway Genes

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000010172	RALBP1	8.68E-08	0.0002418	4.030468	ralA binding protein 1	signal transduction; regulation of GTPase activity; transmembrane transport; small GTPase mediated signal transduction; transport; positive regulation of GTPase activity	intracellular; membrane	protein binding; Ral GTPase binding; Rac GTPase binding; ATPase activity; GTPase activator activity; transmembrane transporter activity
ENSOCUG00000004261	SLC35G1	0.0000844	0.0402239	0.50667	solute carrier family 35, member G1	calcium ion export from cell; cytosolic calcium ion homeostasis	integral component of membrane; membrane; plasma membrane; cytosol; endoplasmic reticulum membrane	protein binding
ENSOCUG00000012918	SYNPR	0.0000131	0.0110808	1.106684	synaptoporin	transport	integral component of membrane; membrane; synaptic vesicle	transporter activity; protein binding
ENSOCUG00000022270	TAS2R14	0.00000977	0.0090742	3.778967	taste receptor, type 2, member 14	G-protein coupled receptor signaling pathway; signal transduction; response to stimulus; sensory perception of taste; detection of chemical stimulus involved in sensory perception of bitter taste	integral component of membrane; membrane	G-protein coupled receptor activity; signal transducer activity; bitter taste receptor activity
ENSOCUG00000008294	UPK1B	0.0000784	0.0383068	3.248255	uroplakin 1B	epithelial cell differentiation	integral component of membrane; membrane; apical plasma membrane; extracellular exosome	
ENSOCUG00000002109	VNN1	0.0000303	0.0194098	1.033339	vanin 1	nitrogen compound metabolic process; single organismal cell-cell adhesion; inflammatory response; acute inflammatory response; innate immune response; negative regulation of oxidative stress-induced intrinsic apoptotic signaling pathway; positive regulation of T cell differentiation in thymus; chronic inflammatory response; pantothenate metabolic	extracellular exosome	hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds; hydrolase activity, acting on carbon-nitrogen (but not peptide) bonds, in linear amides; pantetheine hydrolase activity

Differentially Expressed Genes Loaded ACL & External Control

Rabbit Ensembl Gene	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000020258	5S_rRNA	0.00000624	0.0064445	4.423349	5S ribosomal RNA			
ENSOCUG00000010426	AGFG1	0.0000027	0.003499	1.055737	ArfGAP with FG repeats 1			zinc ion binding; GTPase activator activity
ENSOCUG00000027113	ARL14EPL	0.00000383	0.0043577	3.764038	ADP-ribosylation factor-like 14 effector protein-like			
ENSOCUG00000001523	C12H6orf16	0.00000352	0.0040845	1.341299	Chromosome 12 open reading frame, human C6orf165 (C12H6orf165), transcript variant X1. mRNA			
ENSOCUG00000002767	CEP162	0.0000259	0.0178117	0.890447	centrosomal protein 162kDa	cilium assembly	cytoplasm; axonemal microtubule; centriole; centrosome; nucleoplasm	protein binding
ENSOCUG00000007045	CLP1	1.67E-07	0.0004049	4.639796	cleavage and polyadenylation factor I subunit 1	phosphorylation; mRNA processing; siRNA loading onto RISC involved in RNA interference; mRNA 3'-end processing; tRNA splicing, via endonucleolytic cleavage and ligation; tRNA processing; cerebellar cortex development; targeting of mRNA for destruction involved in RNA interference	nucleus; mRNA cleavage factor complex; tRNA-intron endonuclease complex; cytoplasm; nucleoplasm	transferase activity; kinase activity; nucleotide binding; ATP binding; ATP-dependent polyribonucleotide 5'-hydroxyl-kinase activity; polydeoxyribonucleotide kinase activity; ATP-dependent polydeoxyribonucleotide 5'-hydroxyl-kinase activity
ENSOCUG00000003017	ERICH3	0.0000206	0.0157265	3.375869	glutamate-rich 3			
ENSOCUG00000006154	GINS4	0.00004	0.0240012	-0.49911	GINS complex subunit 4 (Sld5 homolog)	DNA replication; inner cell mass cell proliferation	nucleus; cytoplasm	protein binding
ENSOCUG00000022627	HNRNPA1 (Mouse Orthologue)	0.00000183	0.0027779	3.60961	heterogeneous nuclear ribonucleoprotein A1			nucleotide binding; nucleic acid binding

Differentially Expressed Genes Loaded ACL & External Control

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000023956	HOXD9	0.0000093	0.009012	-0.87204	homeobox D9	regulation of transcription, DNA-templated; transcription, DNA-templated; positive regulation of transcription from RNA polymerase II promoter; anterior/posterior pattern specification; regulation of gene	nucleus	DNA binding; sequence-specific DNA binding; transcription factor activity, sequence-specific DNA binding; RNA polymerase II regulatory region sequence-specific DNA binding; transcriptional repressor activity, RNA
ENSOCUG00000013591	ITLN1 (Mouse Orthologue	4.25E-14	2.16E-10	4.725544	intelectin 1 (galactofuranose binding)			
ENSOCUG00000011556	KCTD18	1.20E-10	4.44E-07	5.9514	potassium channel tetramerization domain containing 18	protein homooligomerization		
ENSOCUG00000013931	LEPROTL1	0.00000164	0.0026161	3.444875	leptin receptor overlapping transcript-like 1			
ENSOCUG00000005200	LOC1000094	0.0000026	0.0034498	-0.57385	lipophilin CS (LOC100009480), mRNA			
ENSOCUG00000029693	LOC1003427	0.00000224	0.0031983	-0.5716	IQ domain-containing protein F5-like			protein binding
ENSOCUG00000023695	LOC1003428	0.0000933	0.0433475	1.119184	40S ribosomal protein S3a	translation; cell differentiation	nucleus; cytoplasm; ribosome; cytosolic small ribosomal subunit; ribonucleoprotein complex; intracellular	structural constituent of ribosome
ENSOCUG00000016686	LOC1003561	0.000079	0.0383068	2.736011	melanoma-associated antigen D4			
ENSOCUG00000016139	MMAA	0.0000244	0.0172025	-0.54772	methylmalonic aciduria			

Differentially Expressed Genes Loaded ACL & External Control

Rabbit Ensembl Gene ID	Rabbit Gene	P-value	Q-Value	B	Rabbit Description	GO Biological Process	GO Cellular Component	GO Molecular Function
ENSOCUG00000008571	MYOM1	0.0000232	0.0169801	3.2322	myomesin 1	muscle contraction; activation of mitophagy in response to mitochondrial depolarization; positive regulation of defense response to virus by host	sarcomere; M band	protein binding; protein homodimerization activity; identical protein binding
ENSOCUG00000028329	SNORD22	2.31E-09	7.56E-06	3.9617	Small nucleolar RNA SNORD22			
ENSOCUG00000014042	SNRPB	7.37E-07	0.001369	3.156418	Small nuclear ribonucleoprotein-		nucleus; ribonucleoprotein complex	RNA binding
ENSOCUG00000014000	TAF7	0.0000293	0.019203	-1.48623	TAF7 RNA polymerase II,	transcription initiation from RNA polymerase II promoter; regulation of	transcription factor TFIID complex; cytoplasm; nucleus; positive	protein binding; thyroid hormone receptor binding; vitamin D receptor
ENSOCUG00000011199	TIMM8A	1.48E-15	8.24E-12	1.870155	translocase of inner	transport; protein transport	mitochondrion; mitochondrial intermembrane space	metal ion binding; protein binding
ENSOCUG00000007954	TPBG	0.00000277	0.0035132	1.537493	trophoblast glycoprotein	positive regulation of synapse assembly	cytoplasm; endoplasmic reticulum; cell surface	protein binding
ENSOCUG00000008740	TTI2	0.00010752	0.0475634	3.875132	TELO2 interacting protein 2		cytoplasm; centrosome; nucleoplasm	binding
ENSOCUG00000019556	U6	0.0000693	0.0351145	0.873067	U6 spliceosomal RNA			
ENSOCUG00000026098	U6	0.0000349	0.0216955	3.265186	U6 spliceosomal RNA			
ENSOCUG00000011756	ZNF358	0.000059	0.0313165	1.723036	zinc finger protein 358			nucleic acid binding; metal ion binding
ENSOCUG00000010370	ZNF605	0.0000026	0.0034498	4.676312	zinc finger protein 605			nucleic acid binding; metal ion binding
ENSOCUG00000005172	ZSCAN30	0.0000285	0.0191628	-6.1404	zinc finger and SCAN domain containing 30	regulation of transcription, DNA-templated; transcription, DNA-templated	nucleus	nucleic acid binding; transcription factor activity, sequence-specific DNA binding; metal ion binding

Appendix II: IACUC Approval Letter

ECU

Animal Care and Use Committee
212 Ed Warren Life Sciences Building | East Carolina University | Greenville, NC 27834-4354
252-744-2436 office | 252-744-2355 fax

January 14, 2020

Zac Domire, PhD
Department of Kinesiology
Ward Sports, MS 158
ECU Brody School of Medicine

Subject: Protocol #P107, original approval date 09/11/19

Dear Dr. Domire:

The amendment to your protocol entitled, "Gene Expression in Response to Mechanical Loading on the Anterior Cruciate Ligament", (AUP #P107) was reviewed by this institution's Animal Care and Use Committee on 01/13/20. The following action was taken by the Committee:

"Approved as submitted"

****Please contact Aaron Hinkle prior to any hazard use****

A copy of your amended protocol is enclosed for your laboratory files. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP/Amendment and are familiar with its contents.**

Sincerely yours,

Jamie DeWitt, Ph.D.
Vice Chair, Animal Care and Use Committee

SM/jm

enclosure

Appendix III: AUP Document

EAST CAROLINA UNIVERSITY
ANIMAL USE PROTOCOL (AUP) FORM
LATEST REVISION APRIL, 2017

Project Title:

Gene Expression in Response to Mechanical Loading on the Anterior Cruciate Ligament

	Principal Investigator	Secondary Contact
Name	Dr. Zac Domire	Brian Diefenbach
Dept.	Kinesiology	Kinesiology
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Pager #	Click here to enter text.	Click here to enter text.
Home Ph #	Click here to enter text.	Click here to enter text.
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For IACUC Use Only

AUP #			
New/Renewal			
Full Review/Date	DR/Date		
Approval Date			
Study Type			
Pain/Distress Category			
Surgery	Survival	Multiple	
Prolonged Restraint			
Food/Fluid Regulation			
Other			
Hazard Approval/Dates	Rad	IBC	EHS
OHP Enrollment			
Mandatory Training			
Amendments Approved			

I. Personnel

A. Principal Investigator(s):

Dr. Zac Domire

B. Department(s):

Kinesiology

C. List all personnel (PI's, co-investigators, technicians, students) that will be working with live animals and describe their qualifications and experience with these specific procedures. If people are to be trained, indicate by whom:

Name/Degree/Certification	Position/Role(s)/ Responsibilities in this Project	Required Online IACUC Training (Yes/No)	Relevant Animal Experience/Training (include species, procedures, number of years, etc.)
Dr. Zac Domire, Ph.D.	PI, Communicates with IACUC, administers tests & interventions, Performs Dissections, Analyzes and Manages Data	Yes	Over 20 years biomechanics research; animal use training at ECU, Penn State, The Mayo Clinic, and Texas Tech University.
Brian Diefenbach, B.S.	Communicates with IACUC, administers tests & interventions, Performs Dissections, Analyzes and Manages Data	Yes	No previous experience, Rodent Handling Training (Completed on 7/19) and Aseptic Surgery Training (Scheduled for 7/24)
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II. Regulatory Compliance

A. Non-Technical Summary

Using language a non-scientist would understand, please provide a clear, concise, and sequential description of animal use. Additionally, explain the overall study objectives and benefits of proposed research or teaching activity to the advancement of knowledge, human or animal health, or good of society. (More detailed procedures are requested later in the AUP.)
Do not cut and paste the grant abstract.

Anterior Cruciate Ligament tears in the knee, due to their common nature and risk of osteoarthritis post-tear, are a long-term issue for many athletes. A stronger ligament may reduce the risk of tears. Many tissues in the body, especially bone, have exhibited adaptive responses to low-magnitude, high frequency mechanical loading. However, the response of ligament to these types of mechanical loads is not well understood. Therefore, the purpose of this study is to identify if ligaments display adaptations that could be expressed though in-vivo high frequency, low magnitude loading. Identifying a mechanism to load a ligament, and the gene response to the loading, may be a pathway to eventually strengthen ligaments, to reduce ACL tears.

To test this hypothesis, New Zealand White Rabbits will be used to determine if there is any gene response to mechanical loading of the ACL. Under anesthesia, rabbits will be subject to 20 minutes of low-magnitude high frequency loading. While the body of the rabbit is supported, one leg of the rabbit will be secured in place, with the shank secured in a leather cinch cast. The ACL will be mechanically loaded by pulling on the cinch-cast along the axis of the ACL, at an oscillating low-magnitude of 3-10N and a frequency of 30 Hz, displacing the shank on the order of millimeters. Following the loading protocol, rabbits will be returned to consciousness, and then sacrificed four hours following the loading protocol, to harvest the ACL for RNA extraction. Prior to loading and sacrifice, anesthetized/sedated rabbits will have a small amount of blood taken for analysis of circulating factors.

B. Ethics and Animal Use

B.1. Duplication

Does this study duplicate existing research? No
If yes, why is it necessary? (note: teaching by definition is duplicative)
Click here to enter text.

B.2. Alternatives to the Use of Live Animals

Are there less invasive procedures, other less sentient species, isolated organ preparation, cell or tissue culture, or computer simulation that can be used in place of the live vertebrate species proposed here? No, the ligament response to mechanotransduction is not well understood, a computer model of the system is not possible. In vitro models of muscle simulation would not allow the investigation of downstream genetic response to loading.

If yes, please explain why you cannot use these alternatives.

Click here to enter text.

B.3. Consideration of Alternatives to Painful/Distressful Procedures

a. Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

All parts of the experiment will be conducted with the rabbits under anesthesia. Additionally, the harvest of the ACL tissue will occur after sacrifice. While the ACL loading may be seen as a potentially distressful procedure, the animals will be anesthetized during the procedure.

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	6-11-2019
Database(s) searched:	PubMed, Google Scholar
Time period covered by the search (i.e. 1975-2013):	All time periods
Search strategy (including scientifically relevant terminology):	Searched based on relevant terminology to: Mechanotransduction, ACL Loading, Rabbit ACL, ACL Injury Prevention, mechanical loading, etc. Consulted previously cited research in peer-reviewed articles that related to these topics.
Other sources consulted:	None

The search for alternatives procedures did not provide any alternatives that would still answer the research question being asked. Due to the novel approach to solve a well-established problem, in-vivo mechanical loading to strengthen and reduce the risk of ACL injuries, there has not been any other studies that are useable as a model or alternative.

Additionally, the potential to use a smaller species than rabbits was considered and searched. However, a smaller species would be harder to load the ACL accurately as well as be harder to harvest. Rabbits knee-complex similarity to humans makes them an ideal candidate for this experiment, and other previous research, due to the ability to make better predictions about the extrapolation of this research to humans. This is why rabbits are often chosen as animal models when studying the knee.

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

Literature searches were performed to try and identify possible alternative procedures. Using PubMed's and Google Scholar's database, searches for "ACL Mechanotransduction" brought in 7 results, and none of them were relevant. The majority of these seven papers involve in-vitro testing of cultured cells, rather than in-vivo testing as this project proposes, and therefore would not allow for a realistic loading environment. To investigate if a similar study has been performed in-vivo, searches for "ACL Mechanotransduction In-Vivo" and "ACL Mechanical Loading In-Vivo" brought back 0 and 63 responses, respectively. However, these studies investigated failure loading where ACL tears occurred, strain percentage of the ACL based on joint position, and the use of human kinematic data to model ACL tears. While these studies are helpful to better understand ACL tears and the biomechanical properties of the ACL, they do not address the research question asked in this study. An additional search of "ACL injury prevention mechanical loading" produced 34 results, and again none of them were relevant. As illustrated in these articles, ACL injury prevention often times involves modifying kinematics of cutting, landing, or jumping, or modifying surrounding muscle activation, rather than looking to strengthen the ACL itself. Based on literature searches, we do not believe that there have been other studies that provide evidence for alternative procedures to answer our research question.

Additionally, we conducted searches to try and refine our procedure. The procedure we are studying is rather unique. Pubmed searches for "ligament loading in vivo gene expression" and "ligament high frequency loading" yielded 12 and 24 results respectively, but none had similar loading protocols. However, there have been similar experiments, using high-frequency, low magnitude loading on bone that provide the rationale for this experiment. A search of "High frequency low magnitude loading bone" produced 66 results. Used on the hind legs of sheep

(Rubin et al., 2002), the ulnas of turkeys (Rubin et al., 1996), the tibias of rats (Turner et al., 1991), and the lower extremity of humans (Rubin et al., 2004), these low-magnitude, high-frequency loads resulted in increased bone mineral density, creation and thickening of trabecular bone, and overall stiffer and stronger bones in the direction of loading. However, an in-vivo experiment similar to these has not been done on the ACL. It would make sense that ligaments, the connective tissue connecting bones together, would also exhibit similar adaptive properties in order to withstand the increased force that a now stronger bone is able to withstand. In these studies on turkeys, sheep, rats, and humans, analgesics were not provided, as the order of magnitude of vibration loading was small. However, to ensure pain and stress do not occur for the animal, and to account for any difference between bone and ligament loading, analgesics will be used in this experiment. Additionally, these studies often have loading durations of 20 minutes, which will be the same duration used in this experiment.

The protocol of this experiment has been designed with the 3 R's in mind. New Zealand White Rabbits will be used during this experiment because of their knee joint complex similarity to humans (*Menetrey et al. 1999)*. For example, rabbits have been used by studies as a model for knee osteoarthritis development (Turhan et al 2019) and for studies examining ACL reconstruction (Chai et al., 2019). This allows us to make better predictions about the extrapolations of this research to human population. Additionally, rabbits were chosen for this research due to the size of their ACL's. Replacement with a less sentient species is not possible because other lab animals such as rats or mice, which would have ACL's that are too small to accurately load or harvest and would have knee anatomy that would less well match human anatomy. To reduce the number of rabbits used for this experiment, each animal will serve as their own control by using the unloaded knee as control. Additionally, to help with reduction, we are using the minimum number of rabbits needed to detect statistical significance. Based on sample size estimates for RNA sequencing data, assuming effect size fold changes consistent with previous mechanotransduction literature of at least 5 times, a sample size of 10 rabbits will be sufficient to detect meaningful changes *(Garlet et al. 2007, Hart et al 2013, Yang et al 2004).* Lastly, due to the lack of understanding on the genetic response in ligaments to mechanical loading, refinement using modeling techniques is not possible, however, during the procedure animals will be anesthetized. While the animals will recover from anesthesia before euthanasia four hours following the loading protocol, the animals will be monitored for pain (increased respiration, irritable, aggression, reluctance to move, etc). Four hours was chosen as a response time based on the time-course for collagen genes hypothesized to be affected by loading to be fully expressed (Majima et al., 2000, Raab-Cullen et al., 1994, Sullivan et al., 2009, Yang et al., 2004). If any signs of pain appear a DCM vet will be consulted and we will follow their recommendations. In this case of pain during the four-hour period, analgesia can be provided. However, if the signs of pain persist, in accordance with the direction of a DCM vet, sacrifice can occur prior to the completion of the four-hour period.

C. Hazardous Agents

1. Protocol related hazards (chemical, biological, or radiological):

Please indicate if any of the following are used in animals and the status of review/approval by the referenced committees:

HAZARDS	Oversight Committee	Status (Approved, Pending, Submitted)/Date	AUP Appendix I Completed?
Radioisotopes	**Radiation**	Click here to enter text.	Choose an item.
Ionizing radiation	**Radiation**	Click here to enter text.	Choose an item.
Infectious agents (bacteria, viruses, rickettsia, prions, etc.)	**IBC**	Click here to enter text.	Choose an item.
Toxins of biological origins (venoms, plant toxins, etc.)	**IBC**	Click here to enter text.	Choose an item.
Transgenic, Knock In, Knock Out Animals---breeding, cross breeding or any use of live animals or tissues	**IBC**	Click here to enter text.	Choose an item.
Human tissues, cells, body fluids, cell lines	**IBC**	Click here to enter text.	Choose an item.
Viral/Plasmid Vectors/Recombinant DNA or recombinant techniques	**IBC**	Click here to enter text.	Choose an item.
Oncogenic/toxic/mutagenic chemical agents	**EH&S**	Click here to enter text.	Choose an item.
Nanoparticles	**EH&S**	Click here to enter text.	Choose an item.
Cell lines, tissues or other biological products injected or implanted in animals	**DCM**	Click here to enter text.	Choose an item.
Other agents		Click here to enter text.	Choose an item.

2. Incidental hazards

Will personnel be exposed to any incidental zoonotic diseases or hazards during the study (field studies, primate work, etc)? If so, please identify each and explain steps taken to mitigate risk:

No

III. Animals and Housing

A. Species and strains:

New Zealand White Rabbits

B. Weight, sex and/or age:

Female, Fully Skeletally Mature. Female animals are proposed as the is a possible gender difference in response. The study sample size is not large enough to explicitly explore a gender difference. Therefore, a single gender design was preferred. Females were chosen as injuries to the ACL are 2-8x more common in females. Skeletally mature animals are proposed as we do not want to confound the results with possible signaling from natural growth in the ligament.

C. Animal numbers: 10

1. Please complete the following table:

Total number of animals in treatment and control groups	**Additional animals (Breeders, substitute animals)**	**Total number of animals used for this project**
10	+ 0	= 10

2. Justify the species and number (use statistical justification when possible) of animals requested:

New Zealand White Rabbits have similar knee joint complexes compared to humans *(Menetrey et al. 2008).* Based on sample size estimates for RNA sequencing data, assuming effect size fold changes consistent with previous mechanotransduction literature of at least 5 times, a sample size of 10 rabbits will be sufficient to detect meaningful changes *(Garlet et al. 2007, Hart et al 2013, Yang et al 2004).*

3. Justify the number and use of any additional animals needed for this study:

No extra animals will be needed

a. For unforeseen outcomes/complications:

Click here to enter text.

b. For refining techniques:

Click here to enter text.

c. For breeding situations, briefly justify breeding configurations and offspring expected:

Click here to enter text.

d. Indicate if following IACUC tail snip guidelines: Choose an item. **(if no, describe and justify)**

Click here to enter text.

4. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitating effects in experimental manipulations? No **(if yes, describe)**

Click here to enter text.

5. Are there any deviations from standard husbandry practices?

No **If yes, then describe conditions and justify the exceptions to standard housing (temperature, light cycles, sterile cages, special feed, prolonged weaning times, wire-bottom cages, etc.):**

Click here to enter text.

6. The default housing method for social species is pair or group housing (including mice, rats, guinea pigs, rabbits, dogs, pigs, monkeys). Is it necessary for animals to be singly housed at any time during the study? No **(If yes, describe housing and justify the need to singly house social species):**

Click here to enter text.

7. Are there experimental or scientific reasons why routine environmental enrichment should not be provided? No

(If yes, describe and justify the need to withhold enrichment)

Click here to enter text.

8. If wild animals will be captured or used, provide permissions (collection permit # or other required information):

No wild animals will be captured

9. List all laboratories or locations outside the animal facility where animals will be used. Note that animals may not stay in areas outside the animal facilities for more than 12 hours without prior IACUC approval. For field studies, list location of work/study site.

All testing will be performed inside the animal facility within the Brody School of Medicine.

IV. Animal Procedures

A. Outline the Experimental Design including all treatment and control groups and the number of animals in each. Tables or flow charts are particularly useful to communicate your design. Briefly state surgical plans in this section. Surgical procedures can be described in detail in IV.S.

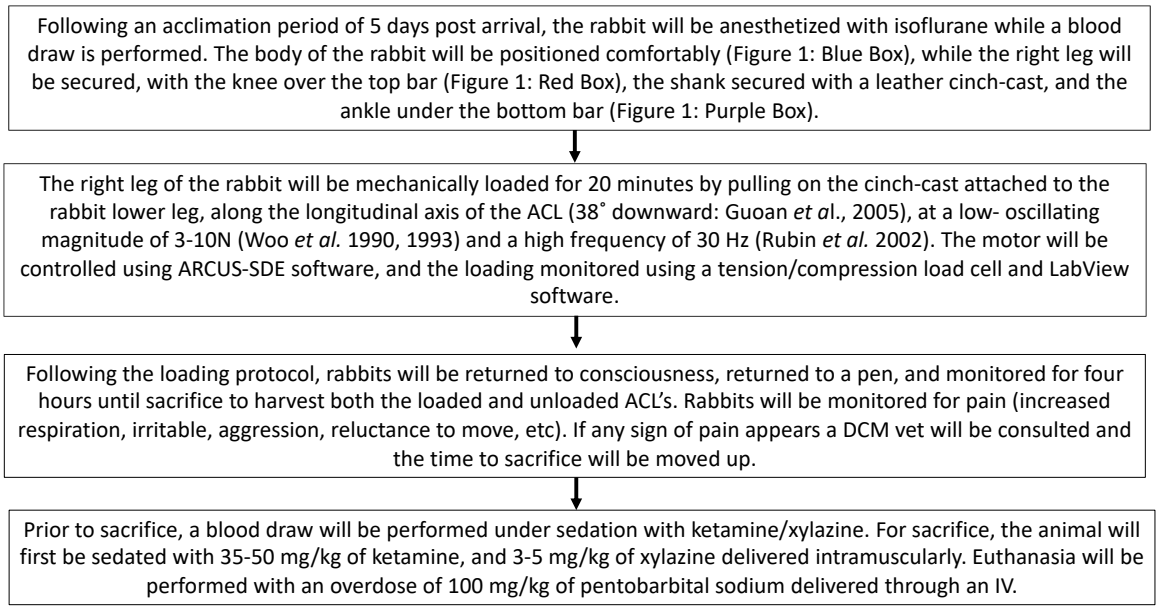
Prior to the procedure, the rabbit will be anesthetized with isoflurane and a blood draw will be performed. The body of the rabbit will be positioned comfortably (Figure 1: Blue Box), while the right leg will be secured, with the knee over the top bar (Figure 1: Red Box), the shank secured with a leather cinch-cast, and the ankle under the bottom bar (Figure 1: Purple Box). The right leg of the rabbit will be mechanically loaded for 20 minutes by pulling on the cinch-cast attached to the rabbit lower leg, along the longitudinal axis of the ACL (38˚ downward: Guoan *et a*l., 2005), at a low-oscillating magnitude of ~3-10N (Woo *et al.* 1990, 1993) and a high frequency of up to

30 Hz (Rubin *et al.* 2002). The motor will be controlled using ARCUS-SDE software, and the loading monitored using a tension/compression load cell and LabView software. Anesthesia will be monitored throughout the procedure by DCM staff.

Following the loading protocol, rabbits will be returned to consciousness, returned to a pen, and monitored for four hours until sacrifice to harvest both the loaded and unloaded ACL's. If any sign of pain appears a DCM vet will be consulted and we will follow their recommendations. For sacrifice, the animal will first be sedated with 35-50 mg/kg of ketamine, and 3-5 mg/kg of xylazine delivered intramuscularly. A second blood draw will be performed under sedation. Euthanasia will be performed with an overdose of 100 mg/kg of pentobarbital sodium delivered through an IV.

Three rabbits will serve as controls. To control for the effects of sitting in the device, these rabbits will spend twenty minutes positioned in the device but will not have their ACL's loaded. These rabbits will be euthanized and have their ACL's harvested and blood drawn the same as the experimentally loaded rabbits.

To comply with the IACUC rules on acclimation, the animals will be housed for at least five days after arrival before the experiment begins. Since the animal will be anesthetized while in the device, acclimation to the testing device is not necessary.



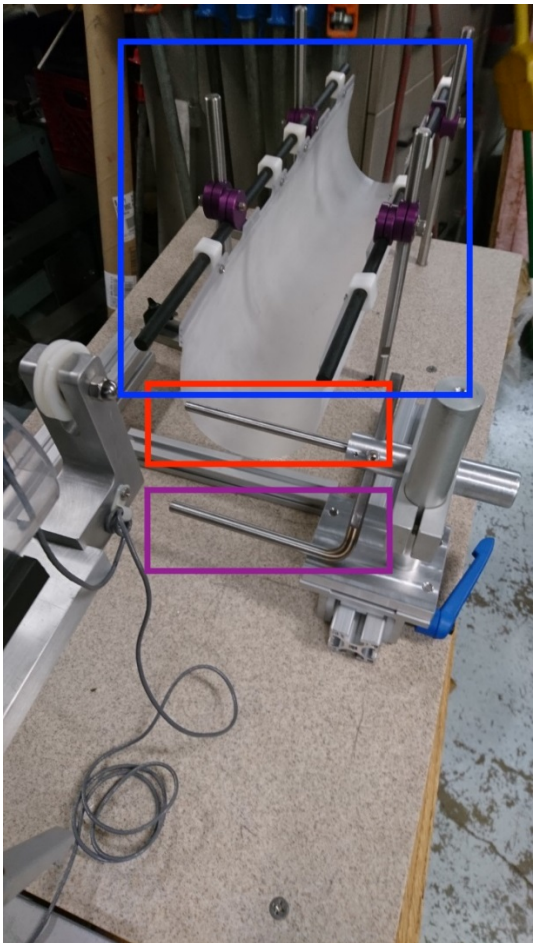


Figure 1: Securing section of the ligament loading device

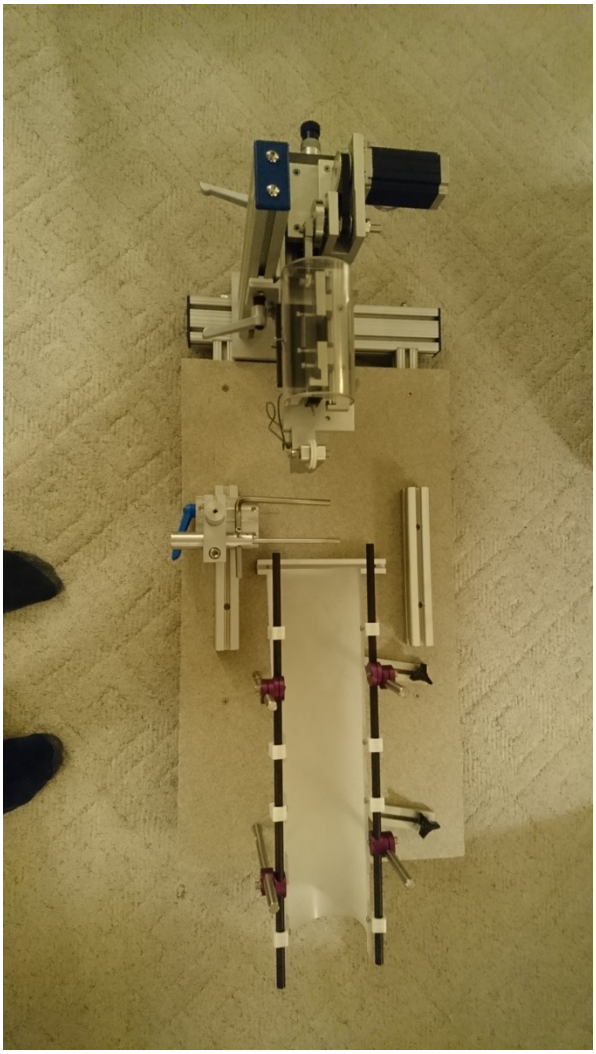


Figure 2: Overview of the ligament loading device. The motor configuration can be seen on the top, with the securing section of the ligament loader on the bottom. Nothing of the animal will interact with the motor section, other than a string attaching the motor section to the cinch-cast.

In sections IV.B-IV.S below, please respond to all items relating to your proposed animal procedures. If a section does not apply to your experimental plans, please leave it blank.

Please refer to DCM and IACUC websites for relevant guidelines and SOPs.

B. Anesthesia/Analgesia/Tranquilization/Pain/Distress Management For Procedures Other than Surgery:

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.
Adequate records describing anesthetic monitoring and recovery must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:
Click here to enter text.

1. **Describe the pre-procedural preparation of the animals:**
   a. **Food restricted for** 0 **hours**
   b. **Food restriction is not recommended for rodents and rabbits and must be justified:**
   Click here to enter text.
   c. Water restricted for 0 **hours**
   d. Water restriction is not recommended in any species for routine pre-op prep and must be justified:
   Click here to enter text.

2. Anesthesia/Analgesia *for Procedures Other than Surgery*

	Agent	**Concentration**	**Dose (mg/kg)**	**Max Volume**	**Route**	**Frequency**	**Number of days administered**
Pre-procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Isoflurane	Click here to enter text.	3-5%	Click here to enter text.	Inhalation	Click here to enter text.	1

Anesthetic	Isoflurane	Click here to enter text.	1-3%	Click here to enter text.	Inhalation	Click here to enter text.	1
Post procedure analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. Reason for administering agent(s):
Anesthesia is provided to restrain the rabbit during the procedure so proper positioning of the lower leg can be achieved, and there will be no muscle guarding against the loading.

4. For which procedure(s):
ACL Loading Protocol

5. Methods for monitoring anesthetic depth:
Noninvasive blood pressure cuff; monitored throughout the loading protocol. The rabbit will be monitored with an anesthesia chart by DCM staff which includes heart rate and respiratory rate.

6. Methods of physiologic support during anesthesia and recovery:
Body temperature is maintained through a DCM approved heating pad

7. Duration of recovery:
Recovery will be allowed for four hours post loading protocol before sacrifice.

8. Frequency of recovering monitoring:
Animal will be monitored continuously until she is returned to her cage

9. Specifically what will be monitored?
Blood pressure, heart rate, temperature

10. When will animals be returned to their home environment?
Animal will be returned to her cage once she is sternal

11. Describe any behavioral or husbandry manipulations that will be used to alleviate pain, distress, and/or discomfort:
During the loading protocol, the body of the rabbit will be secured in a comfortable position. Additionally, the cinch-cast is designed to pull evenly on the entire rabbit shank to help avoid friction effects.

C. Use of Paralytics

1. Will paralyzing drugs be used? No

2. For what purpose:

Click here to enter text.

3. Please provide scientific justification for paralytic use:

Click here to enter text.

4. Paralytic drug:

Click here to enter text.

5. Dose:

Click here to enter text.

6. Method of ensuring appropriate analgesia during paralysis:

Click here to enter text.

D. Blood or Body Fluid Collection

1. Please fill out appropriate sections of the chart below:

No blood of body fluid will be collected.

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Central Ear Artery; lateral saphenous or cephalic veins	23-27 gauge	20 ml	twice	4 hours
Body Fluid Collection	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

E. Injections, Gavage, & Other Substance Administration

1. Please fill out appropriate sections of the chart below:

No injections/infusions, gavages or other substances will be administered.

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (ie two times per day)	Number of days admin (ie for 5 days)	Max dosages (mg/kg)
Injection / Infusion	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Gavage	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

3. **Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:**

a. **Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.**
b. **Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use),**

labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).

Click here to enter text.

F. Prolonged restraint with mechanical devices

Prolonged restraint in this context means *beyond routine care and use procedures* for rodent and rabbit restrainers, and large animal stocks. Prolonged restraint also includes *any* use of slings, tethers, metabolic crates, inhalation chambers, primate chairs and radiation exposure restraint devices.

For our procedure, the animal will be 'restrained' during the mechanical loading, but will be under anesthesia during the 'restraint'.

1. **For what procedure(s):**
   Click here to enter text.
2. **Explain why non-restraint alternatives cannot be utilized:**
   Click here to enter text.
3. **Restraint device(s):**
   Click here to enter text.
4. **Duration of restraint:**
   Click here to enter text.
5. **Frequency of observations during restraint/person responsible:**
   Click here to enter text.
6. **Frequency and total number of restraints:**
   Click here to enter text.
7. **Conditioning procedures:**
   Click here to enter text.
8. **Steps to assure comfort and well-being:**
   Click here to enter text.
9. **Describe potential adverse effects of prolonged restraint and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**
   Click here to enter text.

G. Tumor Studies, Disease Models, Toxicity Testing, Vaccine Studies, Trauma Studies, Pain Studies, Organ or System Failure Studies, Shock Models, etc.

1. **Describe methodology:**

Click here to enter text.

2. **Expected model and/or clinical/pathological manifestations:**

Click here to enter text.

3. **Signs of pain/discomfort:**

Click here to enter text.

4. **Frequency of observations:**

Click here to enter text.

5. **Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):**

Click here to enter text.

H. Treadmills/Swimming/Forced Exercise

1. Describe aversive stimulus (if used):

Click here to enter text.

2. Conditioning:

Click here to enter text.

3. Safeguards to protect animal:

Click here to enter text.

4. Duration:

Click here to enter text.

5. Frequency:

Click here to enter text.

6. Total number of sessions:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

I. Projects Involving Food and Water Regulation or Dietary Manipulation

(*Routine pre-surgical fasting not relevant for this section)*

1. Food Regulation

a. **Amount regulated and rationale:**

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (i.e. recording body weight, body condition, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

2. Fluid Regulation

a. Amount regulated and rationale:

Click here to enter text.

b. Frequency and duration of regulation (hours for short term/weeks or months for long term):

Click here to enter text.

c. Frequency of observation/parameters documented (body weight, hydration status, etc.):

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

3. Dietary Manipulations

a. Compound supplemented/deleted and amount:

Click here to enter text.

b. Frequency and duration (hours for short term/week or month for long term):

Click here to enter text.

c. Frequency of observation/parameters documented:

Click here to enter text.

d. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

J. Endoscopy, Fluoroscopy, X-Ray, Ultrasound, MRI, CT, PET, Other Imaging

1. Describe animal methodology:
Click here to enter text.

2. Duration of procedure:
Click here to enter text.

3. Frequency of observations during procedure:
Click here to enter text.

4. Frequency/total number of procedures:
Click here to enter text.

5. Method of transport to/from procedure area:
Click here to enter text.

6. Describe potential adverse side effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

7. Please provide or attach appropriate permissions/procedures for animal use on human equipment:
Click here to enter text.

K. Polyclonal Antibody Production

1. **Antigen/adjuvant used and justification for adjuvant choice:**
   Click here to enter text.
2. **Needle size:**
   Click here to enter text.
3. **Route of injection:**
   Click here to enter text.
4. **Site of injection:**
   Click here to enter text.
5. **Volume of injection:**
   Click here to enter text.
6. **Total number of injection sites:**
   Click here to enter text.
7. **Frequency and total number of boosts:**
   Click here to enter text.
8. **What will be done to minimize pain/distress:**
   Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

L. Monoclonal Antibody Production

1. Describe methodology:

Click here to enter text.

2. Is pristane used: Choose an item.

Volume of pristane:

Click here to enter text.

3. Will ascites be generated: Choose an item.

i. Criteria/signs that will dictate ascites harvest:

Click here to enter text.

ii. Size of needle for taps:

Click here to enter text.

iii. Total number of taps:

Click here to enter text.

iv. How will animals be monitored/cared for following taps:

Click here to enter text.

4. What will be done to minimize pain/distress:

Click here to enter text.

5. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

M. Temperature/Light/Environmental Manipulations

1. Describe manipulation(s):

Click here to enter text.

2. Duration:

Click here to enter text.

3. Intensity:

Click here to enter text.

4. Frequency:

Click here to enter text.

5. Frequency of observations/parameters documented:

Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

N. Behavioral Studies

1. Describe methodology/test(s) used:

Click here to enter text.

2. Will conditioning occur? If so, describe:

Click here to enter text.

3. If aversive stimulus used, frequency, intensity and duration:

Click here to enter text.

4. Length of time in test apparatus/test situation: *(i.e., each test is ~10 mins)*

Click here to enter text.

5. Frequency of testing and duration of study: *(i.e., 5 tests/week for 6 months)*

Click here to enter text.

6. Frequency of observation/monitoring during test:

Click here to enter text.

7. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

O. Capture with Mechanical Devices/Traps/Nets

1. Description of capture device/method:

Click here to enter text.

2. Maximum time animal will be in capture device:

Click here to enter text.

3. Frequency of checking capture device:

Click here to enter text.

4. Methods to ensure well-being of animals in capture device:

Click here to enter text.

5. Methods to avoid non-target species capture:

Click here to enter text.

6. Method of transport to laboratory/field station/processing site and duration of transport:

Click here to enter text.

7. Methods to ensure animal well-being during transport:
Click here to enter text.

8. Expected mortality rates:
Click here to enter text.

9. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

P. Manipulation of Wild-Caught Animals in the Field or Laboratory

1. **Parameters to be measured/collected:**
Click here to enter text.
2. **Approximate time required for data collection per animal:**
Click here to enter text.
3. **Method of restraint for data collection:**
Click here to enter text.
4. **Methods to ensure animal well-being during processing:**
Click here to enter text.
5. **Disposition of animals post-processing:**
Click here to enter text.

6. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

Q. Wildlife Telemetry/Other Marking Methods

1. Describe methodology (including description of device):
Click here to enter text.

2. Will telemetry device/tags/etc. be removed? Choose an item. **If so, describe:**
Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):
Click here to enter text.

R. Other Animal Manipulations

1. **Describe methodology:**
Click here to enter text.
2. **Describe methods to ensure animal comfort and well-being:**

Click here to enter text.

3. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

S. Surgical Procedures – N/A

All survival surgical procedures must be done aseptically, regardless of species or location of surgery. Adequate records describing surgical procedures, anesthetic monitoring and postoperative care must be maintained for all species. Please see Guidelines for Intra-operative and Intra-procedural Monitoring on the IACUC website.

1. Location of Surgery (Building & Room #):

2. Type of Surgery (check all that are appropriate):

Surgery will not be performed in this experiment. Harvesting of the ACL tissue will occur after sacrifice of the rabbits.

☐ **Non-survival surgery (animals euthanized without regaining consciousness)**

☐ **Major survival surgery (major surgery penetrates and exposes a body cavity or produces substantial impairment of physical or physiologic function)**

☐ **Minor survival surgery**

☐ **Multiple survival surgery**

If yes, provide scientific justification for multiple survival surgical procedures:

Click here to enter text.

3. Describe the pre-op preparation of the animals:

a. **Food restricted for** Click here to enter text. **hours**
b. **Food restricted is not recommended for rodents and rabbits and must be justified:**

   Click here to enter text.

c. **Water restricted for** Click here to enter text. **hours**

d. Water restriction is not recommended in any species for routine pre-op prep and be justified:
Click here to enter text.

4. Minimal sterile techniques will include (check all that apply):
Please refer to DCM Guidelines for Aseptic Surgery for specific information on what is required for each species and type of surgery (survival vs. non-survival).

☐ **Sterile instruments**
How will instruments be sterilized?
Click here to enter text.

If serial surgeries are done, how will instruments be sterilized between surgeries:
Click here to enter text.

☐ **Sterile gloves**
☐ **Mask**
☐ **Cap**
☐ **Sterile gown**
☐ **Sanitized operating area**
☐ **Clipping or plucking of hair or feathers**
☐ **Skin preparation with a sterilant such as betadine**
☐ **Practices to maintain sterility of instruments during surgery**
☐ **Non-survival (clean gloves, clean instruments, etc.)**

5. Describe all surgical procedures:
a. Skin incision size and site on the animal:
Click here to enter text.
b. Describe surgery in detail (include size of implant if applicable):
Click here to enter text.
c. Method of wound closure:
Click here to enter text.
i. Number of layers
Click here to enter text.
ii. Type of wound closure and suture pattern:
Click here to enter text.
iii. Suture type/size/wound clips/tissue glue:

Click here to enter text.

iv. Plan for removing of skin sutures/wound clip/etc:

Click here to enter text.

6. Anesthetic Protocol:

For all procedures, provision of pre-emptive (pre-procedural) analgesia is required, unless specifically exempted by DCM veterinarians. For major survival surgical procedures and extensive non-surgical procedures requiring anesthesia, post-procedural analgesia must be provided for a minimum of 3 full days following anesthetic recovery, unless specifically exempted by DCM veterinarians. Analgesic administration should be continued for at least 1 full day following recovery from minor surgical and non-surgical procedures. Please contact DCM veterinary staff for recommendations and guidance when formulating anesthetic regimens.

a. If anesthesia/analgesia must be withheld for scientific reasons, please provide compelling scientific justification as to why this is necessary:

Click here to enter text.

b. Anesthesia/Analgesia For Surgical Procedures

	Agent	Dose (mg/kg or %)	Volume	Route	Frequency	Number of days administered
Pre-operative analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Pre-anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Anesthetic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Post-operative Analgesic	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.
Other	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.	Click here to enter text.

c. Methods that will be used to monitor anesthetic depth (include extra measures employed when paralyzing agents are used):

Click here to enter text.

d. Methods of physiologic support during anesthesia and immediate post-op period (fluids, warming, etc.):

Click here to enter text.

e. List what parameters are monitored during immediate post-op period. Provide the frequency and duration:

Click here to enter text.

f. Describe any other manipulations that will be used to alleviate pain, distress, and/or discomfort during the immediate post-op period (soft bedding, long sipper tubes, food on floor, dough diet, etc.):

Click here to enter text.

g. List criteria used to determine when animals are adequately recovered from anesthesia and when the animals can be returned to their home environment:

Click here to enter text.

7. Recovery from Surgical Manipulations (after animal regains consciousness and is returned to its home environment)

Click here to enter text.

a. What parameters (behavior, appetite, mobility, wound healing, etc.) will be monitored:

Click here to enter text.

b. How frequently (times per day) will animals be monitored:

Click here to enter text.

c. How long post-operatively (days) will animals be monitored:

Click here to enter text.

8. Surgical Manipulations Affecting Animals

a. Describe any signs of pain/discomfort/functional deficits resulting from the surgical procedure:

Click here to enter text.

b. What will be done to manage any signs of pain or discomfort (include pharmacologic and non-pharmacologic interventions):

Click here to enter text.

c. Describe potential adverse effects of procedures and provide humane endpoints (criteria for either humanely euthanizing or otherwise removing from study):

Click here to enter text.

V. Euthanasia

Please refer to the AVMA Guidelines for the Euthanasia of Animals: 2013 Edition and DCM Guidelines to determine appropriate euthanasia methods.

A. Euthanasia Procedure. ***All investigators, even those conducting non-terminal studies, must complete this section in case euthanasia is required for humane reasons.***

1. Physical Method- If a physical method is used, the animal should be first sedated/anesthetized with CO_2 or other anesthetic agent. If prior sedation is not possible, a scientific justification must be provided:

Click here to enter text.

2. Inhalant Method- Ketamine and Xylazine followed by Pentobarbital Sodium

(if other, describe the agent and delivery method)

The animal will first be sedated with 35-50 mg/kg of ketamine, and 3-5 mg/kg of xylazine delivered intramuscularly. Euthanasia will be performed with an overdose of 100 mg/kg of pentobarbital sodium delivered through an IV.

3. Non-Inhalant Pharmaceutical Method (injectables, MS-222, etc.)- Please provide the following:

a. Agent:

Click here to enter text.

b. Dose or concentration:

Click here to enter text.

c. Route:

Click here to enter text.

B. Method of ensuring death (can be physical method, such as pneumothorax or decapitation for small species and assessment method such as auscultation for large animals):

Pneumothroax by incising on the chest wall.

C. Describe disposition of carcass following euthanasia:

Carcasses will be placed in a designated freezer in the animal facilities and disposed of by Animal Care Services.

I acknowledge that humane care and use of animals in research, teaching and testing is of paramount importance, and agree to conduct animal studies with professionalism, using ethical principles of sound animal stewardship. I further acknowledge that I will perform only those procedures that are described in this AUP and that my use of animals must conform to the standards described in the Animal Welfare Act, the Public Health Service Policy, The Guide For the Care and Use of Laboratory Animals, the Association for the Assessment and Accreditation of Laboratory Animal Care, and East Carolina University.

Please submit the completed animal use protocol form via e-mail attachment to iacuc@ecu.edu. You must also carbon copy your Department Chair.

PI Signature:  **Date: 7/3/2019**

Veterinarian:_____ Date:_____

IACUC Chair: _____ Date:_____

<table>
<tr><th colspan="5">APPENDIX 1-HAZARDOUS AGENTS</th></tr>
<tr><td>Principal Investigator:</td><td colspan="2">Campus Phone:</td><td colspan="2">Home Phone:</td></tr>
<tr><td>IACUC Protocol Number:</td><td colspan="2">Department:</td><td colspan="2">E-Mail:</td></tr>
<tr><td>Secondary Contact:
Department:</td><td>Campus
Phone:</td><td>Home
Phone:</td><td colspan="2">E-Mail:</td></tr>
<tr><td colspan="2">Chemical Agents used:</td><td colspan="3">Radioisotopes used:</td></tr>
<tr><td colspan="2">Biohazardous Agents used:</td><td>Animal
Biosafety Level:</td><td colspan="2">Infectious to
humans?</td></tr>
<tr><td colspan="5">PERSONAL PROTECTIVE EQUIPMENT REQUIRED:</td></tr>
<tr><td colspan="5">Route of Excretion:</td></tr>
<tr><td colspan="5">Precautions for Handling Live or Dead Animals:</td></tr>
<tr><td colspan="5">Animal Disposal:</td></tr>
<tr><td colspan="5">Bedding/Waste Disposal:</td></tr>
<tr><td colspan="5">Cage Decontamination:</td></tr>
<tr><td colspan="5">Additional Precautions to Protect Personnel, Adjacent Research Projects including Animals and the Environment:</td></tr>
<tr><td colspan="3">Initial Approval
Safety/Subject Matter Expert Signature & Date

_____</td><td colspan="2"></td></tr>
</table>