CRUCIATE LIGAMENT PATHOGENESIS

AND ITS ROLE IN THE

INITIATION AND PROGRESSION OF OSTEOARTHRITIS

A Dissertation

Presented to

The Faculty of the Graduate School

University of Missouri-Columbia

In Partial Fulfillment

Of the Requirements for the Degree

Doctor of Philosophy

---

by

LEE ANDREW BRESHEARS

Dr. James L. Cook, Dissertation Supervisor

May 2008

The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

CRUCIATE LIGAMENT PATHOGENESIS AND ITS ROLE IN THE INITIATION AND PROGRESSION OF OSTEOARTHRITIS

Presented by Lee A. Breshears,

a candidate for the degree of Doctor of Philosophy, and hereby certify that, in their opinion, it is worthy of acceptance.

Professor James L. Cook

Professor Derek B. Fox

Professor James L. Tomlinson

Professor James R. Turk

Professor Jeff W. Tyler

DEDICATION

To all my family and friends, I give a heartfelt thank you for the love, support and patience that has sustained me in my time away from Colorado. This work would not have been realized without the unwavering support from Beverly B. Breshears (mother), Lyle L. Breshears (father), and Kurt A. Breshears (brother). Their concern, late night calls, editing, encouragement, financial support and love is what helped me get through the most difficult points in this educational process. There are countless aunts, uncles, cousins, and grandparents who also played a vital role in helping complete these educational goals. A group or five elementary school friends including Elizabeth D. Aybar, Ari P. Ballonoff, Michael A. Heller, Douglas J. Striker and Christopher S. Wells have remained steadfast and supportive through all of the ups and downs over the last 28 years. A close friend from veterinary school, Rance D. Hampton, and from residency, Kim D. Johnson, helped me take life a little less seriously through laughter. Each of you has provided tremendous support over the years. I could not have done it without you, I thank you and I love you!

ACKNOWLEDGEMENTS

The author would like to thank all those involved in the clinical or basic science training that culminated this degree. A multitude of individuals have impacted my career over the years. I can trace my early interest in pathology and molecular techniques to Dr. James C. DeMartini, Professor Emeritus at Colorado State University, who opened a world of exploration in science to me as a young veterinary student. Professor James T. Dormer, Department of Art at Colorado State University, helped me learn to recognize when to step back and see the whole picture. Dr. Steve W. Petersen took a chance with me as a veterinary intern and helped shape many of my clinical interests. Early guidance in the Comparative Orthopaedic Laboratory at the University of Missouri-Columbia was provided by Drs. Keiichi Kuroki and Aaron Stoker. Their guidance was much appreciated as well as that of all of the collaborators in the Comparative Orthopaedic Laboratory and the surgical residents at the Veterinary Medical Teaching Hospital. The author would also like to thank each committee member for their diligence and contributions over the years: Drs. Derek B. Fox, James L. Tomlinson, James R. Turk and Jeff W. Tyler. Finally, I would like to thank Dr. James L. Cook (Jimi), my advisor and mentor, as he has been the most influential individual in my career. Jimi provided steady guidance during my time at Missouri, leading by example, and ensuring his door was **ALWAYS** open, both literally and philosophically speaking. Each of you played a pivotal role in this accomplishment… Thank you!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF NOMENCLATURE

AB-PAS: Alcain Blue-Periodic Acid Schiff

ACL: Anterior Cruciate Ligament

ACL-X: Anterior Cruciate Ligament Transection

ADAMTS: A Disintegrin and Metalloproteinase With Thrombospondin Motifs

APMA: p-Aminophenylmercuric Acetate

CaCL: Caudal Cruciate Ligament

COL: Collagen

COX: Cyclooxygenase

CrCL: Cranial Cruciate Ligament

Ct: Take-Off Point

DMEM: Dulbecco's Modified Eagle's Media

DMMB: Dimethylmethylene Blue

DMOAD: Disease-Modifying Osteoarthritis Drugs

ECM: Extracellular Matrix

EGF: Epidermal Growth Factor

ESS: Epiligamentous Synovial Sheath

EtOH: Ethanol

FN: Fibronectin

GADPH: Glyceraldehydes-3-Phosphate Dehydrogenase

GAG: Glycosaminoglycan

H & E: Hematoxylin and Eosin

HIF: Hypoxia-Inducible Factor

HP: Hydroxyprolene

IFN: Interferon

IL: Interleukin

iNOS: Inducible NO Synthase

LDH: Lactate Dehydrogenase

L-NIL: N-Iminoethyl-L-Lysine

MCL: Medial Collateral Ligament

MFI: Median Fluorescence Intensity

MMP: Matrix Metalloproteinase

MT-MMP: Membrane-Type Matrix Metalloproteinase

NO: Nitric Oxide

NSAID: Non-Steroidal Anti-Inflammatory Drugs

OA: Osteoarthritis

PCL: Posterior Cruciate Ligament

PGE_2 Prostaglandin E_2

PBS: Phosphate Buffered Saline

RA: Rheumatoid Arthritis

SMA: Smooth Muscle Actin

TEM: Transmission Electron Microscopy

TGF: Tissue Growth Factor

TNF: Tumor Necrosis Factor

TPLO: Tibial Plateau Leveling Osteotomy

TKA: Total Knee Arthroplasty

TTA: Tibial Tuberosity Advancement

TUNEL: Terminal deoxynucleatidyl transferase-mediated deoxyuridine triphosphate-nick end labeling

VEGF: Vascular Endothelial Growth Factor

LIST OF TABLES

CRUCIATE LIGAMENT PATHOGENESIS

AND ITS ROLE IN THE

INITIATION AND PROGRESSION OF OSTEOARTHRITIS

Lee A. Breshears

Dr. James L. Cook, Dissertation Supervisor

ABSTRACT

The pathogenesis of cruciate ligament disease and how it may be linked to the initiation and progression of osteoarthritis was investigated using *in vitro* and *in vivo* models. Normal and diseased tissues were initially evaluated to differentially identify genes that may be involved in this disease process. Structural, degradative and inflammatory genes were found to be expressed differentially in these tissues. Gene expression data corresponded well to protein expression when investigated. The role of mechanotransduction in ligament health was investigated. Strain amplitude and duration was found to affect these differentially expressed genes. The Pond-Nuki model was utilized to investigate and confirmed the role of these genes *in vivo.* A novel method of matrix metalloproteinase detection was compared to traditional techniques and found to be superior to techniques currently utilized in the literature. Further investigation of these genes and molecules in cruciate ligament pathogenesis is warranted.

Chapter 1: Introduction

Osteoarthritis (OA) secondary to anterior cruciate ligament (ACL) deficiency is a common orthopaedic problem in dogs and humans. The Pond-Nuki anterior cruciate ligament transection (ACL-X) model is commonly utilized for the study of OA. While abnormal biomechanics are known to play a role in development of OA, the effects of molecular and biochemical alterations in ligament itself in the initiation and progression of OA are poorly understood. Because the ligament is a tissue composed of multiple cell types with a diverse and dynamic extracellular matrix, it is plausible that diseased intra-articular ligament could play a pivotal role in the initiation and exacerbation of secondary OA.

The cruciate ligaments of the knee can be found within the joint cavity and these paired ligaments contribute to the overall stability of the joint. These ligaments are named according to the location of their attachment in the tibia. In humans, the ligament attaching to the tibia in a more anterior position than its paired ligament is known as the anterior cruciate ligament (ACL) and the ligament attaching in a more posterior position is known as the posterior cruciate ligament (PCL). Similarly, in animals, the ligament attaching to the tibia more cranially is known as the cranial cruciate ligament (CrCL) and the ligament attaching more caudally is known as the caudal cruciate ligament (CaCL). For all inclusive purposes the ACL in humans is analogous to the CrCL in animals. The two names for the same ligament arise from the directional terms needed to properly describe anatomy in bipeds as opposed to quadrupeds. Likewise, the PCL is analogous to the CaCL. The location, structure, and function are essentially the same so ACL/CrCL

and PCL/CaCL nomenclature are often used interchangeably. When citing previous studies, this dissertation will utilize the proper anatomical name (ACL or CrCL) in accordance to the species utilized in the study cited. If the discussion involves multiple studies with multiple species or if the discussion is relevant to ACL and CrCL, then the ligament will simply be referred to as cruciate ligament. As often is the case in translational research, findings in one species are often applicable to another and cruciate ligament should be an acceptable term to encompass results across multiple species. Based on their proper anatomical name, the PCL/CaCL could also be included in the reference "cruciate ligament", but this ligament is rarely involved in disease or trauma. So for the sake of simplicity, when this text refers to "cruciate ligament", it encompasses the ACL and the CrCL collectively while excluding the PCL and the CrCL.

The cranial cruciate ligament is a complex intra-articular connective tissue structure comprised of, in it most basic form, cells and extracellular matrix. The primary cell type within the CrCL is the ligament fibrocyte, otherwise known as a ligamentocyte. These ligamentocytes are responsible for the creation and the maintenance of the extracellular matrix (ECM), in which the predominate ECM protein found is collagen type I. Together, these two primary components interact closely on a molecular, biochemical, cellular, and biomechanical level to ensure structure and function of this intra-articular ligament.

Cruciate ligament deficiency is common in both people and dogs. In humans, ACL injury is thought to be primarily traumatic whereas in dogs, CrCL deficiency has traditionally been thought to be a degenerative process. In both species, surgical intervention if often recommended to limit the progression of osteoarthritis. The standard

of care in human medicine has become the placement of intra-articular grafts with the aid of arthroscopy. In recent years, among veterinary surgeons, the choice of surgical intervention is a widely debated topic. More than 20 procedures have been developed, for the management of CrCL deficiency with varying degrees of success. Various types of extra-articular lateral stabilization, the tibial plateau leveling osteotomy (TPLO), and the tibial tuberosity advancement (TTA) are the most common procedures performed today.

In recent years, many surgeons have advocated "thinking of a joint as an organ" rather that individual tissue, each with a defined function. This lineage of thinking implies that tissues within a joint work together, are interdependent on one another, and have the ability to communicate with each other. These tissues are linked to each other as one organ in both disease and in health. Articular cartilage, synovium, ligament, meniscus, and synovial fluid are all make up a joint. Should one tissue become diseased, all others tissues are in some way affected. Indeed, in recent years we have seen a plethora of *in vitro* studies that focused on co-culture models. Authors of these studies have recognized that tissues behave differently when in co-culture and that conclusions from a study can be affected. The trend has been to make these *in vitro* models more "joint-like" by co-culturing two or more tissues. The sheer number of studies in the literature involving co-culture models suggests a recent shift in current orthopaedic basic science. This underscores the importance of considering all intra-articular tissues when addressing joint diseases on both a clinical and basic science level.

Most co-culture models have included articular cartilage and synovium. Few studies have given consideration to other intra-articular structures such as menisci or

ligament. This is surprising given the frequency and severity of disease that results from injury to these structures. As a relatively large intraatricular connective tissue structure, the cruciate ligaments could be one potential source of inflammation and degradative enzymes should they become pathologic. Veterinary surgeons note that the CrCL is often completely resorbed at the time of surgical intervention. There is certainly a mechanism behind this resorption that is not completely understood. Presumably, this resorption occurs through the action of ECM degradative enzymes. It is possible that, during this period of resorption, the ligament serves as a nidus of inflammatory molecules and catabolic enzymes. This brings about an interesting, but very practical question for the veterinary surgeon: does the CrCL have the potential to serve as a source of inflammatory molecules and degradative enzymes that aid in the initiation or the exacerbation of osteoarthritis? If cruciate ligament does have this potential, then a second, equally important question must be considered: is diseased ligament clinically relevant and when should surgical intervention include debridement of this ligament? One currently accepted and common method of addressing CrCL deficiency is to perform a stifle stabilizing osteotomy without full exploration of the joint itself. The traditional reason for joint exploration in the CrCL deficient stifle is to inspect the menisci, as they are commonly damaged in the unstable knee. Current options for joint exploration include a full arthrotomy, a limited caudomedial arthrotomy, and arthroscopic evaluation. The limited caudomedial arthrotomy allows inspection of the meniscus but prevents proper evaluation of all other intra-articular structures, including pathologic ligaments that could serve as a nidus for progression of OA. Yet this approach is commonly used in

practice. Of those surgeons that perform a full arthrotomy or arthroscopy, there are those that do not debride the ligament and leave pathologic ligament *in situ*.

In order to aid intra-operative decision making regarding ligament debridement, a series of studies were developed to investigate whether intra-articular ligament has the potential to serve as a reservoir of proinflammatory and degradative enzymes. The limited knowledge regarding the pathogenesis of secondary OA associated with CrCL deficiency may prevent optimal intervention, as well as the development of novel strategies for the prevention of OA, in ACL pathology. Because the initiation or progression of OA is directly linked to the pathogenesis of CrCL deficiency, a secondary objective was to further characterize healthy and diseased ligament on a molecular and biochemical level.

Chapter 2: Literature Review

The cruciate ligament (ACL = CrCL) is a complex intra-articular but extrasynovial connective tissue structure comprised of, in it most basic form, cells and extracellular matrix. The primary cell type within the CrCL is the ligament fibrocyte, otherwise known as a ligamentocyte. These ligamentocytes are responsible for the creation and the maintenance of the extracellular matrix (ECM). Together, these two primary components interact closely on molecular, biochemical, cellular, and biomechanical levels to ensure structure and function of this intra-articular ligament.

The cells of the cruciate ligament have been well described in the literature. The primary cellular constituent is the ligamentocyte, but the cruciate ligament also contains small amounts of capillaries and nerve fibers.[1-3] Evaluation of CrCL ligamentocytes suggests that the phenotypes that are described for ACL in the human literature are also present in the canine ligament. These phenotypes include fusiform, ovoid, spheroid and intermediary cells.[4-7] Several papers describe transformation of fusiform ligamentocytes to a spheroid cell as part of a pathologic process called chondroid metaplasia.[4,6,7] An initial histologic study that evaluated normal and pathologic ligaments did not identify the spheroid phenotype in the normal control ligaments.[4] However, this is likely because young dogs were utilized in this study. Further studies by the same group later identified these spheroid cells within normal intact ligaments from aged dogs.[8] Human studies have suggested that chondroid transformation to a spheroid phenotype may predispose the ligament to failure.[5,9] However, because the spheroid phenotype has been documented in normal ligaments from dogs, it may be difficult to implicate this chondroid metaplasia as

always being a pathologic process in all ligaments. It is possible that this pathologic finding is a normal remodeling process that occurs secondary to outside factors such as mechanotransduction. Indeed, a recent report that evaluated normal ligaments with electron microscopy from breeds with differing predispositions to ligament rupture found that the fibrocartliage was present in almost all (7/8) racing greyhounds but present within only a few (3/8) ligaments from Labrador retrievers.[10] These authors hypothesize that fibrocartliage may have a protective effect in Greyhounds, but may be indicative of a mild degenerative change in Labrador retrievers. It is unknown whether these spheroid cells were present in any of these samples or whether the fibrocartilagenous appearance of these cells was consistent with findings from other studies. All of these studies shed light on an important question: what defines normal ligament? It is prudent to state that the significance of these spheroid cells, chondroid metaplasia, and fibrocartliage in normal ligaments is not known at this time and may be breed, age and activity dependent. In future studies involving histopathology, it will be important to clarify whether chondroid metaplasia and spheroid cells are present with other signs of degeneration such as loss of ligamentocytes and failure to maintain collagen fibrils and primary collagen bundles.[7]

Aside from ligamentocytes, the other main constituent in the cruciate ligament is the extracellular matrix. Water constitutes at least 60 % of the wet weight of most ligaments and is significantly associated with ground substance (matrix consisting of proteoglycans).[11] Together, water and proteoglycans provide the lubrication and spacing for the gliding function of these ligaments.[12] There are numerous matrix molecules that help ensure the structure and function of normal ligaments. Collagens may comprise up

to 75 % of the ligament's dry weight. After being secreted by ligamentocytes, collagens are assembled into functional fibers. Type I collagen is the primary collagen; with lesser amounts of type III present.[12] Smaller quantities of types V, and VI are also present.[11] Very small amounts of type IV collagen are present and may be involved in linking the ECM with ligamentocytes.[13] When these type IV collagen connections are severed, apoptosis may be initiated in these cells.[13] The proteoglycans are also an important part of this ECM. However, proteoglycans comprise only a small portion of the dry weight (typically less than 1%).[11] These are proteins bound by negatively charged polysaccharide chains referred to as glycosaminoglycans.[12] They include chondroitin and keratin sulfate as well as smaller amounts of dermatan sulfate.[12] Together, these molecules, with their negatively charged chains, expand until restrained by adjacent collagen. This allows for the maintenance of water within the tissue and likely contributes some to the "shock absorbing" properties of the ligament.[12] The CrCL contains only a small amount of elastin (less than 5%) which often cannot be visualized on histological hematoxylin and eosin stained sections of ligament.[7,14] Elastin forms networks interdigitated among collagen fibril fascicles. The glycoproteins (non-collagenous proteins) also play a role in ligament homeostasis. An example of one of these glycoproteins is fibronectin, which may be involved in a cell's interaction within its surrounding environment and ECM.[12,13]

Together, ligamentocytes and the ECM can be observed using light microscopy with standard hematoxylin and eosin stained sections and a variety of other methods. Collagen fibrils within the CrCL are organized into bundles or fascicles.[15] These bundles or fascicles are separated by long narrow spaces that do not contain reticular or elastic

fibers when stained with special connective tissue stains.[7] An interlacing network of collagen fibrils (150-250 nm in diameter) is grouped into fibers (1-20 um in diameter).[15] These fibers are then collected into subfasicular units (100 to 250 um in diameter) and become surrounded by loose connective tissue to form the endotenon.[15] Three to 20 of the subfascicles can form fascicles that can be 250 um to several mm in diameter and are surrounded by epitenon.[15] The entire continuum is enclosed by the paratenon.[15] Ligamentocytes typically occur in long parallel rows of two or three cells and are tightly associated with the surface of these primary collagen bundles.[7,15]

The blood supply of the CrCL has also been well studied. Capillary beds seen in dogs less than 2 years of age were nearly absent in dogs older than 2 years of age.[7] The majority of the blood supply is extraligamentous and very little blood flow to the ligament comes from the ligamentous-osseous attachment.[16] The synovial sheath contains many small vessels that arise from the genicular arteries. These vessels, as well as vessels from the infrapatellar fat pad penetrate the cruciate ligaments.[16] The central core of the ligament does not appear to be as well vascularized as the remaining portion of the ligament. As the CrCL courses from proximal to distal, the cranial medial fascicles are rotated into an outward spiral, and in flexion these fascicles bend around the caudal lateral fascicles.[15] The caudal lateral band forms the bulk of the CrCL.[17] The CrCL has a core region, which is its major axial tissue component, and an epiligamentous region that is more cellular.[18] This epiligamentous region is composed of the synovial intima and underlying loose connective tissue. The latter tissues cover the CrCL except where the CrCL wraps around the caudal cruciate ligament.[7] This synovial intima is composed of a single layer of synoviocytes that rests upon a thin layer of subintimal

areolar tissue. [18] Where the CrCL touches the CaCL, there was no recognizable synovial lining.[7] Finally, the canine cruciate ligament also has nervous tissue. Through the use of a modified gold-chloride technique and other methods, receptors were found within the cranial cruciate ligament, suggesting that ligament has important mechanoreceptive, nociceptive and proprioceptive function.[1-3,19-22]

The physical and biochemical properties of the normal CrCL have been evaluated through several studies. There is evidence that these properties are greatly breed dependent, but the published findings give us insight into the CrCL. Ultimately, the physical and biomechanical properties of the CrCL help explain its anatomy and function.

Many of physical characteristics of the normal CrCL appear to be fairly breed-specific. In two studies by Wingfield et al, the physical characteristics of normal CrCL were outlined in Rottweilers and racing Greyhounds.[23,24] The mean length of the CrCL in Rottweilers was 18.7 mm while it was 18.2 mm in Greyhounds and there was no significant difference between the two lengths. The Rottweiler had greater mean cross-sectional area at proximal, middle, and distal portions of the ligament. This became statistically significant in the distal portion where the mean area in the Rottweiler was 31.17 mm^2 compared to the Greyhound at 21.46 mm^2.[24] There is information available regarding the material properties (properties uninfluenced by length or attachment) of the CrCL. These properties have been evaluated in multiple breeds, flexion angles, and loading protocols. When cranial loading of the tibia was performed to failure, the tensile strength was found to range from 52.6 MPa to 93 MPa, the ultimate strain was found to range from 69.7% to 84.6 %, and the tangent modulus was found to range from 94.1 MPa

to 201.8 MPa depending on the breed and the flexion angle.[24] When loading along the long axis of the ligament was performed to failure, the tensile strength was found to range from 60.8 MPa to 86.2 MPa, the ultimate strain was found to range from 38.1% to 44.2%, and the tangent modulus was found to range from 198.7 MPa to 221.0 MPa, depending on the breed and the flexion angle.[24]

Much more literature is available on the biomechanical properties of the CrCL than on the physical properties. Here again, the means given below are ranges depending on the breed and flexion angles. For cranial tibial loading, ultimate load was found to be between 1389 N and 2130 N. On a per kilogram basis, these numbers came to 33.8 N/Kg to 59.3 N/kg. Ultimate deformation was found to be 11.4 mm to 15.86 mm, linear stiffness was determined to be 148 N/mm to 224.6 N/mm, and energy absorbed at the time of failure ranged from 8.8 Joules to 12.8 Joules.[24] For loading along the long axis of the ligament, ultimate load was found to be between 1421 N and 1781 N. On a per kilogram basis, these numbers came to 39.5 N/Kg to 56.9 N/kg. Ultimate deformation was found to be 6.67 mm to 8.25 mm, linear stiffness was determined to be 263 N/mm to 306.7 N/mm, and energy absorbed at the time of failure ranged from 4.1 Joules to 5.8 Joules.[24] The mode of failure in both axes of loading involved both mid-ligament and tibial avulsion, but never femoral avulsion.[24] The evaluation of these mechanical and material properties revealed that the ultimate load to failure for normal ligament was greatly breed dependent. The Rottweiler required about half as much load per unit body mass as the racing Greyhound. Furthermore, an evaluation of joint stability revealed that the relative contribution of the CrCL increased as the joint flexed.[24] The findings

regarding the biomechanical properties in this study were similar to findings from other studies which the ultimate load to failure was 51 kg/N, 59.4 kg/N, and 44.9 kg/N.[7,25,26]

The etiopathogenesis of cruciate ligament disease in dogs is complex and multifactorial. Early theories suggested that ischemia may play a role the degeneration and eventual rupture of the ligament.[16,27,28] These theories may need to be revisited in future studies. Mutation in collagen genes have been evaluated with microsatellite markers and the collagen genes are unlikely to be involved in the etiopathogenesis.[29] Trauma is no doubt the primary cause of some ruptures and may also be a contributory factor in many other cases. The extent of this contribution has not been specifically been addressed in the literature. Degeneration of the ligament has long been cited as a contributory factor in this disease.[4,6,7,10,30] Genetics was suspected to be a contributory factor by many researchers. This has now been demonstrated in at least one breed, the Newfoundland.[31,32] Obesity is generally thought to be a factor by most surgeons.[33,34] Unfortunately, many epidemiological studies have focused on body weight rather than body condition score or some other methods to consistently define obesity. The increase in weight is thought to increase the amount of strain to which the cruciate ligament is subjected. However, the biology of white adipose tissue may also contribute to ligament metabolism through systemic effects. The tibial plateau angle (TPA) was initially thought to a major cause of cruciate ligament disease.[35] However, recent studies have demonstrated this may not be the case and the role of the TPA in diagnosis and treatment is not entirely understood.[36-47] Further investigation in this area is vital to elucidating the multifactorial etiopathogensis of cruciate ligament disease. Biomechanics may play a role in this disease. Currently, there are no veterinary studies that have considered

muscle strength and flexibility as a potential contributing factor to rupture of the ligament. One human study found increased hamstring flexibility in patients with ACL rupture and the authors suggested that increased flexibility may result in reduced passive protection of the ACL.[48] Several other human studies have looked at muscle strength and co-contraction as a variable.[49] Several studies have suggested an immune-mediated, possibly even an infectious cause for ligament degeneration.[50-56] However, it is unlikely that anti-collagen antibodies are involved in this process if it exists.[30,57,58] Further one study demonstrated similar histopathologic changes in dogs with patella luxation and CrCL disease.[50] This suggests that these changes are the result of osteoarthritis rather than some immune-mediated process. Hormones, in particular, have been implicated in human medicine as playing a role in non-contact injuries.[49,59-65] Little information regarding hormones exist in the canine literature.[66,67] Finally, impingement by the intercondylar notch has been suggested as a contributing factor to this disease.[68-71] Based on the large number of potential factors involved in this disease process, it may inappropriate to suggest that there is a single cause for cruciate ligament disease. It is more likely that a combination of these factors contributes to this disease and that there are different combinations of these factors that contribute to certain clinical presentations of cruciate disease (acute vs. chronic; partial vs. complete). Therefore, it may be important to think of these factors as either initiating or perpetuating factors (Figure 2-1).

Histopathological changes have been identified in both synovium and in the cruciate ligament itself. The histopathological changes within synovium have been reasonably well documented.[18,51,72,73] One caveat, however, is that the mechanisms behind and the temporal sequence of events in which this occurs is much more poorly

understood. Despite the shortcomings of this knowledge, a high risk of rupture is associated with inflammation of the synovium and adaptive or degenerative changes in the cells and matrix of the CrCL.[18] The typical histopathological changes that are associated with the rupture of the CrCL are supported by studies evaluating both natural CrCL disease as well as experimental transection of the CrCL. Histological evaluation of synovium at the time of surgery has been reported in several studies.[28,50,51,74] All of these studies were able to identify a synovitis as the primary pathological process occurring within the evaluated tissue. In some instances the synovitis was also observed with the formation of distinct, nodular aggregates. [74] In all cases, this inflammation was characterized by infiltration of the synovium with plasma cells, lymphocytes and macrophages and synovial thickening.[51] One study found this inflammation, along with these aggregates, was present 36 out of 54 synovial membranes evaluated. [74] The remainder for these patients appeared to have evidence of chronic synovitis with variable numbers of diffuse, mononuclear cells.[74] These typical histopathological changes have also been observed in experimental models involving transection of the CrCL. One such study that utilized experimental transection of the ligament, inflammatory changes in the synovium of the stifle progressed with time and were prominent at 8 weeks postoperatively. These studies also documented subsynovial fibrosis that was greatest at 13 weeks. Inflammation of the synovial membrane and subsynovial tissue was characterized by synovial cell hypertrophy and hyperplasia, plasma cell and lymphocyte infiltration, and increased vascularization of the subsynovial region.[72] The histopathological changes following experimental transection of the cruciate ligament appears to be consistent across species. At 20 weeks following surgery in rats, one study

demonstrated mild to moderate inflammation (synovitis score of 8.5 +/- 2.8) was noted in the ACL transected knees compared to the sham-operated knees (1.2 +/- 0.3) ($p<0.05$). [75] The obvious difficulty with experimental models in CrCL transection is that it is difficult to know what the changes in synovium are resulting from. Are the histopathological changes from the transection of the ligament itself or from the resulting joint instability and subsequent development of osteoarthritis? Most researchers would tend to agree that the later of the two is more likely. Indeed, the Pond-Nuki model, which is the original description of CrCL transection in the dog, has been used as a model of osteoarthritis for more than 30 years.[76] While this may be the case, this lineage of thinking brings to light an important consideration in naturally occurring disease: are these synovial changes present prior to and possibly involved in the rupture of the cruciate ligament or do these changes occur after the ligament ruptures and secondary osteoarthritis ensues? In either instance, synovium is involved in the disease process and we know that some degree of molecular and biochemical cross-talk occurs between tissues present within the joint. This is an interesting question from both a clinical and basic science perspective because understanding this mechanism could help us intervene at the appropriate time-point to prevent this disease from occurring.

Several authors suggest that further investigation is required to answer this question and determine if these immune or inflammatory responses in CrCL rupture joints are the primary factor in ligament rupture or if they develop secondary to ligament rupture and instability.[73] In an attempt to elucidate this "chicken or the egg" scenario, several attempts have been made to further characterize the cells types within the synovium. Specifically, some studies have attempted to identify the type of lymphocytes

present within the synovitis identified in previous work. One such study evaluated biopsies from dogs with OA secondary to CrCL rupture and found there were markedly lower numbers of subsynovial CD5+, CD4+ and CD8+ lymphocytes when compared to normal tissues. T-cells were diffusely distributed. This contrasts the finding in the same study that evaluated synovium from joints affected with rheumatoid arthritis. Here, numerous diffusely and perivascularly distributed CD5+ lymphocytes were found in the subsynovium and CD4+ cells outnumbered CD8+ cells and were more numerous in the perivascular areas. There were also high numbers of alpha beta TCR+ cells when compared with gamma delta TCR+ cells. MHC class II has also been considered as a marker for identification of more specific cells types. In only three of eight OA synovial samples was there an increased percentage of cells expressing MHC class II whereas in all cases of rheumatoid synovial samples there was a marked increase of MHC class II.[50] Other studies have looked at the type of immunoglobulin expressed. One group found that the main cell types in canine synovial biopsies from joints with CrCL rupture were B lymphocytes and plasma cells belonging to the IgG isotype. This group also looked at a variety of adhesion molecules and found that severity of inflammatory cell infiltration positively correlated with the expression these molecules.[51] IgG deposition was four-fold higher and IgM was eight-fold higher in dogs with spontaneous cranial crucial ligament (CrCL) rupture.[73] These findings support the premise that there is an immune component to the inflammation associated with canine CrCL disease. None of these studies adequately address this chicken or egg scenario and fully consider the temporal relationship between rupture of the CrCL, development of OA, and these histopathological findings in synovium.

Aside form histological or immunohistological identification of various cell types, it is theoretically possible to identify cells on a molecular basis. Differential gene expression could potentially be useful in the identification of types of cells. While the literature has primarily reported the use of differential gene expression (differential display or microarray) to help distinguish between pathologic conditions, it could also potentially be used to distinguish cell types.[77-84] One report utilized differential expression to distinguish osteoarthritic vs. rheumatoid arthritic conditions, state of activation (resting vs. cytokine activation), and anatomical location (synovium vs. skin). A total of seven genes were differentially expressed. Four of these genes, TFPI2, GRObeta (CXCL2), MnSOD and GCP-2 (CXCL6), were selectively over-expressed in osteoarthritis fibroblasts rather than rheumatoid fibroblasts.[84] However, what made this study so interesting in regards to identification of cell types was that three other genes (aggrecan, biglycan and caldesmon) were expressed at higher levels in all types of synovial fibroblasts compared with skin fibroblasts even after stimulation with Tumor Necrosis Factor-alpha (TNF-alpha) and intetleukin-1 (IL-1). [84] This last finding supports the premise that cell type could be identified using molecular techniques.

The histopathological changes associated with the rupture of the CrCL are well documented in the literature. Age-related changes within the CrCL of larger dogs have also been documented and are possibly thought to precede ligament rupture. These findings include loss of ligament fibroblasts, progressive degeneration of the ligament fibroblasts, metaplasia of surviving cells from ligament fibroblasts to chondrocytes, and failure to maintain normal collagen fibers and primary collagen bundles.[4,7] In both human and dogs, several ligamentocyte phenotypes have been identified and include:

fusiform, ovoid, and spheroid ligament fibroblasts.[4,9] The spheroid phenotype is thought to be associated with the transformation of these cells to chondrocytes.[5] These spheroid phenotypes can be observed amidst a glossy amorphous extracellular matrix (ECM) in disrupted ligaments.[4] In the study by Hayashi et al, cellularity, vascularity, cellular morphology, and fibrous ECM structure appeared to be similar in both ruptured and intact CrCL epiligamentous regions, but volume of tissue was greater in ruptured ligaments.[8] Ruptured CrCLs demonstrate a significant loss of ligamentocytes in the core region in when compared to normal intact ligaments. This was not the case in the epiligamentous region. The number of spheroid cells also increased in this core section, while the number of fusiform and ovoid cells decreased. Occasional pairs of ligamentocytes can be observed, similar to the cloning that is seen in late-stage osteoarthritis. The loss of crimp, birefringence, and separation of collagen fibers may also occur because of edema within the ligamentous tissue. Many of these alterations were thought to be the result of poor blood flow and tissue hypoxia.[8] The formation of fibrocartilage may not be a disadvantage in some breeds and should not be regarded as a pathological degeneration. Some authors suggest fibrocartilage is formed as a beneficial physiological adaptation to the compression of CCLs caused by tensile stress as a result of the tightening of two twisted bands.[10]

There are many staining techniques, imaging modalities, and other methods that can aid in evaluating the CrCL and ligamentous tissue in general. Some can be utilized for identification of cells, some for cellular metabolism, and others can be utilized for evaluation of extracellular matrix. These can be grouped as histochemical stains, immunohistochemical stains, and miscellaneous modalities. The histochemical stains by

far encompass the largest group of techniques used and are the most widely employed. Investigators should be aware of each of these stains and should consider them for use in future studies.

The most commonly utilized histochemical stain is obviously the Hematoxylin and Eosin stain (H & E). A variety of features important to ligament pathology can be identified using this stain. These include the amount and location of fibrosis, hyalization, cystic degeneration, calcification, mucin accumulation, fibrillation, and fragmentation representing degenerative change. Neovascularization, metaplasia, iron deposition, and proliferation representing changes seen with repeated trauma have also assessed, as well as amyloid deposition and inflammation.[6,7,15] Another commonly utilized stain is the Toludine Blue. This stain helps evaluate glycosaminoglycan distribution within ligament sections. Typically, the overall GAG content in ligament is far less than that of cartilage.[85] Masson's trichrome stain (blue variant) is a stain that aids in the identification of collagen. Normally collagen will stain blue with this stain, but fibroblastic infiltrates can be seen groups of red cells disrupting the collagen bundles.[86] Masson's trichrome stain with circularly polarized light (blue variant) can aid in the identification of collagen fibril birefringence and elongation of the crimping pattern. Utilizing this technique could lend support to the hypothesis that progressive mechanical overload as plays a role in initiating CrCL degeneration.[18]

There are also a variety of less commonly utilized histochemical stains. One of these stains is the Verhoeff-van geison stain which stains for elastin fibers. Elastin is typically absent from tendinous structures and more commonly seen in ligamentous structures.[14] Lactate dehydrogenase (LDH) has been utilized as an objective

histochemical assessment of cell viability.[8] Alcain blue-Periodic acid Schiff (AB-PAS) is a histochemical stain that helps identify mucopolysaccharides which have been identified in some, but not all ligamentocyte perinuclear halos from normal dogs. The accumulation of alcain blue positive mucous substance has been observed in deteriorated collagen fibers of abnormal dogs. This may be morphological evidence of chondroid metaplasia.[6] This stain has also been useful to confirm Mucin formation in areas of voids on H & E stains.[14] Congo red stain, another histochemical stain has been used to detect amyloid, but reported not to be very useful in cruciate ligament disease.[14] Perl's stain is a histochemical stain used to detect hemosiderin deposition. It is apparently only useful only to confirm areas of brown discoloration seen on standard H & E stains that are suspected to be hemosiderin.[14] Safranin Orange may aid in the identification of GAGs, but not commonly used[85] Masson Goldner stain is an additional trichrome stain that is cited in the literature. However, in regards to ligament, its usefulness is poorly outlined in the literature.

Immunohistochemical stains are another broad category of techniques that can be employed to aid in the identification of cells or extracellular matrix. Hypoxia inducible factor-1α (HIF-1α) has been used as a subjective immunohistochemical assessment of metabolic response to tissue hypoxia. This factor can be observed as brown nuclear staining in HIF-1α+ cells.[8] Alpha Smooth muscle actin (SMA) is an interesting stain, particularly in regards to ligament retraction and resorption following rupture. It can be used as a subjective immunohistochemical assessment of crimp disruption and myofibroblasts.[5,9,87-89] S-100 protein is an immunohistochemical marker for cell cycle proliferation. Fine granular substances are sometimes observed as a delicate web-like

structure which were positive for S-100 from normal dogs.[6] Ki-67 antigen is also a marker for cell cycle proliferation and supposedly observed in diseased cruciates, but was poorly defined in the paper's results and discussion section.[6]

There are also miscellaneous techniques that allow for cell identification or assessment of extracellular matrix. Terminal deoxynucleatidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) has been used as a subjective in-situ hybridization stain that assesses apoptosis. The apoptotic cells turn brown.[8] Evaluation of apoptotic cells in this disease is supported by a recent study evaluating apoptosis and nitric oxide (NO). NO has been significantly correlated with cleaved caspase-3 positive cells, a measure of apoptosis.[90] Transmission electron microscopy (TEM) has been utilized in several studies as a completely separate modality to evaluate ligament.[10] More specifically, ligament ultrstructure and pathological abnormalities can be observed including cellular shape, nuclear shape, active cytoplasmic organelles, short surface processes, the presence of a percellular zone, and the presence of abnormal collagen fibrils.[8] Normarski differential interference contrast is a technique delineates the limits of the cytoplasm of individual cells that are not normally discerned. One final technique that was employed early in CrCL research was a tissue clearing technique. The Spaltholz technique was utilized to evaluate CrCL microvasculature.[16]

The behavior and metabolism of cruciate ligament cells is probably best descried using its extra-articular sister ligament the MCL. Much of the basic science research has compared these ligaments because of their differential healing capacity. It has been well documented that the medial collateral ligament (MCL) has a relatively good healing capacity. Its commonly injured intra-articular counterpart, the cruciate ligament, is

clearly not as resilient. At first glance, it is easy to assume that this difference is the result of their anatomic location. However there are a variety of factors that appear to contribute to this discrepancy. Several authors have attempted to classify these factors in a variety of ways: intra-articular factors vs. extra-articular factors, biomechanical factors vs. biochemical factors, or intrinsic vs. extrinsic factors. For the purposes of our discussion, we will classify these factors as intrinsic or extrinsic factors. In other words, factors that contribute to healing that lie inside of the ligament are considered intrinsic and those factors that lie outside of the ligament are termed extrinsic. There is a tremendous body of research that investigates and compares the ACL and MCL on many levels.[91-109] Numerous factors, both intrinsic and extrinsic, have been implicated in the disparity between the ACL and MCL following injury.

Many extrinsic factors have been suggested and some implicated in the healing differential between ACL and MCL. One commonly suggested extrinsic factor is the difference between the vascular anatomy of the ACL and MCL. The MCL has a reasonably well vascularized epiligament with adequate midsubstance penetration.[106,110,111] The blood supply of the ACL on the other hand, is restricted to the small branches of the genicular artery that cover the surface of the ligament.[16] There are almost no vessels penetrating the core of the midsubstance.[16,27,92,106,110] It is also important to consider interspecies variation in vascular anatomy as a variable when using an animal model for investigation.[106]

Angiogenesis and vasodilation have also been proposed as contributory factors to the difference in healing between ACL and MCL. One study evaluated this possibility by measuring a vascular index following injury in the rabbit hemisection model.[93] These

authors found a 6-fold increase in vascular index for MCL whereas only a 2-fold increase in vascular index in for ACL.[93] They also evaluated overall blood flow and found an 8-fold increase in vascular index for MCL whereas blood flow for ACL remained unchanged.[93] True vasodilation and nutrition are also factors that have been proposed and are indirectly related to angiogenesis and blood flow. Vasodilation and nutrition were not evaluated in this study and have not been evaluated in the literature. Functionally, it appeared that there was prominent angiogenesis that also resulted in the formation of new blood vessels in the MCL. The limited angiogenesis in the ACL failed to make any improvements in flow. Could it be that the cruciate ligament's inability to make improvements in blood flow is a result of the limited availability of new sources of vessels?[16] Owing that the ACL did show some ability (2-fold increase) to initiate angiogenesis, it seems more plausible that intra-articular conditions are limiting this response. The lack of blood vessels is probably not a limiting response.[93] Clearly, the MCL has the superior capacity to mount angiogenesis and mobilize blood supply which is crucial for healing to occur. This then, could be a major extrinsic factor influencing the difference in healing potential between the ACL and MCL.

There are no published studies, and very little evidence, that directly evaluates the difference in biomechanics between the MCL and the ACL. Yet most of the literature cites biomechanical differences exerted on the two ligaments as a plausible factor in their ability to heal. These biomechanical differences are often vaguely discussed in the introduction sections of papers evaluating MCL and ACL without any citation. Indeed, it seems very likely that the different biomechanical environments involving these two ligaments do influence their ability to heal. However, it also seems like an experimental

design nightmare to try and set up a study to evaluate this possibility in a controlled manner. Is there any evidence to support biomechanics as a contributory extrinsic factor? The only possible evidence found in performing this literature review is indirectly from the veterinary literature in abstract form. Arthroscopic evaluation was performed in 16 dogs (22 stifles) having a partial tear of the CrCL.[112] All dogs received TPLO as part of an ongoing clinical study. Of the 22 ligaments that sustained partial tears of the cranial cruciate ligament, 19 were intact during the second look arthroscopy that took place anywhere from 3 to 33 after the first scoping procedure.[112] The authors suggested that the improved appearance of the cranial cruciate ligament at second-look arthroscopic examination supports the theory that TPLO reduces strain on the cranial cruciate ligament.[112] This provides some minimal evidence that if the biomechanical environment can be altered, then progressive rupture of the ligament can be halted and quiescence of the joint achieved. This supports biomechanics as a potentially important extrinsic factor contributing to the differential healing of the cruciate ligament and MCL.

Many intrinsic factors have been suggested and some implicated for the healing differential in ACL and MCL. Perhaps the best studied is the role of nitric oxide and there is a tremendous body of research regarding this molecule. There is some evidence that nitiric oxide (NO) may be produced in different amounts by different ligaments and then, in turn, NO may also influence the metabolism differently in ligaments. Some insight can be gained from considering a rabbit study that evaluated healing in ACL vs. MCL. One important finding from this study was that not all ligamentocytes respond to stimuli in the same manner. In this study by Cao, et al, anterior cruciate ligaments (ACL), posterior cruciate ligaments (PCL), and medial

collateral ligaments (MCL) were collected, processed, and grown to confluency in monolayer cell culture. These cultures were then stimulated with IL-1β. The ACL and PCL generated large amounts of NO. The MCL, in contrast, produce very little NO.[94] This provides some evidence that the role of NO may differ depending the ligament evaluated, its location, or both. To further support this, ACLs synthesized NO spontaneously whereas the MCL never did so.[94] Not only could the production of NO be unique in different joints, but its mechanism of action once NO has been produced could be different. When NO was administered to explant cultures, it prevented collagen synthesis on both the ACL and PCL, but had very little influence on the MCL.[94] In a separate study, the susceptibility to apoptosis was between ACL and MCL cells after treatment with sodium nitroprusside, a NO donor. Apoptosis was evaluated by a variety of means but ultimately the ACL cells were more prone to apoptosis at 1 mM compared to MCL cells. So it appears, on both cellular and tissue levels that ligaments are behaving differently at different sites in the body in response to NO. Indeed, this has lead some researchers to implicate NO a possible mediator of the differential healing capacities of the MCL and ACL.[94,103]

Nitric oxide (NO) is thought to be important in the initiation and progression of inflammatory and degenerative joint diseases in general.[113] NO produced within a joint is derived from arginine, its production is mediated by inducible NO synthase (iNOS).[114] Other isoforms, such endothelial NO synthase (eNOS), might also be present in joint-associated endothelium. One study found the eNOS dysregulation is likely involved in the overall oxidative stress in rheumatoid arthritis.[115] This enzyme can be found in small amounts in normal tissues, but can also be found in pathologic states such as immune-

mediated inflammatory joint diseases or OA.[114] This enzyme has also been experimentally induced by activation with pro-inflammatory cytokines such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α.[114,116,117] Inhibition of collagen and proteoglycans production by cartilage chondrocytes leads to degradation is probably partially mediated by NO.[118] Perhaps most intriguing, at least in regards to disease associated with the CrCL, is that inhibition of this enzyme can cause modulation of the OA changes associated with the Pond-Nuki model.[119]

Several studies have looked at NO production in the canine CrCL, but the findings from these studies have further complicated the exact role of NO in ligament health and disease. Some authors have alluded that NO may have a physiologic role in the CrCL because similar concentrations of NO were detected in CrCL and cartilage explants from control dogs (normal ligaments).[120] Surprisingly, when these authors look at diseased tissues, NO was decreased in pathologic CrCLs when compared to normal CrCLs.[120] One might first suggest that this indicates that NO derived from ligament likely does not play a major role in ligament pathogenesis. However, one most also consider that the duration of disease in this study was not considered, other than the mean duration of lameness was found to be 10 weeks. Furthermore one might also suggest that NO produced by ligament likely does not play a role in the initiation or progression of OA. However, a primary histologic feature of CrCL disease is decreased cellularity within the core of the CrCL. This study also did not evaluate the degree of cellularity in their explant samples. There could have been a smaller number of viable cells in the pathologic ligament, leading to decreased NO production. Ultimately, this studied demonstrated the enormous capacity of cruciate ligament to produce NO.

Some effort has been made to clarify the role of NO and the duration of disease by using only normal tissues. In a study by Riitano et al, explant cultures of intact CCLs that were previously shown to produce iNOS-induced NO could be stimulated by a combination of combination of interluekin-1 and tumor necrosis factor-α to increase production of iNOS, NO, and MMP.[121] Other studies have indicated that NO is involved in the activation of MMPs and revealed a relationship between NO and MMP in the stifle joint. Selective inhibition of NO production decreases MMP concentrations in cartilage.[119] These findings have furthered one hypothesis previously presented: matrix-degrading proteinases produced within the CrCL and activated through NO could be responsible ligament pathogenesis. Through NO, the activation of MMPs or other degradative enzymes could predispose the ligament for failure under normal loads.

This lineage of thinking has encouraged some groups to consider the use iNOS inhibitors to slow degradation of the collagen network and proteoglycans of the CrCL and/or in OA. One study evaluated the effectiveness of a selective inhibitor of iNOS, L-NIL, in attenuating the progression of experimental OA. The data suggest that L-NIL may act by reducing the activity of metalloproteases in cartilage and the production of IL-1beta by synovium, both of which are known to play a major role in the pathophysiology of OA structural changes.[118] Another study evaluated doxycycline as an inhibitor of NO production. The findings in this study indicate that doxycycline inhibits NO production in cartilage in dogs with CrCL rupture. The authors suggested that doxycycline may have a role in the treatment of canine OA by inhibiting NO production.[122]

The role of NO in other joints also remains unclear. However, there is some evidence that NO may act differently on different ligaments. This likely depends on

several factors including the ligament microenvirnment, the ligamentous blood supply, relative hypoxia, the type of force the ligament is subjected to (compressive forces, tensile forces, or both), whether the ligament is encompassed and closely associated with a synovial sheath and whether or not the ligament is extra-articular or intra-articular. To my knowledge, the majority of these have not been evaluated in the canine literature. However, some insight could be gained from considering a rabbit study that evaluated healing in several different ligaments. One important finding from this study was that not all ligamentocytes respond to stimuli in the same manner. In this study by Cao, et al, anterior cruciate ligaments (ACL), posterior cruciate ligaments (PCL), and medial collateral ligaments (MCL) were collected, processed, and grown to confluency in monolayer cell culture. These cultures were then stimulated with IL-1β. The ACL and PCL generated large amounts of NO. The MCL, in contrast, produce very little NO.[94] This provides some evidence that the role of NO may differ depending the ligament evaluated, its location, or both. To further support this, ACLs synthesized NO spontaneously whereas the MCL never did so.[94] Not only could the production of NO be unique in different joints, but its mechanism of action once NO has been produced could be different. When NO was administered to explant cultures, it prevented collagen synthesis on both the ACL and PCL, but had very little influence on the MCL.[94] So it appears, on both the cellular and the tissue level, that NO is behaving differently at different sites in the body. Further eNOS has been shown to be intrical in mediating angiogenesis: NO may regulate aspect of vascular endothelial growth factor (VEGF) signaling.[123] Indeed, this has lead some researchers to implicate NO a possible mediator of the differential healing capacities of the MCL and ACL.[94]

Aside from NO, there are more basic intrinsic factors that have been well studied and include the rate of cell proliferation and the rate of cell migration. One study found that migration from 45 to 134 hrs in culture, was 6-12 times greater for MCL cells than that for the ACL cells.[96] The large difference in cell numbers at early times of culture was due to the more rapid MCL cell migration out of the explants and not to a difference in the rate of cell proliferation.[96] This was later confirmed by additional studies that evaluated migration and proliferation of these cells.[99,124] In one study, the authors found that the outgrowth from cells from ACL explants was slower than from MCL explants.[124] This was confirmed with DNA synthesis measured in terms of [3H]thymidine incorporation of both log phase and confluent cultures.[124] Western blot analysis of cellular proteins revealed higher actin content in ACL cells than in MCL cells.[124] The authors also utilized an in vitro wound closure assay and found that ACL cultures were occupied partially by single cells in a nonconfluent fashion. In contrast, the wounded zone in the MCL cultures was nearly covered by the cells.[124] All of these data supports the theory that proliferation and migration rates are significant factors in the differential ability of MCL and ACL to heal.

Adhesion ability is also an intrinsic factor that has been evaluated in the literature, primarily through the study of integrins. The integrins are a family of cell surface receptors that mediate adhesion, migration, and interaction with extracellular matrix, all of which are critical to wound healing. In one study, a partial laceration was surgically created in rabbit MCL and ACL.[125] Immunohistochemistry was performed on sections from the ligaments at various time points and a substantial increase in staining for several subunits was observed for MCL.[125] In marked contrast, the ACL did not demonstrate a

comparable alteration of integrin expression from baseline levels.[125] The findings from this study lend support to the theory that integrins and adhesion are intrinsic factors that could affect ligament healing. Other studies have looked at cell adhesiveness more directly. One group utilized a micropipette aspiration technique to determine the forces needed to separate ACL or MCL cells from a fibronectin-coated surface.[126,127] Delivery of exogenous tropomodulin, an actin-filament capping protein, into MCL fibroblasts significantly increased adhesion strength.[126] As a follow-up portion to this experiment, it was found that tropomodulin's monoclonal antibody significantly decreased cell adhesiveness.[126] This was not the case for ACL cells where tropomodulin significantly reduced adhesion, whereas its mAb had no effect. [126] The results from this study demonstrate yet another way in which the differential healing of MCL and ACL could be explained.

Another possible intrinsic factor that could explain differential healing is the response to growth factors and hormones. One study compared the differential effects of epidermal growth factor (EGF) on adhesion and proliferation of ACL and MCL fibroblasts. MCL cells showed a significant increase in proliferation upon stimulation with EGF compared to ACL cells when cultured in fibronectin (FN) coated wells.[102] Following exposure to EGF, ACL and MCL cells responded by increasing their adhesion strength. MCL cells responded to all concentrations of EGF while ACL cells appeared to have a threshold concentration after which EGF effects plateau.[102] The results found in this study elucidated EGF's role in adhesion and proliferation, two factors outlined above that may contribute to the differential healing response between ACL and MCL. VGEF has also been implicated in a variety of function in the joint including the modulation of

various isoforms during cruciate ligament maturation[128], graft healing and remodeling[129-132], degree of laxity[133] and the invasion of vessels during the formation of osteophytes.[134] Aside from growth factors, the numerous paper that have looked estrogen and its role in the metabolism and pathogenesis in humans.[59-63] Human female athletes are 3-8 times as likely as there male counterparts to rupture their ligament.[49,59,64,65] The phases of the menstrual cycle and its phases have been evaluated and their relation to incidence of ACL injury has been described as "equivocal and controversial."[49] The influence of hormones has not been looked closely in veterinary medicine, but some researchers have suggested that adipokines may play a role in pathogenesis.[66]

There is a small body of evidence to suggest that the MCL and the ACL have a different ability to produce certain collagens under different loads. One in vitro study evaluated ACL cells and MCL cells under cyclic strain. Whereas anterior cruciate ligament fibroblasts responded to cyclic strains by expression of higher levels of type-I collagen message with very little increase in type-III collagen, medial collateral ligament fibroblasts demonstrated significant increases in type-III collagen mRNA.[98] MCL cells demonstrated almost no significant increase in type-I collagen.[98] Furthermore, differences in responses by fibroblasts from the two ligaments were detected between the two strain magnitudes. For instance, 7.5 % strains induced a time-dependent increase in type-III collagen mRNA levels in medial collateral ligament fibroblasts whereas 5 % strains did not.[98] These differences in collagen production imply that they may be involved in the differential healing capacities of the ACL and MCL.

There are other potential factors that may play a role in the differential healing observed between the MCL and ACL, but have been poorly studied. Some of these

include lack of apposition of the healing ends, joint instability, factors in the local environment, and degree of apoptosis following injury. Most of these have only been mentioned in the literature and have not had more detailed study devoted to their potential mechanisms. It is likely that together, all of these contribute, to some degree, to the healing disparity noted in these two ligaments and further study may produce a more unified mechanism for differential response to injury and healing of these two ligaments.

The degradative molecules are thought to be responsible for ligament pathogenesis are the same molecules that have the potential to leak from the ligament into the synovial fluid; these molecules have been extensively studied and could cause the initiation or progression osteoarthritis. There are numerous studies that implicate the matrix metalloproteinases in the ligament pathogenesis. The collagenases have been identified in CrCL on both the gene and the protein level.[121,135-140] The gelatinases have also been characterized in a similar manner[109,138,141] as have the cathepsins and other enzymes.[56,138,142,143] The documentation that these degradative enzymes are present within the ligament is evidence that supports complete ligament debridement.

Treatment of cruciate ligament deficiency involves both medical and surgical intervention. Medical management includes, first and foremost, weight management. Other important avenues of medical management include the administration of non-steroidal anti-inflammatory drugs (NSAIDs), disease-modifying osteoarthritis drugs (DMOADs), non-concussive activity and physical therapy. Surgical intervention is commonly used to address cruciate insufficiency. However, most surgeons are not completely satisfied with current techniques for addressing this disease. The are more than 20 techniques in the literature design to address this disease.[70,144-169] Indeed, a

plethora of surgical interventions implies that none of the procedures is completely effective at addressing all of the components of the disease process. This is exactly the case with cruciate insufficiency. Surgeons are unsatisfied with invariable progression of OA following surgical intervention, displeased the failure of surgical techniques to address all biomechanical abnormalities or disenchanted with the severity of complications associated with some surgical techniques. Comparison surgical techniques have not definitively suggested one technique as superior.[170-173] The future may bring new technique including those involving tissue engineering[87,95,174-182], modifications of grafting procedures[3,85,145,146,183-190], growth factors[100,179-181,191-198], gene therapy[199-201] and stem cells[182,193,202-207].

Despite the surgical technique utilized, progression of osteoarthritis occurs in all cases.[38,208-222] Why this progressive OA cannot be halted is not entirely known but has been assumed to be due abnormal joint biomechanics and kinematics. Little thought has been given to the possibility of ligament itself contributing to OA. With the advent of the TPLO many veterinary surgeons stopped performing arthrotomies to inspect and deride the cranial cruciate ligament (CrCL) as well as to inspect other potential intra-articular pathology. Whether to provide complete debridement of the CrCL at the time of surgery is a frustrating and difficult question to answer. If there were conclusive studies that indicate leaving or debriding the ligament was more appropriate, then there would not be so much debate. The current best evidence in the literature neither supports nor dismisses the need for ligament debridement. Evaluation of this evidence is complicated by the fact that there appears to be a lack of basic science evidence that suggests leaving the CrCL and a lack of clinical evidence for providing debridement. Some surgeons have started to

utilize a limited caudal medial arthrotomy to address meniscal tears.[167,217] However, this approach prevents any evaluation or debridement of the ligaments because direct visualization cannot be achieved through this approach. One aim of the present dissertation was to provide basic science evidence that the ligament is capable of releasing degradative molecules and inflammatory mediators that could contribute to the progression of OA. If this basic science evidence could be demonstrated, then surgeons should consider complete debridement of the ligament. The author would like to point out however, that demonstrating theses molecules are released from ligament does not necessarily mean that ligament should be debrided in all cases. Further research may be needed to identify which cases debridement should be provided and which cases are amendable to ligamentous remodeling without substantial progression of OA as a result of leaving the ligament *in situ*.

To date there is no clinical evidence that supports the complete debridement of these ligaments. There are no clinical studies that definitively show that dogs that receive ligament debridement have less OA or have better function then dogs in which the ligament was left *in situ*. Likewise there is very little basic science evidence that supports leaving the ligament intact within the stifle joint, which seems difficult to justify based on the overwhelming evidence of harmful molecules that have been documented to be present within the ligament. There is however, some clinical evidence that suggests that partial tears should be left un-debrided *in situ*. Some evidence comes from a clinical study that evaluated partial tears within the joint. Arthroscopy was performed in 16 dogs (22 stifles). Of the 22 ligaments that sustained partial tears of the cranial cruciate ligament, 19 were intact during the second-look arthroscopy.[112] This strongly suggests

that some ligaments do indeed have the ability to normalize themselves following a traumatic event. In other words, once the process of degradation is initiated, the ligament is not necessarily committed to continue down this path of degradation and eventual complete resorption. It is possible that the identified molecules of degradation are not necessarily part of a pathologic process, but part of a remodeling process. Even if the literature eventually demonstrates that there is far greater degradative shift in these pathologic ligaments, the mere presence of these molecules does not automatically mean that they are involved in osteoarthritis or any disease process for that matter. Given enough time the ligaments may remodel and re-achieve a state of normal turnover. This is somewhat supported by the observation that on second-look arthroscopy these joints appeared fairly quiescent. Indeed, the villous hypertrophy present at the time of an acute ligament tear subsided at follow-up examination as evidenced by a decrease in the severity of synovitis.[112] Again, the authors suggested that the improved appearance of the cranial cruciate ligament supports the theory that TPLO reduces strain on the cranial cruciate ligament.[112] It is also possible that the period of relative inactivity, and not the surgical procedure itself, allows enough time for the ligament to remodel. One explanation for all this evidence is that there may be specific clinical indications for both debridement and leaving the ligament in situ. These indications would need to be identified in future studies. Ultimately, the disparity between both arguments in term of clinical and basic science research indicates that additional studies should and need to be performed in order to optimize treatment of CrCL disease.

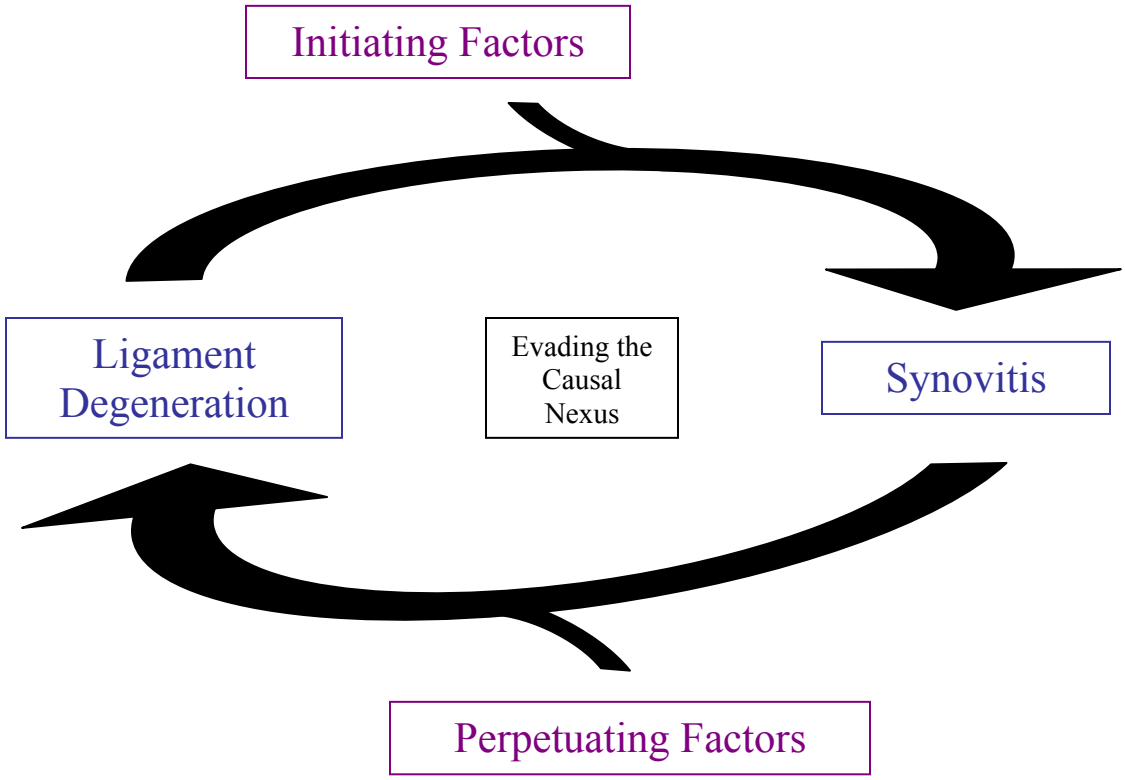

Figure 2-1: Evading the casual nexus. There is debate as to whether arthritic changes precede ligament rupture or follow it and some investigators refer to this as the proverbial chicken or the egg scenario. It is unlikely that one pathologic process (ligament degeneration or synovitis) is solely responsible for or leads to the other process. Instead, it may be important to evade this causal nexus by identify initiating and perpetuating factors in this disease process. Initiating factor might include factors such as genetics, conformation and obesity. Perpetuating factors might include chronic instability, degradative enzymes, and inflammation. Initiating and perpetuating factors are not necessarily independent of each other. It is more likely that ligament degeneration and synovitis are interrelated and one contributes to the other in a perpetual cycle.

References

1. Yahia LH, Newman NM, St-Georges M. Innervation of the canine cruciate ligaments. A neurohistological study. Anatomia, Histologia, Embryologia: Veterinary Medicine Series C 1992;21(1):1-8.

2. Arnoczky SP. Anatomy of the anterior cruciate ligament. Clin Orthop Relat Res 1983(172):19-25.

3. Arnoczky SP. Biology of ACL reconstructions: what happens to the graft? Instr Course Lect 1996;45:229-33.

4. Hayashi K, Frank JD, Dubinsky C, Zhengling H, Markel MD, Manley PA, et al. Histologic changes in ruptured canine cranial cruciate ligament. Vet Surg 2003;32(3):269-77.

5. Murray MM, Martin SD, Martin TL, Spector M. Histological changes in the human anterior cruciate ligament after rupture. J Bone Joint Surg Am 2000;82-A(10):1387-97.

6. Narama I, Masuoka-Nishiyama M, Matsuura T, Ozaki K, Nagatani M, Morishima T. Morphogenesis of degenerative changes predisposing dogs to rupture of the cranial cruciate ligament. J Vet Med Sci 1996;58(11):1091-7.

7. Vasseur PB, Pool RR, Arnoczky SP, Lau RE. Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs. Am J Vet Res 1985;46(9):1842-54.

8. Hayashi K, Frank JD, Hao Z, Schamberger GM, Markel MD, Manley PA, et al. Evaluation of ligament fibroblast viability in ruptured cranial cruciate ligament of dogs. Am J Vet Res 2003;64(8):1010-6.

9. Murray MM, Spector M. Fibroblast distribution in the anteromedial bundle of the human anterior cruciate ligament: the presence of alpha-smooth muscle actin-positive cells. J Orthop Res 1999;17(1):18-27.

10. Comerford EJ, Tarlton JF, Wales A, Bailey AJ, Innes JF. Ultrastructural differences in cranial cruciate ligaments from dogs of two breeds with a differing predisposition to ligament degeneration and rupture. J Comp Pathol 2006;134(1):8-16.

11. Comerford EJ, Innes JF, Tarlton JF, Bailey AJ. Investigation of the composition, turnover, and thermal properties of ruptured cranial cruciate ligaments of dogs. Am J Vet Res 2004;65(8):1136-41.

12. Woo SLY, Buckwalter JA. Injury and repair of the musculoskeletal soft tissues : workshop, Savannah, Georgia, June 1987. Park Ridge, Ill. :: American Academy of Orthopaedic Surgeons; 1988.

13. Innes JF. Personal Communication. In. San Diego, CA; 2005.

14. Stubbs G, Dahlstrom J, Papantoniou P, Cherian M. Correlation between macroscopic changes of arthrosis and the posterior cruciate ligament histology in the osteoarthritic knee. ANZ J Surg 2005;75(12):1036-40.

15. Heffron LE, Campbell JR. Morphology, histology and functional anatomy of the canine cranial cruciate ligament. Veterinary Record 1978;102(13):280-283.

16. Arnoczky SP, Rubin RM, Marshall JL. Microvasculature of the cruciate ligaments and its response to injury. An experimental study in dogs. Journal of Bone and Joint Surgery 1979;61A(8):1221-1229.

17. Arnoczky SP, Marshall JL. The cruciate ligaments of the canine stifle: an anatomical and functional analysis. Am J Vet Res 1977;38(11):1807-14.

18. Hayashi K, Manley PA, Muir P. Cranial cruciate ligament pathophysiology in dogs with cruciate disease: a review. J Am Anim Hosp Assoc 2004;40(5):385-90.

19. Arcand MA, Rhalmi S, Rivard CH. Quantification of mechanoreceptors in the canine anterior cruciate ligament. Int Orthop 2000;24(5):272-5.

20. Halata Z, Wagner C, Baumann KI. Sensory nerve endings in the anterior cruciate ligament (Lig. cruciatum anterius) of sheep. Anat Rec 1999;254(1):13-21.

21. Koch B, Kurriger G, Brand RA. Characterisation of the neurosensory elements of the feline cranial cruciate ligament. J Anat 1995;187 (Pt 2):353-9.

22. Krogsgaard MR, Dyhre-Poulsen P, Fischer-Rasmussen T. Cruciate ligament reflexes. J Electromyogr Kinesiol 2002;12(3):177-82.

23. Wingfield C, Amis AA, Stead AC, Law HT. Cranial cruciate stability in the rottweiler and racing greyhound: an in vitro study. J Small Anim Pract 2000;41(5):193-7.

24. Wingfield C, Amis AA, Stead AC, Law HT. Comparison of the biomechanical properties of Rottweiler and racing Greyhound cranial cruciate ligaments. Journal of Small Animal Practice 2000;41(7):303-307.

25. Alm A, Ekstrom H, Stromberg B. Tensile strength of the anterior cruciate ligament in the dog. Acta Chirurgica Scandinavica - Supplementum 1974;445:15-23.

26. Gupta BN, Brinker WO, Subramanian KN. Breaking strength of cruciate ligaments in the dog. Journal of the American Veterinary Medical Association 1969;155(10):1586-8.

27. Scapinelli R. Vascular anatomy of the human cruciate ligaments and surrounding structures. Clin Anat 1997;10(3):151-62.

28. Tirgari M. Changes in the canine stifle joint following rupture of the anterior cruciate ligament. Journal of Small Animal Practice 1978;19(1):17-26.

29. Temwichitr J, Hazewinkel HA, van Hagen MA, Leegwater PA. Polymorphic microsatellite markers for genetic analysis of collagen genes in suspected collagenopathies in dogs. J Vet Med A Physiol Pathol Clin Med 2007;54(9):522-6.

30. de Rooster H, de Bruin T, van Bree H. Morphologic and functional features of the canine cruciate ligaments. Vet Surg 2006;35(8):769-80.

31. Wilke VL, Conzemius MC, Rothschild MF. SNP detection and association analyses of candidate genes for rupture of the cranial cruciate ligament in the dog. Anim Genet 2005;36(6):519-21.

32. Wilke VL, Conzemius MG, Kinghorn BP, Macrossan PE, Cai W, Rothschild MF. Inheritance of rupture of the cranial cruciate ligament in Newfoundlands. J Am Vet Med Assoc 2006;228(1):61-4.

33. Duval JM, Budsberg SC, Flo GL, Sammarco JL. Breed, sex, and body weight as risk factors for rupture of the cranial cruciate ligament in young dogs. J Am Vet Med Assoc 1999;215(6):811-4.

34. Whitehair JG, Vasseur PB, Willits NH. Epidemiology of cranial cruciate ligament rupture in dogs. J Am Vet Med Assoc 1993;203(7):1016-9.

35. Morris E, Lipowitz AJ. Comparison of tibial plateau angles in dogs with and without cranial cruciate ligament injuries. J Am Vet Med Assoc 2001;218(3):363-6.

36. Baroni E, Matthias RR, Marcellin-Little DJ, Vezzoni A, Stebbins ME. Comparison of radiographic assessments of the tibial plateau slope in dogs. Am J Vet Res 2003;64(5):586-9.

37. Fettig AA, Rand WM, Sato AF, Solano M, McCarthy RJ, Boudrieau RJ. Observer variability of tibial plateau slope measurement in 40 dogs with cranial cruciate ligament-deficient stifle joints. Veterinary Surgery. 2003;32(5):471-8.

38. Fujita Y, Hara Y, Ochi H, Nezu Y, Harada Y, Yogo T, et al. The possible role of the tibial plateau angle for the severity of osteoarthritis in dogs with cranial cruciate ligament rupture. J Vet Med Sci 2006;68(7):675-9.

39. Havig ME, Dyce J, Kowaleski MP, Reynolds LR, Budsberg SC. Relationship of tibial plateau slope to limb function in dogs treated with a lateral suture technique

for stabilization of cranial cruciate ligament deficient stifles. Vet Surg 2007;36(3):245-51.

40. Odders JW, Jessen CR, Lipowitz AJ. Sequential measurements of the tibial plateau angle in large-breed, growing dogs. Am J Vet Res 2004;65(4):513-8.

41. Osmond CS, Marcellin-Little DJ, Harrysson OL, Kidd LB. Morphometric assessment of the proximal portion of the tibia in dogs with and without cranial cruciate ligament rupture. Vet Radiol Ultrasound 2006;47(2):136-41.

42. Reif U, Probst CW. Comparison of tibial plateau angles in normal and cranial cruciate deficient stifles of Labrador retrievers. Vet Surg 2003;32(4):385-9.

43. Shahar R, Milgram J. Biomechanics of tibial plateau leveling of the canine cruciate-deficient stifle joint: a theoretical model. Vet Surg 2006;35(2):144-9.

44. Talaat MB, Kowaleski MP, Boudrieau RJ. Combination tibial plateau leveling osteotomy and cranial closing wedge osteotomy of the tibia for the treatment of cranial cruciate ligament-deficient stifles with excessive tibial plateau angle. Vet Surg 2006;35(8):729-39.

45. Warzee CC, Dejardin LM, Arnoczky SP, Perry RL. Effect of tibial plateau leveling on cranial and caudal tibial thrusts in canine cranial cruciate-deficient stifles: an in vitro experimental study. Vet Surg 2001;30(3):278-86.

46. Wilke VL, Conzemius MG, Besancon MF, Evans RB, Ritter M. Comparison of tibial plateau angle between clinically normal Greyhounds and Labrador Retrievers with and without rupture of the cranial cruciate ligament. J Am Vet Med Assoc 2002;221(10):1426-9.

47. Zeltzman PA, Pare B, Johnson GM, Zeltzman V, Robbins MA, Gendreau CL. Relationship between age and tibial plateau angle in dogs with cranial cruciate rupture. J Am Anim Hosp Assoc 2005;41(2):117-20.

48. Boden BP, Dean GS, Feagin JA, Jr., Garrett WE, Jr. Mechanisms of anterior cruciate ligament injury. Orthopedics 2000;23(6):573-8.

49. Hewett TE, Myer GD, Ford KR. Anterior cruciate ligament injuries in female athletes: Part 1, mechanisms and risk factors. Am J Sports Med 2006;34(2):299-311.

50. Hewicker-Trautwein M, Carter SD, Bennett D, Kelly DF. Immunocytochemical demonstration of lymphocyte subsets and MHC class II antigen expression in synovial membranes from dogs with rheumatoid arthritis and degenerative joint disease. Veterinary Immunology & Immunopathology 1999;67(4):341-57.

51. Lemburg AK, Meyer-Lindenberg A, Hewicker-Trautwein M. Immunohistochemical characterization of inflammatory cell populations and

adhesion molecule expression in synovial membranes from dogs with spontaneous cranial cruciate ligament rupture. Veterinary Immunology & Immunopathology 2004;97(3-4):231-40.

52. Muir P, Manley PA, Hao Z. Collagen fragmentation in ruptured canine cranial cruciate ligament explants. Vet J 2006;172(1):121-8.

53. Muir P, Manley PA, Hao Z. COL-3 inhibition of collagen fragmentation in ruptured cranial cruciate ligament explants from dogs with stifle arthritis. Vet J 2007;174(2):403-6.

54. Muir P, Oldenhoff WE, Hudson AP, Manley PA, Schaefer SL, Markel MD, et al. Detection of DNA from a range of bacterial species in the knee joints of dogs with inflammatory knee arthritis and associated degenerative anterior cruciate ligament rupture. Microb Pathog 2007;42(2-3):47-55.

55. Muir P, Schaefer SL, Manley PA, Svaren JP, Oldenhoff WE, Hao Z. Expression of immune response genes in the stifle joint of dogs with oligoarthritis and degenerative cranial cruciate ligament rupture. Vet Immunol Immunopathol 2007;119(3-4):214-21.

56. Muir P, Schamberger GM, Manley PA, Hao Z. Localization of cathepsin K and tartrate-resistant acid phosphatase in synovium and cranial cruciate ligament in dogs with cruciate disease. Vet Surg 2005;34(3):239-46.

57. de Bruin T, de Rooster H, van Bree H, Cox E. Evaluation of anticollagen type I antibody titers in synovial fluid of both stifle joints and the left shoulder joint of dogs with unilateral cranial cruciate disease. Am J Vet Res 2007;68(3):283-9.

58. de Rooster H, Cox E, van Bree H. Prevalence and relevance of antibodies to type-I and -II collagen in synovial fluid of dogs with cranial cruciate ligament damage. Am J Vet Res 2000;61(11):1456-61.

59. Beynnon BD, Johnson RJ, Braun S, Sargent M, Bernstein IM, Skelly JM, et al. The relationship between menstrual cycle phase and anterior cruciate ligament injury: a case-control study of recreational alpine skiers. Am J Sports Med 2006;34(5):757-64.

60. Komatsuda T, Sugita T, Sano H, Kusakabe T, Watanuki M, Yoshizumi Y, et al. Does estrogen alter the mechanical properties of the anterior cruciate ligament? An experimental study in rabbits. Acta Orthop 2006;77(6):973-80.

61. Rau MD, Renouf D, Benfield D, Otto DD, Thornton GM, Raso VJ, et al. Examination of the failure properties of the anterior cruciate ligament during the estrous cycle. Knee 2005;12(1):37-40.

62. Seneviratne A, Attia E, Williams RJ, Rodeo SA, Hannafin JA. The effect of estrogen on ovine anterior cruciate ligament fibroblasts: cell proliferation and collagen synthesis. Am J Sports Med 2004;32(7):1613-8.

63. Slauterbeck J, Clevenger C, Lundberg W, Burchfield DM. Estrogen level alters the failure load of the rabbit anterior cruciate ligament. J Orthop Res 1999;17(3):405-8.

64. Shultz SJ, Sander TC, Kirk SE, Perrin DH. Sex differences in knee joint laxity change across the female menstrual cycle. J Sports Med Phys Fitness 2005;45(4):594-603.

65. Adachi N, Nawata K, Maeta M, Kurozawa Y. Relationship of the menstrual cycle phase to anterior cruciate ligament injuries in teenaged female athletes. Arch Orthop Trauma Surg 2008;128(5):473-8.

66. Innes J. Do hormones play a role in canine cruciate disease? J Small Anim Pract 2003;44(11):520.

67. Slauterbeck JR, Pankratz K, Xu KT, Bozeman SC, Hardy DM. Canine ovariohysterectomy and orchiectomy increases the prevalence of ACL injury. Clin Orthop Relat Res 2004(429):301-5.

68. Aiken SW, Kass PH, Toombs JP. Intercondylar notch width in dogs with and without cranial cruciate ligament injuries. Veterinary and Comparative Orthopaedics and Traumatology 1995;8(3):128-132.

69. Comerford EJ, Tarlton JF, Avery NC, Bailey AJ, Innes JF. Distal femoral intercondylar notch dimensions and their relationship to composition and metabolism of the canine anterior cruciate ligament. Osteoarthritis Cartilage 2005.

70. Fitch RB, Montgomery RD, Kincaid SA, Hatchcock JT, Milton JL, Garrett PD, et al. The effect of intercondylar notchplasty on the normal canine stifle. Vet Surg 1995;24(2):156-64.

71. Fitch RB, Montgomery RD, Milton JL, Garrett PD, Kincaid SA, Wright JC, et al. The intercondylar fossa of the normal canine stifle an anatomic and radiographic study. Vet Surg 1995;24(2):148-55.

72. Lipowitz AJ, Wong PL, Stevens JB. Synovial membrane changes after experimental transection of the cranial cruciate ligament in dogs. American Journal of Veterinary Research 1985;46(5):1166-70.

73. Lawrence D, Bao S, Canfield PJ, Allanson M, Husband AJ. Elevation of immunoglobulin deposition in the synovial membrane of dogs with cranial cruciate ligament rupture. Veterinary Immunology & Immunopathology 1998;65(1):89-96.

74. Galloway RH, Lester SJ. Histopathological evaluation of canine stifle joint synovial membrane collected at the time of repair of cranial cruciate ligament rupture. Journal of the American Animal Hospital Association 1995;31(4):289-94.

75. Jean YH, Wen ZH, Chang YC, Huang GS, Lee HS, Hsieh SP, et al. Increased concentrations of neuro-excitatory amino acids in rat anterior cruciate ligament-transected knee joint dialysates: a microdialysis study. Journal of Orthopaedic Research 2005;23(3):569-75.

76. Pond MJ, Nuki G. Experimentally-induced osteoarthritis in the dog. Ann Rheum Dis 1973;32(4):387-8.

77. Bauer D, Muller H, Reich J, Riedel H, Ahrenkiel V, Warthoe P, et al. Identification of differentially expressed mRNA species by an improved display technique (DDRT-PCR). Nucleic Acids Res 1993;21(18):4272-80.

78. Bluteau G, Gouttenoire J, Conrozier T, Mathieu P, Vignon E, Richard M, et al. Differential gene expression analysis in a rabbit model of osteoarthritis induced by anterior cruciate ligament (ACL) section. Biorheology 2002;39(1-2 Special Issue SI):247-258.

79. Ito T, Kito K, Adati N, Mitsui Y, Hagiwara H, Sakaki Y. Fluorescent differential display: arbitrarily primed RT-PCR fingerprinting on an automated DNA sequencer. FEBS Lett 1994;351(2):231-6.

80. Liang P, Pardee AB. Differential display. A general protocol. Mol Biotechnol 1998;10(3):261-7.

81. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 1992;257(5072):967-71.

82. Muller-Ladner U, Judex M, Justen HP, Wessinghage D, Welsh J, McClelland M, et al. Analysis of gene expression patterns in rheumatoid synovial fibroblasts using RAP-PCR for differential display. Medizinische Klinik 1999;94(4):228-232.

83. Patel IR, Attur MG, Patel RN, Stuchin SA, Abagyan RA, Abramson SB, et al. TNF-alpha convertase enzyme from human arthritis-affected cartilage: isolation of cDNA by differential display, expression of the active enzyme, and regulation of TNF-alpha. Journal of Immunology 1998;160(9):4570-9.

84. Scaife S, Brown R, Kellie S, Filer A, Martin S, Thomas AM, et al. Detection of differentially expressed genes in synovial fibroblasts by restriction fragment differential display. Rheumatology 2004;43(11):1346-52.

85. Jackson DW. The Anterior cruciate ligament : current and future concepts. New York :: Raven Press; 1993.

86. Van Hoogmoed LM, Agnew DW, Whitcomb M, Hyde DW, MacDonald MH, Snyder JR. Ultrasonographic and histologic evaluation of medial and middle patellar ligaments in exercised horses following injection with ethanolamine oleate and 2% iodine in almond oil. Am J Vet Res 2002;63(5):738-43.

87. Murray MM, Martin SD, Spector M. Migration of cells from human anterior cruciate ligament explants into collagen-glycosaminoglycan scaffolds. J Orthop Res 2000;18(4):557-64.

88. Murray MM, Spector M. The migration of cells from the ruptured human anterior cruciate ligament into collagen-glycosaminoglycan regeneration templates in vitro. Biomaterials 2001;22(17):2393-402.

89. Murray MM, Weiler A, Spindler KP. Interspecies variation in the fibroblast distribution of the anterior cruciate ligament. Am J Sports Med 2004;32(6):1484-91.

90. Gyger O, Botteron C, Doherr M, Zurbriggen A, Schawalder P, Spreng D. Detection and distribution of apoptotic cell death in normal and diseased canine cranial cruciate ligaments. Vet J 2007;174(2):371-7.

91. Bhargava MM, Beavis AJ, Edberg JC, Warren RF, Attia ET, Hannafin JA. Differential expression of integrin subunits in canine knee ligament fibroblasts. J Orthop Res 1999;17(5):748-54.

92. Bray RC, Leonard CA, Salo PT. Vascular physiology and long-term healing of partial ligament tears. J Orthop Res 2002;20(5):984-9.

93. Bray RC, Leonard CA, Salo PT. Correlation of healing capacity with vascular response in the anterior cruciate and medial collateral ligaments of the rabbit. J Orthop Res 2003;21(6):1118-23.

94. Cao M, Stefanovic-Racic M, Georgescu HI, Fu FH, Evans CH. Does nitric oxide help explain the differential healing capacity of the anterior cruciate, posterior cruciate, and medial collateral ligaments? Am J Sports Med 2000;28(2):176-82.

95. Cooper JA, Jr., Bailey LO, Carter JN, Castiglioni CE, Kofron MD, Ko FK, et al. Evaluation of the anterior cruciate ligament, medial collateral ligament, achilles tendon and patellar tendon as cell sources for tissue-engineered ligament. Biomaterials 2006;27(13):2747-54.

96. Geiger MH, Green MH, Monosov A, Akeson WH, Amiel D. An in vitro assay of anterior cruciate ligament (ACL) and medial collateral ligament (MCL) cell migration. Connective Tissue Research 1994;30(3):215-24.

97. Hannafin JA, Attia ET, Warren RF, Bhargava MM. Characterization of chemotactic migration and growth kinetics of canine knee ligament fibroblasts. J Orthop Res 1999;17(3):398-404.

98. Hsieh AH, Tsai CM, Ma QJ, Lin T, Banes AJ, Villarreal FJ, et al. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. J Orthop Res 2000;18(2):220-7.

99. Kobayashi K, Healey RM, Sah RL, Clark JJ, Tu BP, Goomer RS, et al. Novel method for the quantitative assessment of cell migration: a study on the motility of rabbit anterior cruciate (ACL) and medial collateral ligament (MCL) cells. Tissue Engineering 2000;6(1):29-38.

100. Lee J, Harwood FL, Akeson WH, Amiel D. Growth factor expression in healing rabbit medial collateral and anterior cruciate ligaments. Iowa Orthop J 1998;18:19-25.

101. Marui T, Niyibizi C, Georgescu HI, Cao M, Kavalkovich KW, Levine RE, et al. Effect of growth factors on matrix synthesis by ligament fibroblasts. Journal of Orthopaedic Research 1997;15(1):18-23.

102. McKean JM, Hsieh AH, Sung KL. Epidermal growth factor differentially affects integrin-mediated adhesion and proliferation of ACL and MCL fibroblasts. Biorheology 2004;41(2):139-52.

103. Murakami H, Shinomiya N, Kikuchi T, Fujikawa K, Nemoto K. Differential sensitivity to NO-induced apoptosis between anterior cruciate and medial collateral ligament cells. Journal of Orthopaedic Science 2005;10(1):84-90.

104. Ross SM, Joshi R, Frank CB. Establishment and comparison of fibroblast cell lines from the medial collateral and anterior cruciate ligaments of the rabbit. In Vitro Cell Dev Biol 1990;26(6):579-84.

105. Sung KL, Kwan MK, Maldonado F, Akeson WH. Adhesion strength of human ligament fibroblasts. Journal of Biomechanical Engineering 1994;116(3):237-42.

106. Wallace CD, Amiel D. Vascular assessment of the periarticular ligaments of the rabbit knee. J Orthop Res 1991;9(6):787-91.

107. Witkowski J, Yang L, Wood DJ, Sung KL. Migration and healing of ligament cells under inflammatory conditions. Journal of Orthopaedic Research 1997;15(2):269-77.

108. Yang L, Tsai CM, Hsieh AH, Lin VS, Akeson WH, Sung KL. Adhesion strength differential of human ligament fibroblasts to collagen types I and III. Journal of Orthopaedic Research 1999;17(5):755-62.

109. Zhou D, Lee HS, Villarreal F, Teng A, Lu E, Reynolds S, et al. Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts. J Orthop Res 2005;23(4):949-57.

110. Bray RC, Fisher AW, Frank CB. Fine vascular anatomy of adult rabbit knee ligaments. J Anat 1990;172:69-79.

111. Bray RC, Rangayyan RM, Frank CB. Normal and healing ligament vascularity: a quantitative histological assessment in the adult rabbit medial collateral ligament. J Anat 1996;188 (Pt 1):87-95.

112. Beale BS, Hulse DA. Second-Look arthroscopy: What happens after TPLO. In: *2005 American College of Veterinary Surgeons Veterinary Symposium* 2005; San Diego, CA; 2005. p. 474-477.

113. Evans CH, Stefanovic-Racic M, Lancaster J. Nitric oxide and its role in orthopaedic disease. Clin Orthop Relat Res 1995(312):275-94.

114. Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96(5):2357-63.

115. Haruna Y, Morita Y, Komai N, Yada T, Sakuta T, Tomita N, et al. Endothelial dysfunction in rat adjuvant-induced arthritis: vascular superoxide production by NAD(P)H oxidase and uncoupled endothelial nitric oxide synthase. Arthritis Rheum 2006;54(6):1847-55.

116. Pelletier JP, Mineau F, Ranger P, Tardif G, Martel-Pelletier J. The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation. Osteoarthritis Cartilage 1996;4(1):77-84.

117. Grabowski PS, Macpherson H, Ralston SH. Nitric oxide production in cells derived from the human joint. Br J Rheumatol 1996;35(3):207-12.

118. Pelletier JP, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, et al. Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. Arthritis & Rheumatism 1998;41(7):1275-86.

119. Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, et al. Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. Arthritis Rheum 2000;43(6):1290-9.

120. Spreng D, Sigrist N, Jungi T, Busato A, Lang J, Pfister H, et al. Nitric oxide metabolite production in the cranial cruciate ligament, synovial membrane, and articular cartilage of dogs with cranial cruciate ligament rupture. Am J Vet Res 2000;61(5):530-6.

121. Riitano MC, Pfister H, Engelhardt P, Neumann U, Reist M, Zurbriggen A, et al. Effects of stimulus with proinflammatory mediators on nitric oxide production and matrix metalloproteinase activity in explants of cranial cruciate ligaments obtained from dogs. Am J Vet Res 2002;63(10):1423-8.

122. Jauernig S, Schweighauser A, Reist M, Von Rechenberg B, Schawalder P, Spreng D. The effects of doxycycline on nitric oxide and stromelysin production in dogs with cranial cruciate ligament rupture. Vet Surg 2001;30(2):132-9.

123. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest 1997;100(12):3131-9.

124. Nagineni CN, Amiel D, Green MH, Berchuck M, Akeson WH. Characterization of the intrinsic properties of the anterior cruciate and medial collateral ligament cells: an in vitro cell culture study. Journal of Orthopaedic Research 1992;10(4):465-75.

125. Schreck PJ, Kitabayashi LR, Amiel D, Akeson WH, Woods VL, Jr. Integrin display increases in the wounded rabbit medial collateral ligament but not the wounded anterior cruciate ligament. Journal of Orthopaedic Research 1995;13(2):174-83.

126. Sung KL, Yang L, Whittemore DE, Shi Y, Jin G, Hsieh AH, et al. The differential adhesion forces of anterior cruciate and medial collateral ligament fibroblasts: effects of tropomodulin, talin, vinculin, and alpha-actinin. Proceedings of the National Academy of Sciences of the United States of America 1996;93(17):9182-7.

127. Sung KL, Whittemore DE, Yang L, Amiel D, Akeson WH. Signal pathways and ligament cell adhesiveness. Journal of Orthopaedic Research 1996;14(5):729-35.

128. Hofstaetter JG, Saad FA, Sunk IG, Bobacz K, Friehs I, Glimcher MJ. Age-dependent expression of VEGF isoforms and receptors in the rabbit anterior cruciate ligament. Biochim Biophys Acta 2007;1770(7):997-1002.

129. Kohno T, Ishibashi Y, Tsuda E, Kusumi T, Tanaka M, Toh S. Immunohistochemical demonstration of growth factors at the tendon-bone interface in anterior cruciate ligament reconstruction using a rabbit model. J Orthop Sci 2007;12(1):67-73.

130. Kanazawa T, Soejima T, Murakami H, Inoue T, Katouda M, Nagata K. An immunohistological study of the integration at the bone-tendon interface after reconstruction of the anterior cruciate ligament in rabbits. J Bone Joint Surg Br 2006;88(5):682-7.

131. Petersen W, Unterhauser F, Pufe T, Zantop T, Sudkamp NP, Weiler A. The angiogenic peptide vascular endothelial growth factor (VEGF) is expressed

during the remodeling of free tendon grafts in sheep. Arch Orthop Trauma Surg 2003;123(4):168-74.

132. Petersen W, Pufe T, Zantop T, Tillmann B, Mentlein R. Hypoxia and PDGF have a synergistic effect that increases the expression of the angiogenetic peptide vascular endothelial growth factor in Achilles tendon fibroblasts. Arch Orthop Trauma Surg 2003;123(9):485-8.

133. Yoshikawa T, Tohyama H, Katsura T, Kondo E, Kotani Y, Matsumoto H, et al. Effects of local administration of vascular endothelial growth factor on mechanical characteristics of the semitendinosus tendon graft after anterior cruciate ligament reconstruction in sheep. Am J Sports Med 2006;34(12):1918-25.

134. Hashimoto S, Creighton-Achermann L, Takahashi K, Amiel D, Coutts RD, Lotz M. Development and regulation of osteophyte formation during experimental osteoarthritis. Osteoarthritis Cartilage 2002;10(3):180-7.

135. Attia EH, R; Bhargava M, Hannafin J. Gene Expressiion of Collagen I, Collagen III, Alpha-Smooth Muscle Actin and MMP-1 in a Rabbit Partial ACL Transection Model *52nd Annual Meeting of the Orthopaedic Research Society* 2006(Paper 1130).

136. Foos MJ, Hickox JR, Mansour PG, Slauterbeck JR, Hardy DM. Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament. J Orthop Res 2001;19(4):642-9.

137. Lavagnino M, Arnoczky SP, Tian T, Vaupel Z. Effect of Amplitude and Frequency of Cyclic Tensile Strain on the Inhibition of MMP-1 mRNA Expression in Tendon Cells: An In Vitro Study. Connect Tissue Res 2003;44(3-4):181-7.

138. Muir P, Danova NA, Argyle DJ, Manley PA, Hao Z. Collagenolytic protease expression in cranial cruciate ligament and stifle synovial fluid in dogs with cranial cruciate ligament rupture. Vet Surg 2005;34(5):482-90.

139. Spindler KP, Clark SW, Nanney LB, Davidson JM. Expression of collagen and matrix metalloproteinases in ruptured human anterior cruciate ligament: an in situ hybridization study. J Orthop Res 1996;14(6):857-61.

140. Amiel D, Billings E, Jr., Harwood FL. Collagenase activity in anterior cruciate ligament: protective role of the synovial sheath. J Appl Physiol 1990;69(3):902-6.

141. Volk SW, Kapatkin AS, Haskins ME, Walton RM, D'Angelo M. Gelatinase activity in synovial fluid and synovium obtained from healthy and osteoarthritic joints of dogs. Am J Vet Res 2003;64(10):1225-33.

142. Barrett JG, Hao Z, Graf BK, Kaplan LD, Heiner JP, Muir P. Inflammatory changes in ruptured canine cranial and human anterior cruciate ligaments. Am J Vet Res 2005;66(12):2073-80.

143. Muir P, Hayashi K, Manley PA, Colopy SA, Hao Z. Evaluation of tartrate-resistant acid phosphatase and cathepsin K in ruptured cranial cruciate ligaments in dogs. Am J Vet Res 2002;63(9):1279-84.

144. Arnoczky SP, Tarvin GB, Marshall JL, Saltzman B. The over-the-top procedure: a technique for anterior cruciate ligament substitution in the dog. Journal of the American Animal Hospital Association 1979;15(3):283-290.

145. Arnoczky SP, Warren RF, Ashlock MA. Replacement of the anterior cruciate ligament using a patellar tendon allograft. An experimental study. J Bone Joint Surg Am 1986;68(3):376-85.

146. Arnoczky SP, Warren RF, Minei JP. Replacement of the anterior cruciate ligament using a synthetic prosthesis. An evaluation of graft biology in the dog. Am J Sports Med 1986;14(1):1-6.

147. Dieterich HF. Repair of anterior cruciate ligament rupture using a modified lateral and medial retinacular imbrication technique. Vet Med Small Anim Clin 1974;69(12):1519-26.

148. Flo GL. Modification of the lateral retinacular imbrication technique for stabilizing cruciate ligament injuries. Journal of the American Animal Hospital Association 1975;11(5):570-576.

149. Guenego L, Zahra A, Madelenat A, Gautier R, Marcellin-Little DJ, Hulse D. Cranial cruciate ligament rupture in large and giant dogs. A retrospective evaluation of a modified lateral extracapsular stabilization. Vet Comp Orthop Traumatol 2007;20(1):43-50.

150. Jerram RM, Walker AM, Warman CG. Proximal tibial intraarticular ostectomy for treatment of canine cranial cruciate ligament injury. Vet Surg 2005;34(3):196-205.

151. Korvick DL, Johnson AL, Schaeffer DJ. Surgeons' preferences in treating cranial cruciate ligament ruptures in dogs. J Am Vet Med Assoc 1994;205(9):1318-24.

152. Kudnig ST. Intra-articular replacement. Aust Vet J 2000;78(6):384-5.

153. LaPrade RF, Terry GC, Montgomery RD, Curd D, Simmons DJ. Winner of the AlbertTrillat Young Investigator Award. The effects of aggressive notchplasty on the normal knee in dogs. Am J Sports Med 1998;26(2):193-200.

154. Leighton RL. Preferred method of repair of cranial cruciate ligament rupture in dogs: a survey of ACVS diplomates specializing in canine orthopedics. American College of Veterinary Surgery. Vet Surg 1999;28(3):194.

155. Lewis DG. A modified tendon transfer technique for stabilising the canine stifle joint after rupture of the cruciate ligament(s). Veterinary Record 1974;94(No.1):3-8.

156. Lewis DG. An evaluation of 62 cases of cruciate rupture stabilised using the modified tendon transfer technique. Veterinary Record 1974;94(No.2):32-38.

157. Mitton GR, Ireland WP, Runyon CL. Evaluation of the instantaneous centers of rotation of the stifle before and after repair of torn cruciate ligament by use of the over-the-top technique in dogs. Am J Vet Res 1991;52(10):1731-7.

158. Monnet E, Schwarz PD, Powers B. Popliteal tendon transposition for stabilization of the cranial cruciate ligament deficient stifle joint in dogs: an experimental study. Vet Surg 1995;24(6):465-75.

159. Saidi K, Beauchamp P, Laurin CA. Prosthetic replacement of the anterior cruciate ligament in dogs. Can J Surg 1976;19(6):547-9.

160. Shires PK, Hulse DA, Liu W. The under-and-over fascial replacement technique for anterior cruciate ligament rupture in dogs: a retrospective study. Journal of the American Animal Hospital Association 1984;20(1):69-77.

161. Smith GK, Torg JS. Fibular head transposition for repair of cruciate-deficient stifle in the dog. J Am Vet Med Assoc 1985;187(4):375-83.

162. Paatsama S. Ligament Injuries of the Canine Stifle Joint: A Clinical and Experimental Study [Thesis]. Helsinki: Veterinary College; 1952.

163. O'Donoghue DH, Rockwood CA, Jr., Frank GR, Jack SC, Kenyon R. Repair of the anterior cruciate ligament in dogs. J Bone Joint Surg Am 1966;48(3):503-19.

164. Strande A. Replacement of the anterior cruciate ligament in the dog. J Small Anim Pract 1966;7(5):351-9.

165. Hohn RB, Miller JM. Surgical correction of rupture of the anterior cruciate ligament in the dog. J Am Vet Med Assoc 1967;150(10):1133-41.

166. Gupta BN, Brinker WO. Anterior cruciate ligament prosthesis in the dog. J Am Vet Med Assoc 1969;154(9):1057-61.

167. Slocum B, Slocum TD. Tibial plateau leveling osteotomy for repair of cranial cruciate ligament rupture in the canine. Veterinary Clinics of North America, Small Animal Practice 1993;23(4):777-795.

168. Dennler R, Kipfer NM, Tepic S, Hassig M, Montavon PM. Inclination of the patellar ligament in relation to flexion angle in stifle joints of dogs without degenerative joint disease. Am J Vet Res 2006;67(11):1849-54.

169. Schwandt CS, Bohorquez-Vanelli A, Tepic S, Hassig M, Dennler R, Vezzoni A, et al. Angle between the patellar ligament and tibial plateau in dogs with partial rupture of the cranial cruciate ligament. Am J Vet Res 2006;67(11):1855-60.

170. Ballagas AJ, Montgomery RD, Henderson RA, Gillette R. Pre- and postoperative force plate analysis of dogs with experimentally transected cranial cruciate ligaments treated using tibial plateau leveling osteotomy. Vet Surg 2004;33(2):187-90.

171. Conzemius MG, Evans RB, Besancon MF, Gordon WJ, Horstman CL, Hoefle WD, et al. Effect of surgical technique on limb function after surgery for rupture of the cranial cruciate ligament in dogs. J Am Vet Med Assoc 2005;226(2):232-6.

172. Marsolais GS, Dvorak G, Conzemius MG. Effects of postoperative rehabilitation on limb function after cranial cruciate ligament repair in dogs. J Am Vet Med Assoc 2002;220(9):1325-30.

173. Moore KW, Read RA. Cranial cruciate ligament rupture in the dog--a retrospective study comparing surgical techniques. Aust Vet J 1995;72(8):281-5.

174. Aiken SW, Badylak SF, Toombs JP, Shelbourne KD, Hiles MC, Lantz GC, et al. Small intestinal submucosa as an intra-articular ligamentous graft material - a pilot study in dogs. Veterinary & Comparative Orthopaedics & Traumatology 1994;7(3):124-128.

175. Brody GA, Eisinger M, Arnoczky SP, Warren RF. In vitro fibroblast seeding of prosthetic anterior cruciate ligaments. A preliminary study. Am J Sports Med 1988;16(3):203-8.

176. Cooper JA, Jr., Sahota JS, Gorum WJ, 2nd, Carter J, Doty SB, Laurencin CT. Biomimetic tissue-engineered anterior cruciate ligament replacement. Proc Natl Acad Sci U S A 2007;104(9):3049-54.

177. Gentleman E, Livesay GA, Dee KC, Nauman EA. Development of ligament-like structural organization and properties in cell-seeded collagen scaffolds in vitro. Ann Biomed Eng 2006;34(5):726-36.

178. Laitinen O, Pohjonen T, Tormala P, Saarelainen K, Vasenius J, Rokkanen P, et al. Mechanical properties of biodegradable poly-L-lactide ligament augmentation device in experimental anterior cruciate ligament reconstruction. Arch Orthop Trauma Surg 1993;112(6):270-4.

179. Murray MM, Forsythe B, Chen F, Lee SJ, Yoo JJ, Atala A, et al. The effect of thrombin on ACL fibroblast interactions with collagen hydrogels. J Orthop Res 2006;24(3):508-15.

180. Murray MM, Spindler KP, Ballard P, Welch TP, Zurakowski D, Nanney LB. Enhanced histologic repair in a central wound in the anterior cruciate ligament with a collagen-platelet-rich plasma scaffold. J Orthop Res 2007;25(8):1007-17.

181. Murray MM, Spindler KP, Devin C, Snyder BS, Muller J, Takahashi M, et al. Use of a collagen-platelet rich plasma scaffold to stimulate healing of a central defect in the canine ACL. J Orthop Res 2006;24(4):820-30.

182. Noth U, Schupp K, Heymer A, Kall S, Jakob F, Schutze N, et al. Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel. Cytotherapy 2005;7(5):447-55.

183. Arnoczky SP, Tarvin GB, Marshall JL. Anterior cruciate ligament replacement using patellar tendon. An evaluation of graft revascularization in the dog. J Bone Joint Surg Am 1982;64(2):217-24.

184. Cabaud HE, Feagin JA, Rodkey WG. Acute anterior cruciate ligament injury and augmented repair. Experimental studies. Am J Sports Med 1980;8(6):395-401.

185. Cabaud HE, Feagin JA, Rodkey WG. Acute anterior cruciate ligament injury and repair reinforced with a biodegradable intraarticular ligament. Experimental studies. Am J Sports Med 1982;10(5):259-65.

186. Jackson DW, Grood ES, Arnoczky SP, Butler DL, Simon TM. Cruciate reconstruction using freeze dried anterior cruciate ligament allograft and a ligament augmentation device (LAD). An experimental study in a goat model. Am J Sports Med 1987;15(6):528-38.

187. Jackson DW, Grood ES, Arnoczky SP, Butler DL, Simon TM. Freeze dried anterior cruciate ligament allografts. Preliminary studies in a goat model. Am J Sports Med 1987;15(4):295-303.

188. Jackson DW, Grood ES, Cohn BT, Arnoczky SP, Simon TM, Cummings JF. The effects of in situ freezing on the anterior cruciate ligament. An experimental study in goats. J Bone Joint Surg Am 1991;73(2):201-13.

189. McMaster WC. A histologic assessment of canine anterior cruciate substitution with bovine xenograft. Clin Orthop Relat Res 1985(196):196-201.

190. Vasseur PB, Stevenson S, Gregory CR, Rodrigo JJ, Pauli S, Heitter D, et al. Anterior cruciate ligament allograft transplantation in dogs. Clinical Orthopaedics & Related Research 1991(269):295-304.

191. Amiel D, Nagineni CN, Choi SH, Lee J. Intrinsic properties of ACL and MCL cells and their responses to growth factors. Medicine & Science in Sports & Exercise 1995;27(6):844-51.

192. DesRosiers EA, Yahia L, Rivard CH. Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. J Orthop Res 1996;14(2):200-8.

193. Lee IC, Wang JH, Lee YT, Young TH. Development of a useful technique to discriminate anterior cruciate ligament cells and mesenchymal stem cells--the application of cell electrophoresis. J Biomed Mater Res A 2007;82(1):230-7.

194. Lee J, Green MH, Amiel D. Synergistic effect of growth factors on cell outgrowth from explants of rabbit anterior cruciate and medial collateral ligaments. J Orthop Res 1995;13(3):435-41.

195. Meaney Murray M, Rice K, Wright RJ, Spector M. The effect of selected growth factors on human anterior cruciate ligament cell interactions with a three-dimensional collagen-GAG scaffold. J Orthop Res 2003;21(2):238-44.

196. Murray MM, Bennett R, Zhang X, Spector M. Cell outgrowth from the human ACL in vitro: regional variation and response to TGF-beta1. J Orthop Res 2002;20(4):875-80.

197. Murray MM, Spindler KP, Abreu E, Muller JA, Nedder A, Kelly M, et al. Collagen-platelet rich plasma hydrogel enhances primary repair of the porcine anterior cruciate ligament. J Orthop Res 2007;25(1):81-91.

198. Spindler KP, Murray MM, Detwiler KB, Tarter JT, Dawson JM, Nanney LB, et al. The biomechanical response to doses of TGF-beta 2 in the healing rabbit medial collateral ligament. J Orthop Res 2003;21(2):245-9.

199. Pascher A, Steinert AF, Palmer GD, Betz O, Gouze JN, Gouze E, et al. Enhanced repair of the anterior cruciate ligament by in situ gene transfer: evaluation in an in vitro model. Mol Ther 2004;10(2):327-36.

200. Woo SL, Abramowitch SD, Kilger R, Liang R. Biomechanics of knee ligaments: injury, healing, and repair. J Biomech 2006;39(1):1-20.

201. Woo SL, Jia F, Zou L, Gabriel MT. Functional tissue engineering for ligament healing: potential of antisense gene therapy. Ann Biomed Eng 2004;32(3):342-51.

202. Agung M, Ochi M, Yanada S, Adachi N, Izuta Y, Yamasaki T, et al. Mobilization of bone marrow-derived mesenchymal stem cells into the injured tissues after intraarticular injection and their contribution to tissue regeneration. Knee Surg Sports Traumatol Arthrosc 2006;14(12):1307-14.

203. Ge Z, Goh JC, Lee EH. Selection of cell source for ligament tissue engineering. Cell Transplant 2005;14(8):573-83.

204. Ge Z, Goh JC, Lee EH. The effects of bone marrow-derived mesenchymal stem cells and fascia wrap application to anterior cruciate ligament tissue engineering. Cell Transplant 2005;14(10):763-73.

205. Kanaya A, Deie M, Adachi N, Nishimori M, Yanada S, Ochi M. Intra-articular injection of mesenchymal stromal cells in partially torn anterior cruciate ligaments in a rat model. Arthroscopy 2007;23(6):610-7.

206. Rodkey WG, Arnoczky SP, Steadman JR. Healing of a surgically created partial detachment of the posterior cruciate ligament using marrow stimulation: an experimental study in dogs. J Knee Surg 2006;19(1):14-8.

207. Van Eijk F, Saris DB, Riesle J, Willems WJ, Van Blitterswijk CA, Verbout AJ, et al. Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. Tissue Eng 2004;10(5-6):893-903.

208. Chu Q, Lopez M, Hayashi K, Ionescu M, Billinghurst RC, Johnson KA, et al. Elevation of a collagenase generated type II collagen neoepitope and proteoglycan epitopes in synovial fluid following induction of joint instability in the dog. Osteoarthritis Cartilage 2002;10(8):662-9.

209. Elkins AD, Pechman R, Kearney MT, Herron M. A retrospective study evaluating the degree of degenerative joint disease in the stifle joint of dogs following surgical repair of anterior cruciate ligament rupture. Journal of the American Animal Hospital Association 1991;27(5):533-540.

210. Girling SL, Bell SC, Whitelock RG, Rayward RM, Thomson DG, Carter SC, et al. Use of biochemical markers of osteoarthritis to investigate the potential disease-modifying effect of tibial plateau levelling osteotomy. J Small Anim Pract 2006;47(12):708-14.

211. Gordon WJ, Conzemius MG, Riedesel E, Besancon MF, Evans R, Wilke V, et al. The relationship between limb function and radiographic osteoarthrosis in dogs with stifle osteoarthrosis. Vet Surg 2003;32(5):451-4.

212. Innes JF, Barr AR, Sharif M. Efficacy of oral calcium pentosan polysulphate for the treatment of osteoarthritis of the canine stifle joint secondary to cranial cruciate ligament deficiency. Vet Rec 2000;146(15):433-7.

213. Innes JF, Costello M, Barr FJ, Rudorf H, Barr AR. Radiographic progression of osteoarthritis of the canine stifle joint: a prospective study. Vet Radiol Ultrasound 2004;45(2):143-8.

214. Innes JF, Sharif M, Barr AR. Relations between biochemical markers of osteoarthritis and other disease parameters in a population of dogs with naturally acquired osteoarthritis of the genual joint. Am J Vet Res 1998;59(12):1530-6.

215. Innes JF, Sharif M, Barr AR. Changes in concentrations of biochemical markers of osteoarthritis following surgical repair of ruptured cranial cruciate ligaments in dogs. Am J Vet Res 1999;60(9):1164-8.

216. Lazar TP, Berry CR, deHaan JJ, Peck JN, Correa M. Long-term radiographic comparison of tibial plateau leveling osteotomy versus extracapsular stabilization for cranial cruciate ligament rupture in the dog. Vet Surg 2005;34(2):133-41.

217. Lineberger JA, Allen DA, Wilson ER, Tobias TA, Shaiken LG, Shiroma JT, et al. Comparison of radiographic arthritic changes associated with two variations of tibial plateau leveling osteotomy. Vet Comp Orthop Traumatol 2005;18(1):13-7.

218. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33(9):1406-15.

219. Rayward RM, Thomson DG, Davies JV, Innes JF, Whitelock RG. Progression of osteoarthritis following TPLO surgery: a prospective radiographic study of 40 dogs. J Small Anim Pract 2004;45(2):92-7.

220. Trumble TN, Billinghurst RC, McIlwraith CW. Correlation of prostaglandin E2 concentrations in synovial fluid with ground reaction forces and clinical variables for pain or inflammation in dogs with osteoarthritis induced by transection of the cranial cruciate ligament. Am J Vet Res 2004;65(9):1269-75.

221. Tsutsumi A, Suzuki E, Adachi Y, Murata H, Goto D, Kojo S, et al. Expression of tristetraprolin (G0S24) mRNA, a regulator of tumor necrosis factor-alpha production, in synovial tissues of patients with rheumatoid arthritis. Journal of Rheumatology 2004;31(6):1044-9.

222. Vasseur PB, Berry CR. Progression of stifle osteoarthrosis following reconstruction of the cranial cruciate ligament in 21 dogs. Journal of the American Animal Hospital Association 1992;28(2):129-136.

Chapter 3: Evaluation of gene expression patterns in normal and pathologic cranial cruciate ligament explants

The primary drive behind this study was to investigate whether intra-articular ligament has the potential to serve as a reservoir of pro-inflammatory and degradative enzymes. Therefore, it seemed logical to first examine the most basic molecules that control disease and health: the gene. There are a variety of genes that are presumed to be involved in either the initiation or progression of osteoarthritis. Many of these same genes are suspected to play significant roles in the health and disease of ligaments. Gene expression in cruciate ligament has not been studied in-depth in either humans or animals. At the time this study was initiated, there were no published reports on gene expression in cruciate ligament in dogs. Since this time, one study utilizing standard PCR[1] and another utilizing MicroArray with quantitative PCR[2] have evaluated gene expression in normal and diseased ligaments. In humans, the earliest report broadly characterizing gene expression in cruciate ligament revealed expression of nine MMPs and four TIMPs.[3] Normal human tissue is difficult to obtain and ligaments from this study were collected from patients receiving total knee replacements; joints already affected by osteoarthritis. Interpretation of the role of these genes in disease of ligament and its role in secondary osteoarthritis was limited in this study. The degree that gene expression may have been altered by the primary pathologic process of OA could not be ascertained because no control of cohorts were used. Based on the difficulties in including appropriate controls and cohorts for human studies, translational research is

advantageous because it may help bridge our understanding of cruciate ligament deficiency in both dogs and humans.

In this study, our first step was to identify genes of interest. This could be accomplished through differential display[4-7], MicroArray[2], or in the case of the present study, a review of the literature. Genes of interest fell under one of three categories: signaling, synthesis, or degradation. Perhaps the most well known signaling molecule thought to be involved in cruciate ligament disease is nitric oxide (NO).[8-22] Cyclooxygenase 2 is another potentially important inflammatory molecule that was considered. Anabolic molecules included the collagens.[23-28] Finally, degradative molecules were selected based upon their level of involvement in OA. [29-32] Specific catabolic molecules included MMPs, a group of zinc-dependant endopeptidases known to be involved in connective tissue metabolism and an aggrecanase.[33]

The purpose of this initial study was to compare normal and pathologic CrCL explants from both normal dogs and dogs with spontaneously occurring CrCL deficiency, respectively. Selection of a number of genes thought to be important in health and disease of cruciate ligaments and characterization of these genes through real-time PCR were performed in this study for two major reasons. First, identification of genes in ligament tissue that are known to be important in disease brings into question whether ligament itself could be contributing to either the initiation or progression of OA. Second, it establishes an important baseline for further exploration of CrCL pathogenesis and possibly provides clues for elucidating the etiology of this disease. Our null hypothesis for this study was that the gene expression of selected molecules known to be

involved in health and disease of articular tissues will not differ between normal and diseased CrCL tissues from dogs or between explants in culture over time.

Materials and Methods:

Cranial cruciate ligaments were harvested from normal (control) and CrCL deficient (affected) dogs. Normal CrCL tissues were harvested from adult (2-6 years of age) dogs (n=10) from various breeds weighing 25-45 kg, which had no clinical, radiographic, or gross evidence of CrCL deficiency or other orthopaedic disease. All normal dogs were euthanized for reasons unrelated to this study. Affected CrCL tissues were excised from adult dogs (n=10) presenting for surgical treatment for CrCL deficiency and were documented to have CrCL pathology based on clinical, radiographic and gross findings. Each of these dogs was age and weight matched to the control group.

Tissue and Media Collection

All ligaments were closely inspected at the time of surgery or euthanasia. During surgery, pathologic ligaments that had completely ruptured and had undergone massive resorption of the substance of the ligament were not included in this study. Enough ligament tissue to section the ligament and perform both gene expression and explant culture was needed. Therefore, diseased ligaments included in this study were moderate or substantial partial tears in which any remaining ligament could be considered grossly abnormal and pathologic. Mild partial tears or tears in which the majority of the ligament remained (and appeared grossly normal) were not included in this study. One half of the excised ligament was immediately snap-frozen and placed into -80° C storage for

subsequent molecular analysis. The remaining ligament was immediately placed into *Ligamentocyte Media.* This specialized media consisted of Dulbecco's Modified Eagle's Media (DMEM)[a] with 10% fetal bovine serum, 0.002% Penicillin, 100 ug/mL Streptomycin, 25 ug/mL Amphoterocin B, 0.002% L-Ascorbate[b], 0.01% L-glutamine. The flasks were incubated at 37˚C in 5% CO2 and 95% humidity with sterile medium change performed every 3 days. These explants were incubated for 3 days at 37° C with 5 % CO2 and 95 % humidity. On day three, the media was decanted, frozen in a -20° C freezer, and replaced with fresh media. Following an additional three days of incubation, the media was collected and frozen at -20° C as a separate aliquot. At the end of six days, the ligament explants were then snap-frozen and stored at -80° C for eventual molecular analysis.

RNA extraction

Total RNA was extracted using the Trispin method as previously described. [34] Ligament explants were pulverized while still frozen using a custom-made pulverizer that could be maintained under liquid nitrogen. The pulverized tissue was homogenized through the use of a mini-bead beater[c]. The bead beater utilized 3.2 mm stainless steel beads[c] and Trizol® reagent at high speed for 2 minutes. Following centrifugation, the supernatant was transferred for continued processing and the pellet was discarded. The supernatant from each sample, containing the mRNA of interest, was washed with 200 ul of chloroform[b]. An upper aqueous phase and a lower organic phase were separated by high speed centrifugation. The upper phase was transferred to a new tube and the lower organic phase was stored at -80◦C. Ethanol (EtOH)[b] was vortexed with the upper

aqueous phase to a final concentration of 35%. The extraction was performed with an RNeasy® Mini-Kit[d]. The upper aqueous phase/ethanol admixture was passed though the RNeasy® mini-column to facilitate the binding of the mRNA to the column filter. Any flow-through was collected and saved. The bound mRNA was washed with RW1 buffer. In order to prevent any DNA contamination, the bound mRNA was treated with a mixture of 10 ul DNase 1[d] and 80 ul RDD buffer at room temperature for 15 minutes. The column was then washed with RW1 buffer. Another two washes were performed with RPE buffer. Any flow-through was collected and saved. As a final step, the mRNA was eluted from the column using 30 ul RNase-free water and centrifugation.

Determination of RNA Yield

For each sample, 5 ul of the eluted RNA was added to 95 ul of TE buffer (pH 7.8) and utilized for determination of RNA concentration using a RiboGreen® RNA Quantitation Kit.[e] The diluted RNA for each sample was placed in individual wells of a 96-well plate and mixed with 100 ul of the ribogreen dye diluted 1:2000 in TE buffer. The RNA concentration was determined using an internal standard, a spectrophotometer and KC4 absorbance software.[f]

Reverse Transcription

Reverse transcription was accomplished through the use of 500 ng of total RNA, as determined by spectrophotometry, and Stratascript™ RNaseH reverse transcriptase[g]. The 500 ng of sample RNA was mixed to a final volume of 16 ul with DEPC water and 10 pM of random primers[h]. Following incubation at 68°C for 5 minutes the reaction was

placed on ice for 3 minutes. At this time 4 ul of a reaction mixture was added to bring the total volume to 20 ul. The reaction mixture contained 1 ul of Stratascript™ enzyme, 1 ul of 10mM dNTPs[i] and 2 ul 10X Stratascript™ buffer. Each 20 ul reaction was incubated for at 45°C fore 90 minutes and held at 4°C using a PE GeneAmp 9700[j] until the sample was diluted to 200 ul using RNase-free water. Each sample cDNA was stored at -20°C until utilization for real-time PCR.

Real-time PCR

A QuantiTect SYBR Green PCR kit[d] and a Rotor-Gene 3000™ Real-time PCR thermocycler[k] was used to accomplished real-time polymerase chain reaction. Each 20 ul reaction mixture included 0.1 ul of HK-UNG, 1 ul of 0.3 ul forward primer, 1 ul of 0.3 reverse primer, 4 ul of diluted sample cDNA, 4 ul of RNase-free water and 10 ul of 2X Quantitect™ SYBR Green master mix. The thermocycler settings including minutes at 35°C, 15 minutes at 94°C followed by 40 cycles of 3 major phases of PCR:

Melting: 5 seconds at 94°C

Annealing: 10 seconds at 57°C

Extension: 15 seconds at 72°C

The "Take-Off" point (Ct) (Fig. 3-1), PCR amplification efficiency and the melt curve analysis was performed using the thermocycler software (Rotor-Gene Version 5.0)[k] A melt curve analysis was performed from 69°C to 95°C at 470nm/510nm (excitation/emission) for SYBR Green to verify specific amplification and validate PCR data (Fig. 3-2). Analysis of each sample was performed in duplicate for a single housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GADPH) as well as

collagen 1 (COL1), collagen 2 (COL2), collagen 3 (COL3, collagen 6 (COL6), inducible nitric oxide synthase (INOS), cyclooxygenase 2 (COX2), matrix metalloproteinase 1 (MMP-1), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 3 (MMP-3), matrix metalloproteinase 9 (MMP-9), matrix metalloproteinase 13 (MMP-13) and aggrecanase 2 (ADAMTS-5) using specific forward and reverse oligonucleotide primers (Table 3-1).

Statistical Analysis

Relative levels of gene expression were determined using QGene and expressed as a ratio to GAPDH. The relative expression statistical tool, REST-XL, was used to assess for differences in gene expression. [35,36]

Results

Significant differences were detected between normal and pathologic ligaments and in almost all cases the change was more than 5-fold. In some cases, the fold change was greater than 200. Pathologic ligaments collected immediately following excision at surgery demonstrated significantly more gene expression of COL 2, COL 3, MMP 3, and MMP 13 as well as significantly less gene expression of INOS compared to normal ligaments. After 6 days of culture, pathologic ligaments demonstrated significantly more gene expression for COL2 and significantly less gene expression of MMP-1, COX2 and INOS when compared to normal ligament cultured for 6 days.

Additionally, significant differences were evident over time in cell culture. Normal ligament explants cultured for 6 days revealed a significant down-regulation of

COL 1 and up-regulation of MMP-3, and MMP-13, COX-2 and INOS. When cultured over time, pathologic ligaments revealed a significant up-regulation of COL2 and a significant down-regulation of COL2, COL3, COL6 and MMP-1 (Table 3-2).

Discussion

The experimental design of this study provided important data for the pathogenesis of cruciate ligament disease. Ligaments were analyzed at time 0 and this provided important *in vivo* data for both normal ligament metabolism and for diseased ligaments. The ligaments were also cultured over time and this provides insight into the degree of change these ligaments are capable of when placed into an unfavorable environment. The *in vitro* portion of this study also contributes to the basic science literature for ligament metabolism.

In vivo (Time 0) Comparison of Gene Expression in Normal and Pathologic Ligaments

Perhaps the most anticipated results were those generated from comparing gene expression in normal dogs to age and weight-matched with dogs with cruciate ligament disease. A 3-fold increase in COL2 in pathologic ligaments is consistent with the current literature that describes transformation of fusiform ligamentocytes to a spheroid phenotype as part of a pathologic process called chondroid metaplasia.[37-39] Because chondroid metaplasia may also be correlated with COL2, it was a gene of interest to this study. Other studies have suggested that the intercondylar notch may be involved in CrCL pathogenesis and have suggested that the chondroid metaplasia may be the result of ligamentous compression.[40,41] The authors postulated that compression of ligament may

result in increased collagen remodeling and increased sulphated GAG deposition. This, in turn, could lead to reduced structural integrity of the ligament.[40] The increase in COL2 provides some evidence that, in addition to assuming a chondroid phenotype, these ligamentocytes are also embracing metabolism that is chondroid. Certainly, it is possible that the cruciate ligament is involved in an enthesiopathy, where normal adaptive mechanisms turn pathological with continued compressive loads over time. COL3 also demonstrated a 22-fold increase in relative gene expression. The primary reason for evaluating this gene was because it has traditionally been thought of as a repair collagen.[42,43] Three overlapping phases of ligament healing have been described in the literature: inflammatory, reparative, and remodeling.[42,43] The increase in collagen III mRNA suggests that the cruciate ligament is indeed attempting to, but cannot, bridge the gap as described in the reparative phase. Collagen III is thought to be quite active during this phase.[42,43]

These data also suggest that pathologic ligaments have assumed a degradative shift as indicated by the upregulation of the MMPs. Gene expression analysis of MMP-3 revealed a 16-fold increase over normal ligaments. MMP-3 is a stromelysin and has been a molecule that has received a great deal of attention. It has been studied primarily for its role in cartilage and OA rather than in ligamentous tissue itself.[12,44-48] The increase in gene expression of degradative molecules such as MMP-3 could be responsible for substantial remodeling within ligament and may be an important factor in the disease mechanism leading to failure of the CrCL. It is also possible that ligament itself plays an important role in the initiation of OA. The pathophysiology of OA has been more thoroughly studied.[12,44-48] Fujita *et. al*. found elevations in pro-inflammatory cytokines,

MMP-3 activity and S-GAG content in synovial fluid from canine stifle joints with naturally acquired CrCL disease.[49] In that study, it is not apparent from which tissues these molecules are being produced. MMP-3 has been presumed to come from cartilage, but it is possible that this enzyme could be originating from synovium, ligament and/or meniscus. It is very likely that pathogenic molecules could come from multiple tissue sources within the joint only to be later measured in synovial fluid. An argument against the role of MMP-3 in these disease processes is that, unlike collagenases and gelatinases, it is unable to digest the triple helical regions of interstitial collagens.[50] MMP-3's primary substrates include collagen Types III, IV, V, VII, IX, X, and XI. MMP-3 also has activity against gelatin Type I, elastin, aggrecan, fibronectin, vitronectin, laminin, entactin, and tenascin, decorin, fibrillin and osteonectin.[33] Note this extensive list did not include collagen type I or II, the primary collagen present in ligament and cartilage respectively, as a substrate for MMP-3. However, one other unique property of MMP-3 is that it is known to have a pivotal role in activating other MMPs. The active form of MMP-3 has the ability to convert proMMPs-1, -8, -9 and -13 to their active forms.[33,51] It is possible these active enzymes engage in degradation while MMP-3 remains behind the scenes as an instigative molecule. Aside from MMP-3, there was a 13-fold increase in MMP-13 gene expression and this MMP does has an affinity for both collagen I and II.[33,51] A recent study evaluated MMP-13 gene expression in human ACL, patellar tendon, semitendinosus/gracilis tendon, and grafted ligament. These researchers found expression of MMP-13 in both normal patellar tendon and semitendinosus/gracilis tendon, but greater amounts were found in normal ACL.[52] The highest amounts of relative gene expression for MMP-13 were found in reconstructed graft that had utilized

either the patellar tendon or the semitendinosus/gracilis tendon. The authors postulated that MMP-13 might play a role a role in ligamentization.[52] This study confirms the presence of MMP-13 in these tissue and demonstrates that it may be involved in health and disease of orthopaedic structures other than cartilage. A previous report from Amiel *et al.* demonstrated that there was active collagenase in ligament after rupture and that collagenase may contribute to the rapid resorption of the ligament stumps.[53] At the time of publication, differentiation between types of collagenases in that study was likely not possible.

The expression of INOS was decreased in pathologic ligaments by 3-fold. At first, it seems surprising that the enzyme supposedly responsible for a tremendous amount of pain and inflammation would be decreased in diseased ligament. The role of NO in a variety of processes, including those involving orthopaedic health and disease, is well established, but its exact contribution is not entirely understood.[9] In a study by Spreng *et al*. the CrCL was shown to be capable of producing large amounts of NO.[21] Gene expression was not evaluated in that study, but our results support the author's finding that ruptured CrCL produced less NO than did intact ligaments.[21] One logical explanation for this disparity is nothing more than a temporal one. All dogs in our study had cruciate ligament disease for some time before presentation. This time frame was guessed to be greater than one month, but ultimately cannot be known exactly because of the insidious onset of the this disease. Dogs presenting with chronic disease could have once had intra-articular ligament that was producing substantial amounts of NO. Then, as the joint begins to resolve some of this inflammation, expression of INOS begins to

wane. A chronically affected ligament may simply have run out of the ability and/or stimulus for producing large amounts of NO.

Gene Expression in Normal Ligaments: The Effect of In Vitro Explant Culture

Examination of gene expression in explant culture was performed in the present study primarily to serve as a baseline for future studies and to gain insight into the metabolism ligament. This metabolism may involve the production of molecules that can then leach from the diseased ligament into the synovial fluid. These molecules can then act directly or indirectly on other intra-articular tissues inciting anabolism, catabolism, inflammation or other processes. Relative to its normal *in vivo* location and function within the joint, ligament likely considers an *in vitro* environment adverse. Placing normal ligament into this situation will no doubt cause metabolic changes. Interpretation of these changes can provide insight into how ligament behaves *in vivo*. This is especially so when we can compare the cultured explant to ligament used at the initiation of the study. Indeed, when the day 6 gene expression analysis was performed in normal ligaments, we found that there was an 8-fold down-regulation in collagen 1, the primary collagen of ligament. This was despite the addition of ascorbic acid in ligamentocyte media, which is necessary for the hydroxylation of proline during collagen synthesis.[54] Generally speaking, this decrease in collagen I expression is likely from the abnormal *in vitro* environment into which the ligament was placed. This could be from any one of a variety or a combination of factors. Specifically, the ligamentocyte media in which the ligament was placed, the loss of blood supply, the loss of a mechanical stimulus and a host of other factors could have contributed to this change.

The most striking changes in relative gene expression in the present study were the 222-fold and a 48-fold increases in MMP-3 and MMP-13 expression, respectively, by normal ligament over time in culture. Both of these elevations could be associated with the adverse *in vitro* environment as speculated above. While purely speculative, one explanation for this increase in degradative enzymes is the loss of tensile force applied to the ligament explant once it was placed in culture. Mechanotransduction is the process by which cells convert mechanical stimulus into cellular activity and eventually evoke a response. A great deal of research has focused on mechanotransductive pathways[22,31,55-60] and based on this literature, these gene expression changes are not surprising. In our study, no *in vitro* strain was applied to any of these ligaments and the application of physiologic strain is known to be important in the maintenance of health in ligament. Noyes *et al*. demonstrated a decrease in the maximum failure load and energy absorbed by cruciate ligament (39 per cent and 32 per cent respectively) after 8 weeks of whole-body immobilization[58] The immobilization period was only 8 weeks, but it took 12 months for the immobilized ligaments to completely recover. Therefore, the degradative shift found in the present study, as evidenced by a decrease in anabolic gene expression and an increase in catabolic gene expression, supports those *in vivo* data well.

Signaling molecules were also up-regulated over 6 days in explant culture. A 46-fold up-regulation of COX2 and an 8-fold up-regulation INOS were apparent. This further supports the finding, mentioned above, that normal ligaments produce substantially more NO than ruptured ligaments *in vitro*. Corroboration between protein expression from the study by Spreng *et al*.[21] and gene expression data from the present study provides convincing evidence that normal ligaments have an enormous capacity to

up-regulate INOS and produce NO with little post-transcriptional, post-translational, or inhibitory regulation. To the authors knowledge, there is no information in the literature about the evaluation of COX2 gene expression by RT-PCR.

Gene Expression in Pathologic Ligaments: The Effect of In Vitro Explant Culture

Following culture for 6 days, diseased ligament demonstrated several changes in collagen gene expression. Collagen I was down-regulated 14-fold, collagen II was up-regulated 6-fold, collagen III was down-regulated 160-fold, and collagen VI was down-regulated 6-fold. The most obvious aberration among the collagens was the substantial change in collagen III. Analysis of pathologic ligament at time 0 (*in vivo*) revealed increased collagen III expression that, as stated above, is thought to be part of an attempt by the ligament to repair itself.[42,43] It is theorized that upon placement of pathologic ligament in explant culture, this reparative process comes to an abrupt halt and a massive down-regulation of collagen III ensues, as we have detected here.

A 14-fold increase in MMP-3 was also detected, although this upregulation was not nearly as great as that seen for normal ligament. It is suspected that MMP-3 is up-regulated in pathologic ligaments and therefore cannot be up-regulated to the same extent as normal ligament. This lineage of thinking is supported by the increase in MMP-3 expression that was found at time 0 (*in vivo*). This scenario suggests that there may an expression plateau for MMP-3 above which it cannot be increased, despite the ligament having been placed into the seemingly adverse culture environment.

In vitro (Day 6) Comparison of Gene Expression in Normal and Pathologic Ligaments

When compared to normal ligaments, pathologic ligaments revealed an increase collagen II gene expression at day 6. A 7-fold increase in collagen II is more difficult to explain than other significant differences detected in this study. It could simply be that pathologic ligament, having assumed a chondroid phenotype, is more readily capable of producing collagen II mRNA. A substantial 131-fold decrease in MMP-1 gene expression was also detected and is equally difficult to explain. It would be logical to theorize that MMP-1 gene expression was prominent in pathologic ligaments and was down regulated over the six days in culture, but this could not be confirmed in our analysis of pathologic ligaments over time. It might also be possible that normal ligaments greatly increased the expression of MMP-1 over time. This also could not be confirmed in our analysis over normal ligaments in explant culture over time. Therefore, one explanation is that both of these processes are occurring at lower levels and neither one alone could produce a significant difference in our statistical analysis. Indeed, we anticipated more differential results with MMP-1 in this study, but there appeared to be a great deal of variation associated in MMP-1 expression when compared to other molecules of interest.

Finally, when compared to normal ligaments, pathologic ligaments revealed a tremendously lower gene expression in COX2 and INOS at day 6. COX2 had an 11-fold decrease and INOS had a 26-fold decrease in gene expression. These results are more readily explainable. Over the 6 days in culture, a down-regulation of these genes in pathologic ligaments was not detected. However, substantial up-regulation of the genes was detected in normal ligaments. So, the lower expression seen in pathologic ligaments

when compared to normal ligaments is likely due to the dramatic upregulation of COX-2 and INOS in normal ligaments as they respond adversely to *in vitro* stimuli. These finding are strongly supported by studies evaluating INOS on the protein level and resultant production of NO.[11,12,14,15,19,21] Normal ligaments were found to produce substantial amounts of NO over ruptured ligaments,[21] a finding supported by the present study.

Comparison of In Vivo Results with Previously Reported Literature

The *in vivo* results reported above are probably of interest to surgeons and basic science researchers alike. Only a handful of reports evaluate the molecules that our group evaluated on the gene level.[1-3,61] Regarding structural molecules, our findings were consistent with those reported in human ligament[61] and in canine ligament[1,2] with a few minor discrepancies. In ruptured ligaments, we found a 22-fold increase in collagen III gene expression over normal ligaments that was consistent with human ligament [61] and a 16-fold increase previously found in canine ligament[2]. We were unable to confirm an increase in collagen I in addition to collagen III as some studies have demonstrated.[2,61] We were able to show a small 3-fold increase in collagen II gene expression that has not previously been directly reported by other groups. It was interesting to note that we found increased gene expression in MMP-3 and MMP-13, but not MMP-1,-2,-9. This sharply contrasts previously reported literature that has demonstrated a 25-fold and 492-fold increase in MMP-2 and MMP-9, respectively. [2] Other groups had also reported involvement of MMP-2 and MMP-9 through standard RT-PCR.[1] It is difficult to explain this discrepancy based on the magnitude of expression in these two genes from this study.

In a study by Muir *et al*., MMP-2 and MMP-9 were detected only in ruptured ligaments and not normal ligaments from young or aged dogs.[1] The present study documented MMP-2 and MMP-9 in normal and pathologic ligaments through the use of real-time PCR which is a sensitive method. Several possibilities exist that might explain these disparate results. Ligaments collected in the study by Clement *et al*.[2] and Muir *et al*.[1] were complete ruptures. The ligaments collected in the present study were classified as partial, but clearly diseased with little or no contribution to the stability of the stifle joint. Selection of these ligaments in each of these studies may account for this difference through two possibilities. One consideration is purely a temporal one. It is possible that these partial ligaments were collected earlier in the disease process, and had the owner delayed surgical intervention for their pet, these partial ruptures would have progressed to complete ruptures. Upregulation of MMP-2 and MMP-9, the gelatinases, may occur later in the temporal progression of ligaments from partial rupture to complete rupture to retraction[62]/resorption of ligament ends. This could explain why MMP-2 and MMP-9 gene expression were not increased in our study. MMP-2 has greater potential to be involved in CrCL pathogenesis and the initiation and progression of OA because it is capable of digesting collagens I, II and III. MMP-9, on the other hand, does not readily digest collagen I, II, or III.[33,63] A second possibility is that these ligaments are not at unique stages in disease progression but, in fact, represent two separate entities, or subsets of CrCL disease. It is possible these partial tears may result from a different set of pathological circumstances than do the complete tears. Furthermore, all of these studies including the present study have failed to address duration of disease. This is a difficult factor to control. Patients presenting to our hospital may have presented acutely

or may have had chronic disease associated with their stifle joint. Owners often are not reliable sources for information about the onset of disease. Therefore, a clinical population for the study of CrCL pathophysiology is not ideal. However, it may be the best option at this time. While the Pond-Nuki model has existed for more than 30 years for the study of osteoarthritis[64], no such model exists for ligament itself and its pathogenesis. Therefore, the opportunity to study the progression of disease cannot be studied under controlled situations in a manner that OA pathogenesis has been for the last 30 years.

Our MMP-13 data was consistent with previously reported data in which it was detected in human ligament[3] and quantified in dogs as having a 22-fold increase over ruptured ligaments[2]. MMP-3 has not been extensively studied in ligament, but we found a significant increase in gene expression that has previously not been reported. Together with previously published studies, this analysis of gene expression provides some convincing evidence on the gene level, that the CrCL itself, in addition to mechanical instability, may be involved in orchestrating the initiation and progression of OA.

Footnotes

[a] Gibco BRL, Grand Island, NY.
[b] Sigma Chemical Co, St. Louis, MO.
[c] Biospec Products, Bartlesville, OK.
[d] Qiagen, Valencia, CA.
[e] Molecular Probes, Eugene OR.
[f] Bio-Tek, Winooski, VT.
[g] Stratagene, La Jolla, CA.
[h] Integrated DNA Technologies, Coralville, CA.
[i] Promega, Madison, WI.
[j] Applied Biosystems, Foster City, CA.
[k] Corbett Research, Sydney Australia

Category	mRNA Target	Primer Type	Oligonucleotides (5'- 3')	Amplicon Size	Relevance
Housekeeping					
	GADPH	Forward	GTGACTTCAACAGTGACACC	152	Used for relative gene expression analysis
		Reverse	CCTTGGAGGCCATGTAGACC		
Synthesis					
	COL1	Forward	TGCACGAGTCACACTGGAGC	124	Involved in the maintenance of normal extracellular matrix
		Reverse	ATGCCGAATTCCTGGTCTGG		
	COL3	Forward	GGCCTGTCAGAACATCACAT	142	
		Reverse	CACCTTCATTTGACCCCATC		
	COL2	Forward	GGCCTGTCTGCTTCTTGTAA	127	Involved in repair or ligament metaplasia
		Reverse	ATCAGGTCAGGTCAGCCATT		
	COL6	Forward	TGCCGAGTACCAGCCAGAAC	118	
		Reverse	CTGCTGAAGACTGTCTGAAC		
Degradation					
	MMP-1	Forward	CCTAGAACCGTGAAGAGCAT	150	A broad group of zinc-dependant endopetidases including collagenases, gelatinases, and stromelysins suspected to be involved in ligament degeneration and known to be involved in extracellular matrix degradation
		Reverse	CAGGAAAGTCAGCTGCTATC		
	MMP-2	Forward	GCTCGTGCCTTCCAAGTCTG	114	
		Reverse	GGTATCCATCTCCATGCTCC		
	MMP-3	Forward	ATGGCATCCAGTCCCTGTAT	161	
		Reverse	AAAGAACAGGAACTCTCCCC		
	MMP-9	Forward	ACACAGGCACGTCGGTGGTA	157	
		Reverse	ACGGTCTGCGTCTTCACGTC		
	MMP-13	Forward	TCTGGTCTTCTGGCTCATGC	141	
		Reverse	GGTCAAGACCTAAGGAGTGG		
	ADAMTS-5	Forward	TGACTTCTTGCATGGCATGG	120	
		Reverse	CTGGCATGGCTGGTGACTGA		
Signaling					
	COX-2	Forward	ACACTCTACCACTGGCATCC	196	Involved in degradation, inflammation, and mechanotransduction
		Reverse	GCTACTTGTTGTACTGCAGC		
	INOS	Forward	GCTATGCTGGCTACCAGATG	139	
		Reverse	ATCAGCCTGCAGCACCAGAG		

Table 3-1. Summary of canine specific oligonucleotides primers of genes of interest

	Pathologic Ligament Compared to Normal Ligament		Gene Expression Over Time (6 days) in Culture	
	at Day 0	**at Day 6**	**Normal Ligament**	**Pathologic Ligament**
Synthesis				
COL 1	ns	ns	↓ 8.213 (p=0.001)	↓ 14.247 (p=0.001)
COL 2	↑ 3.274 (p=0.025)	↑ 7.201 (p=0.018)	ns	↑ 5.591 (p=0.018)
COL 3	↑ 22.072 (p=0.0415)	ns	ns	↓ 160.177 (p=0.001)
COL 6	ns	ns	ns	↓ 5.635 (p=0.0075)
Degradation				
MMP-1	ns	↓ 130.468 (p=0.001)	ns	ns
MMP-2	ns	ns	ns	ns
MMP-3	↑ 15.988 (p=0.0245)	ns	↑ 221.829 (p=0.001)	↑ 14.382 (p=0.039)
MMP-9	ns	ns	ns	ns
MMP-13	↑ 12.736 (p=0.0375)	ns	↑ 47.964 (p=0.0075)	ns
ADAMTS-5	ns	ns	ns	ns
Signaling				
COX-2	ns	↓ 115.133 (p=0.023)	↑ 45.988 (p=0.001)	ns
INOS	↓ 3.624 (p=0.033)	↓ 25.617 (p=0.0235)	↑ 7.719 (p=0.001)	ns

Table 3-2. Summary of relative gene expression: ↑ = Increased Expression, ↓ Decreased Expression. P-value given in parentheses.

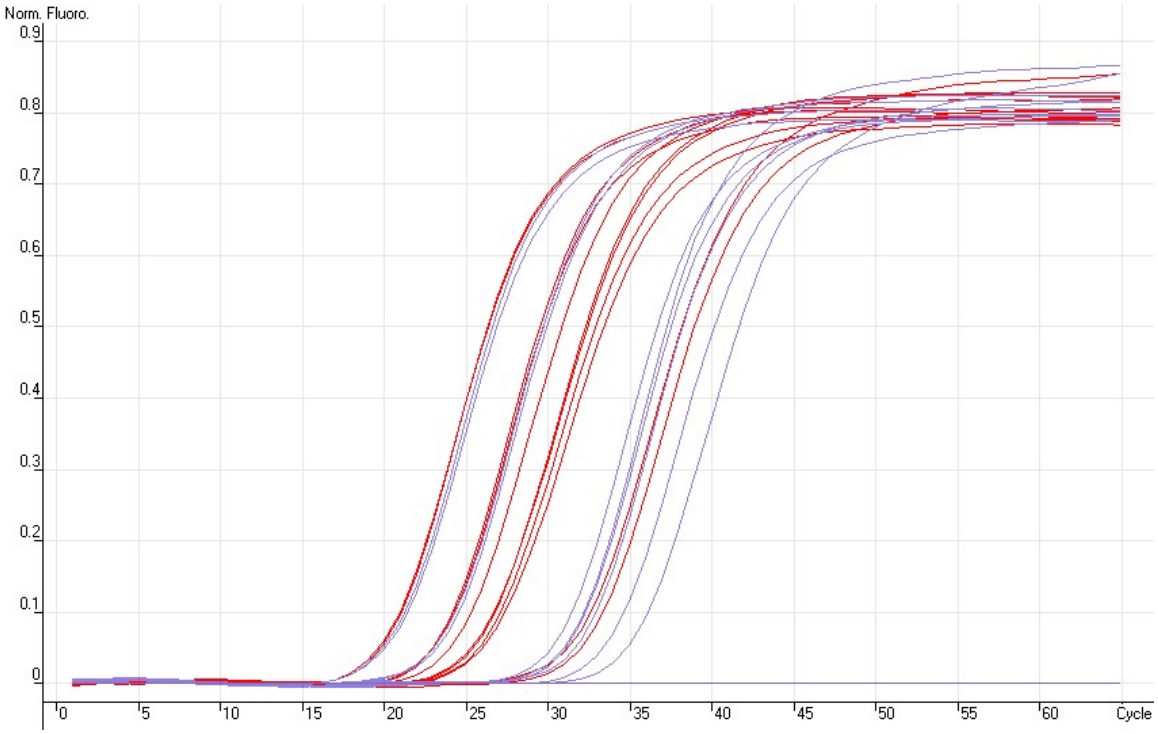

Figure 3-1. Typical take-off points for samples in Real-Time RT-PCR. MMP-2 (red) and MMP-9 (purple) are shown here. X-axis = Number of PCR cycles, Y-axis = Normal Fluorescence.

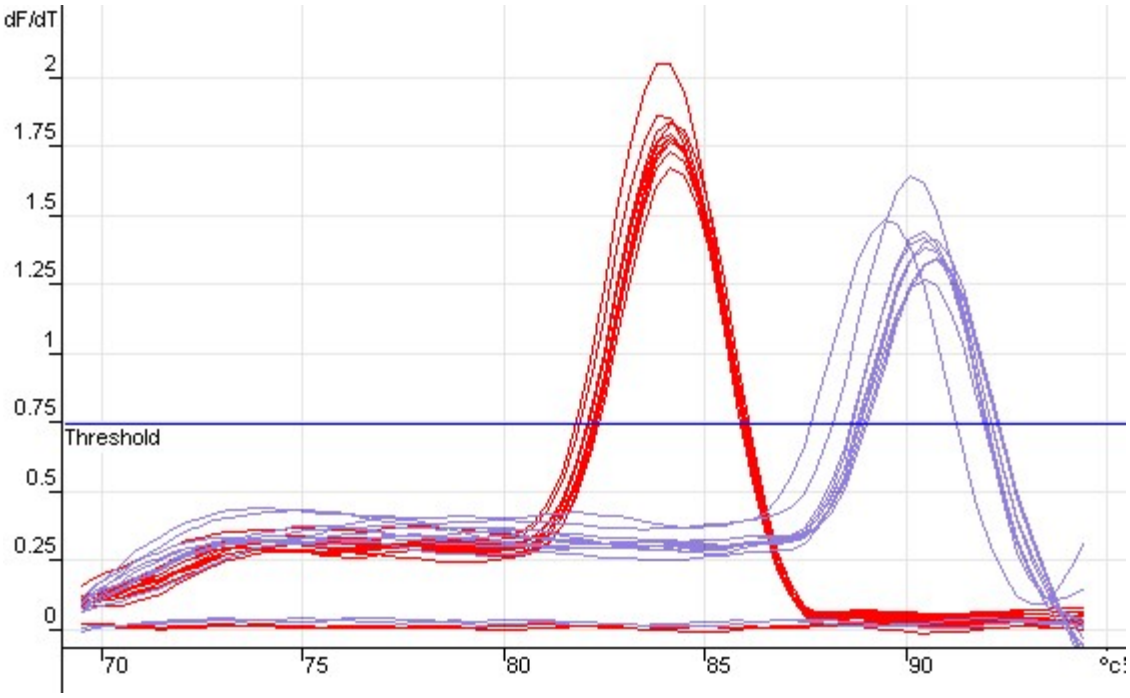

Figure 3-2. A typical melt curve in Real-Time RT-PCR utilized for verifying specific amplification (MMP vs. primer dimer). MMP-2 (red) and MMP-9 (purple) are shown here along with the flatline negative controls. MMP-2 was amplified and the amplicon verified by the correct melting temperature of MMP-2 and MMP-9 at 84 ºC and 90.5 ºC, respectively. X-axis = degrees Celsius, Y-axis = derivative of fluorescence over temperature.

References

1. Muir P, Danova NA, Argyle DJ, Manley PA, Hao Z. Collagenolytic protease expression in cranial cruciate ligament and stifle synovial fluid in dogs with cranial cruciate ligament rupture. Vet Surg 2005;34(5):482-90.

2. Clements DN, Carter SD, Innes JF, Ollier WE, Day PJ. Gene expression profiling of normal and ruptured canine anterior cruciate ligaments. Osteoarthritis Cartilage 2008;16(2):195-203.

3. Foos MJ, Hickox JR, Mansour PG, Slauterbeck JR, Hardy DM. Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament. J Orthop Res 2001;19(4):642-9.

4. Bluteau G, Gouttenoire J, Conrozier T, Mathieu P, Vignon E, Richard M, et al. Differential gene expression analysis in a rabbit model of osteoarthritis induced by anterior cruciate ligament (ACL) section. Biorheology 2002;39(1-2 Special Issue SI):247-258.

5. Ito T, Kito K, Adati N, Mitsui Y, Hagiwara H, Sakaki Y. Fluorescent differential display: arbitrarily primed RT-PCR fingerprinting on an automated DNA sequencer. FEBS Lett 1994;351(2):231-6.

6. Muller-Ladner U, Judex M, Justen HP, Wessinghage D, Welsh J, McClelland M, et al. Analysis of gene expression patterns in rheumatoid synovial fibroblasts using RAP-PCR for differential display. Medizinische Klinik 1999;94(4):228-232.

7. Scaife S, Brown R, Kellie S, Filer A, Martin S, Thomas AM, et al. Detection of differentially expressed genes in synovial fibroblasts by restriction fragment differential display. Rheumatology 2004;43(11):1346-52.

8. Cao M, Stefanovic-Racic M, Georgescu HI, Fu FH, Evans CH. Does nitric oxide help explain the differential healing capacity of the anterior cruciate, posterior cruciate, and medial collateral ligaments? Am J Sports Med 2000;28(2):176-82.

9. Evans CH, Stefanovic-Racic M, Lancaster J. Nitric oxide and its role in orthopaedic disease. Clin Orthop Relat Res 1995(312):275-94.

10. Grabowski PS, Macpherson H, Ralston SH. Nitric oxide production in cells derived from the human joint. Br J Rheumatol 1996;35(3):207-12.

11. Gyger O, Botteron C, Doherr M, Zurbriggen A, Schawalder P, Spreng D. Detection and distribution of apoptotic cell death in normal and diseased canine cranial cruciate ligaments. Vet J 2007;174(2):371-7.

12. Jauernig S, Schweighauser A, Reist M, Von Rechenberg B, Schawalder P, Spreng D. The effects of doxycycline on nitric oxide and stromelysin production in dogs with cranial cruciate ligament rupture. Vet Surg 2001;30(2):132-9.

13. Louis E, Remer KA, Doherr MG, Neumann U, Jungi T, Schawalder P, et al. Nitric oxide and metalloproteinases in canine articular ligaments: a comparison between the cranial cruciate, the medial genual collateral and the femoral head ligament. Vet J 2006;172(3):466-72.

14. Murakami H, Shinomiya N, Kikuchi T, Fujikawa K, Nemoto K. Differential sensitivity to NO-induced apoptosis between anterior cruciate and medial collateral ligament cells. Journal of Orthopaedic Science 2005;10(1):84-90.

15. Murakami H, Shinomiya N, Kikuchi T, Yoshihara Y, Nemoto K. Upregulated expression of inducible nitric oxide synthase plays a key role in early apoptosis after anterior cruciate ligament injury. J Orthop Res 2006;24(7):1521-34.

16. Pelletier JP, Jovanovic D, Fernandes JC, Manning P, Connor JR, Currie MG, et al. Reduced progression of experimental osteoarthritis in vivo by selective inhibition of inducible nitric oxide synthase. Arthritis & Rheumatism 1998;41(7):1275-86.

17. Pelletier JP, Jovanovic DV, Lascau-Coman V, Fernandes JC, Manning PT, Connor JR, et al. Selective inhibition of inducible nitric oxide synthase reduces progression of experimental osteoarthritis in vivo: possible link with the reduction in chondrocyte apoptosis and caspase 3 level. Arthritis Rheum 2000;43(6):1290-9.

18. Pelletier JP, Mineau F, Ranger P, Tardif G, Martel-Pelletier J. The increased synthesis of inducible nitric oxide inhibits IL-1ra synthesis by human articular chondrocytes: possible role in osteoarthritic cartilage degradation. Osteoarthritis Cartilage 1996;4(1):77-84.

19. Riitano MC, Pfister H, Engelhardt P, Neumann U, Reist M, Zurbriggen A, et al. Effects of stimulus with proinflammatory mediators on nitric oxide production and matrix metalloproteinase activity in explants of cranial cruciate ligaments obtained from dogs. Am J Vet Res 2002;63(10):1423-8.

20. Sakurai H, Kohsaka H, Liu MF, Higashiyama H, Hirata Y, Kanno K, et al. Nitric oxide production and inducible nitric oxide synthase expression in inflammatory arthritides. J Clin Invest 1995;96(5):2357-63.

21. Spreng D, Sigrist N, Jungi T, Busato A, Lang J, Pfister H, et al. Nitric oxide metabolite production in the cranial cruciate ligament, synovial membrane, and articular cartilage of dogs with cranial cruciate ligament rupture. Am J Vet Res 2000;61(5):530-6.

22. Wiseman M, Henson F, Lee DA, Bader DL. Dynamic compressive strain inhibits nitric oxide synthesis by equine chondrocytes isolated from different areas of the cartilage surface. Equine Veterinary Journal 2003;35(5):451-456.

23. de Rooster H, de Bruin T, van Bree H. Morphologic and functional features of the canine cruciate ligaments. Vet Surg 2006;35(8):769-80.

24. Everts V, van der Zee E, Creemers L, Beertsen W. Phagocytosis and intracellular digestion of collagen, its role in turnover and remodelling. Histochem J 1996;28(4):229-45.

25. Eyre DR, Paz MA, Gallop PM. Cross-linking in collagen and elastin. Annu Rev Biochem 1984;53:717-48.

26. Frank CB, Hart DA, Shrive NG. Molecular biology and biomechanics of normal and healing ligaments--a review. Osteoarthritis & Cartilage 1999;7(1):130-40.

27. Garnero P, Delmas PD. Biomarkers in osteoarthritis. Curr Opin Rheumatol 2003;15(5):641-6.

28. Petrigliano FA, McAllister DR, Wu BM. Tissue engineering for anterior cruciate ligament reconstruction: a review of current strategies. Arthroscopy 2006;22(4):441-51.

29. Dreier R, Wallace S, Fuchs S, Bruckner P, Grassel S. Paracrine interactions of chondrocytes and macrophages in cartilage degradation: articular chondrocytes provide factors that activate macrophage-derived pro-gelatinase B (pro-MMP-9). J Cell Sci 2001;114(Pt 21):3813-22.

30. Hegemann N, Wondimu A, Ullrich K, Schmidt MF. Synovial MMP-3 and TIMP-1 levels and their correlation with cytokine expression in canine rheumatoid arthritis. Vet Immunol Immunopathol 2003;91(3-4):199-204.

31. Lavagnino M, Arnoczky SP, Tian T, Vaupel Z. Effect of Amplitude and Frequency of Cyclic Tensile Strain on the Inhibition of MMP-1 mRNA Expression in Tendon Cells: An In Vitro Study. Connect Tissue Res 2003;44(3-4):181-7.

32. Zhou D, Lee HS, Villarreal F, Teng A, Lu E, Reynolds S, et al. Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts. J Orthop Res 2005;23(4):949-57.

33. Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH. Matrix metalloproteinases and their clinical applications in orthopaedics. Clin Orthop Relat Res 2004(428):272-85.

34. Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 1997;22(6):1082-6.

35. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.

36. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30(9):e36.

37. Hayashi K, Frank JD, Dubinsky C, Zhengling H, Markel MD, Manley PA, et al. Histologic changes in ruptured canine cranial cruciate ligament. Vet Surg 2003;32(3):269-77.

38. Narama I, Masuoka-Nishiyama M, Matsuura T, Ozaki K, Nagatani M, Morishima T. Morphogenesis of degenerative changes predisposing dogs to rupture of the cranial cruciate ligament. J Vet Med Sci 1996;58(11):1091-7.

39. Vasseur PB, Pool RR, Arnoczky SP, Lau RE. Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs. Am J Vet Res 1985;46(9):1842-54.

40. Comerford EJ, Tarlton JF, Avery NC, Bailey AJ, Innes JF. Distal femoral intercondylar notch dimensions and their relationship to composition and metabolism of the canine anterior cruciate ligament. Osteoarthritis Cartilage 2005.

41. Fitch RB, Montgomery RD, Kincaid SA, Hatchcock JT, Milton JL, Garrett PD, et al. The effect of intercondylar notchplasty on the normal canine stifle. Vet Surg 1995;24(2):156-64.

42. Frank C, Woo SL, Amiel D, Harwood F, Gomez M, Akeson W. Medial collateral ligament healing. A multidisciplinary assessment in rabbits. Am J Sports Med 1983;11(6):379-89.

43. Woo SL, Abramowitch SD, Kilger R, Liang R. Biomechanics of knee ligaments: injury, healing, and repair. J Biomech 2006;39(1):1-20.

44. Fox DB, Cook JL. Synovial fluid markers of osteoarthritis in dogs. J Am Vet Med Assoc 2001;219(6):756-61.

45. Hegemann N, Kohn B, Brunnberg L, Schmidt MF. Biomarkers of joint tissue metabolism in canine osteoarthritic and arthritic joint disorders. Osteoarthritis Cartilage 2002;10(9):714-21.

46. Kuroki K, Cook JL, Kreeger JM, Tomlinson JL. The effects of TIMP-1 and -2 on canine chondrocytes cultured in three-dimensional agarose culture system. Osteoarthritis Cartilage 2003;11(9):625-35.

47. Trumble TN, Trotter GW, Oxford JR, McIlwraith CW, Cammarata S, Goodnight JL, et al. Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. Am J Vet Res 2001;62(9):1467-77.

48. Spreng D, Sigrist N, Busato A, Rechenberg Bv, Schawalder P. Stromelysin activity in canine cranial cruciate ligament rupture. Veterinary and Comparative Orthopaedics and Traumatology 1999;12(4):159-165.

49. Fujita Y, Hara Y, Nezu Y, Schulz KS, Tagawa M. Proinflammatory cytokine activities, matrix metalloproteinase-3 activity, and sulfated glycosaminoglycan content in synovial fluid of dogs with naturally acquired cranial cruciate ligament rupture. Vet Surg 2006;35(4):369-76.

50. Nagase H. Stromelysin 1. In: Barrett A, Rawlings N, Woessner J, editors. Handbook of Proteolytic Enzymes. London: Academic Press; 1998. p. 1172-1178.

51. Woessner JF. Matrix metalloproteinases and TIMPs. Oxford ; New York :: Oxford University Press; 2000.

52. Roseti L, Buda R, Cavallo C, Desando G, Facchini A, Grigolo B. Ligament repair: a molecular and immunohistological characterization. Journal of Biomedical Materials Research Part A 2008;84(1):117-27.

53. Amiel D, Billings E, Jr., Harwood FL. Collagenase activity in anterior cruciate ligament: protective role of the synovial sheath. J Appl Physiol 1990;69(3):902-6.

54. Ballock T, O'Keefe R. Growth and Development of the Skelaton. In: Buckwalter JA, Einhorn TA, Simon SR, American Academy of Orthopaedic S, editors. Orthopaedic basic science : biology and biomechanics of the musculoskeletal system. 2nd ed. ed. Rosemont, IL :: American Academy of Orthopaedic Surgeons; 2000.

55. Arnoczky SP, Tian T, Lavagnino M, Gardner K, Schuler P, Morse P. 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 2002;20(5):947-52.

56. Chano T, Tanaka M, Hukuda S, Saeki Y. Mechanical stress induces the expression of high molecular mass heat shock protein in human chondrocytic cell line CS-OKB. Osteoarthritis & Cartilage 2000;8(2):115-119.

57. Henshaw DR, Attia E, Bhargava M, Hannafin JA. Canine ACL fibroblast integrin expression and cell alignment in response to cyclic tensile strain in three-dimensional collagen gels. J Orthop Res 2006;24(3):481-90.

58. Noyes FR. Functional properties of knee ligaments and alterations induced by immobilization: a correlative biomechanical and histological study in primates. Clin Orthop Relat Res 1977(123):210-42.

59. Peverali FA, Basdra EK, Papavassiliou AG. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. Molecular Medicine. 2001;7(1):68-78.

60. Vanwanseele B, Eckstein F, Knecht H, Stussi E, Spaepen A. Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement. Arthritis Rheum 2002;46(8):2073-8.

61. Lo IK, Marchuk LL, Hart DA, Frank CB. Comparison of mRNA levels for matrix molecules in normal and disrupted human anterior cruciate ligaments using reverse transcription-polymerase chain reaction. J Orthop Res 1998;16(4):421-8.

62. Murray MM, Martin SD, Martin TL, Spector M. Histological changes in the human anterior cruciate ligament after rupture. J Bone Joint Surg Am 2000;82-A(10):1387-97.

63. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92(8):827-39.

64. Pond MJ, Nuki G. Experimentally-induced osteoarthritis in the dog. Ann Rheum Dis 1973;32(4):387-8.

Chapter 4: Characterization of matrix metalloproteinases and inflammatory mediators in explant media from normal and pathologic cranial cruciate ligaments

In Chapter 3, gene expression in normal and pathologic ligaments was evaluated *in vivo* and *in vitro*. A logical next question to ask is whether this identified gene expression then translates into protein expression. One might further inquire as to whether these proteins are latent or active and if they have the ability to be released from their tissue of origin to migrate and affect other intra-articular structures. The most important of these structures is articular cartilage and synovium. For this reason, protein expression is the focus of this chapter. It is not known from the current literature if ligament has the ability to produce molecules, degradative or inflammatory, that are released from the mid-substance into synovial fluid where they target other tissues. Providing evidence that ligament has this ability is central to the idea that ligament itself, and not just the biomechanical alterations that result from the loss of ligament integrity, can contribute the health and disease of the joint. By taking into account this potential ligamentous contribution to joint health, one considers both the biology of the ligament and the mechanics of the ligament. This is crucial when thinking of a joint as an "organ."

At the initiation of this study, our analysis of gene expression data described in chapter 3 was not complete. There was however, convincing evidence in the literature that gelatinases play a significant role in a variety of physiologic and pathologic conditions and may be involved in ligament pathophysiology.[1-9] This study was designed to further characterize MMP 2 and 9 (Gelatinase A and B respectively) gene and protein expression in normal and pathologic ACL. Our null hypothesis was that

degradative and inflammatory molecules would not differ between explant media collected from normal and pathologic ligaments. Characterization of these degradative enzymes and inflammatory mediators may help implicate or absolve their role in the pathogenesis of ACL disease and its role in the initiation/progression of OA.

Materials and Methods

As described in chapter 3, cranial cruciate ligaments were harvested from normal (control) and CrCL deficient (affected) dogs. Based on clinical, radiographic or gross evidence, excised ligaments were classified as normal (n=10) or pathologic (n=10). The ligament was excised and one half was immediately snap-frozen and stored for analysis as described in Chapter 3. The other half of the excised ligament was immediately placed in tissue culture using *Ligamentocyte Media.*

Tissue Culture and Media Collection

The media used in this study was the media collected for evaluation of gene expression as outlined in Chapter 3. All ligaments were closely inspected at the time of surgery or euthanasia. Recall that in chapter 3, these ligaments were described as diseased ligaments that were near-complete ruptures or substantial partial tears in which any remaining ligament could be considered grossly abnormal and pathologic. Mild partial tears or tears in which the majority of the ligament remained (and appeared grossly normal) were not included in this study. Explant culture was performed as

outlined in Chapter 3 with *Ligamentocyte Media.* The biochemical analysis was performed on all samples (n=40) as described below.

Glycosaminoglycan (GAG) Assay

Total sulfated GAG content in explant media was determined using the dimethylmethylene blue (DMMB) assay[10]. Five ul of each sample was added to 245 ul of DMMB. GAG concentration was determined using serial dilution of a known concentration of chondroitin sulfate to create a standard curve. A spectrophotometer with KC4 absorbance software[a] was used to determine absorbance and concentration of GAG in the ligamentocyte media. Cultured explants varied in size especially between normal and ruptured ligaments. In order to standardize these results, values were reported as ug/ml per gram of wet ligament. All samples were run in duplicate.

Hydroxyprolene (HP) Assay

Total collagen content was determined using a colorimetric method to measure the hydroxyprolene (HP) content[11]. Determination of this content was accomplished through the use of a hydroxyprolene standard[b] prepared from a stock solution and placed in a 96-well plate[b]. A 100 ul aliquot from each sample was utilized for this experiment. For each sample, hydrolysis was accomplished by sealing the plate with a silicon mat[c] compressed with a C-Clamp[d] and autoclaved at 120°C for 20 minutes. Each 100 ul sample was mixed with 400 ul of chloramine T and incubated at room temperature for 25 minutes. Following incubation, 400 ul of Ehrlich's aldehyde reagent was added to each well. The wells were gently mixed and the chromophore was developed by incubating

the sample at 65°C for 20 minutes. When ready, a smaller 200 ul aliquot was placed into a new 96-well plate and a spectrophotometer with KC4 absorbance software[a] was used to determine absorbance at 550 nm. A standard curve was constructed and concentration of HP in the ligamentocyte media was determined and reported as ug/ml per gram of wet ligament. All samples were run in duplicate.

The Griess Assay for Nitric Oxide

Nitric oxide (NO) production was determined by measuring the nitrite (NO2-) concentration in the conditioned media using a spectrophotometer and the Griess Reagent System[e].[12] Sulfanilamide (1%) was incubated with 50 ul of each sample or sodium nitrate serial standard at room temperature for 5 minutes while protected from light. A second incubation was performed for 5 minutes at room temperature while protected from light after the addition of 50 ul of 0.1% N-napthylethylenediamine dihydrochloridec in distilled water. The contents of each well were transferred to a new 96-well plate and absorbance was measured at 530 nm with a spectrophotometer that utlized KC4 absorbance software[a]. A standard curve was constructed and the concentration of NO in the ligamentocyte media was determined and reported as nM per gram of wet ligament. All samples were run in duplicate.

Prostaglandin E2 (PGE_2) ELISA Assay

Prostaglandin E2 (PGE2) content in ligamentocyte media was quantitated by Biotrak Enzymeimmunoassay System according to the manufacture's protocol. The concentration of PGE2 was determined through the use of a standard curve and

concentrations were reported as picograms/ul per gram of wet ligament. All biochemical evaluations were performed in duplicate.

Gelatin Zymography

Total protein content was determined using the Bradford protein quantitation assay[f] and reported as ug/ml. A standard 40 ug of protein from each media sample and a data spreadsheet[g] was utilized to bring each lane to 10 ul with deionized water and 5 ul Tris-Glycine SDS Sample Buffer (2X). Gelatin zymography was performed using pre-cast commercial gels (Novex®)[h]. Each gel was electrophoresed with a molecular marker (SeeBlue®)[h] and a commercially available control containing MMP 2 and 9. Gel electrophoresis was performed for 2 hours at 120 volts and 48 mAmps. A few selected samples were run for 4 hours in order to resolve bands more efficiently. Resolved protein was renatured and the gels were developed according to the manufacture's protocol. The gels were scanned and analyzed using the specialized imaging software Fovea Professional[i]. Digested gelatin was visualized as a clear band and activity was expressed as a percentage of MMP control intensity.

Enzyme Activity Assay for MMP-2 and MMP-9

Both active and total MMP activities were evaluated. A standard volume of sampled media was utilized in plates coated with MMP 2 and 9 antibodies. A total of four wells were utilized for this portion of the experiment: 2 for detection of pro-enzyme and 2 for the detection of total enzyme, each in duplicate. Detection of total MMP (pro and active forms) was facilitated with the addition of p-Aminophenylmercuric Acetate

(APMA). Plates were incubated overnight at 6° C to allow binding of the MMP. The next day, the plates were washed and further incubated at 37° C with 50 ul of substrate and 50 ul of detection enzyme. The addition of 50 ul of p-Aminophenylmercuric Acetate (APMA) allowed for detection of total MMP by activating the pro-enzyme in 2 of 4 wells for each sample. Plates were read with a dynamic protocol at time 0 and at 3 hours at 405 nm using a spectrophotometer and KC4 absorbance software[a]. A standard curve was constructed and activity was expressed as ng/ul.

Statistical Analysis

Biochemical data were analyzed by ANOVA with significance at $p<0.05$. Zymography and activity assays were analyzed using a student's T-test with significance at $p < 0.05$.

Results

Explant ligaments produced significantly more NO on day three (495.4 uM/ul/g) when compared to the same ligament on day 6 at (131.0 uM/ul/g). There were no other statistically significant differences detected for the biochemical analyses (Table 4-1). Zymography did not reveal any significant differences between normal and pathologic ligaments or between time points. The activity assays did reveal a few significant differences (Table 4-2). Total MMP 2 in explant media was found to be 266.5 ng/ul in normal ligaments at day 6. This was significantly higher ($p=0.049$) than the value obtained 3 days earlier (156.7 ng/ul). Day 6 normal ligaments also demonstrated significantly higher ($p=0.038$) levels of total MMP-2 than pathologic ligaments sampled

at the same period of time (167.4 ng/ml). Total MMP-9 in explant media in pathologic ligaments at day 6 was 11.6 ng/ul. This was significantly higher than for normal ligaments during the same time frame (2.9 ng/ul) ($p=0.015$). Total MMP-9 was also significantly higher ($p=0.015$) in explant media from pathologic ligaments at day 6 when compared to day 3 (4.4 ng/ul). No other significant differences were found using the activity assays for any other group.

Discussion

The biochemical analysis revealed that all 4 factors of interest could be detected in the culture media. This of course implies that the explant is capable of producing and then releasing these molecules into the media. A similar scenario may occur *in vivo*. Pathologic ligaments released the greatest amount of GAG into the culture media, but this was not found to be significantly different from other groups. Pathologic ligaments also released more HP than did normal ligaments. However, the amount of HP released was quite variable and a statistical significance was not reached. PGE_2 appeared to be consistent among each group except for pathologic ligaments at day 3. The release of NO into media has previously been demonstrated *in vitro*.[13] The level of NO that was produced in this study was similar to that found in previous studies. Spreng *et al*. concluded that the CrCL is capable of producing substantial amounts of NO.[13] In that study, the authors emphasized that the importance of their finding was not completely known, but more recent evidence by the same authors seems to suggest that their finding may hold great significance. Recent data from this group revealed that there was a moderate, but significant correlation with NO and caspase-positive cells evaluated by

immunohistochemistry.[14] Generally, previous studies have focused on 3 days of culture for measurement of NO. Our study demonstrated that NO production decreases from day 3 to day 6. Ligament explants produced significantly more NO at the day three time point when compared to the day 6 time point. In chapter 3, we suggested that the lower INOS gene expression seen in pathologic ligaments when compared to normal ligaments was likely due to the dramatic upregulation of INOS in normal ligaments as they respond adversely to *in vitro* stimuli. This corresponds well with measured levels of NO in the media. However, less NO production was found at day 6 than at day 3. This could be because gene expression was actually decreasing from day 3 to days 6, but it is not possible to know because gene expression was not performed at day 3. It may also simply be that it takes time for the NO that is produced in the ligament to diffuse into the media. Owing to the small size of the NO molecule, this is unlikely. More probable explanations include substantial post-transcriptional or post translation regulation of INOS or its activity. Interestingly, one study found that cells staining positively for INOS were often the cells identified as having undergone chondroid metaplasia.[15] There is also a reasonable body of work showing that NO differentially affects ACL and MCL.[16-18] These study have demonstrated that NO had a tremendous effect on collagen synthesis[16] and NO-induced apoptosis[17,18]. Mechanistic studies on this phenomenon were able to show that the p38 MAPK pathway is likely responsible for triggering apoptosis.[18] Using a well-designed study, the authors demonstrated that a p38 MAPK inhibitor resulted in less apoptosis.[18]

One of the major findings in the present study was the detection of both MMP-2 and MMP-9 in every sample evaluated. This is an important finding because it

demonstrates that ligament can produce these molecules and subsequently release them into their surrounding environment. Detection of mRNA on the gene level and enzyme at the protein level provides reasonable evidence that cruciate ligament is metabolically active in regard to MMP-2 and MMP-9. Previous studies support the presence of these molecules in ligament.[2-5,9] These data suggest that pathologic CCLs were less adversely affected by *in vitro* culture than normal CCLs with respect to MMP 2 and 9 expression and activity. In general, MMP 2 and 9 levels were higher for normal CCLs than pathologic CCLs after culture. These levels further increased with time in culture, but significance was not reached. This may be due to the fact that pathologic ligaments have already been exposed to adverse conditions within an osteoarthritic joint diminishing the effects of culture. Therefore, the *in vitro* culture system used in the present study may be inappropriate for study of the roles of MMP-2 and MMP-9 in CCL pathology *in vivo*. Another explanation for these findings is that MMP-2 and MMP-9 may not play significant roles in CrCL disease in dogs. Comerford *et al*. has put together a body of work that has demonstrated that MMP-2 does appear to be involved in CrCL pathogenesis.[2-4] At first glance, it is tempting to assume that results from these studies dispute the findings in the present study. However, it is important to make some distinctions between these studies and the present study. It is equally important to make a distinction between the focus of these investigations: ligament pathogenesis as opposed to OA that follows rupture of the ligament. The studies by Comerford *et al*. assessed ligament itself whereas the current study evaluated MMP activity in the supernatant of explant cultures. This was intentional in that we were interested to know if the ligament was capable of releasing MMP into its surroundings. This question is more relevant in

answering whether ligament itself can contribute to the OA that follows rupture. So, it may be that MMP-2 is produced in ligament, but is unable make its way into the culture media.

The MMP-2 data at the protein level in the present study correlates well with our gene expression data presented in Chapter 3. For MMP-2 and MMP-9, our *in vivo* gene expression data was not able to show much difference between normal and ruptured ligaments and more directly disagrees with work performed on the protein level. This too can be explained. The ligaments collected in the present study were all partial tears whereas the ligaments collected from Comerford *et al*. were completely ruptured. So, once again, it is possible that MMP-2 and MMP-9 are not involved partial tears. Ligaments collected as partial tears may be earlier in the stage of disease or may be the result of an entirely separate disease process than ligament tears presenting as complete. A major limitation of all of these studies was that no attempt was made to classify these ligaments as acute or chronic or to provide some other method for addressing stage of disease. Failure to address stage of disease has been cited as a concern in previous studies[2,19] and is frequently a topic of discussion. Some researchers suggest that the changes seen in the synovium are primary changes.[20] There is debate as to whether arthritic changes precede ligament rupture or follow it. This is causal nexus and some investigators refer to this as the proverbial chicken or the egg scenario[21-23]. Failure to adequately address or perhaps quantify the stage of disease continues to be a major and debilitating limitation in the study of CrCL pathogenesis and the OA that invariably is associated with it. This is compounded by the fact that an *in vivo* disease model mimicking the progressive deterioration and eventual rupture of the ligament itself does

not exist. The lack of such a model is, in part, because the risk factors behind CrCL ligament pathogenesis are not entirely understood. In order to completely understand cranial cruciate ligament disease, it may be important to evade this causal nexus. It is less likely that either ligament degeneration or synovitis is solely responsible for the other and for eventual rupture of the ligament. Instead, it may be important to identify initiating and perpetuating factors in this disease process. It is more likely that ligament degeneration and synovitis are interrelated and one contributes to the other in a perpetual cycle (Figure 2-1).

There were some interesting subjective findings from the zymography that was performed in this study. The gel electrophoresis was performed over two hours. In selected gels, electrophoresis was performed over 4 hours. In these gels, additional bands were resolved. For MMP-9 one band can be expected at approximately 92 kDA and possibly another at 84 kDa depending on the sample. These bands represent the pro and cleaved forms for MMP-9 respectively. In the 4 hour gels in this study, two separate bands were observed at each of the 92 and 84 kDa positions. This phenomenon was not appreciated for MMP-2. One explanation for this finding is that there are two variants of post-translational modification of MMP-9. The bands are extremely close together and had a 4-hour electrophoresis never been performed, these bands would not have been observed as they overlap on the 2-hour zymogram (Figure 4-2). This suggests that they are the same protein with only very subtle changes in size. Perhaps there are multiple cleavage sites for MMP-9 while there is only one for MMP-2. It would have been interesting to verify these results with a corresponding western blot, but the amount of available media became a limiting factor. For this reason, the discussion above is nothing

more than speculative as we could not rule out some other MMP that has activity against gelatin as a possibility. Another interesting finding in the gelatin zymography was rather large bands at approximately 225 kDa. These are suspected to be homo-dimers of MMP-9 that have been previously reported.[24] A 130 kDa hetero-dimer of MMP-9 was not observed in any of our samples.[24]

The *in vivo* data from gene expression data implicated MMP-3 and MMP-13 as more significant in CCL pathogenesis. These enzymes were not evaluated in this study. It would have been interesting to evaluate these enzymes more closely but the amount of media available for this type of study limited our ability to be comprehensive. ELISAs and activity assays would have required more media than available to us at the time this study was performed. Additionally, having seen the data from this study, it would have been interesting to look at protein expression in the explants in addition to the supernatant. This would have required using a portion of the 6-day explant for protein expression and a portion for gene expression. At the time, we were more concerned about our ability to recovery enough mRNA to perform RT-PCR. As it turns out, ligament tissue yields a relatively large amount of mRNA using the technique described above. This study demonstrated that a variety of active molecules are produced in the CrCL and have the ability to migrate from the mid-substance of the ligament. Therefore, the role of ligament in the health and disease of the stifle joint cannot be readily ruled out. Limitations of the present study include the absence of load on cultured explants, the limited breadth of genes and proteins examined, the inability to address the stage of

disease in pathologic ligaments, and the relatively short study period. Future work should attempt to address some of these limitations.

---

[a] Bio-Tek, Winooski, VT.
[b] Fischer Scientific, Hampton, NH
[c] Corning Costar, Costar, NY.
[d] Lowes, Columbia, MO
[e] Promega Corporation, Madison, WI
[f] Bio-Rad Laboratories, Hercules, CA
[g] Excel, Microsoft Corporation, Redmond, WA
[h] Invitrogen, Carlsbad, CA
[i] Reindeer Graphics, Asheville, NC

	Normal Ligament Explant Culture		Pathologic Ligament Explant Culture	
	at Day 3	**at Day 6**	**at Day 3**	**at Day 6**
GAG	955.7	690.564	1337.488	850.900
HP	166.7	137.6	734.6	906.417
PGE2	6662.5	6559.9	6364.4	3942.219
NO	495.4*	131.0*	324.4	86.7

Table 4-1. Summary of biochemical evaluation in explant media from normal and pathologic ligaments at days 3 and 6. *Significant difference at $p < 0.05$.

MMP	Group	Activity Assay		Zymography	
		Active MMP ng/ul	Total MMP ng/ul	Active MMP - Band Intensity	Pro MMP- Band Intensity
MMP-2	Normal Day 3	5.4 (± 6.1)	156.7† (± 41.7)	0	26.5 (± 23.3)
	Normal Day 6	9.9 (± 9.3)	266.5†* (± 98.2)	0.1 (± 5.6)	24.8 (± 24.3)
	Pathologic Day 3	4.1 (± 5.6)	129.9 (± 94.5)	0.4 (± 1.2)	11.0 (± 12.1)
	Pathologic Day 6	5.2 (± 6.1)	167.4* (± 99.7)	0.4 (± 1.4)	10.1 (± 10.7)
MMP-9	Normal Day 3	0.9 (± 0.7)	3.1 (± 3.2)	0	8.8 (± 8.3)
	Normal Day 6	0.6 (± 0.8)	2.9† (± 4.0)	0.8 (± 1.1)	9.3 (± 5.5)
	Pathologic Day 3	1.0 (± 0.9)	4.4* (± 4.4)	2.3 (± 5.4)	11.9 (± 11.6)
	Pathologic Day 6	0.7 (± 0.8)	11.6†* (± 9.3)	0.7 (± 1.3)	10.0 (± 11.6)

Table 4-2: Summary of zymography and activity assays of explant media from normal and pathologic ligaments at days 3 and 6 reported as a median value. *† Significant differences within columns.

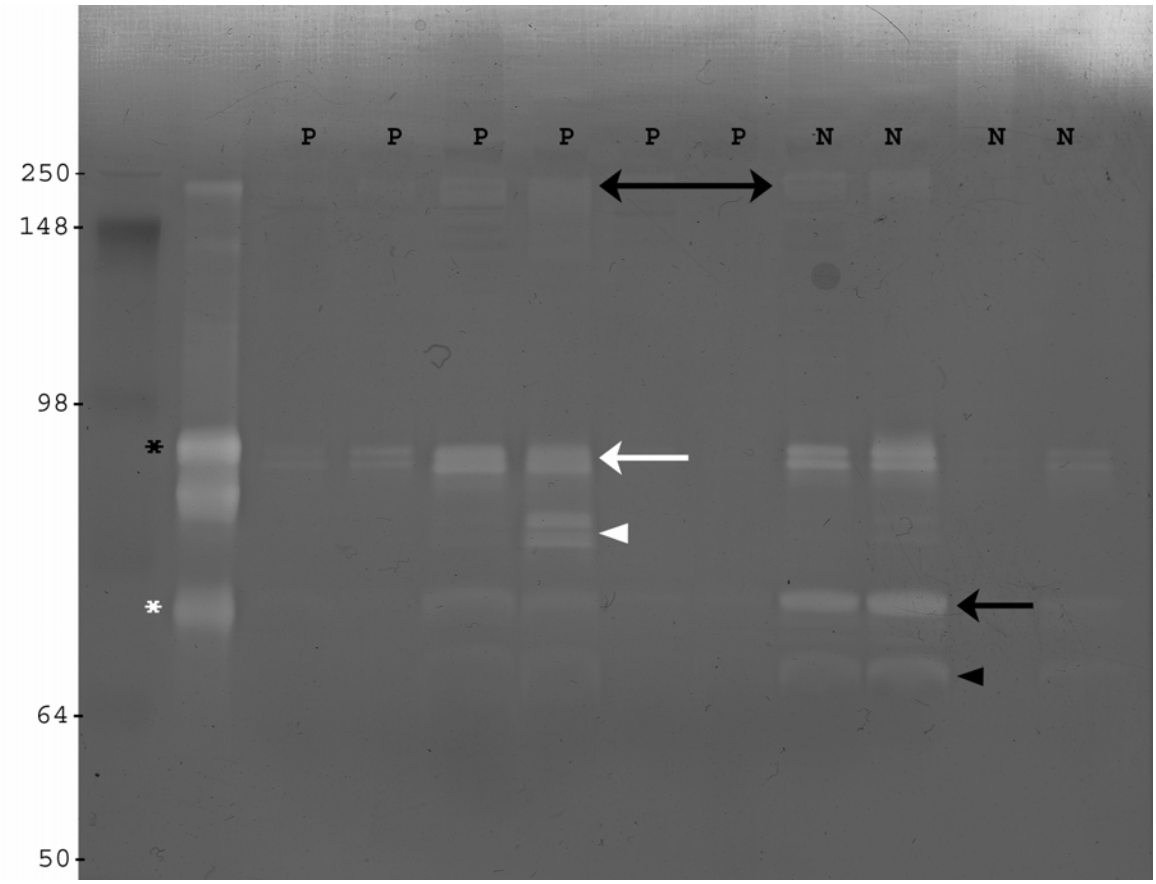

Figure 4-1: A typical zymogram of supernatant from both normal and pathologic ligaments. Gel electrophoresis was performed for 4 hours. The first lane is the molecular marker with kDa levels labeled on the left. The second lane is the MMP standard with the 92-kDa gelatinase, pro-MMP-9 (black asterisk), and 72-kDa gelatinase, pro-MMP-9 (white asterisk). The remaining lanes are labeled P for pathologic supernatant or N for normal supernatant. The zymogram sample lanes demonstrate pro-MMP-9 at 92 kDa (white arrow), active MMP-9 at 84 kDa (white arrowhead), pro-MMP-2 at 72 kDa (black arrow) and active MMP-2 at 67 kDa (black arrowhead). Note that some of these bands appear to be resolving into two separate bands as a result of the long 4-hour electrophoresis time. Note also the presence of clear bands (black double ended arrow) at approximately 225 kDa which are presumed to be either MMP dimers or less likely MMP-TIMP Complexes. The previously reported hetrodimer at 130 kDa was not observed.[24]

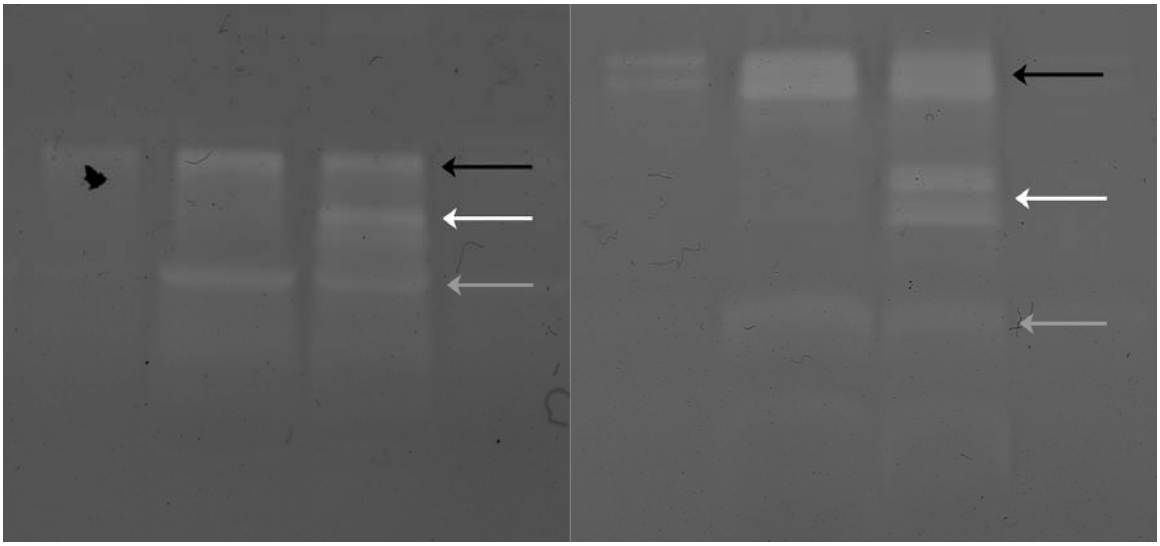

Figure 4-2: Comparison of the 2 (left) and 4 (right) hour zymograms on the same samples. The 92 kDa band (black arrow) corresponds to pro-MMP-9. The 84 kDa band (white arrowhead) corresponds to active MMP-9. The 72 kDa band (grey arrow) corresponds to MMP-2. Note that each MMP-9 bands on the right appear to be resolving into two separate bands on the left. The significance of this finding is not known.

References

1. Bolcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. Journal of Dental Research. 2000;79(9):1712-6.

2. Comerford EJ, Innes JF, Tarlton JF, Bailey AJ. Investigation of the composition, turnover, and thermal properties of ruptured cranial cruciate ligaments of dogs. Am J Vet Res 2004;65(8):1136-41.

3. Comerford EJ, Tarlton JF, Avery NC, Bailey AJ, Innes JF. Distal femoral intercondylar notch dimensions and their relationship to composition and metabolism of the canine anterior cruciate ligament. Osteoarthritis Cartilage 2005.

4. Comerford EJ, Tarlton JF, Innes JF, Johnson KA, Amis AA, Bailey AJ. Metabolism and composition of the canine anterior cruciate ligament relate to differences in knee joint mechanics and predisposition to ligament rupture. J Orthop Res 2005;23(1):61-6.

5. Muir P, Danova NA, Argyle DJ, Manley PA, Hao Z. Collagenolytic protease expression in cranial cruciate ligament and stifle synovial fluid in dogs with cranial cruciate ligament rupture. Vet Surg 2005;34(5):482-90.

6. Thrailkill KM, Moreau CS, Cockrell G, Simpson P, Goel R, North P, et al. Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology. Clin Chem Lab Med 2005;43(12):1392-9.

7. Trumble TN, Trotter GW, Oxford JR, McIlwraith CW, Cammarata S, Goodnight JL, et al. Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. Am J Vet Res 2001;62(9):1467-77.

8. Volk SW, Kapatkin AS, Haskins ME, Walton RM, D'Angelo M. Gelatinase activity in synovial fluid and synovium obtained from healthy and osteoarthritic joints of dogs. Am J Vet Res 2003;64(10):1225-33.

9. Zhou D, Lee HS, Villarreal F, Teng A, Lu E, Reynolds S, et al. Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts. J Orthop Res 2005;23(4):949-57.

10. Farndale RW, Buttle DJ, Barrett AJ. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochem Biophys Acta 1986;883(2):173-7.

11. Reddy GK, Enwemeka CS. A simplified method for the analysis of hydroxyproline in biological tissues. Clin Biochem 1996;29(3):225-9.

12. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 1982;126(1):131-8.

13. Spreng D, Sigrist N, Jungi T, Busato A, Lang J, Pfister H, et al. Nitric oxide metabolite production in the cranial cruciate ligament, synovial membrane, and articular cartilage of dogs with cranial cruciate ligament rupture. Am J Vet Res 2000;61(5):530-6.

14. Gyger O, Botteron C, Doherr M, Zurbriggen A, Schawalder P, Spreng D. Detection and distribution of apoptotic cell death in normal and diseased canine cranial cruciate ligaments. Vet J 2007;174(2):371-7.

15. Riitano MC, Pfister H, Engelhardt P, Neumann U, Reist M, Zurbriggen A, et al. Effects of stimulus with proinflammatory mediators on nitric oxide production and matrix metalloproteinase activity in explants of cranial cruciate ligaments obtained from dogs. Am J Vet Res 2002;63(10):1423-8.

16. Cao M, Stefanovic-Racic M, Georgescu HI, Fu FH, Evans CH. Does nitric oxide help explain the differential healing capacity of the anterior cruciate, posterior cruciate, and medial collateral ligaments? Am J Sports Med 2000;28(2):176-82.

17. Murakami H, Shinomiya N, Kikuchi T, Fujikawa K, Nemoto K. Differential sensitivity to NO-induced apoptosis between anterior cruciate and medial collateral ligament cells. Journal of Orthopaedic Science 2005;10(1):84-90.

18. Murakami H, Shinomiya N, Kikuchi T, Yoshihara Y, Nemoto K. Upregulated expression of inducible nitric oxide synthase plays a key role in early apoptosis after anterior cruciate ligament injury. J Orthop Res 2006;24(7):1521-34.

19. Aiken SW, Kass PH, Toombs JP. Intercondylar notch width in dogs with and without cranial cruciate ligament injuries. Veterinary and Comparative Orthopaedics and Traumatology 1995;8(3):128-132.

20. Muir P, Schaefer SL, Manley PA, Svaren JP, Oldenhoff WE, Hao Z. Expression of immune response genes in the stifle joint of dogs with oligoarthritis and degenerative cranial cruciate ligament rupture. Vet Immunol Immunopathol 2007;119(3-4):214-21.

21. Steffey MA, Miura N, Todhunter RJ, Nykamp SG, Freeman KP, Scarpino V, et al. The potential and limitations of cartilage-specific (V+C)(-) fibronectin and cartilage oligomeric matrix protein as osteoarthritis biomarkers in canine synovial fluid. Osteoarthritis Cartilage 2004;12(10):818-25.

22. Fingeroth JM. Views design of cranial cruciate ligament disease study as flawed. Am J Vet Res 2007;68(5):458; author reply 458.

23. de Bruin T, de Rooster H, van Bree H, Cox E. Evaluation of anticollagen type I antibody titers in synovial fluid of both stifle joints and the left shoulder joint of dogs with unilateral cranial cruciate disease. Am J Vet Res 2007;68(3):283-9.

24. Makowski GS, Ramsby ML. Use of citrate to minimize neutrophil matrix metalloproteinase-9 in human plasma. Anal Biochem 2003;322(2):283-6.

Chapter 5: The effect of cyclic tensile load on gene expression patterns in canine cranial cruciate ligamentocytes

In chapters three and four, one study limitation outlined was that the ligaments were cultured as explants without any applied load. As previously discussed, studies from the late 1970s demonstrated the importance of everyday loading for ultimate health of ligament.[1] It is not known how exactly, if at all, this lack of physiologic load affected our ligament explants. There is a plethora of information on mechanotransduction and its role in various physiologic and pathologic processes. [1-8] However, only a smaller number of studies evaluate the role of mechanotransduction in canine CrCL.[4,9] Therefore, the objective of the study presented in this chapter was to evaluate the effects of cyclic tensile load on molecules that had been identified in chapters three and four as potentially involved in the pathogenesis of cruciate ligament disease. The specific aim was to delineate the effect of no load, physiologic load, or supraphysiologic load on the expression of genes suspected to be involved in ligament degeneration or the initiation of osteoarthritis.

The limited knowledge available regarding the response of cruciate ligament to mechanical stimuli prevents the development of novel strategies for diagnosis, treatment, and prevention of secondary OA. Chapter 3 and 4 demonstrated significant differences between normal and diseased cruciate ligaments in explant culture. Our gene and protein expression might have been different had tensile load been applied to these explants. This would have been a technical challenge, but has been performed before in at least one study.[10] Rather than evaluate cruciate ligament on the tissue level, we elected to evaluate

the effects of load on the cellular level through monolayer culture. The culture of cruciate ligament cells in monolayer has been more extensively studied than tissue culture.[4,10-36] Therefore, we felt that beginning our approach to assessing mechanotransductive effects on the cellular level would be most productive. Although the exact mechanism of canine CrCL deficiency is unknown, research suggests that pathology is the result of ligament remodeling secondary to adaptive or degenerative changes.[37-41] Changes identified in deficient CrCLs include loss of fibroblasts, chondroid transformation of remaining fibroblasts to a spheroid phenotype, and disruption of the extracellular matrix.[38] These changes may be a response to hypoxia secondary to microinjury or poor blood flow.[37,42] Mechanotransduction is involved in a variety of processes and may also play a role in these pathologic changes in CrCL.[6,8,43,44] The objective of the proposed study was to investigate the effects of cyclic tensile load on CrCL cells. It is generally accepted that tensile load plays a significant role in maintaining homeostasis.[32,45,46] However, a detailed understanding of this mechanotransduction and its role in cruciate ligament pathogenesis remains unclear. Tensile load has been applied to human anterior cruciate ligamentocytes in monolayer culture using specialized culture wells that stretch individual cells. An increase in collagen I synthesis in these cells over unloaded ligamentocytes suggests that the application of load is an important factor in the regulation of collagen synthesis.[32] Understanding the effect of load in this *in vitro* environment is a first step in understanding mechanotransduction in CrCL homeostasis, pathogenesis, and the initiation of osteoarthritis *in vivo*. This knowledge will aid the clinical study of CrCL deficiency, development of disease models, and will lead to the development of novel

prophylactic and therapeutic strategies. Our null hypothesis was the gene expression of MMP-3 and collagen 1 genes would not differ between loaded and unloaded canine cranial cruciate ligamentocytes.

Methods

Cranial cruciate ligaments were harvested from normal dogs. The dogs had no clinical, radiographic or gross evidence of cruciate ligament disease. Animals euthanized for reasons unrelated to this study and free of any orthopedic disease based on orthopaedic examination and radiographs were eligible for inclusion in this study. All ligaments were closely inspected at the time of surgery or euthanasia. The ligament was excised and immediately placed in tissue culture using *Ligamentocyte Media* until processing. As a separate parallel study and for comparison, medial collateral ligaments were harvested in a similar manner.

Tissue Processing and Culture

Ligament tissues were aseptically diced into 1-2 mm^3 pieces. The CrCL tissue was digested with sterile Type 1A Clostridial Collagenase solution, at a concentration of 7.5mg/mL of media for eight hours. CrCL fibroblasts were then cultured in Dulbecco's Modified Eagle's Media (DMEM)[a] with 10% fetal bovine serum, 0.002% Penicillin, 100 ug/mL Streptomycin, 25 ug/mL Amphoterocin B, 0.002% L-Ascorbate[b], 0.01% L-glutamine. The flasks were incubated at 37˚C in 5% CO2 and 95% humidity with sterile medium change performed every 3 days during the strain regimes. Only passage 2 cells were used for mechanical loading.

Strain regimes

After reaching approximately 80-90% confluency, cells were washed with phosphate-buffered saline and exposed to TrypLE Express[c] solution for five minutes. Counted cells were seeded into Flexcell culture plates[d] that contain a flexible collagen 1 coated membrane at a concentration of 3 x 10^5 cells/ml of media (Figure 5-1). Following a 24-hour period of attachment to the collagen-coated plates, cyclic tensile strain was applied using a FlexCell FX4000-T™[d] (Figure 5-2). This system applied uniaxial strain to the seeded cells using a vacuum to deform the flexible membrane. Seeded cells received tensile load using a sinusoidal strain profile at 0.5 Hz (30 cycles/min) according to the following groups:

0 % strain amplitudes (subphysiologic) for 24 and 48 hours

4 % strain amplitudes (physiologic) for 24 and 48 hours

8 % strain amplitudes (supraphysiologic) for 24 and 48 hours

The cells cultured at 0 % were essentially under parallel conditions in identical Flexcell plates, but not subjected to strain. Because no strain was applied, these served as controls for this *in vitro* study. However, it is important to note that normal human ligament strains have been documented to be approximately 4 %.[47,48] So while the 0% strain served as the control baseline group, the 4% group was most analogous to what is normal in the ligament itself during everyday activity.[47,48] The 8% group was most representative of loads that are supraphysiologic but not so high as to approach strain associated with traumatic rupture. Culture media and cell monolayers were collected at

the initiation of the strain regime as well as 24 and 48 hours following the application of tensile load.

RNA Extraction from Monolayer

Total RNA was extracted using the Trispin method as previously described with some modifications for flexible membrane cultured cells.[49] Ligamentocyte media was first removed and stored at 4°C. The monolayers were washed once with Phosphate Buffered Saline (PBS). One ml of Trizol® was added to each Flexcell well and the cells were dislodged from the membrane using a sterile cell scrapper. The cells and Trizol® were placed in a 2ml O-ring vial along with three 3.2 mm stainless steel beads[c]. The cells were homogenized though the use of a mini-bead beater[e] at high speed for 30 seconds and the sample was incubated at room temperature for 10 minutes. Each sample was washed with 200 ul of chloroform[b] and vortexed. Following a short incubation of 2 minutes at room temperature an upper aqueous phase and a lower organic phase were separated by high speed centrifugation at 2°C and 12,000 rpm for 10 minutes. The upper phase was transferred to a new 1.5 ml RNA-free tube containing 3 ul of linear acrylamide, 0.5 ml isopropyl alcohol and vortexed. The interphase and lower organic phase was discarded. After 10 minutes, the tube containing the upper aqueous phase was centrifuged at 2°C and 12,000 rpm for 10 minutes. The supernatant was decanted and the small amount of fluid in the tip of the tube was removed with a pipette. One ml of 75% ethanol (EtOH)[b] was added, the tube was vortexed and centrifuged at 2°C and 7500 rpm for 5 minutes. The supernatant was decanted and the tube was given a short centrifugation for 5 seconds. Any remaining ethanol was removed with a small pipette.

The tube was dried for five minutes and a miniscule white pellet containing the mRNA could be visualized at the bottom of the centrifuge tube. To each tube, 20 ul of RNAse-free water was added to each sample. DNA contamination was addressed through the use of a TurboDNA-free™ Kit[f] according to the manufacture's recommendations. To each sample 2 ul (.1 volume of RNA) of 10X TURBO DNase Buffer and 1 ul of TURBO DNAase was added and incubated for 30 minutes. Following incubation, 2 ul (.1 volume of RNA) of a DNase inactivation reagent was added and mixed well. An incubation of 2 minutes at room temperature with occasional agitation was performed. The tube was centrifuged at 10,000 rpm for 1.5 minutes and the supernatant containing the mRNA was transferred to a new RNase-free tube.

Determination of RNA Yield

For each sample, 5 ul of the mRNA was added to 95 ul of TE buffer (pH 7.8) and utilized for determination of RNA concentration using a RiboGreen® RNA Quantitation Kit.[g] The diluted RNA for each sample was placed in individual wells of a 96-well plate and mixed with 100 ul of the ribogreen dye diluted 1:2000 in TE buffer. The RNA concentration was determined using an internal standard, a spectrophotometer and KC4 absorbance software.[h]

Reverse Transcription

Reverse transcription was accomplished through the use of 500 ng of total RNA, as determined by the spectrophotometer, and a Stratascript™ RNaseH reverse transcriptase[i]. The 500 ng of sample RNA was mixed to a final volume of 16 ul with

DEPC water and 10 pM of random primers[j]. Following incubation at 68°C for 5 minutes the reaction was placed on ice for 3 minutes. At this time 4 ul of a reaction mixture was added to bring the total volume to 20 ul. The reaction mixture contained 1 ul of Stratascript™ enzyme, 1 ul of 10mM dNTPs[k] and 2 ul 10X Stratascript™ buffer. Each 20 ul reaction was incubated at 45°C for 90 minutes and held at 4°C using a PE GeneAmp 9700[l] until the sample was diluted to 200 ul using RNase-free water. Each sample cDNA was stored at -20°C until utilization for real-time PCR.

Real-time PCR

A QuantiTect SYBR Green PCR kit[m] and a Rotor-Gene 3000™ Real-time PCR thermocycler[n] was used to accomplished real-time polymerase chain reaction. Each 20 ul reaction mixture included 0.1 ul of HK-UNG, 1 ul of 0.3 ul forward primer, 1 ul of 0.3 reverse primer, 4 ul of diluted sample cDNA, 4 ul of RNase-free water and 10 ul of 2X Quantitect™ SYBR Green master mix. The thermocycler settings including minutes at 35°C, 15 minutes at 94°C followed by 40 cycles of 3 major phases of PCR:

Melting: 5 seconds at 94°C

Annealing: 10 seconds at 57°C

Extension: 15 seconds at 72°C

The "Take-Off" point (Ct), PCR amplification efficiency and the melt curve analysis was performed using the thermocycler software (Rotor-Gene Version 5.0)[k] A melt curve analysis was performed from 69°C to 95°C at 470nm/510nm (excitation/emission) for SYBR Green to verify specific amplification and validate PCR data. Analysis of each sample was performed in duplicate for a single housekeeping gene, glyceraldehydes-3-

phosphate dehydrogenase (GADPH) as well as collagen 1 (COL1), collagen 3 (COL3) and matrix metalloproteinase 3 (MMP-3) using specific forward and reverse oligonucleotide primers (Table 3-1).

Statistical Analysis

Relative levels of gene expression were determined using QGene and expressed as a ratio to GAPDH. The relative expression statistical tool, REST-XL, was used to assess for differences in gene expression. [50,51]

Results

A total of 19 CrCL ligaments were processed for this study. Of these, only 6 survived the processing and collagenase digestion into cell culture and were designated passage 0. In sharp contrast, of the 19 MCL harvested, all 19 survived processing. When comparing to MCL cells with CrCL cells, several unique differences were observed. First, during processing, all CrCL samples demonstrated very poor pelleting during centrifugation. Instead, a thick amorphous substance was found throughout the supernatant. In addition, during the initial stages of culture in flasks, CrCL cells demonstrated only fair attachment while their MCL counterparts demonstrated strong attachment. Slow growth (only 20 % confluent by 1 week) was observed for CrCL cells while all MCL monolayers were nearly confluent by 1 week in culture (Figure 5-3). Lastly, CrCL cultures demonstrated a linear arrangement of cells (a string of cells) in association with this observed poor attachment and growth.

Both duration and amplitude of strain were evaluated in this study. Compared to cells collected at the initiation of the strain regime, cells collected after 24 hours of 8% strain downregulated collagen 1 and up-regulated MMP 3 expression by a factor of 1.443 (P=.0455) and 45.084 (P=.001), respectively. Likewise, cells collected after 48 hours of culture under 8% strain downregulated collagen 1 and up-regulated MMP-3 expression by a factor of 2.895 (P=.001) and 49.316 (P=.0075), respectively. Compared to cells collected after 24 hours of culture under 8% strain, cells collected at 48 hours downregulated collagen 1 by a factor of 2.007 (P=.006). Compared to unloaded (0% strain) CrCL cells, cells loaded with 8% strain demonstrated a 16.4 fold increase (P=.001) in MMP-3 expression at 24 hours and a 2.256 fold decrease (P=.0035) in collagen 1 expression at 48 hours. Compared to cells loaded at 4% strain, cells loaded with 8% strain demonstrated a 29.551 fold increase (P=.005) in MMP 3 expression at 24 hours. There were no significant differences found for collagen 3 at any time point or between strain regimes.

Discussion

Of the 38 ligaments that were processed for initial culture, 13 of these ligaments did not continue to grow in the *in vitro* culture. All of the non-surviving cultures were derived from CrCL. This study used a clostridial collagenase to digest minced tissue for initial culture and we suspect that this enzyme may have harsh and detrimental effect on ligamentocytes from the CrCL. Several observations support this claim. First, the protocol for establishing these cell lines was used successfully on MCL tissue. 100% of the MCL tissues survived processing and established proliferative cell lines. This

suggests a differential sensitivity of CrCL cells to the clostridial collagenase when compared to MCL cells. Differential behavior and expression between cruciate ligament cells and MCL cells is well documented in the literature.[9,13,16,18-20,28,31,34-36,52-60] Given this information it would not be surprising if the CrCL cell had a sensitivity to collagenase. It is possible that the collagenase so significantly disrupts the bond between the ECM and the ligamentocyte that the cell can no longer remain viable. Anoikis is the induction of a type of apoptosis of cells caused by loss of contact with extracellular matrix. Such a scenario has been established in periodontal ligaments in association with inflammation. Dai *et al*. was able to demonstrate that inflammation-associated breakdown products of fibronectin will induce anoikis.[61] They were also able to rescue the cells from anoikis through co-incubation with intact fibronectin, vitronectin and, to a lesser degree, collagen type I.[61] Second, the observation of the thick amorphous substance in the centrifuge tubes during processing should not be overlooked. This finding, while subjective, was present in nearly all of the tubes containing CrCL tissue. It appeared as though the clostridial collagenase was able to fully dislodge MCL cells into the surrounding fluid during agitation whereas the collagenase did not liberate the CrCL from their matrix fully. Upon centrifugation of the digested tissue, thick amorphous debris was found in the supernatant of digested CrCL tissue. There was generally only a small pellet in the tip of the centrifuge tube in these digests. In contrast, the tubes containing MCL digest offered a large pellet for subsequent cell culture. When this amorphous debris was observed under an inverted microscope, sheets of CrCL cells could be seen as layers of these linear arrangements. Within this linear arrangement, rounded ligamentocytes linked end to end on a string could be observed (Figure 5-4).

Perhaps the CrCL is not sensitive to collagenase, but rather resistant to collagenase digestion. The incomplete digestion prevents the liberation of the cells and contributes to the ultimate failure of the cell culture. The significance of these linear arrays is not entirely known other than it was associated with our inability to achieve viable monolayers. Our study was initiated in the Summer of 2005 and shortly after the observation of the linear array of cells in our digests, other research groups with interest in CrCL have described these linear arrangement of these cells after digestion.[62] Innes also cited a study in which these linear arrays were isolated from canine tendon.[63] In this study, the peri-cellular matrix associated with these linear arrangement of cells was identified as versican, collagen type VI, and Fibrillin-2.[63] Innes has theorized, based on preliminary data, that cell death (anoikis) occurs should this pericellular matrix be severed.[62] If this theory is indeed correct, it could provide a molecular mechanism to support a theory of mechanical overuse secondary to repetitive microtrauma as a contributing factor to CrCL disease. These theories have been set forth in the study of tendon overuse injury and some mechanistic pathways have been worked out.[2] It is possible that either the duration or the concentration of the collagenase digestion was enough to adversely affect our CrCL cells, but not our MCL cells. This finding must also be considered as a potential limitation of the study because this digestion may have affected or perhaps even selected for a particular phenotype in our CrCL cultures. Only 6 cultures survived, so perhaps these were the heartiest of the CrCL cells that were able to eventually establish culture.

Both duration and amplitude of the strain applied appeared to affect collagen and MMP expression in canine CrCL cells. Collagen type I gene expression was consistently

down-regulated over time at a supraphysiologic strain of 8%. This finding held true for both the 24 hour and 48 hour time points. The cells exposed to an amplitude of 8% strain also demonstrated less collagen I expression when compared to cell in parallel at 0% strain. Because most literature describes an upregulation of collagen I with the application of load[18,32,64], a discussion of the down-regulation observed in the present study is difficult. The most logical explanation is that this down-regulation occurred as a result of something that was different between the present study and previous reports. Our study was the first such study to evaluate collagen metabolism on the gene level in the dog. Unlike previous studies on collagen expression, our study evaluated metabolism to 48 hours. It is possible that cells initially respond to strain by increasing collagen metabolism, but follow this upregulation with substantial down-regulation of collagen I. Time dependent changes like these have been demonstrated in other studies.[10,36] We may have caught this gene expression as it was decreasing over time and it would have been interesting to have evaluated gene expression at 12 hours. The study by Kim *et al*. demonstrated an up-regulation of collagen 1 expression, but these cells were harvested from ligaments collected from total knee replacements.[64] These ligaments were exposed to an osteoarthritic joint for an unknown period of time. It is possible that the cell culture was a welcome environment compared to the rigors of an arthritic joint. This scenario is supported by evidence in chapter 3 outlining how normal ligament responded much more adversely to tissue culture than pathologic ligament. The study by Hseih *et al*. performed northern blotting for mRNA, but the type of strain used in this study was equibiaxial[18], different from the uniaxial utilized in the present study. The study by Toyoda *et al*. performed on rabbits demonstrated increased collagen expression on the protein level.[32]

The point here is that there are a variety of differences between the present study and previous studies (Table 5-3). Any one of these differences could have contributed to our decrease in collagen 1 gene expression. In terms of supraphysiologic strains, a decrease in collagen 1 could make sense if one assumes that too much stimulus could cause cells to react adversely. Furthermore, ligamentocytes must first breakdown ECM in order to then produce new ECM and ultimately strengthen the tissue. This may be why we observed a small decrease in collagen I synthesis at the 48 hours mark. One must also consider that we may have selected, as described above, for a certain ligamentocyte phenotype during our processing of ligament. In the future, we might recommend using the island outgrowth technique for establishment of cell culture as describe by several groups.[36,64,65]

MMP-3 was also affected by the 8% loading regime. A larger up-regulation in the MMP-3 was seen at the 24 and 48 hour time-points when compared to time 0. At the 24 hour mark, loaded cells demonstrated significantly more MMP-3 expression than cells in parallel without any strain. This was not demonstrated at the 48 hour mark. Little information in the literature exists about MMP-3 in response to a mechanical stimulus in ligament or fibroblasts in general. One possibility is that MMP-3 up-regulation occurs as a result a perceived need by the cell to initiate the turnover of ECM. Other MMPs have been studied following mechanical injury defined at 12 or 14% strain.[36] Those authors demonstrated an increase in MMP-2 in a time and amplitude dependent manner.[36] These findings together support a theory of turnover in the these cells.

No significant differences were found for collagen 3 expression. This both supports and conflicts with previous work.[18,32,64] Our work was consistent with the work

of Toyoda *et al*. in which the authors detected no change in the collagen 3 synthesis after cyclical strain.[32] Two other studies showed an increase in collagen 3 gene expression.[18,64]

These results should be considered in light of the limitations of this study. These data provide evidence of cellular responses to strain, but the in vitro nature of this study fails to consider the extracellular matrix or its interactions with ligamentocytes. Furthermore, the techniques that have been recommended for the culture of human anterior cruciate ligaments and used in this study may select for certain ligament phenotypes that can survive collagenase digestion. Because 68% of the ligaments did not survive processing, other culturing techniques for human and canine ligament fibroblasts may be better. Indeed, several studies utilized the tissue outgrowth method for establishment of cell culture. An 8% strain regimen was selected as a supraphysiologic protocol to mimic loads that would be expected to damage normal ligament. The small downregulation in collagen 1 and the large upregulation in MMP 3 provide evidence that there are changes in the gene expression in both structural proteins and degradative enzymes. This evidence of mechanotransduction supports claims, on a cellular level, that ultimate failure of the cruciate ligament may be, in part, due to cellular injury and abnormal adaptation to supraphysiologic strain. The amplitude of strain applied in the present study appeared to affect gene expression as evidenced by a significant increase in MMP-3 expression between 4% and 8% strain regimes. These findings suggest that the collagens and MMPs may be involved in normal extracellular matrix turnover leading to successful adaptation under physiologic strains. Conversely, these structural and degradative molecules may be involved in unsuccessful adaptation, ligament degeneration and ligament failure under supraphysiologic levels of strain.

[a] Gibco BRL, Grand Island, NY.
[b] Sigma Chemical Co, St. Louis, MO.
[c] Invitrogen, Carlsbad, CA
[d] Flexcell International, Hillsborough, NC
[e] Biospec Products, Bartlesville, OK.
[f] Applied Biosystems/Ambion, Austin, TX.
[g] Molecular Probes, Eugene OR.
[h] Bio-Tek, Winooski, VT.
[i] Stratagene, La Jolla, CA.
[j] Integrated DNA Technologies, Coralville, CA.
[k] Promega, Madison, WI.
[l] Applied Biosystems, Foster City, CA.
[m] Qiagen, Valencia, CA.
[n] Corbett Research, Sydney Australia
[o] Country Machine and Plastics, San Diego, CA.

	Day 4		Day 8	
Stifle #	CrCL	MCL	CrCL	MCL
1	1-2	50-70	0-1	95-100
2	4-6	50-70	15-20	95-100
3	0-1	70-80	0	95-100
4	0-1	40-60	0	95-100
5	0-1	40-60	0-1	95-100
6	0-1	40-60	0	95-100
7	0-1	60-80	0	95-100
8	0-1	60-70	0	95-100
9	10-15	50-70	10-20	95-100
10	0-1	60-70	0	95-100
11	4-6	30-40	3-10	90-100
12	5-10	50-60	7-10	95-100
13	0-1	40-60	0	85-95
14	0-1	20-30	0	95-100
15	0-1	40-60	0	90-100
16	0-1	60-70	0	95-100
17	8-12	50-70	5-10	95-100
18	15-20	20-30	70-80	90-100

Table 5-1. Summary of CrCL and MCL monolayer confluency over 8 days. Note the tremendous growth of MCL cells compare the slow and sometimes non-existent growth of CrCL cells.

	24 hours vs. Time 0	**48 hours vs. Time 0**	**48 hours vs. 24 hours**
Collagen 1	↓ 1.433 (p=0.0455)	↓ 2.895 (p=0.0455)	↓ 2.007 (p=0.006)
Collagen 3	ns	ns	ns
MMP-3	↑ 45.084 (p=0.0375)	↑ 49.316 (p=0.0075)	ns

Table 5-2. Summary of gene expression for cranial cruciate ligamentocytes under 8% strain.

	Loaded vs. Unloaded at 24 hours	**Loaded vs. Unloaded at 48 hours**
Collagen 1	ns	↓ 2.256 (p=0.0035)
Collagen 3	ns	ns
MMP-3	↑ 16.4 (p=0.001)	ns

Table 5-3. The effect of amplitude (0% vs. 8%) on gene expression for cranial cruciate ligamentocytes.

Year	Cell Type	Species	Cell Seeding (cells/well) and Culture Technique	Strain Unit	Type of Strain	Plate Substrate	Amount of Strain	Freq	Duration of Strain	Expression Evaluated	Level	Major Findings	Reference
1998	CrCL	Rabbit	4×10^5 collagenase digestion	Custom	Non-uniform uniaxial	Silicone	-2 % at Center 17% at Edge	0.17 Hz	24 hours	Collagen I and Collagen III	Protein – Collagen Assay	Increased collagen 1 synthesis; No change for Collagen III synthesis	Toyoda *et al.*[32]
2000	ACL and MCL	Human	# NA collagenase digestion	Flexcell[d]	Equi-biaxial	Collagen I Coated	0 % 5 % 7.5%	1 Hz	0, 0.5, 1, 2, 4, 16, and 24 hours	Collagen I and Collagen III	Gene – Northern Blot	Consistent increase in collagen III over 24 hrs in MCL; Significant increase in collagen I at some time points in ACL	Hseih *et al.*[18]
2002	ACL (OA – TKA)	Human	# NA outgrowth technique	Custom	Uniaxial	Collagen I Coated	10 %	0.17 Hz	24 hours	Collagen I and Collagen III	Gene – RT-PCR	Collagen I and III increased; results ablated with TGF-β1 Antibody	Kim *et al.*[64]
2005	ACL and MCL	Human	5×10^5 outgrowth technique	Country Machines and Plastics[o]	Equi-biaxial	Silicone	0% 6% 12%		0, 4, 8, 12, and q 24 hours for 9 days	MMP-2	Protein – Zymography Western Blot and Activity Assay.	MMP-2 increase in a time and amplitude dependant manner	Zhou *et al.*[36]
2006	CrCL and MCL	Canine	4×10^4 outgrowth technique	Flexcell[d]	Uniaxial	Type I Collagen or Fibro-nectin	5 %	0.1 Hz	2 or 22 hours daily for 3 days	Integrin subunits β1 and α5	Protein – Flow Cytometry	Increase expression of integrin subunits β1 and α5 in all fibroblasts with in MCL cultures having greater magnitude	Hannafin *et al.*[65]
-	CrCL and (MCL)	Canine	3×10^5 collagenase digestion	Flexcell[d]	Uniaxial	Type I Collagen	0% 4 % 8 %	0.5 Hz	24 and 48 hours	Collagen I, Collagen III and MMP-3	Gene – Real Time RT-PCR	Small decrease in collagen I, a large increase in MMP-3 and no change in collagen III	Present Study

Table 5-4. Summary of selected *in vitro* studies evaluating gene expression in of ligament cells in monolayer after cyclic tensile load

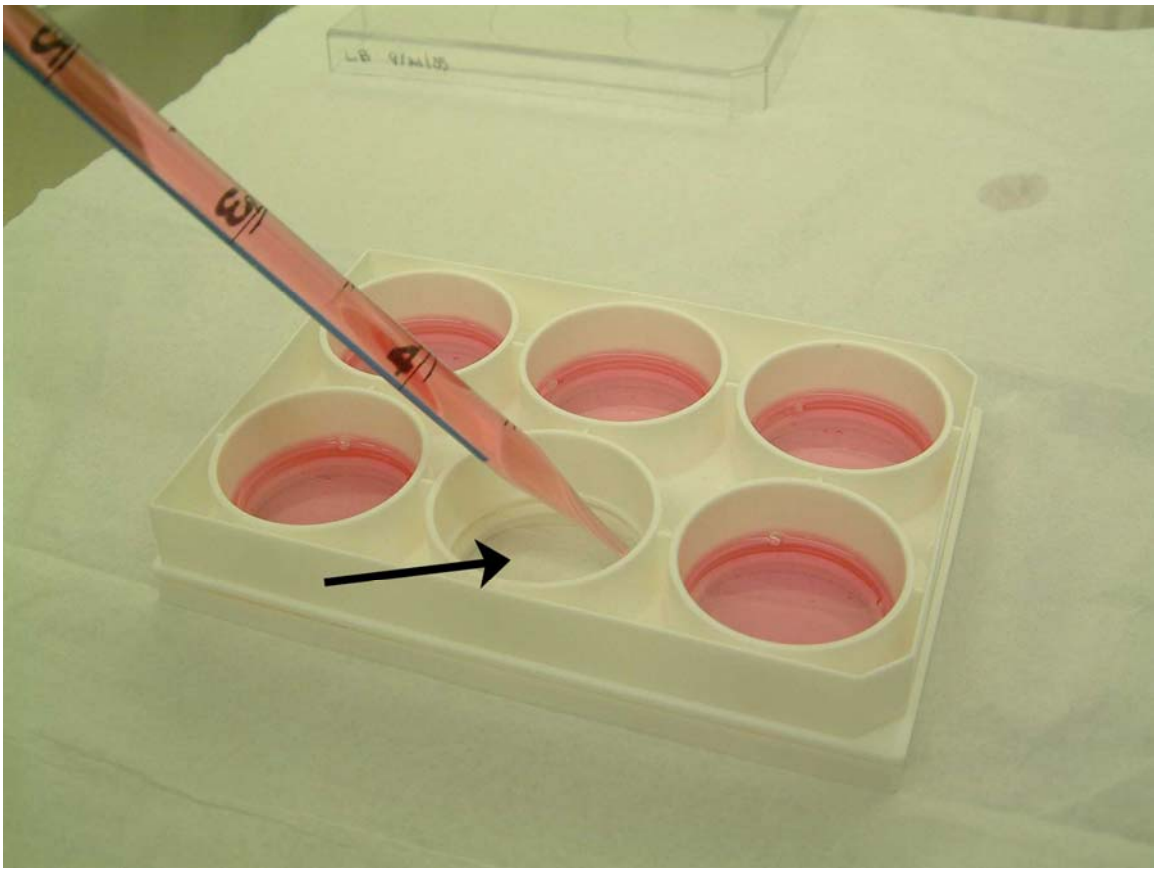

Figure 5-1. Seeding CrCL cells to the collagen coated flexible membranes (black arrow).

Figure 5-2. The Flexcell[d] Tension Apparatus with two filler plates and two culture plates ready for tensioning for 24 and 48 hours.

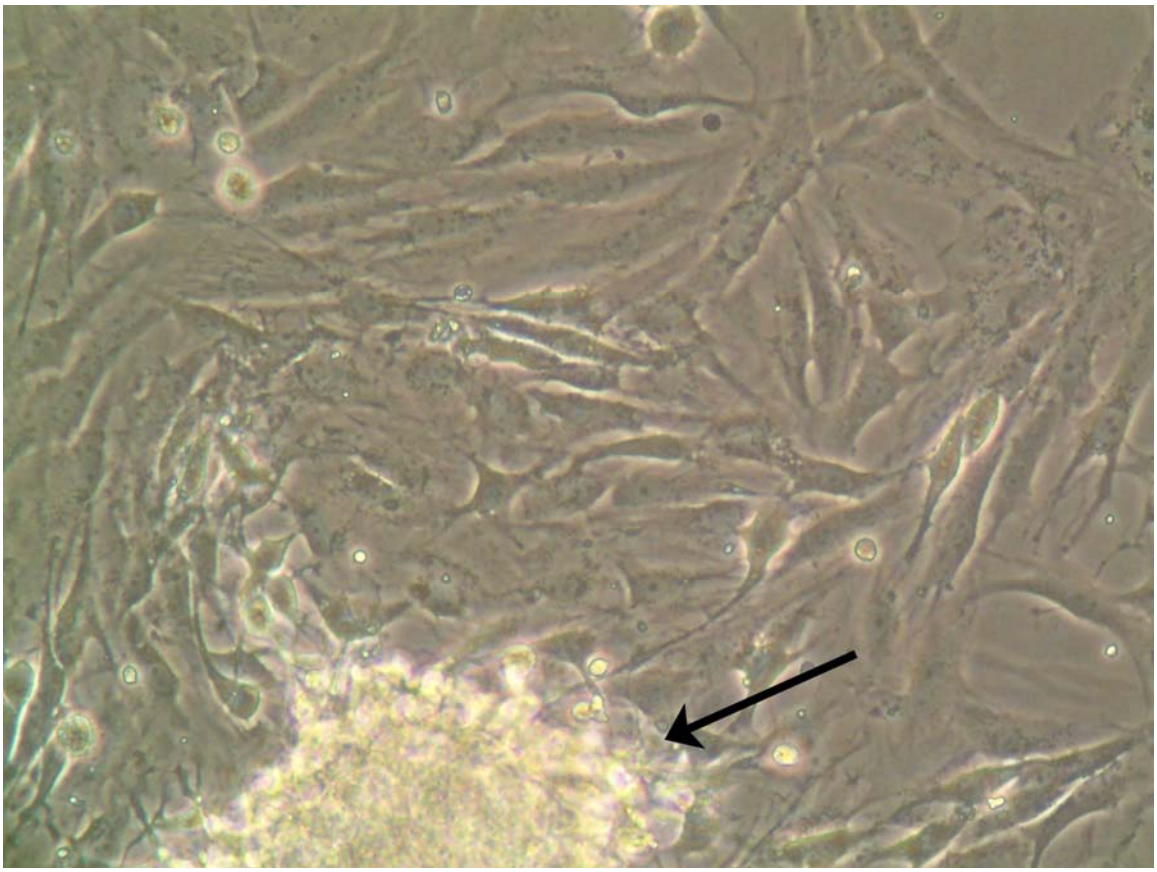

Figure 5-3. Medial collateral ligaments following 8 days of culture. Unlike their CrCL counterparts, these cultures are nearly confluent. MCL cells appear to be flourishing from individual cell growth as well as from small islands of MCL tissue (black arrow).

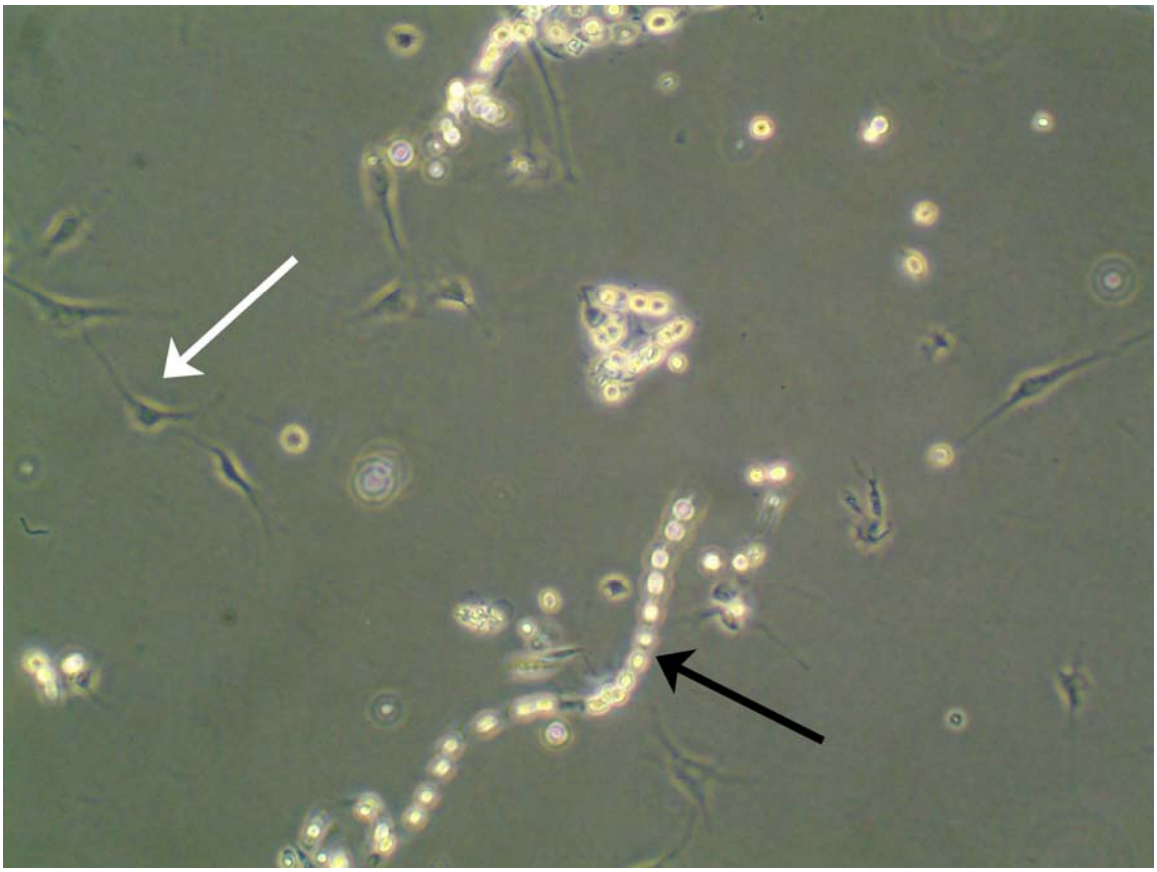

Figure 5-4: Cranial cruciate ligament cells after 8 days of culture. Note the overall poor growth of the monolayer, scattered cell and the linear array of cells

References

1. Noyes FR. Functional properties of knee ligaments and alterations induced by immobilization: a correlative biomechanical and histological study in primates. Clin Orthop Relat Res 1977(123):210-42.

2. Arnoczky SP, Tian T, Lavagnino M, Gardner K, Schuler P, Morse P. 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 2002;20(5):947-52.

3. Chano T, Tanaka M, Hukuda S, Saeki Y. Mechanical stress induces the expression of high molecular mass heat shock protein in human chondrocytic cell line CS-OKB. Osteoarthritis & Cartilage 2000;8(2):115-119.

4. Henshaw DR, Attia E, Bhargava M, Hannafin JA. Canine ACL fibroblast integrin expression and cell alignment in response to cyclic tensile strain in three-dimensional collagen gels. J Orthop Res 2006;24(3):481-90.

5. Lavagnino M, Arnoczky SP, Tian T, Vaupel Z. Effect of Amplitude and Frequency of Cyclic Tensile Strain on the Inhibition of MMP-1 mRNA Expression in Tendon Cells: An In Vitro Study. Connect Tissue Res 2003;44(3-4):181-7.

6. Peverali FA, Basdra EK, Papavassiliou AG. Stretch-mediated activation of selective MAPK subtypes and potentiation of AP-1 binding in human osteoblastic cells. Molecular Medicine. 2001;7(1):68-78.

7. Vanwanseele B, Eckstein F, Knecht H, Stussi E, Spaepen A. Knee cartilage of spinal cord-injured patients displays progressive thinning in the absence of normal joint loading and movement. Arthritis Rheum 2002;46(8):2073-8.

8. Wiseman M, Henson F, Lee DA, Bader DL. Dynamic compressive strain inhibits nitric oxide synthesis by equine chondrocytes isolated from different areas of the cartilage surface. Equine Veterinary Journal 2003;35(5):451-456.

9. Hannafin JA, Attia ET, Warren RF, Bhargava MM. Characterization of chemotactic migration and growth kinetics of canine knee ligament fibroblasts. J Orthop Res 1999;17(3):398-404.

10. Hsieh AH, Sah RL, Paul Sung KL. Biomechanical regulation of type I collagen gene expression in ACLs in organ culture. J Orthop Res 2002;20(2):325-31.

11. Amiel D, Nagineni CN, Choi SH, Lee J. Intrinsic properties of ACL and MCL cells and their responses to growth factors. Medicine & Science in Sports & Exercise 1995;27(6):844-51.

12. Cao M, Stefanovic-Racic M, Georgescu HI, Fu FH, Evans CH. Does nitric oxide help explain the differential healing capacity of the anterior cruciate, posterior cruciate, and medial collateral ligaments? Am J Sports Med 2000;28(2):176-82.

13. Cooper JA, Jr., Bailey LO, Carter JN, Castiglioni CE, Kofron MD, Ko FK, et al. Evaluation of the anterior cruciate ligament, medial collateral ligament, achilles tendon and patellar tendon as cell sources for tissue-engineered ligament. Biomaterials 2006;27(13):2747-54.

14. DesRosiers EA, Yahia L, Rivard CH. Proliferative and matrix synthesis response of canine anterior cruciate ligament fibroblasts submitted to combined growth factors. J Orthop Res 1996;14(2):200-8.

15. Ge Z, Goh JC, Lee EH. Selection of cell source for ligament tissue engineering. Cell Transplant 2005;14(8):573-83.

16. Geiger MH, Green MH, Monosov A, Akeson WH, Amiel D. An in vitro assay of anterior cruciate ligament (ACL) and medial collateral ligament (MCL) cell migration. Connective Tissue Research 1994;30(3):215-24.

17. Gentleman E, Livesay GA, Dee KC, Nauman EA. Development of ligament-like structural organization and properties in cell-seeded collagen scaffolds in vitro. Ann Biomed Eng 2006;34(5):726-36.

18. Hsieh AH, Tsai CM, Ma QJ, Lin T, Banes AJ, Villarreal FJ, et al. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. J Orthop Res 2000;18(2):220-7.

19. Marui T, Niyibizi C, Georgescu HI, Cao M, Kavalkovich KW, Levine RE, et al. Effect of growth factors on matrix synthesis by ligament fibroblasts. Journal of Orthopaedic Research 1997;15(1):18-23.

20. McKean JM, Hsieh AH, Sung KL. Epidermal growth factor differentially affects integrin-mediated adhesion and proliferation of ACL and MCL fibroblasts. Biorheology 2004;41(2):139-52.

21. Meaney Murray M, Rice K, Wright RJ, Spector M. The effect of selected growth factors on human anterior cruciate ligament cell interactions with a three-dimensional collagen-GAG scaffold. J Orthop Res 2003;21(2):238-44.

22. Muir P, Manley PA, Hao Z. COL-3 inhibition of collagen fragmentation in ruptured cranial cruciate ligament explants from dogs with stifle arthritis. Vet J 2007;174(2):403-6.

23. Murray MM, Bennett R, Zhang X, Spector M. Cell outgrowth from the human ACL in vitro: regional variation and response to TGF-beta1. J Orthop Res 2002;20(4):875-80.

24. Murray MM, Forsythe B, Chen F, Lee SJ, Yoo JJ, Atala A, et al. The effect of thrombin on ACL fibroblast interactions with collagen hydrogels. J Orthop Res 2006;24(3):508-15.

25. Murray MM, Martin SD, Spector M. Migration of cells from human anterior cruciate ligament explants into collagen-glycosaminoglycan scaffolds. J Orthop Res 2000;18(4):557-64.

26. Nagineni CN, Amiel D, Green MH, Berchuck M, Akeson WH. Characterization of the intrinsic properties of the anterior cruciate and medial collateral ligament cells: an in vitro cell culture study. Journal of Orthopaedic Research 1992;10(4):465-75.

27. Pascher A, Steinert AF, Palmer GD, Betz O, Gouze JN, Gouze E, et al. Enhanced repair of the anterior cruciate ligament by in situ gene transfer: evaluation in an in vitro model. Mol Ther 2004;10(2):327-36.

28. Ross SM, Joshi R, Frank CB. Establishment and comparison of fibroblast cell lines from the medial collateral and anterior cruciate ligaments of the rabbit. In Vitro Cell Dev Biol 1990;26(6):579-84.

29. Salo P, Frank C, Marchuk L. Synovial fluid does not inhibit collagen synthesis. Bovine cruciate ligament studied in vitro. Acta Orthop Scand 1990;61(6):570-4.

30. Schmidt CC, Georgescu HI, Kwoh CK, Blomstrom GL, Engle CP, Larkin LA, et al. Effect of growth factors on the proliferation of fibroblasts from the medial collateral and anterior cruciate ligaments. J Orthop Res 1995;13(2):184-90.

31. Sung KL, Yang L, Whittemore DE, Shi Y, Jin G, Hsieh AH, et al. The differential adhesion forces of anterior cruciate and medial collateral ligament fibroblasts: effects of tropomodulin, talin, vinculin, and alpha-actinin. Proceedings of the National Academy of Sciences of the United States of America 1996;93(17):9182-7.

32. Toyoda T, Matsumoto H, Fujikawa K, Saito S, Inoue K. Tensile load and the metabolism of anterior cruciate ligament cells. Clin Orthop 1998(353):247-55.

33. Van Eijk F, Saris DB, Riesle J, Willems WJ, Van Blitterswijk CA, Verbout AJ, et al. Tissue engineering of ligaments: a comparison of bone marrow stromal cells, anterior cruciate ligament, and skin fibroblasts as cell source. Tissue Eng 2004;10(5-6):893-903.

34. Witkowski J, Yang L, Wood DJ, Sung KL. Migration and healing of ligament cells under inflammatory conditions. Journal of Orthopaedic Research 1997;15(2):269-77.

35. Yang L, Tsai CM, Hsieh AH, Lin VS, Akeson WH, Sung KL. Adhesion strength differential of human ligament fibroblasts to collagen types I and III. Journal of Orthopaedic Research 1999;17(5):755-62.

36. Zhou D, Lee HS, Villarreal F, Teng A, Lu E, Reynolds S, et al. Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts. J Orthop Res 2005;23(4):949-57.

37. Hayashi K, Frank JD, Hao Z, Schamberger GM, Markel MD, Manley PA, et al. Evaluation of ligament fibroblast viability in ruptured cranial cruciate ligament of dogs. Am J Vet Res 2003;64(8):1010-6.

38. Hayashi K, Frank JD, Dubinsky C, Zhengling H, Markel MD, Manley PA, et al. Histologic changes in ruptured canine cranial cruciate ligament. Vet Surg 2003;32(3):269-77.

39. Hayashi K, Manley PA, Muir P. Cranial cruciate ligament pathophysiology in dogs with cruciate disease: a review. J Am Anim Hosp Assoc 2004;40(5):385-90.

40. Riitano MC, Pfister H, Engelhardt P, Neumann U, Reist M, Zurbriggen A, et al. Effects of stimulus with proinflammatory mediators on nitric oxide production and matrix metalloproteinase activity in explants of cranial cruciate ligaments obtained from dogs. Am J Vet Res 2002;63(10):1423-8.

41. Spreng D, Sigrist N, Jungi T, Busato A, Lang J, Pfister H, et al. Nitric oxide metabolite production in the cranial cruciate ligament, synovial membrane, and articular cartilage of dogs with cranial cruciate ligament rupture. Am J Vet Res 2000;61(5):530-6.

42. Arnoczky SP. Blood supply to the anterior cruciate ligament and supporting structures. Orthop Clin North Am 1985;16(1):15-28.

43. Kletsas D, Basdra EK, Papavassiliou AG. Effect of protein kinase inhibitors on the stretch-elicited c-Fos and c-Jun up-regulation in human PDL osteoblast-like cells. Journal of Cellular Physiology. 2002;190(3):313-21.

44. Bolcato-Bellemin AL, Elkaim R, Abehsera A, Fausser JL, Haikel Y, Tenenbaum H. Expression of mRNAs encoding for alpha and beta integrin subunits, MMPs, and TIMPs in stretched human periodontal ligament and gingival fibroblasts. Journal of Dental Research. 2000;79(9):1712-6.

45. Cabaud HE, Feagin JA, Rodkey WG. Acute anterior cruciate ligament injury and augmented repair. Experimental studies. Am J Sports Med 1980;8(6):395-401.

46. Viidik A. Elasticity and tensile strength of the anterior cruciate ligament in rabbits as influenced by training. Acta Physiol Scand 1968;74(3):372-80.

47. Beynnon BD, Fleming BC. Anterior cruciate ligament strain in-vivo: a review of previous work. J Biomech 1998;31(6):519-25.

48. Fleming BC, Beynnon BD. In vivo measurement of ligament/tendon strains and forces: a review. Ann Biomed Eng 2004;32(3):318-28.

49. Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 1997;22(6):1082-6.

50. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.

51. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30(9):e36.

52. Bhargava MM, Beavis AJ, Edberg JC, Warren RF, Attia ET, Hannafin JA. Differential expression of integrin subunits in canine knee ligament fibroblasts. J Orthop Res 1999;17(5):748-54.

53. Bray RC, Butterwick DJ, Doschak MR, Tyberg JV. Coloured microsphere assessment of blood flow to knee ligaments in adult rabbits: effects of injury. J Orthop Res 1996;14(4):618-25.

54. Bray RC, Leonard CA, Salo PT. Vascular physiology and long-term healing of partial ligament tears. J Orthop Res 2002;20(5):984-9.

55. Bray RC, Leonard CA, Salo PT. Correlation of healing capacity with vascular response in the anterior cruciate and medial collateral ligaments of the rabbit. J Orthop Res 2003;21(6):1118-23.

56. Kobayashi K, Healey RM, Sah RL, Clark JJ, Tu BP, Goomer RS, et al. Novel method for the quantitative assessment of cell migration: a study on the motility of rabbit anterior cruciate (ACL) and medial collateral ligament (MCL) cells. Tissue Engineering 2000;6(1):29-38.

57. Lee J, Harwood FL, Akeson WH, Amiel D. Growth factor expression in healing rabbit medial collateral and anterior cruciate ligaments. Iowa Orthop J 1998;18:19-25.

58. Murakami H, Shinomiya N, Kikuchi T, Fujikawa K, Nemoto K. Differential sensitivity to NO-induced apoptosis between anterior cruciate and medial collateral ligament cells. Journal of Orthopaedic Science 2005;10(1):84-90.

59. Sung KL, Kwan MK, Maldonado F, Akeson WH. Adhesion strength of human ligament fibroblasts. Journal of Biomechanical Engineering 1994;116(3):237-42.

60. Wallace CD, Amiel D. Vascular assessment of the periarticular ligaments of the rabbit knee. J Orthop Res 1991;9(6):787-91.

61. Dai R, Iwama A, Wang S, Kapila YL. Disease-associated fibronectin matrix fragments trigger anoikis of human primary ligament cells: p53 and c-myc are suppressed. Apoptosis 2005;10(3):503-12.

62. Innes J. Imaging of Cruciate Ligament Cells - What Can It Teach Us? In: 2005 ACVS Veterinary Symposium; 2005 October 25-26, 2005; San Diego, CA; 2005. p. 472-473.

63. Ritty TM, Roth R, Heuser JE. Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly. Structure 2003;11(9):1179-88.

64. Kim SG, Akaike T, Sasagaw T, Atomi Y, Kurosawa H. Gene expression of type I and type III collagen by mechanical stretch in anterior cruciate ligament cells. Cell Structure & Function 2002;27(3):139-44.

65. Hannafin JA, Attia EA, Henshaw R, Warren RF, Bhargava MM. Effect of cyclic strain and plating matrix on cell proliferation and integrin expression by ligament fibroblasts. J Orthop Res 2006;24(2):149-58.

Chapter 6: *In vivo* determination of gene expression patterns in cranial cruciate ligaments, caudal cruciate ligaments, and medial collateral ligaments in the Pond-Nuki model

In chapters 3 and 4, the biology of cruciate ligaments was examined using explant culture. In chapter 5, mechanical deformation of cell monolayer was used to investigate the effect of mechanics on cruciate ligament cells. When attempting to answer any orthopaedic question one must consider two major arms: biology and mechanics. The Pond-Nuki model involves CrCL transection and has been utilized for more than 30 years for the study of OA.[1-11] Traditionally, the pathogenesis of OA following transection of the cranial cruciate ligament was thought to be from abnormal biomechanics resulting from the loss of ligament function in joint stability and kinematics. Little consideration has been given to the potential biologic ramifications of the intra-articular transected ligament in the Pond-Nuki. Few studies have evaluated the role of the CrCL itself or other ligament, such as the caudal cruciate ligaments (CaCL), in the pathogenesis of OA in this model. Additionally, previous studies have noted significant differences between CrCL and medial collateral ligaments (MCL) in terms of their healing capacity, cell morphology, growth in culture, adhesiveness, migration, response to growth factors, recruitment of blood supply, as well as their gene and protein expression.[12-29] We hypothesize that ligament itself may be contributing to the initiation and progression of OA in this model as well as in naturally occurring disease. The purpose of this study was to compare gene expression patterns in CrCL, CaCL, and MCL in dogs using the Pond-Nuki model. These patterns may provide insight into the pathogenesis of secondary OA

in terms of the relative contributions of abnormal biomechanics and abnormal ligament physiology. An understanding of these relative contributions may aid in the development of novel or adjunctive treatment modalities for CCL-associated OA.

Materials and Methods

Experimental Dogs

With institutional ACUC approval, four purpose-bred hound mix dogs were utilized for this study including 2 that received arthroscopic transection of the CrCL, one dog that received arthroscopic transection of the CaCL, and one dog that received a sham operation involving arthroscopy, brief probing of the CrCL and joint lavage. Arthroscopic surgery in the contralateral limb was not performed in any dog so as to allow these stifles to serve as internal controls. The dogs were recovered, monitored, and given additional opioid pain support as needed. Non-steroidal anti-inflammatory drugs were not given. The dogs were then allowed unrestricted use of the limbs for a total of 8 weeks.[8]

Tissue Collection

The experimental animals were euthanized at eight weeks and the CrCL, CaCL, and MCL from both the surgical and non-surgical stifles were collected. Care was taken to remove epiligamentous synovial sheath in the CrCL and the CaCL as well as fat from the MCL. A total of 24 ligaments were collected in this study.

RNA extraction

Total RNA was extracted using the Trispin method as previously described. [30] Ligament explants were pulverized while still frozen using homemade pulverizer that could be maintained under liquid nitrogen. The pulverized tissue was homogenized though the use of a mini-bead beater[a]. The bead beater utilized 3.2 mm stainless steel beads[c] and Trizol® reagent at high speed for 2 minutes. Following centrifugation, the supernatant was transferred for continued processing and the pellet was discarded. The supernatant from each sample, containing the mRNA of interest, was washed with 200 ul of chloroform[b]. An upper aqueous phase and a lower organic phase were separated by high speed centrifugation. The upper phase was transferred to a new tube and the lower organic phase was stored at -80◦C. Ethanol (EtOH)[b] was vortexed with the upper aqueous phase to a final concentration of 35%. The extraction was performed with an RNeasy® Mini-Kit[c]. The upper aqueous phase/ethanol admixture was passed though the RNeasy® mini-column to facilitate the binding of the mRNA to the column filter. Any flow-through was collected and saved. The bound mRNA was washed with RW1 buffer. In order to prevent any DNA contamination, the bound mRNA was treated with a mixture of 10 ul DNase 1[d] and 80 ul RDD buffer at room temperature for 15 minutes. The column was then washed with RW1 buffer. Another two washes were performed with RPE buffer. Any flow-through was collected and saved. As a final step, the mRNA was eluted from the column using 30 ul RNase-free water and centrifugation.

Determination of RNA Yield

For each sample, 5 ul of the eluted RNA was added to 95 ul of TE buffer (pH 7.8) and utilized for determination of RNA concentration using a RiboGreen® RNA

Quantitation Kit.[d] The diluted RNA was placed for each sample was placed in individual wells of a 96-well plate and mixed with 100 ul of the ribogreen dye diluted 1:2000 in TE buffer. The RNA concentration was determined using an internal standard, a spectrophotometer and KC4 absorbance software.[e]

Reverse Transcription

Reverse transcription was accomplished through the use of 500 ng of total RNA, as determined by the spectrophotometer, and a Stratascript™ RNaseH reverse transcriptase[f]. The 500 ng of sample RNA was mixed to a final volume of 16 ul with DEPC water and 10 pM of random primers[g]. Following incubation at 68°C for 5 minutes the reaction was placed on ice for 3 minutes. At this time 4 ul of a reaction mixture was added to bring the total volume to 20 ul. The reaction mixture contained 1 ul of Stratascript™ enzyme, 1 ul of 10mM dNTPs[h] and 2 ul 10X Stratascript™ buffer. Each 20 ul reaction was incubated for 90 minutes at 45°C and held at 4°C using a PE GeneAmp 9700[i] until the sample was diluted to 200 ul using and RNase-free water. Each sample cDNA was stored at -20°C until utilization for real-time PCR.

Real-time PCR

A QuantiTect SYBR Green PCR kit[d] and a Rotor-Gene 3000™ Real-time PCR thermocycler[j] was used to accomplished real-time polymerase chain reaction. Each 20 ul reaction mixture included 0.1 ul of HK-UNG, 1 ul of 0.3 ul forward primer, 1 ul of 0.3 reverse primer, 4 ul of diluted sample cDNA, 4 ul of RNase-free water and 10 ul of 2X

Quantitect™ SYBR Green master mix. The thermocycler settings including minutes at 35°C, 15 minutes at 94°C followed 40 cycles of 3 major phases of PCR:

Melting: 5 seconds at 94°C

Annealing: 10 seconds at 57°C

Extension: 15 seconds at 72°C

The "Take-Off" point (Ct) (Fig. 3-1), PCR amplification efficiency and the melt curve analysis was performed using the thermocycler software (Rotor-Gene Version 5.0)[k] A melt curve analysis was performed from 69°C to 95°C at 470nm/510nm (excitation/emission) for SYBR Green to verify specific amplification and validate PCR data (Fig. 3-2). Analysis of each sample was performed in duplicate for a single housekeeping gene, glyceraldehydes-3-phosphate dehydrogenase (GADPH) as well as collagen 1 (COL1), collagen 2 (COL2), collagen 3 (COL3, collagen 6 (COL6), inducible nitric oxide synthase (INOS), cyclooxygenase 2 (COX2), matrix metalloproteinase 1 (MMP-1), matrix metalloproteinase 2 (MMP-2), matrix metalloproteinase 3 (MMP-3), matrix metalloproteinase 9 (MMP-9), matrix metalloproteinase 13 (MMP-13) and aggrecanase 2 (ADAMTS-5) using specific forward and reverse oligonucleotide primers (Table 3-1).

Statistical Analysis

Relative levels of gene expression were determined using QGene and expressed as a ratio to GAPDH. The relative expression statistical tool, REST-XL, was used to asses for differences in gene expression. [31,32]

Results

A descriptive approach to these data was used to highlight important findings because of the high number of comparisons in this study. Increased gene expression in transected ligaments (both CrCL and CaCL) was observed for collagen type I that was not observed in the sham or any of the contralateral controls. There was more collagen type II gene expression in the normal ligaments in the contralateral stifles than in the transected ligaments. These increases were not identified the control joint from our sham operated dog. Collagen type III demonstrated a similarly level high level of gene expression, but these increases were also observed in ligament from our sham operated stifle. Increased MMP-1 and MMP-2 gene expression in transected ligaments (both CrCL and CaCL) was observed without concurrent increased expression in the sham joint or any of the control ligaments. The gene expression profile of MMP-3 was unique. MMP-3 appeared increased and this increase was not observed in the sham operated stifle. However, none of the ligaments from the control stifles demonstrated this increase except for the sham operated dog. Increased MMP-9 gene expression was observed for CrCL. The same increases in sham operated and contralateral control ligaments were not appreciated. MMP-9 expression for the caudal cruciate ligament appeared somewhat elevated but no more so the contralateral control ligament. Increased MMP-13 gene expression was observed for CrCL, but not for CaCL, when compared to the sham and controls. Finally, the MCLs of CrCL transected stifles, but not CaCL transected stifles demonstrated increased collagen type I expression that was not observed in the sham or the contralateral control ligaments.

Discussion

It was not a surprise to observe increased levels of collagen I in all of our transected ligaments, regardless of anatomic origin. They most likely explanation for this finding is that the ligament is attempting to remodel despite its complete transection. In an interesting *in situ* hybridization study in rabbits an increased alpha 1 (I) procollagen was detected in CrCL near the site of experimental transection. This finding held true throughout the 28-day study period but was highest on day 14.[33] Our gene expression data was derived from dogs 8 weeks out from their experimental transection and suggests that this upregulation of collagen remains elevated as long as the ligament is making attempts at repair. It was interesting that collagen type II gene expression in the normal ligaments was greater in the contralateral stifles than in the transected ligaments. This suggests that some at least some basal level of collagen type II expression is present in normal ligaments and that following transection, there is a down-regulation of this expression. It is not known whether collagen II gene expression can be linked to the chondroid metaplasia that has been reported in multiple histologic studies of cruciate ligament.[34-39] It has been suggested that fibrocartilage formation may be a response to compression of the two bands under load and is potentially physiologic[40] and not pathologic[34-39] as described by other investigators. An initial evaluation of collagen type III demonstrated a similar level high level of gene expression in healing ligament and this would have correlated well with previously reported literature. [41,42] An increase in collagen type III in our ligaments that were attempting to heal would fit nicely with the

reported stages of ligamentous healing. [41,42] However, because this increase was also observed in ligament from our sham operated stifle, we must consider that this change may be due to out arthroscopic approach rather than the transection of the ligaments. It is possible that this one ligament corresponds to an outlying data point, but the evaluation of more ligaments would be necessary to resolve this disparity.

Differential gene expression was also observed for degradative enzymes. MMP-1 was elevated in all transected ligaments, regardless of type and without concurrent elevations in the sham or control ligaments. MMP-1 gene expression has previously been detected in patients whose ACLs were harvested during total knee replacements.[43] In the dog, MMP-1 gene expression has been reported in older dogs and dogs with CrCL rupture.[44] Taken together, these findings suggests that MMP-1 is involved in at least the resorption of the ligament stump and possibly the initiation of OA. MMP-2 had a nearly identical pattern of expression as MMP-1. MMP-2 has been more extensively studied in ligament and synovial fluid than MMP-1.[29,44-49] Our data contributes to the consensus that MMP-2 is an active molecule in ligaments and synovial fluid, but is unique in demonstrating the role of MMP-2 in experimentally transected ligaments. This finding contrasts our data from Chapter 3 in which MMP-2 and MMP-9 were detected but no significant differences were found between experimental groups. Recall however, that the ligaments evaluated in chapter 3 and 4 were partial tears and that MMP-2 and MMP-9 were hypothesized to become more relevant late in this disease process. Our data supports this hypothesis because we were able to detect substantial change over the sham and control ligaments at a time point 8 weeks following disruption of the ligament. Initial evaluation of MMP-3 appeared promising as it increased and this increase was not

observed in the sham operated stifle. This would have fit nicely with data from previous chapters. However, we also found that the control ligament sham operated dog was elevated. Our first thought was that this increased expression might be due to the dog placing more weight in the limb contralateral to the surgical leg. However, we would have expected an increase in the contralateral controls and this was not the case. Therefore, it becomes difficult for us to provide any plausible explanation for this unusual finding and demonstrates the need for the evaluation of additional ligaments.

MMP-9 demonstrated a slightly different gene expression pattern. Increased in this gene was observed for transected CrCL but not for the CaCL. There was no elevation in the corresponding sham operated and contralateral control ligaments. It is difficult to explain differential gene expression between types of ligaments: CrCL and CaCL. One possibility is there could be some intrinsically inherent difference between the CrCL and the CaCL. As discussed in the introduction, profound differences have been found between the ACL and the MCL, but very little is known about difference between the PCL/CaCL and other ligaments. MMP-9 has been demonstrated in ruptured ligaments, but was not found in normal ligaments from either young or old dogs.[44] In our previous study in Chapter 3, we were able to detect MMP-9 in both normal and partially ruptured ligaments in all cases, but no significant difference was found between groups. Here again, this may have been the result of a temporal factor in this disease process. MMP-9 may have been detected in these experimentally transected ligaments because they were 8 weeks from the time of injury. These gene expression data from this study are also supported by a 492-fold increase in MMP-9 gene-expression in ruptured ligaments over normal ligaments in Labradors.[50] Evaluation of MMP-13 revealed increased gene

expression for CrCL and is consistent with observation from other studies.[44,50] Some of the most interesting results from the present study centered on evaluation of MCLs. The MCLs of CrCL transected stifles demonstrated increased collagen type I expression that was not observed in the sham or the contralateral control ligaments. What was so interesting was that the MCL from the CaCL transected stifle did not demonstrate this increase. We believe this to be a classic example of differentiation between the biology and mechanics involving the stifle joint. Unlike the CaCL, the MCL lies outside the joint and is therefore protected from any change that may occur in the stifle as a result of ligament transection (biology) whether CrCL or CaCL. Therefore, assuming that there is not any systemic factor that is significantly involved in ligament pathology, any change that occurs in the MCL ligament can safely be attributed the abnormal weight bearing and ambulation (mechanics) that ensues following transection. Intuitively, this makes sense as when the CrCL transection increases the amount of strain on the MCL. As this happens, it is logical to predict an increase in collagen expression, which is exactly what was demonstrated. By transecting the CrCL the MCL responds by remodeling in an attempt to stabilize the stifle; it is a remodeling response secondary to abnormal biomechanics and cranial tibial thrust. Transection of the CaCL did not result in this increase in collagen expression because it does not result in these same biomechanical forces that place strain on the MCL.

Overall, these data suggest that the ligament itself may leach degradative enzymes into synovial fluid and exacerbate the ensuing OA. In clinical practice, many surgeons perform a limited caudomedial approach during the tibial plateau leveling osteotomy that allows for meniscal release, but precludes joint exploration and ligament debridement.

These data suggest that failure to debride a completely ruptured cruciate ligament may not optimize treatment for this common orthopedic disease. These data also suggest that OA in the Pond-Nuki model is the result of both joint instability and abnormal ligament physiology. Results from this pilot study warrant further investigation in a larger number of dogs to delineate the role of the ligament in the initiation and progression of osteoarthritis.

---

[a] Biospec Products, Bartlesville, OK.
[b] Sigma Chemical Co, St. Louis, MO
[c] Qiagen, Valencia, CA.
[d] Molecular Probes, Eugene OR.
[e] Bio-Tek, Winooski, VT.
[f] Stratagene, La Jolla, CA.
[g] Integrated DNA Technologies, Coralville, CA.
[h] Promega, Madison, WI.
[i] Applied Biosystems, Foster City, CA.
[j] Corbett Research, Sydney Australia

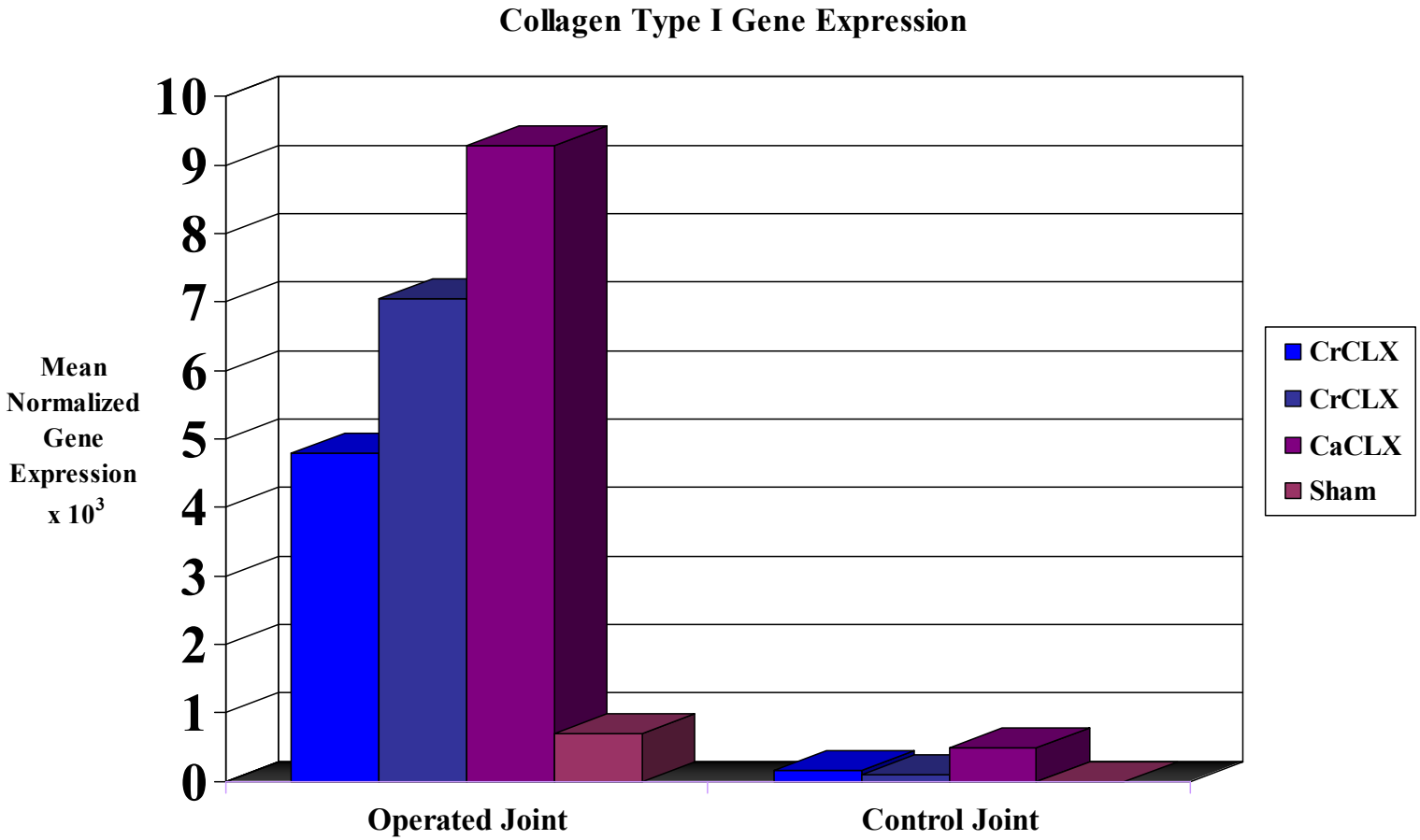

Figure 6-1. Graphical representation of collagen type I: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

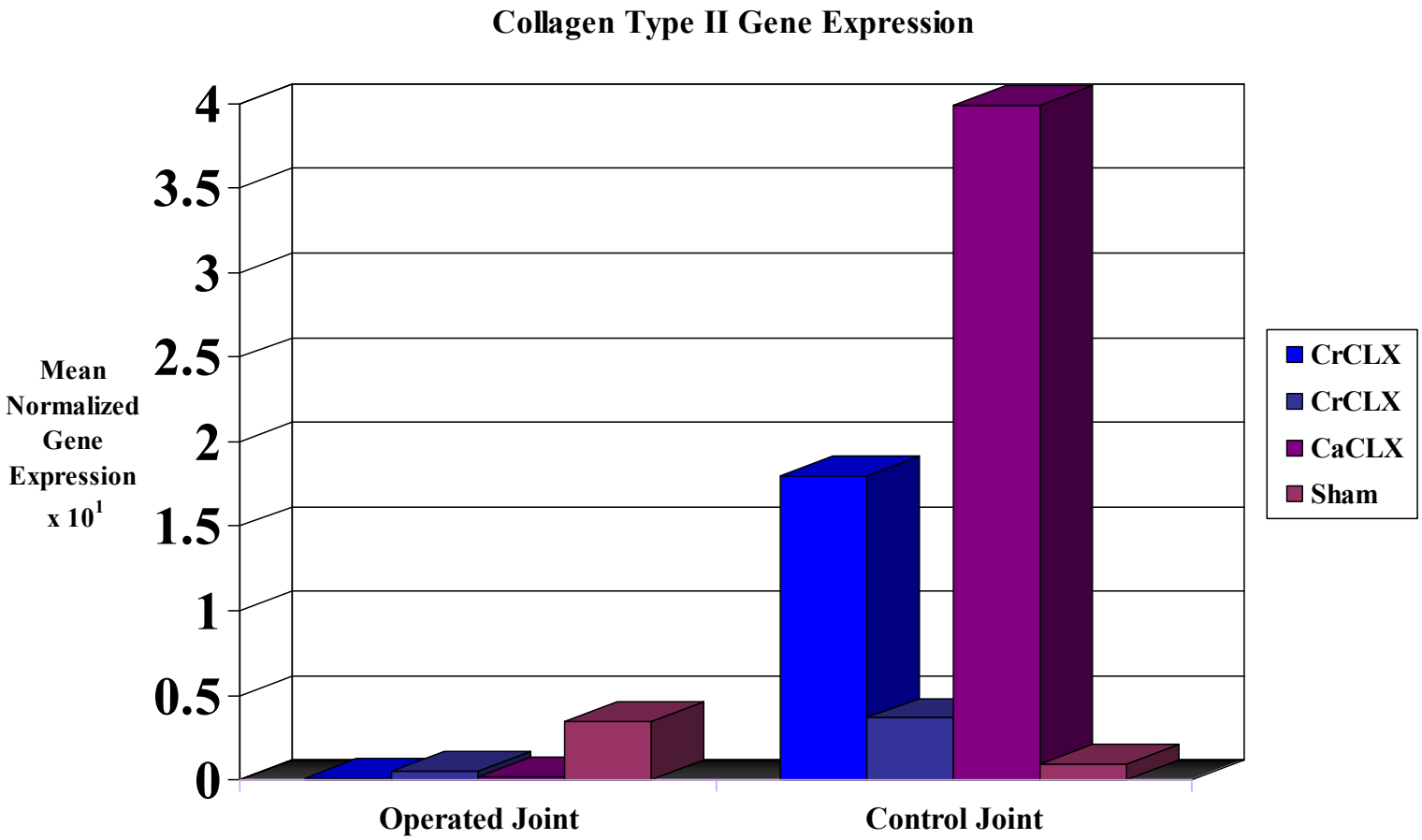

Figure 6-2. Graphical representation of collagen type II: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

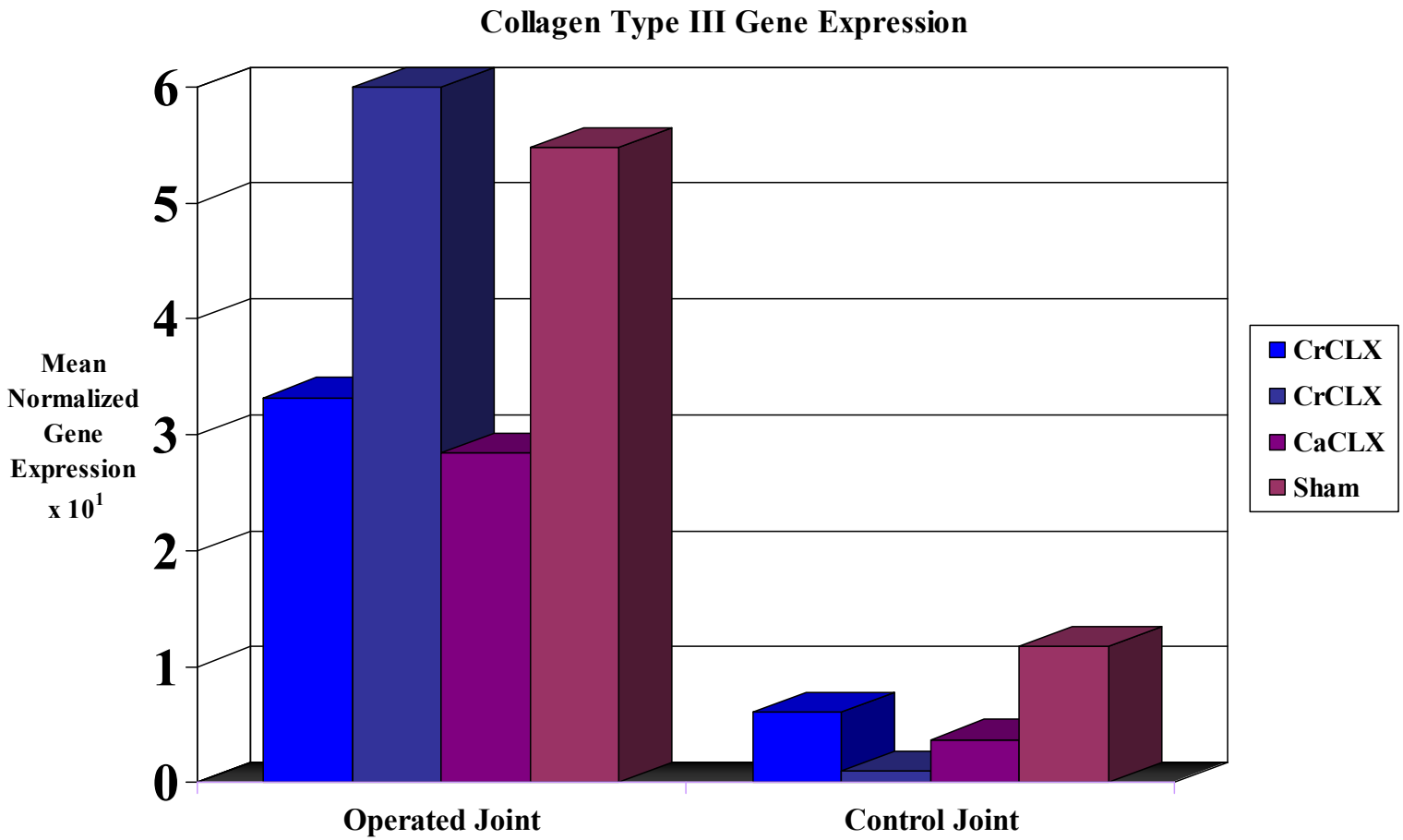

Figure 6-3. Graphical representation of collagen type III: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

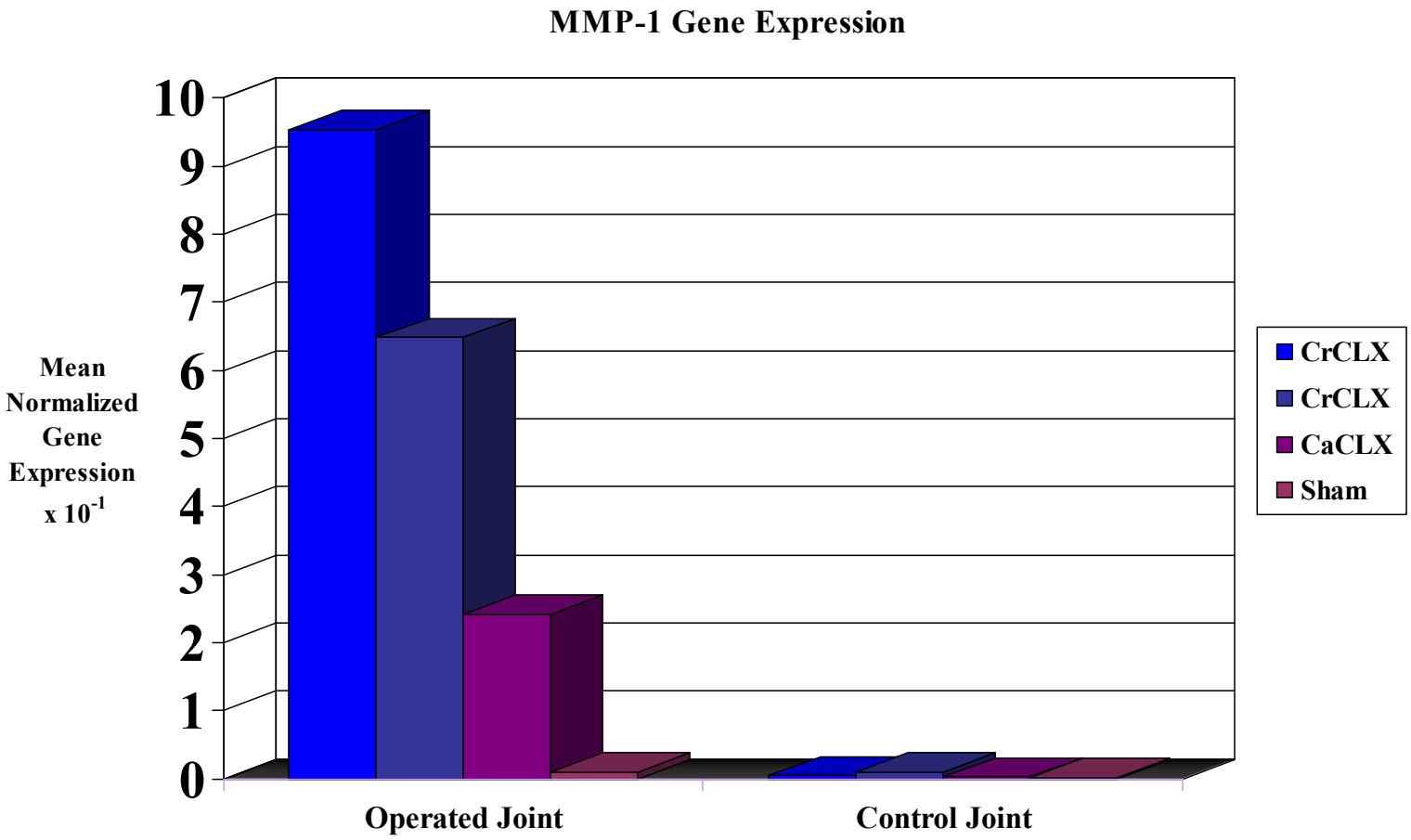

Figure 6-4. Graphical representation of MMP-1: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

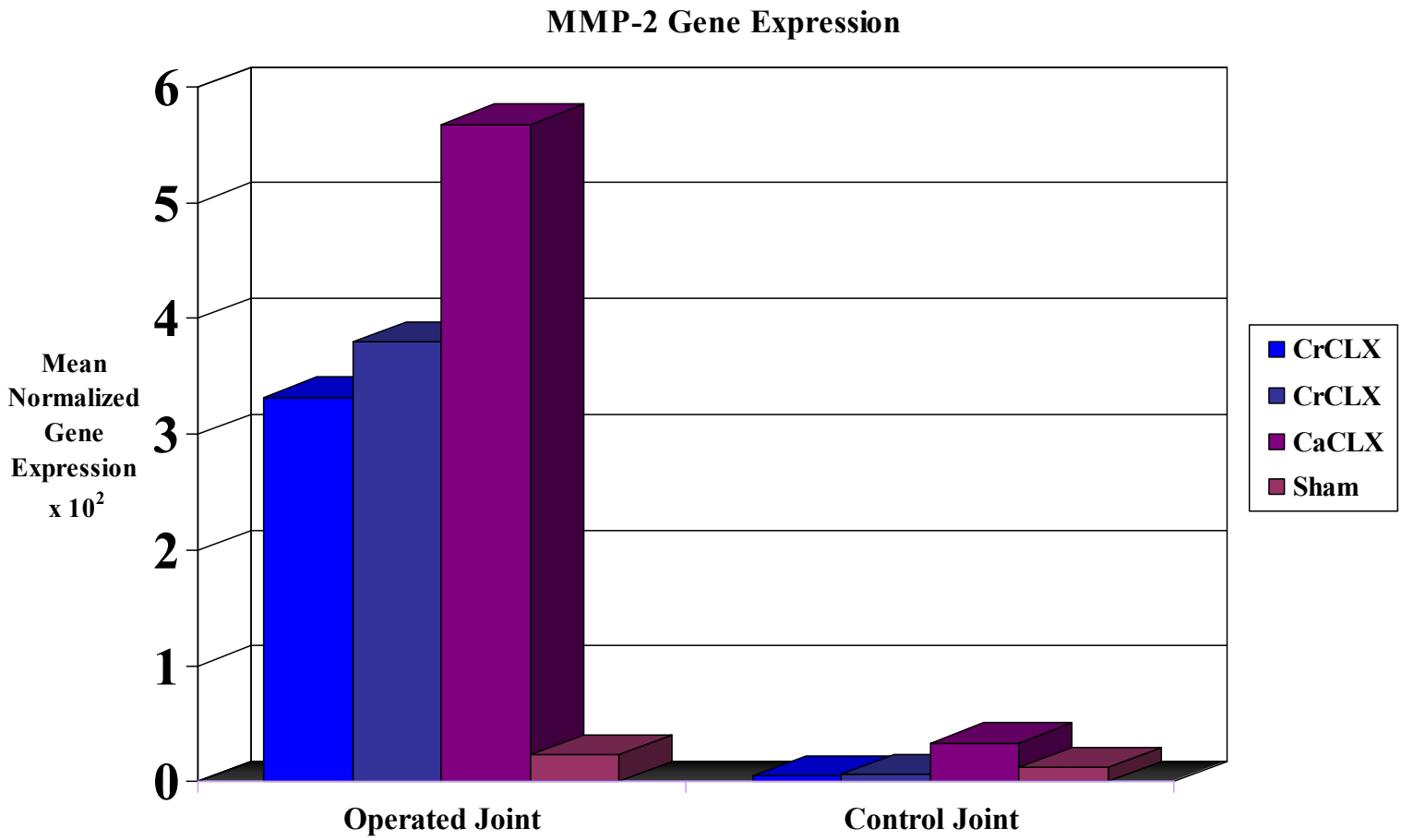

Figure 6-5. Graphical representation of MMP-2: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

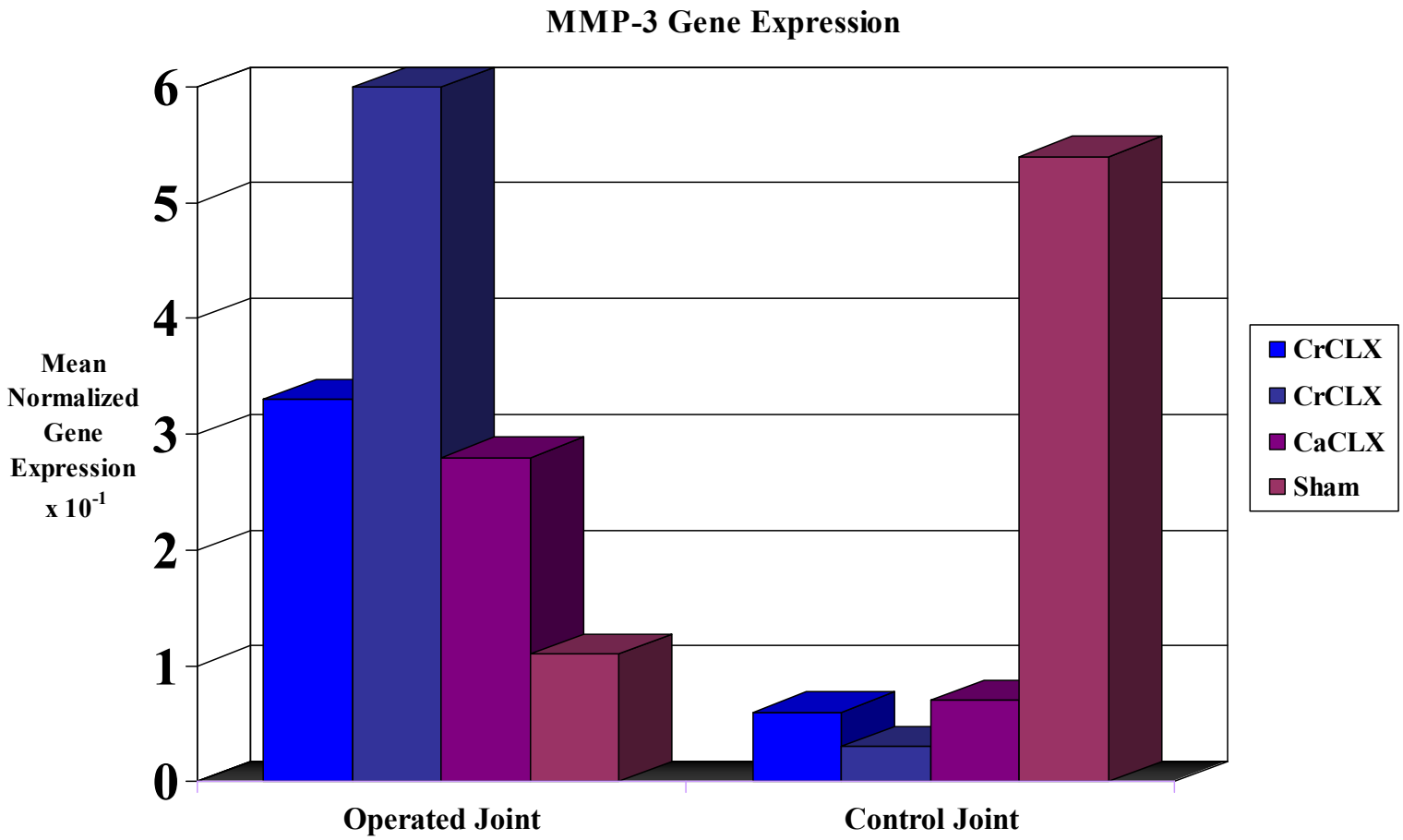

Figure 6-6. Graphical representation of MMP-3: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

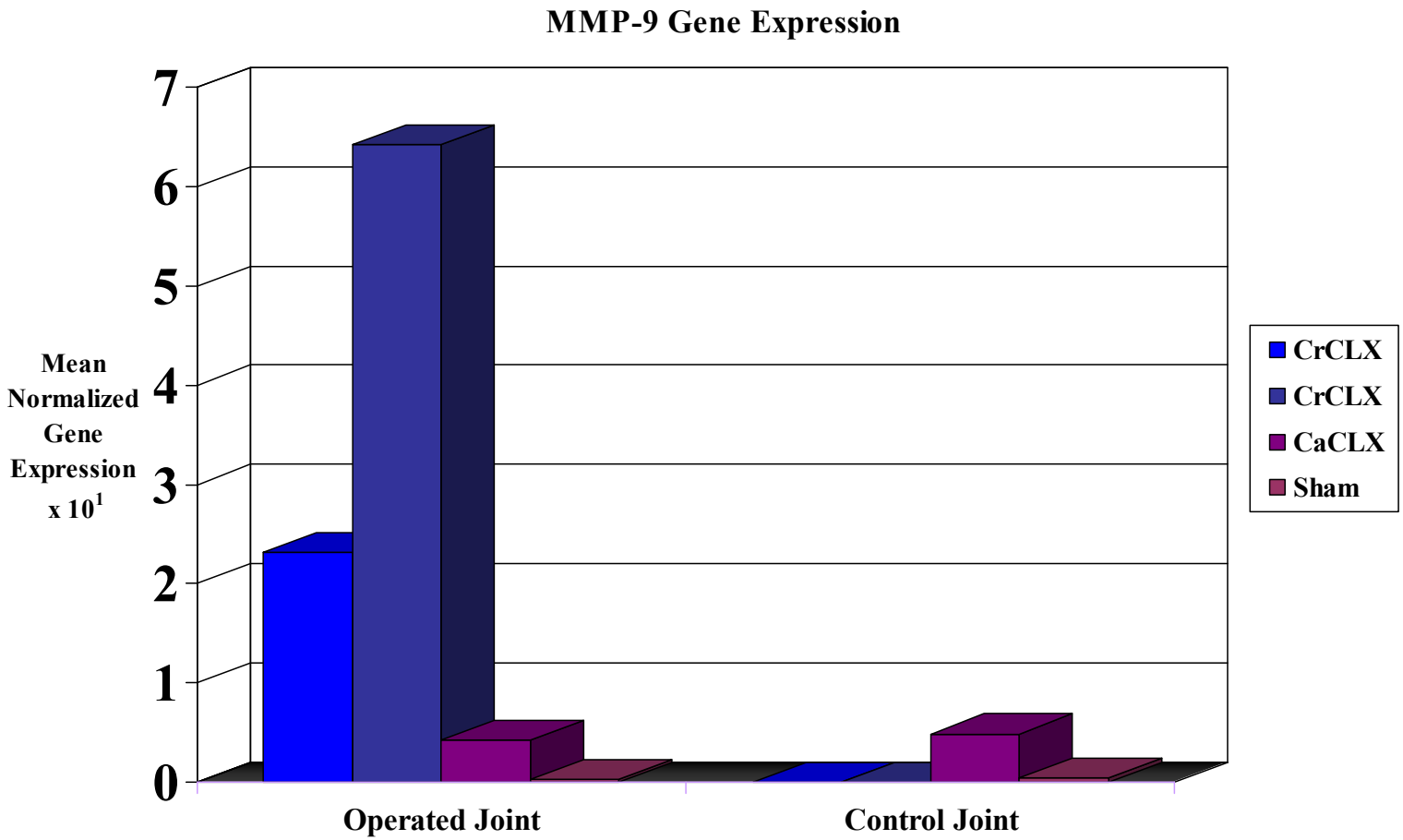

Figure 6-7. Graphical representation of MMP-9: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

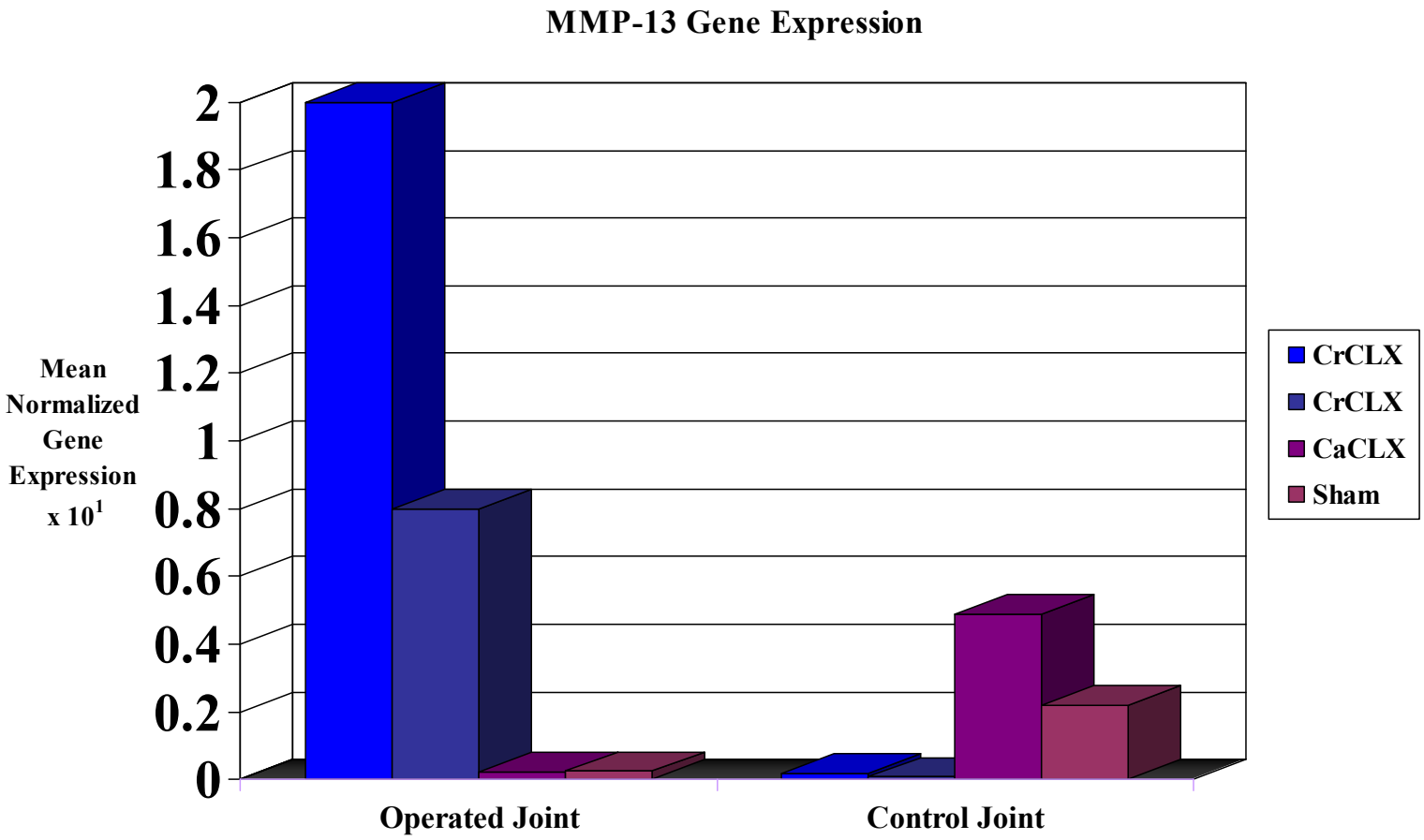

Figure 6-8. Graphical representation of MMP-13: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3.

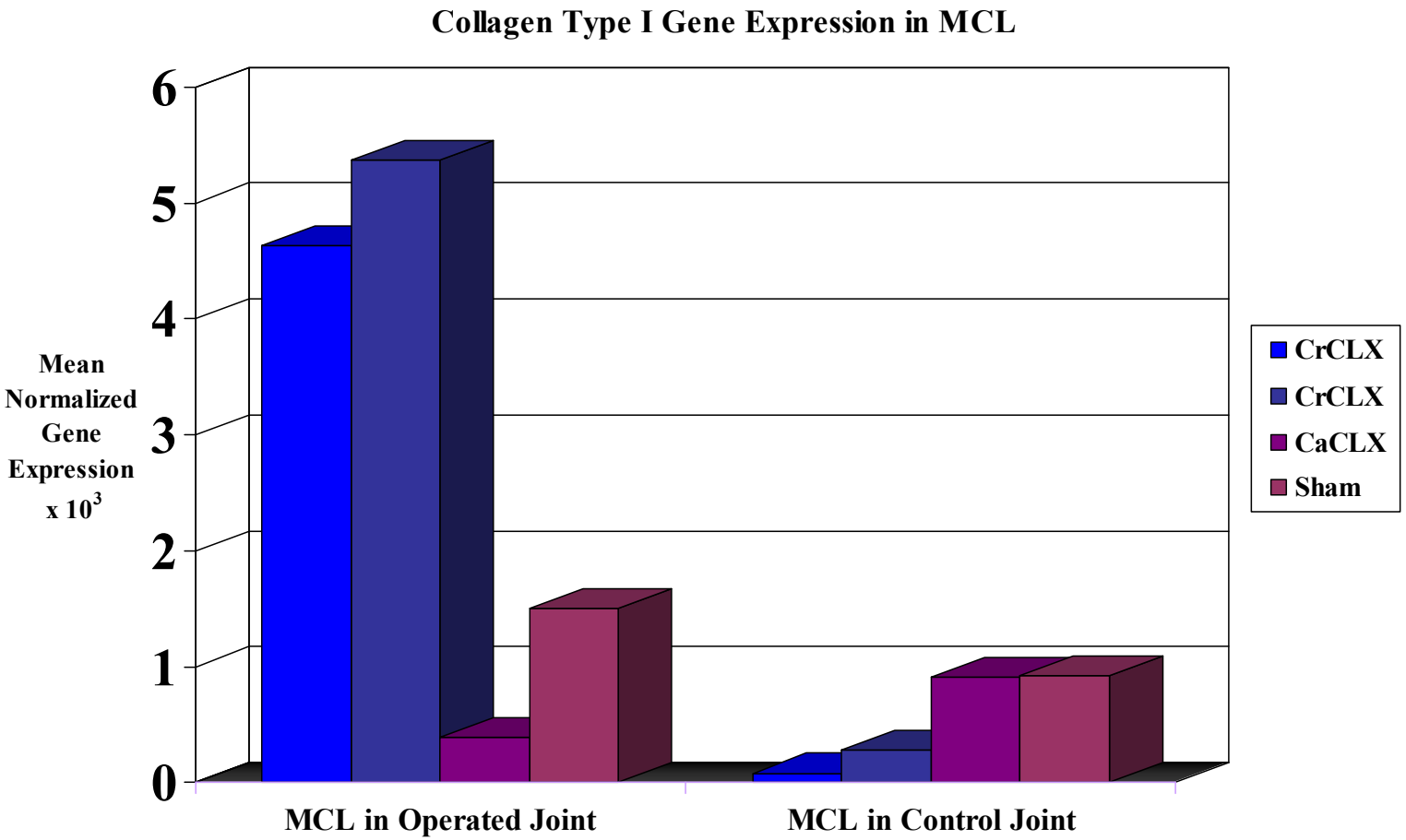

Figure 6-9. Graphical representation of collagen type I in MCL: mean normalized gene expression data for operated and control joints expressed as copies of collagen I per copies of GADPH x 10^3

References

1. Pelletier JP, Martel-Pelletier J, Ghandur-Mnaymneh L, Howell DS, Woessner JF, Jr. Role of synovial membrane inflammation in cartilage matrix breakdown in the Pond-Nuki dog model of osteoarthritis. Arthritis & Rheumatism 1985;28(5):554-61.

2. Pond MJ, Nuki G. Experimentally-induced osteoarthritis in the dog. Ann Rheum Dis 1973;32(4):387-8.

3. Attia EH, R; Bhargava M, Hannafin J. Gene Expressiion of Collagen I, Collagen III, Alpha-Smooth Muscle Actin and MMP-1 in a Rabbit Partial ACL Transection Model *52nd Annual Meeting of the Orthopaedic Research Society* 2006(Paper 1130).

4. DeGroot J, Verzijl N, Wenting-van Wijk MJ, Jacobs KM, Van El B, Van Roermund PM, et al. Accumulation of advanced glycation end products as a molecular mechanism for aging as a risk factor in osteoarthritis. Arthritis & Rheumatism 2004;50(4):1207-15.

5. Francis DA, Millis DL, Head LL. Bone and lean tissue changes following cranial cruciate ligament transection and stifle stabilization. J Am Anim Hosp Assoc 2006;42(2):127-35.

6. Funakoshi Y, Hariu M, Tapper JE, Marchuk LL, Shrive NG, Kanaya F, et al. Periarticular ligament changes following ACL/MCL transection in an ovine stifle joint model of osteoarthritis. J Orthop Res 2007;25(8):997-1006.

7. Myers SL, Brandt KD, O'Connor BL, Visco DM, Albrecht ME. Synovitis and osteoarthritic changes in canine articular cartilage after anterior cruciate ligament transection. Effect of surgical hemostasis. Arthritis Rheum 1990;33(9):1406-15.

8. Palmoski MJ, Brandt KD. Immobilization of the knee prevents osteoarthritis after anterior cruciate ligament transection. Arthritis Rheum 1982;25(10):1201-8.

9. Pardy CK, Matyas JR, Zernicke RF. Doxycycline effects on mechanical and morphometrical properties of early- and late-stage osteoarthritic bone following anterior cruciate ligament injury. J Appl Physiol 2004;97(4):1254-60.

10. Trumble TN, Billinghurst RC, McIlwraith CW. Correlation of prostaglandin E2 concentrations in synovial fluid with ground reaction forces and clinical variables for pain or inflammation in dogs with osteoarthritis induced by transection of the cranial cruciate ligament. Am J Vet Res 2004;65(9):1269-75.

11. Marijnissen AC, van Roermund PM, TeKoppele JM, Bijlsma JW, Lafeber FP. The canine 'groove' model, compared with the ACLT model of osteoarthritis. Osteoarthritis & Cartilage 2002;10(2):145-55.

12. Abramowitch SD, Yagi M, Tsuda E, Woo SL. The healing medial collateral ligament following a combined anterior cruciate and medial collateral ligament injury--a biomechanical study in a goat model. J Orthop Res 2003;21(6):1124-30.

13. Bhargava MM, Beavis AJ, Edberg JC, Warren RF, Attia ET, Hannafin JA. Differential expression of integrin subunits in canine knee ligament fibroblasts. J Orthop Res 1999;17(5):748-54.

14. Bray RC, Leonard CA, Salo PT. Vascular physiology and long-term healing of partial ligament tears. J Orthop Res 2002;20(5):984-9.

15. Bray RC, Leonard CA, Salo PT. Correlation of healing capacity with vascular response in the anterior cruciate and medial collateral ligaments of the rabbit. J Orthop Res 2003;21(6):1118-23.

16. Geiger MH, Green MH, Monosov A, Akeson WH, Amiel D. An in vitro assay of anterior cruciate ligament (ACL) and medial collateral ligament (MCL) cell migration. Connective Tissue Research 1994;30(3):215-24.

17. Hannafin JA, Attia ET, Warren RF, Bhargava MM. Characterization of chemotactic migration and growth kinetics of canine knee ligament fibroblasts. J Orthop Res 1999;17(3):398-404.

18. Hsieh AH, Tsai CM, Ma QJ, Lin T, Banes AJ, Villarreal FJ, et al. Time-dependent increases in type-III collagen gene expression in medical collateral ligament fibroblasts under cyclic strains. J Orthop Res 2000;18(2):220-7.

19. Kobayashi K, Healey RM, Sah RL, Clark JJ, Tu BP, Goomer RS, et al. Novel method for the quantitative assessment of cell migration: a study on the motility of rabbit anterior cruciate (ACL) and medial collateral ligament (MCL) cells. Tissue Engineering 2000;6(1):29-38.

20. Lee J, Harwood FL, Akeson WH, Amiel D. Growth factor expression in healing rabbit medial collateral and anterior cruciate ligaments. Iowa Orthop J 1998;18:19-25.

21. Marui T, Niyibizi C, Georgescu HI, Cao M, Kavalkovich KW, Levine RE, et al. Effect of growth factors on matrix synthesis by ligament fibroblasts. Journal of Orthopaedic Research 1997;15(1):18-23.

22. McKean JM, Hsieh AH, Sung KL. Epidermal growth factor differentially affects integrin-mediated adhesion and proliferation of ACL and MCL fibroblasts. Biorheology 2004;41(2):139-52.

23. Murakami H, Shinomiya N, Kikuchi T, Fujikawa K, Nemoto K. Differential sensitivity to NO-induced apoptosis between anterior cruciate and medial collateral ligament cells. Journal of Orthopaedic Science 2005;10(1):84-90.

24. Ross SM, Joshi R, Frank CB. Establishment and comparison of fibroblast cell lines from the medial collateral and anterior cruciate ligaments of the rabbit. In Vitro Cell Dev Biol 1990;26(6):579-84.

25. Sung KL, Kwan MK, Maldonado F, Akeson WH. Adhesion strength of human ligament fibroblasts. Journal of Biomechanical Engineering 1994;116(3):237-42.

26. Wallace CD, Amiel D. Vascular assessment of the periarticular ligaments of the rabbit knee. J Orthop Res 1991;9(6):787-91.

27. Witkowski J, Yang L, Wood DJ, Sung KL. Migration and healing of ligament cells under inflammatory conditions. Journal of Orthopaedic Research 1997;15(2):269-77.

28. Yang L, Tsai CM, Hsieh AH, Lin VS, Akeson WH, Sung KL. Adhesion strength differential of human ligament fibroblasts to collagen types I and III. Journal of Orthopaedic Research 1999;17(5):755-62.

29. Zhou D, Lee HS, Villarreal F, Teng A, Lu E, Reynolds S, et al. Differential MMP-2 activity of ligament cells under mechanical stretch injury: an in vitro study on human ACL and MCL fibroblasts. J Orthop Res 2005;23(4):949-57.

30. Reno C, Marchuk L, Sciore P, Frank CB, Hart DA. Rapid isolation of total RNA from small samples of hypocellular, dense connective tissues. Biotechniques 1997;22(6):1082-6.

31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(9):e45.

32. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30(9):e36.

33. Wiig ME, Amiel D, Ivarsson M, Nagineni CN, Wallace CD, Arfors KE. Type I procollagen gene expression in normal and early healing of the medial collateral and anterior cruciate ligaments in rabbits: an in situ hybridization study. J Orthop Res 1991;9(3):374-82.

34. Hayashi K, Frank JD, Dubinsky C, Zhengling H, Markel MD, Manley PA, et al. Histologic changes in ruptured canine cranial cruciate ligament. Vet Surg 2003;32(3):269-77.

35. Muir P, Hayashi K, Manley PA, Colopy SA, Hao Z. Evaluation of tartrate-resistant acid phosphatase and cathepsin K in ruptured cranial cruciate ligaments in dogs. Am J Vet Res 2002;63(9):1279-84.

36. Narama I, Masuoka-Nishiyama M, Matsuura T, Ozaki K, Nagatani M, Morishima T. Morphogenesis of degenerative changes predisposing dogs to rupture of the cranial cruciate ligament. J Vet Med Sci 1996;58(11):1091-7.

37. Riitano MC, Pfister H, Engelhardt P, Neumann U, Reist M, Zurbriggen A, et al. Effects of stimulus with proinflammatory mediators on nitric oxide production and matrix metalloproteinase activity in explants of cranial cruciate ligaments obtained from dogs. Am J Vet Res 2002;63(10):1423-8.

38. Vasseur PB, Pool RR, Arnoczky SP, Lau RE. Correlative biomechanical and histologic study of the cranial cruciate ligament in dogs. Am J Vet Res 1985;46(9):1842-54.

39. Zachos TA, Arnoczky SP, Lavagnino M, Tashman S. The effect of cranial cruciate ligament insufficiency on caudal cruciate ligament morphology: An experimental study in dogs. Vet Surg 2002;31(6):596-603.

40. Comerford EJ, Tarlton JF, Wales A, Bailey AJ, Innes JF. Ultrastructural differences in cranial cruciate ligaments from dogs of two breeds with a differing predisposition to ligament degeneration and rupture. J Comp Pathol 2006;134(1):8-16.

41. Frank C, Woo SL, Amiel D, Harwood F, Gomez M, Akeson W. Medial collateral ligament healing. A multidisciplinary assessment in rabbits. Am J Sports Med 1983;11(6):379-89.

42. Woo SL, Abramowitch SD, Kilger R, Liang R. Biomechanics of knee ligaments: injury, healing, and repair. J Biomech 2006;39(1):1-20.

43. Foos MJ, Hickox JR, Mansour PG, Slauterbeck JR, Hardy DM. Expression of matrix metalloprotease and tissue inhibitor of metalloprotease genes in human anterior cruciate ligament. J Orthop Res 2001;19(4):642-9.

44. Muir P, Danova NA, Argyle DJ, Manley PA, Hao Z. Collagenolytic protease expression in cranial cruciate ligament and stifle synovial fluid in dogs with cranial cruciate ligament rupture. Vet Surg 2005;34(5):482-90.

45. Comerford EJ, Innes JF, Tarlton JF, Bailey AJ. Investigation of the composition, turnover, and thermal properties of ruptured cranial cruciate ligaments of dogs. Am J Vet Res 2004;65(8):1136-41.

46. Comerford EJ, Tarlton JF, Avery NC, Bailey AJ, Innes JF. Distal femoral intercondylar notch dimensions and their relationship to composition and metabolism of the canine anterior cruciate ligament. Osteoarthritis Cartilage 2005.

47. Comerford EJ, Tarlton JF, Innes JF, Johnson KA, Amis AA, Bailey AJ. Metabolism and composition of the canine anterior cruciate ligament relate to

differences in knee joint mechanics and predisposition to ligament rupture. J Orthop Res 2005;23(1):61-6.

48. Trumble TN, Trotter GW, Oxford JR, McIlwraith CW, Cammarata S, Goodnight JL, et al. Synovial fluid gelatinase concentrations and matrix metalloproteinase and cytokine expression in naturally occurring joint disease in horses. Am J Vet Res 2001;62(9):1467-77.

49. Volk SW, Kapatkin AS, Haskins ME, Walton RM, D'Angelo M. Gelatinase activity in synovial fluid and synovium obtained from healthy and osteoarthritic joints of dogs. Am J Vet Res 2003;64(10):1225-33.

50. Clements DN, Carter SD, Innes JF, Ollier WE, Day PJ. Gene expression profiling of normal and ruptured canine anterior cruciate ligaments. Osteoarthritis Cartilage 2008;16(2):195-203.

Chapter 7: Detection and evaluation of matrix metalloproteinases involved in cruciate ligament disease using multiplex technology

In Chapter 4, detection of MMPs was accomplished with both zymography and activity assays. While these experiments provided useful information and insight into the possible mechanisms that may ultimately be involved in diseased ligament, they are not without their limitations. The handling of the zymograms themselves is difficult as they are delicate and prone to tearing. While zymography is a sensitive technique, the imaging that is required for this technique makes it semi-quantitative at best. Furthermore the substrates of these gels, gelatin, limits the detection of MMPs to all except the gelatinases (MMP-2 and MMP-9). Other MMPs of interest, namely MMP-1, MMP-3, and MMP-13 cannot be evaluated by this technique. This was a major consideration as all three of these enzymes were suspected of contributing to joint pathology. In particular MMP-3 appeared to be implicated in pathologic ligaments so we were interested to know if this enzyme could migrate from the ligament midsubstance into its surrounding culture medium. If MMP dimers and MMP-TIMP complexes were present in sufficient quantities, they may detract from the overall intensities of the pro or active bands discerned on the zymogram. Indeed, extra bands were seen on the gels from Chapter 4. Only 5 samples, in duplicate, can be run in a single gel. For this reason, inconsistencies between gels and between runs may contribute to error. Perhaps most frustrating is that performing this technique on a large number of samples takes a great deal of time. It may be appropriate for a small number of samples, but becomes cumbersome in larger studies. The activity assay is an improvement over zymography as

they are quantitative. However, these assays, like Zymography, are time consuming. Both techniques used relatively large volumes of sample. This becomes problematic when many MMPs were of interest because each assay required the same large volume for evaluation of each MMP. In chapter 4, this was a major problem as we essentially ran out of media to continue any further analysis. The problems listed above contributed to the authors' interest in pursuing more efficient more efficient means to evaluate MMPs.

Multiplex Bead Technology[a] is offered by the Luminex Corporation and provides potential resolutions to these problems associated with zymography and activity assays. The primary objective of this study was to demonstrate that MMPs could be detected using Multiplex Bead Technology. By definition, multiplexing broadly quantifies the MMPs in media from explant cultures. Secondary objectives included comparing this data to previously reported MMP gene expression data and to compare this multiplex method of MMP detection with other established methods currently in use by our laboratory, namely zymography and activity assays. Our null hypothesis was that MMPs would not differ between normal and pathologic explant media over time. Characterization of these degradative enzymes may implicate or absolve particular MMPs in the pathogenesis of ACL disease and its role in the initiation/progression of OA.

Materials and Methods

Tissue and Media Collection

Cranial cruciate ligaments were harvested from normal (control) and affected (ACL deficient) dogs. Based on clinical, gross, or radiographic evidence of ACL deficiency, excised ligaments were classified as normal (n=10) or pathologic (n=10). The ligament was excised and one third was immediately placed in tissue culture. Culture media was sampled on days 3 and 6.

Multiplex Bead Analysis

MMP 1, 2, 3, 9, and 13 were quantified in explant media using a commercially available kit[b]. Median fluorescence was determined through the use of a multiplexing machine that utilizes flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry[a]. Briefly, biomarkers and MMP standards were resuspended in assay buffer. Serial dilution of these standards was performed. Then, 25 ul of standard, quality controls or sample were added to each well of a 96-well plate. Twenty-five ul the bead solution was also placed in these well. The plate was sealed and covered with aluminum foil to prevent penetration by light. Gentile agitation on a plate shaker at 48C was provided during overnight incubation. The next day, two washings of 200ul each were performed in each well. A vacuum filter aided in the removal this buffer wash. Twenty-five ul of a detection antibody cocktail was added into each well. The plate was incubated at room temperature for 1.5 h. To each well 25 ul of a streptavidin–phycoerythrin solution was added and incubated at room temperature for 30 minutes. The plate was then analyzed on a Luminex 100 analyzer[a] and Median Fluorescence Intensity (MFI) was measured. Using the standards and appropriate curve-fitting software[a], concentration was determined for each MMP. All measurements were performed in

duplicate and these values were normalized to the wet weight of ligament. The results were reported as pg/ul media per gram of ligament.

Statistical Analysis

Statistical analysis was performed with the aid of statistical software (SigmaStat 3.5). MMP concentrations were normalized using wet weight and analyzed by a Mann-Whitney Rank Sum test with significance set at $p < 0.05$. MMP concentrations were further analyzed using zymographic and enzyme activity data previously reported by our group using a Pearson Product Moment Correlation.

Results

Detection of MMP-1, MMP-2, MMP-3, MMP-9 and MMP-13

MMP-1 was rarely detected in any of the samples. When it was detected, it was found only in small amounts. By day 3 in culture, both normal and pathologic ligaments released equivalent amounts of MMP 2 into the surrounding media and no significant difference was detected. However, by day six, normal ligaments demonstrated more than three times the amount of MMP 2 in culture media than pathologic ligaments ($p=0.006$). Pathologic ligaments also demonstrated significantly lower levels of MMP 3 in culture media than normal ligaments at day 3 ($p=0.035$) and on day 6 ($p=.05$). No other significant differences between groups were found for MMP 9. Twice and four times as much MMP 13 was detected in media from pathologic ligaments compared to normal

ligaments on days 3 and 6 respectively, but MMP 13 levels varied greatly and significance was not reached (Table 7-1).

Evaluation of MMP Multiplexing: Correlations to Zymography and Activity Assays

For active MMP 2, a weak positive correlation ($r=0.27$) between activity assays and zymography was demonstrated. For total MMP 2, a significant ($p<.001$) and strong positive correlation ($r=0.78$) between the multiplex assays and the activity assays was observed. For active MMP 9, a significant ($p<0.001$) and moderately strong ($r=0.65$) correlation between activity assays and zymography was demonstrated. For total MMP 9, a significant ($p<0.001$) and moderately strong ($r=0.64$) correlation between activity assays and zymography was observed (Table 7-2).

Discussion

The results from this study demonstrate that MMP multiplexing is a reliable, efficient, and sample-sparing method of MMP quantification for multiple enzymes of interest. The results also demonstrate that this method is very dependent on the antibody selected for detection of the enzyme of interest. Each of the MMPs of interest was detected consistently except MMP-1. Several explanations exist for this finding. Our analysis of gene expression revealed that MMP-1 mRNA was detected in all ligaments, regardless of their normal or pathologic status, so we cannot conclude that gene expression was obliterated. It is possible that the ligaments were not translating MMP-1 mRNA into protein or that there is some other post-transcription/translational regulation.

Initially, we suspected that MMP-1 might be produced in ligament but remains within its mid-substance. This would provide some evidence that ligament may not have the ability to affect adjacent tissues within the joint. However, there is evidence that this is not the case. All other MMPs were detected in the explant media thus demonstrating that the majority of the MMPs evaluated in this study do have this ability. One major advantage of an *in vitro* experiment is that it allows for a more mechanistic and controlled approach to the study of disease. For In this explant culture system there were only two components: ligament and media. We did not have multiple tissues contributing to an overall amount of MMP as we would *in vivo*. If we are able to measure MMP in the media after culture, then there is only one place it could have come from: ligament. In an *in vivo* study, for instance, one that evaluated joint fluid, it would be difficult to say where the MMP came from. Indeed synovium, cartilage, tendon, fat, meniscus and synovial fluid cells may have all contributed to the concentration of MMP within joint fluid. So, to say that one MMP out of all MMPs measured does not have the ability to diffuse into the media seems unlikely, but one we must still consider. Trans-membrane MMPs exist (MT-MMPs), but MMP-1 is not one of them. A more likely explanation is that the human MMP-1 antibody that we have utilized in our multiplexing does not cross react with canine MMP-1. Other studies in our lab utilizing multiplex technology have also found consistently low MMP-1 when found at all (unpublished data). Therefore, it is likely that detection of MMP-1 through multiplexing with this antibody is not possible for canine tissue.

MMP-2 was found in media from both normal and pathologic ligaments. As the ligaments remained in culture it became evident that the normal ligaments were

responding to the adverse in vitro environment by producing substantially more MMP-2. Again, pathologic ligaments may have a limited capacity to increase MMP-2 when compared to normal ligaments as a result of chronic exposure to a disease joint. By day 3 in culture, both normal and pathologic ligaments released equivalent amounts of MMP 2 into the surrounding media and no significant difference was detected. However, by day 6, normal ligaments demonstrated more than three times the amount of MMP 2 in culture media than pathologic ligaments. More significant differences were found with MMP-3. MMP-3 was consistently found to be significantly more elevated in normal explant media than in pathologic explant media throughout the study. This demonstrates an enormous capacity of the normal ligament to produce this degradative molecule and get it to its surrounding environment. This correlated extremely well with our gene expression in normal ligaments that showed a massive 222-fold up-regulation of MMP-3 in normal ligaments over the six days in culture compared to only 14-fold upregulation by pathologic ligaments. Because MMP-3 is a molecule that has the ability to cleave other MMP to their active state[1-3], it is possible that MMP-3 has a central role to both CrCL pathogenesis and initiation of OA. MMP-3 gene expression was found to be increased in older hounds when compared to younger dogs[4], further supporting the role of this MMP in the health and disease of cruciate ligament. Another study implicated MMP-3 as a potential biomarker in canine rheumatoid arthritis. In this study, dogs with CrCL served as controls and the amount of MMP-3 in theses clinical cases was substantially less than synovial fluid from RA patients.[5] It is possible that this increase in MMP-3 is mediated through IL-1β as cartilage explants have been shown to release MMP-3 in response to this molecule.[6] Less MMP-3 in OA cases than in RA cases does

not necessarily equate with MMP-3 having no involvement in disease. Like all studies involving CrCL disease, there was no way to stringently control for the duration of disease as owners observation of the onset of clinical signs can be prone to error. Furthermore, the slow and insidious onset of CrCL disease complicates the owner's ability to accurately define the onset of clinical signs. Indeed, conflicting reports of MMP-3 and its relation to lameness duration exist. One study demonstrated a positive correlation with MMP-3 and the duration of lameness[7] while another study revealed an inverse correlation with MMP-3 and the duration of clinical signs[8]. Our data, on the gene and protein level, suggests that MMP-3 is elevated early in the disease process and taper to lower, yet elevated levels after the initial insult. This is supported by a study that sampled synovial fluid within the first day after trauma. MMP-3 concentrations were found to increase 25-fold in the first day after trauma.[9] This study evaluated both ACL and meniscal tissue and interestingly found that MMP-3 was higher for ligament over the first 1-6 months following injury.[9] Furthermore, the levels of MMP-3 dropped after this time frame to level similar to patients with meniscal injury.[9] The data from this study seems to implicate ligament itself itself, as opposed to other structures in the joint, in the production and release of MMP-3. Smaller amounts of MMP-9 and MMP-13 were also found and although there were no significant differences found between groups, the identification of these degradative enzymes suggests that we cannot dismiss cruciate ligament biology as a contributor to the health and disease of any joint.

As a final analysis of MMP multiplex we compared this method to the two previously utilized methods of MMP detected. We utilized a Pearson product correlation to detect strength of correlations among methods. It is important to ensure that one

compares apples to apples and oranges to oranges. Recall that the multiplexing performed in this chapter measured total MMP. Therefore we were able to compare this method to total MMP from our activity assays in Chapter 4, but we were unable to directly compare any multiplex data to zymography. Zymography allows for detection of both pro and active forms of gelatinases, but not both together. Therefore it would be inappropriate to compare pro or active data from zymography to total enzyme detected by multiplexing. Likewise, it would only be appropriate to correlate active MMP concentration from the activity assay to the active band in a zymogram. As such, there is no way to appropriately compare the pro-enzyme band in zymography to any of the other two methods of MMP determination. Zymography, therefore, remains the only method of semi-quantitative detection of pro-MMP. It also remains the only method of direct visualization and method of estimation of the true activity of MMP. Together our data provide some evidence that zymography is fickle for the quantification of MMPs. This finding is supported by a study by Quesada *et al*. that showed zymography was sensitive but quantitation problems compromised its value.[10] Nonetheless, for reasons outlined in Chapter 4, it remains a cumbersome and limited method in the author's opinion. We found that there was very strong correlation between multiplexing and activity assays. This provides evidence that these two methods are equally effective at quantifying MMPs. As expected, there was poor correlation with the activity assays and zymography. Multiplexing MMPs is a relatively new method with most references in the literature published after 2006.[11-18] It is important to note that this multiplexing allows for detection of pro-, mature, and TIMP-1-complexed forms of MMP[18] and does so with less than 0.5% cross-reactivity between the different MMPs.[15] Because multiplexing is

reliable, sample-sparing, easy to use, quantitative, time saving and allows for the detection of multiple analytes, it should always be considered an option when evaluating MMPs.

[a] Luminex Corporation, Austin, TX.
[b] R&D Systems Inc., Minneapolis, MN

	Normal Day 3	Normal Day 6	Pathologic Day 3	Pathologic Day 6
MMP 1	2.0	2.8	0.7	1.4
MMP 2	66,920	101,163*	49,022	31,887*
MMP 3	70,739*	69,423†	40,966*	38,907 †
MMP 9	511	1,137	421	614
MMP 13	6,658	4,666	13,449	11,272

Table 7-1. Summary of results for MMP multiplexing: reported as pg/ul media per gram of ligament. $p \leq 0.05$* † within rows.

	Multiplex assays vs. Activity Assays	Zymography vs. Activity Assays
MMP 2	r = 0.78*	r = 0.27
MMP 9	r = 0.64*	r = 0.65*

Table 7-2. Correlation coefficients found between measures of MMP-2 and MMP-9 in chapters 4 and 7. *$p < 0.05$.

References

1. Bramono DS, Richmond JC, Weitzel PP, Kaplan DL, Altman GH. Matrix metalloproteinases and their clinical applications in orthopaedics. Clin Orthop Relat Res 2004(428):272-85.

2. Woessner JF. Matrix metalloproteinases and TIMPs. Oxford ; New York :: Oxford University Press; 2000.

3. Dreier R, Wallace S, Fuchs S, Bruckner P, Grassel S. Paracrine interactions of chondrocytes and macrophages in cartilage degradation: articular chondrocytes provide factors that activate macrophage-derived pro-gelatinase B (pro-MMP-9). J Cell Sci 2001;114(Pt 21):3813-22.

4. Muir P, Danova NA, Argyle DJ, Manley PA, Hao Z. Collagenolytic protease expression in cranial cruciate ligament and stifle synovial fluid in dogs with cranial cruciate ligament rupture. Vet Surg 2005;34(5):482-90.

5. Hegemann N, Wondimu A, Ullrich K, Schmidt MF. Synovial MMP-3 and TIMP-1 levels and their correlation with cytokine expression in canine rheumatoid arthritis. Vet Immunol Immunopathol 2003;91(3-4):199-204.

6. Cook JL, Anderson CC, Kreeger JM, Tomlinson JL. Effects of human recombinant interleukin-1beta on canine articular chondrocytes in three-dimensional culture. Am J Vet Res 2000;61(7):766-70.

7. Fujita Y, Hara Y, Nezu Y, Schulz KS, Tagawa M. Proinflammatory cytokine activities, matrix metalloproteinase-3 activity, and sulfated glycosaminoglycan content in synovial fluid of dogs with naturally acquired cranial cruciate ligament rupture. Vet Surg 2006;35(4):369-76.

8. Hegemann N, Kohn B, Brunnberg L, Schmidt MF. Biomarkers of joint tissue metabolism in canine osteoarthritic and arthritic joint disorders. Osteoarthritis Cartilage 2002;10(9):714-21.

9. Lohmander LS, Roos H, Dahlberg L, Hoerrner LA, Lark MW. Temporal patterns of stromelysin-1, tissue inhibitor, and proteoglycan fragments in human knee joint fluid after injury to the cruciate ligament or meniscus. J Orthop Res 1994;12(1):21-8.

10. Quesada AR, Barbacid MM, Mira E, Fernandez-Resa P, Marquez G, Aracil M. Evaluation of fluorometric and zymographic methods as activity assays for stromelysins and gelatinases. Clin Exp Metastasis 1997;15(1):26-32.

11. Olle EW, Ren X, McClintock SD, Warner RL, Deogracias MP, Johnson KJ, et al. Matrix metalloproteinase-9 is an important factor in hepatic regeneration after partial hepatectomy in mice. Hepatology 2006;44(3):540-9.

12. Thrailkill KM, Moreau CS, Cockrell G, Simpson P, Goel R, North P, et al. Physiological matrix metalloproteinase concentrations in serum during childhood and adolescence, using Luminex Multiplex technology. Clin Chem Lab Med 2005;43(12):1392-9.

13. Bonfield TL, Swaisgood CM, Barna BP, Farver CF, Kavuru MS, Thomassen MJ. Elevated gelatinase activity in pulmonary alveolar proteinosis: role of macrophage-colony stimulating factor. J Leukoc Biol 2006;79(1):133-9.

14. Addabbo F, Mallamaci F, Leonardis D, Tripepi R, Tripepi G, Goligorsky MS, et al. Searching for biomarker patterns characterizing carotid atherosclerotic burden in patients with reduced renal function. Nephrol Dial Transplant 2007;22(12):3521-6.

15. Jung K. Matrix metalloproteinase-8 and tissue inhibitor of metalloproteinase-1 in serum do not reflect the analytes circulating in blood. Arterioscler Thromb Vasc Biol 2008;28(3):e15-6; author reply e17.

16. Tuomainen AM, Nyyssonen K, Laukkanen JA, Tervahartiala T, Tuomainen TP, Salonen JT, et al. Serum matrix metalloproteinase-8 concentrations are associated with cardiovascular outcome in men. Arterioscler Thromb Vasc Biol 2007;27(12):2722-8.

17. Warstat K, Pap T, Klein G, Gay S, Aicher WK. Co-activation of synovial fibroblasts by laminin-111 and transforming growth factor-beta induces expression of matrix metalloproteinases 3 and 10 independently of nuclear factor-kappaB. Ann Rheum Dis 2008;67(4):559-62.

18. Grab DJ, Nyarko E, Barat NC, Nikolskaia OV, Dumler JS. Anaplasma phagocytophilum-Borrelia burgdorferi coinfection enhances chemokine, cytokine, and matrix metalloprotease expression by human brain microvascular endothelial cells. Clin Vaccine Immunol 2007;14(11):1420-4.

VITA

Lee A. Breshears was born August 6, 1974 in Denver, Colorado. After graduating from Cherry Creek High School in 1993, he pursued studies in microbiology and received a Bachelor of Science from Colorado State University in 1997. He continued studies at Colorado State University and received a Doctor of Veterinary Medicine in 2001. He completed a general rotating internship in small animal medicine and surgery at the University of Minnesota in 2002 and a private practice surgical internship at Veterinary Specialists of Nevada in 2003. Upon completing those internships, he began a small animal surgical residency at the University of Missouri-Columbia and finished as a clinical instructor in 2007. At the completion of this dissertation in 2008, he has accepted a small animal surgery locum tenens position at Massey University in North Palmerson, New Zealand.