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Characterization of proteoglycan 4 and hyaluronan composition and function of ovine synovial fluid following knee surgery

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Characterization of Proteoglycan 4 and Hyaluronan Composition and Function of Ovine Synovial Fluid Following Knee Surgery

by

Kristen Barton

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SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Characterization of Proteoglycan 4 and Hyaluronan Composition and Function of Ovine Synovial Fluid Following Knee Surgery" submitted by Kristen Barton in partial fulfilment of the requirements of the degree of Masters of Science.

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Abstract

Osteoarthritis is a degenerative joint disease involving the breakdown of articular cartilage, which is common after injury or with aging. Cartilage lubrication is a vital mechanism for the protection and maintenance of joints. Proteoglycan 4 (PRG4), a glycoprotein present in synovial fluid (SF), contributes to the boundary lubrication of cartilage and maintenance of the joint. PRG4 also acts synergistically with hyaluronan (HA), another molecule present in SF, as cartilage boundary lubricants.

The objective of this thesis was to determine 1) PRG4 and HA concentration, 2) HA molecular weight (MW) distribution, 3) cartilage lubricating ability, and 4) the isoelectric point (pI) of PRG4 in SF from surgical sham (SHAM), anterior cruciate ligament (ACL)/medial collateral ligament (MCL) transection, and lateral meniscectomy (MEN) in a post-knee injury ovine model at 20 weeks. SHAM (n=5), ACL/MCL transection (n=6), and MEN (n=5) ovine SF (oSF) was collected at euthanization 20 weeks after surgery, with the left joint serving as the non-operative control (CTRL). PRG4 and HA concentration in oSF was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) and HA MW distribution by agarose gel electrophoresis. A cartilage-cartilage friction test under boundary lubrication conditions was used to assess the cartilage lubricating ability of oSF. The pI of PRG4 was determined by two-dimensional (2D) gel electrophoresis.

PRG4 and HA concentration in SHAM, ACL/MCL, and MEN oSF was similar in comparison to the contralateral CTRL oSF. The HA MW distribution in the SHAM, ACL/MCL, and MEN oSF for all ranges were similar with respect to the contralateral CTRL oSF. The kinetic coefficient of friction in phosphate buffered saline was
significantly higher than all groups, both operated, CTRL oSF, and bovine SF in all cases, which was lower and similar. The pI of PRG4 is ~4.0-4.4 and the pI of PRG4 may have shifted to a higher pI in MEN oSF, in compared to contralateral CTRL oSF. These results suggest that lubricant composition and function was normal 20 weeks post-knee surgery in this model and 2D electrophoresis may provide insight into the structure of PRG4 glycosylations.
Preface

Chapter 2 is based on the following scientific manuscript:


*A portion of the thesis is based on a scientific manuscript, and therefore has some repetition in the introduction and methods sections of Chapter 2.*

Chapter 2 was presented on February 3-7, 2012 at the Orthopaedic Research Society Annual Meeting in San Francisco, USA.
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### List of Symbols, Abbreviations, and Nomenclature

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>ACL</td>
<td>Anterior cruciate ligament</td>
</tr>
<tr>
<td>ACL/MCL</td>
<td>Anterior cruciate ligament and medial collateral ligament transection</td>
</tr>
<tr>
<td>AM</td>
<td>Anterior medial anterior cruciate ligament bundle</td>
</tr>
<tr>
<td>bSF</td>
<td>Bovine synovial fluid</td>
</tr>
<tr>
<td>CS</td>
<td>Chondroitin sulfate side chain</td>
</tr>
<tr>
<td>CTRL</td>
<td>Contralateral control</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>HA</td>
<td>Hyaluronan</td>
</tr>
<tr>
<td>HA’se</td>
<td>Hyaluronidase</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilized pH gradient</td>
</tr>
<tr>
<td>MCL</td>
<td>Medial collateral ligament</td>
</tr>
<tr>
<td>MEN</td>
<td>Meniscectomy surgery</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>OA</td>
<td>Osteoarthritis</td>
</tr>
<tr>
<td>oSF</td>
<td>Ovine synovial fluid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with 0.1% Tween-20</td>
</tr>
<tr>
<td>PEX</td>
<td>Hemopexin domain</td>
</tr>
<tr>
<td>PL</td>
<td>Posterior lateral anterior cruciate ligament bundle</td>
</tr>
<tr>
<td>PRG4</td>
<td>Proteoglycan 4</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>rh-PRG4</td>
<td>Recombinant human proteoglycan 4</td>
</tr>
<tr>
<td>SAPL</td>
<td>Surface active phospholipids</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial Fluid</td>
</tr>
<tr>
<td>SHAM</td>
<td>Sham surgery (open arthrotomy)</td>
</tr>
<tr>
<td>SMB</td>
<td>Somatomedin B domain</td>
</tr>
<tr>
<td>TBST</td>
<td>0.1% Tween in tris buffered saline</td>
</tr>
<tr>
<td>Tps</td>
<td>Pre-sliding durations</td>
</tr>
<tr>
<td>µ</td>
<td>Frictional coefficient</td>
</tr>
<tr>
<td>µ&lt;sub&gt;static,Neq&lt;/sub&gt;</td>
<td>Static friction; the resistance to start up motion</td>
</tr>
<tr>
<td>&lt;µ&lt;sub&gt;kinetic,Neq&lt;/sub&gt;</td>
<td>Kinetic friction; the resistance to steady state motion</td>
</tr>
<tr>
<td>1D</td>
<td>One-dimensional</td>
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<tr>
<td>2D</td>
<td>Two-dimensional</td>
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CHAPTER 1: INTRODUCTION

The thesis consists of 4 chapters, outlined as follows:

Chapter 1 an introduction to the question addressed herein and provides a review of scientific and non-scientific literature related to the investigation. Chapter 1 begins with a description of the causes and prevalence of OA. A description of articular cartilage structure, function, and composition, followed by a description of boundary lubrication are explained. The components of SF, including PRG4 and HA, are explained in detail. Ligament injury, specifically ACL and MCL, and meniscal injury are explained and described in relation to the development of OA. Lastly, the choice for an ovine animal model in this study is explained.

Chapter 2 reproduces a manuscript entitled “Characterization of Proteoglycan 4 and Hyaluronan Composition and Function of Ovine Synovial Fluid Following Knee Surgery”. This manuscript will be submitted to the Journal of Orthopaedic Research, June 2012. The contents of this manuscript address the first, second, and third aims of the thesis by determining changes in lubricant composition and function of a post-knee injury ovine model by a sandwich ELISA, agarose gel electrophoresis, and an in-vitro cartilage-on-cartilage friction test.

Chapter 3 addresses the fourth aim of the investigation by using 2D gel electrophoresis as an initial assessment of PRG4 glycosylations. Chapter 3 explains the 2D electrophoresis methodology development and describes the pI of PRG4 in a post-knee injury ovine model.
Finally, Chapter 4 summarizes the background and main findings of the current work, and provides future directions for research in this area.

1.1 Osteoarthritis

Osteoarthritis (OA) is a degenerative joint disease involving the breakdown of the cartilage that covers the ends of the articular joint-bone interface (Gelber et al., 2000). OA can be classified into two categories: primary OA and secondary OA. The main difference between primary and secondary OA is the underlying cause of the cartilage breakdown. Primary OA is the normal “wear and tear” of the articular cartilage surface with age, whereas secondary OA is usually attributed to a specific identifiable cause, such as trauma to the joint, obesity, disease, or genetics. OA causes pathological changes in the cartilage, synovium, and bone and can lead to an exposed bone-on-bone interaction causing extreme pain, thus reducing the quality of life of many individuals (Hui et al., 2011). OA symptoms may include joint pain, stiffness, loss of range of motion, and inflammation. OA is a multifactorial disease that may include a number of contributing factors including genetics, developmental, biomechanical, inflammatory, and metabolic abnormalities (Figure 1.1.1) (Coles et al., 2010) (Van de Velde et al., 2009).

OA is the most common form of arthritis. It is prevalent, especially in elderly populations, and can be detrimental to quality of life as it restricts the mobility of millions of patients globally. Recent estimates suggest that 27 million people over the age of 25 suffer from OA in the United States (Lawrence et al., 2008). Approximately 10% of Canadians (3 million people) are affected by OA (Statistics Canada, 2010). Two major underlying risk factors that are increasing the prevalence, and subsequent economic
burden, of OA are aging and obesity (Bitton, 2009). Although less common in adults aged under 40 years, OA may be becoming a greater burden to the health care system since anterior cruciate ligament (ACL) injuries are occurring more frequent in younger populations. OA shows an increase in incidence with increase in age over 60 years and has been known to progress with a stronger prevalence in women than men (Felson, 2003) (Sabatini et al., 2004). Other risk factors for OA include sex, joint disorder, abnormal joint alignment, muscle weakness, inflammatory arthritis, trauma, or injury (Felson, 2003) (Wildner & Sangha, 2000).

Figure 1.1.1 Multiple factors influence the degradation of articular cartilage and subchondral bone leading to the development of OA (modified from Minas, 2011).
OA has a large impact on patient disability and healthcare costs in developed countries. The economic burden is attributable to disability, comorbid disease, and treatment expense (Bitton, 2009). In 2007, in the United States, estimated costs related to OA alone were in the excess of $60 billion (Waddell, 2007). In 2002, the mean annual healthcare cost to a Canadian patient was $12200, which was majority of lost wages and assistance from a caregiver (Gupta et al., 2005). Additional studies have reported similar societal and personal costs due to OA (Lapsley et al., 2001) (Reginster, 2002) (Wildner & Sangha, 2000). It is estimated that by 2015 in Canada, OA will result in $195 billion in direct and indirect costs (Beaton et al., 2010).

Mohr (2000) classified the morphogenesis of OA into three stages: primary, secondary, and tertiary. The primary event is thought to involve the initial cartilage destruction and breakdown where the chondrocytes die either by necrosis or apoptosis, allowing the cartilage surface to become fibrillated (Mohr, 2000). During the early stages of OA, softening, fibrillation and fissuring changes become apparent to the articular surface of cartilage, as well erosion and ulcerations of cartilage substance (Angel et al., 2009) (Young et al., 2006). Changes in the cells occur at cellular and molecular levels, leading to functional and structural loss of articular cartilage (Klug & Weseloh, 2000). This loss may be initiated with degradative enzymes by the synovium and chondrocytes (Teeple et al., 2010). Pathologic features of OA in cartilage include matrix edema, proteoglycan depletion, chondrocyte necrosis and proliferation, and active calcification (Pritzker, 2003). Proteolytic degradation of the extracellular matrix of the superficial cartilage results in decreased structural integrity (Young et al., 2006). The cartilage matrix is broken down by matrix metalloproteinases, enzymes that degrade type II
collagen and proteoglycans, and these are elevated in OA and joint injury (Angel et al., 2009) (Ishiguro et al., 1999) (Tchetverikov et al., 2005). Inflammatory cytokines, including interleukin-1β, tumor necrosis factor-α, and interleukin-6, alter joint homeostasis by increasing metalloproteinase production, thus affecting cartilage breakdown (Angel et al., 2009). Interleukin-1β primarily initiates matrix degradation and both tumor necrosis factor-α and interleukin-6 initiate the inflammatory response (Angel et al., 2009). OA is very difficult to identify at early time points in humans. Early signs of knee OA can be seen via histology in the superficial layers of the hyaline cartilage where there is loss of proteoglycans and softening of the cartilage (Reichel & Hein, 2000).

The secondary event in OA progression is thought to be disease development in the subchondral bone and synovium (Mohr, 2000). OA progresses to deeper regions of the articular cartilage, leading to complete loss of cartilage (Coles et al., 2010). This stage includes osteonecrosis, or bone necrosis, and synovitis, which involve inflammatory synovial reactions and molecular degradation of synovial fluid (SF) products. In later OA, cartilage degradation leads to loss of non-calcified and calcified cartilage (Reichel & Hein, 2000). The tertiary events in OA progression include bone and synovial tissue remodelling (Mohr, 2000). The gross OA degradation process is demonstrated in Figure 1.1.2.
Figure 1.1.2 Progression of OA in a human knee. A normal healthy knee is shown on the left; with minimal articular cartilage degradation. The development of OA is shown across the spectrum, with severe articular cartilage degradation and loss shown on the right (modified from Sah, 2009).

The ability to characterize OA is improving, however, the classification of OA can be difficult. Basic clinical OA criteria include pain on motion, pain at rest, morning stiffness, range of motion limitation, and bony enlargement and expansion (Flores & Hochberg, 2003). More comprehensive procedures include radiographs and computed tomography scans that are used to detect changes in the joint surface and bone deformity. Radiographic criteria for OA include formation of osteophytes on joint margins or ligaments, periarticular ossicles, narrowing of the joint space, cystic areas, and altered shape of the bone ends (Flores & Hochberg, 2003). Magnetic resonance imaging can detect articular cartilage damage or loss as well as subchondral bone changes.

Unfortunately, there are presently no cures for OA nor any treatments that are effective in slowing OA progression. Depending on the stage of degeneration, various options may be available to patients: biotherapeutics and drug therapies, tissue engineered cartilage, and total-joint arthroplasty. The motivation of this research is to focus on reducing the rate of OA when it is at an early stage and when there is still
potential to intervene. Extensive research is being performed to develop tissue-engineered cartilage to allow joint repair before bone-on-bone contact occurs. Tissue engineering involves the use of cells, regulators, and matrices to synthesize tissues (Nehrer & Spector, 2000). Tissue engineering still needs to be developed further as the biomechanical properties of tissue engineered cartilage are insufficient to withstand the physiological mechanical loads in the body (Koopman & Moreland, 2005). However, once full bone-on-bone contact is achieved, the joint is usually treated via total joint arthroplasty to replace the damaged knee joint.

1.2 Articular Cartilage

Hyaline articular cartilage covers the ends of long bones providing a smooth surface for low-friction and low-wear articulation with another bone (Levangie & Norkin, 2001) (Schmidt et al., 2007). Articular cartilage consists of matrix that includes type II collagen fibers, proteoglycans, ions, and water (Pritzker, 2003). Chondrocytes make up the majority of the cellular component of articular cartilage. Chondrocytes produce HA and proteoglycan, which are essential for development of cartilage and maintenance of the extracellular matrix. Cartilage matrix is biphasic, where the fluid phase flows upon mechanical deformation of its solid phase (Minas & Glowacki, 2011). The articular cartilage is aneural and avascular and it is nourished by subchondral blood vessels or synovial fluid (SF). Articular cartilage surfaces that are in contact, but are not bound together, make up a synovial joint (Figure 1.2.1). Synovial tissue lines the fibrous capsule, covering the intra-articular structures except the cartilage (Walker & Helewa,
2004). Fibrous ligaments, such as the ACL and medial collateral ligament (MCL), reinforce the joint capsule and SF lubricates the joint.

Articular cartilage slides and moves relative to an opposing tissue surface in a synovial fluid lubricated cavity. During loading of the knee joint, articular cartilage is subject to compressive, tensile, and shear forces. With such loading, the articular cartilage transfers forces from one bone to the other and can withstand enormous forces that are several times an individual's body weight. Articular cartilage displays exceptional viscoelastic biomechanical properties that allow for the transmission of load across the joint while minimizing peak stresses (Angel et al., 2009). With OA, articular cartilage becomes softer and more susceptible to injury during loading. With late stage OA progression, the articular cartilage deteriorates completely and loading is transmitted directly through the bone-on-bone contact area.
Figure 1.2.1 The components of the human knee (Skalak & Chien, 1987).
1.3 Joint Lubrication

Healthy articular cartilage provides low-friction with SF and wear-resistant properties that allows synovial joint to move. Its movement is facilitated through a combination of lubrication mechanisms: fluid mediated lubrication and boundary lubrication (Figure 1.3.1).

Variables affecting joint lubrication include cartilage surface compliance, roughness, protein lubricants, viscosity of lubricating fluid, and the loading environment (Walker & Helewa, 2004). Proteoglycans and glycosaminoglycans are hydrophilic and function to regulate the movement of water within the cartilage matrix, which is essential for lubrication of the joint. The “slipperiness” of human joints is measured by the coefficient of friction ($\mu$), which is the ratio of the tangential friction force to the normal force. A higher coefficient of friction corresponds with higher friction in the system (Walker & Helewa, 2004). The two coefficients of friction that were calculated in this
thesis were static friction ($\mu_{\text{static},\text{Neq}}$), the resistance to the start-up of motion, and kinetic friction ($<\mu_{\text{kinetic},\text{Neq}}>$), the resistance to steady-state motion (Schmidt et al., 2007).

\[
\mu_{\text{static},\text{Neq}} = \frac{\frac{|t_1|_{\text{max}}}{R_{\text{eff}}}}{N_{\text{Eq}}}
\]

\[
<\mu_{\text{kinetic},\text{Neq}}> = \frac{\frac{|t_1|}{R_{\text{eff}}}}{N_{\text{Eq}}}
\]

**Figure 1.3.2** Equations to calculate static friction ($\mu_{\text{static},\text{Neq}}$) and kinetic friction ($<\mu_{\text{kinetic},\text{Neq}}>$) friction coefficients.

### 1.3.1 Fluid Mediated Lubrication

Fluid mediated lubrication includes hydrostatic lubrication, hydrodynamic lubrication, elastohydrodynamic lubrication, and boosted lubrication (Figure 1.3.1.). Hydrostatic lubrication involves load-bearing surfaces that remain separated by a pressurized fluid film, which bears significant portions of the load with little resistance to shear forces (Levangie & Norkin, 2001). Hydrodynamic lubrication occurs when there is a wedge of fluid created as nonparallel opposing surfaces move against each other. Elastohydrodynamic lubrication occurs when elastic deformation maintains the pressurized fluid film (Levangie & Norkin, 2001). Boosted lubrication occurs when water is forced into the cartilage, leaving lubricant molecules concentrated at the cartilage surface (Levangie & Norkin, 2001).
1.3.2 Boundary Lubrication

Boundary lubrication involves a thin layer of a variety of molecules that form a molecular surface layer or gel film at the articular surface. Boundary lubrication occurs through surface-to-surface contact and friction is mediated by the lubricant molecules at the surface (Levangie & Norkin, 2001). Boundary lubrication generally occurs at high loads and slow velocities (Figure 1.3.1) (Levangie & Norkin, 2001). Boundary lubrication is an important and operative lubrication mechanism for the protection and maintenance of joint health (Elsaid et al., 2006).

Proteoglycan 4 (PRG4) and Hyaluronan (HA) are molecules that are present in SF, both of which adsorb to the articular cartilage and play a vital role in boundary lubrication. HA and PRG4 lubricant molecules have shown to decrease $\mu_{\text{kinetic,Neq}}$ independently, but provide synergistic effects when used in combination (Figure 1.3.2.1) (Schmidt et al., 2007). The boundary lubricating ability of SF has been shown to decrease after acute ACL injury and return to normal after several months (Jay et al., 2004).
Figure 1.3.2.1 Kinetic $\mu_{\text{kinetic}, \text{Neq}}$ friction coefficients of HA, PRG4, surface active phospholipids (SAPL), and bovine SF (Schmidt et al., 2007).

1.4 Synovial Fluid (SF) Components

SF is the critical joint lubricant at the interface between the articular cartilage surfaces. SF is an ultrafiltrate of blood plasma that coats articular cartilage within the joint capsule and lubricates the synovial joint, thus reducing friction. SF is an extremely viscous fluid that is normally clear or straw coloured (Hui et al., 2011). One of the main functions of SF is to maintain joint lubrication thus reducing friction in joint articulation, specifically resisting motion to shear loading. Additionally, the SF provides nourishment for the articular cartilage and facilitates the transport of nutrients and metabolites. In
healthy knee joints, there is approximately 1.0-2.5 mL of SF in the joint (Ropes et al., 1994) (Walker & Helewa, 2004).

SF contains plasma proteins, cells, cytokines and growth factors, and lubricant molecules. Plasma proteins, mainly consisting of albumin and globulins, increase in content and concentration with OA (Ropes et al., 1940). Normal human levels of albumin and globulins are 15-28 mg/mL and have been shown to increase after acute injury (Ropes et al., 1940) (Mazzucco et al., 2004). Albumin acts to regulate osmotic pressure of SF (Walker & Helewa, 2004). Plasma protein content and concentration in SF is increased with OA (Hui et al., 2011). In terms of cells, some leukocytes are present in normal SF, however erythrocytes are absent. White blood cell concentration can increase ten-fold with OA and a hundred-fold in acute knee injury, in comparison to normal human SF levels (Fye, 2001). Some cytokines, both proinflammatory and anti-inflammatory, and growth factors in SF are essential for regulatory functions and maintaining joint homeostasis. Major anabolic growth factors in the SF include transforming growth factor-β1 and insulin growth factor-1. With OA and injury, cytokine and growth factor levels are increased compared to normal human SF (Hui et al., 2011). Lastly, lubricant molecules PRG4 and HA are the primary lubricant macromolecules in normal SF.

1.4.1 Proteoglycan 4 (PRG4)

PRG4 is a friction-reducing, boundary lubricating anti-adhesive coating on the surface of articular cartilage that prevents adhesion of synovial cells to the surface and protects the joint from tissue wear and breakdown (Flannery et al., 2009). PRG4, also
known as lubricin, is a mucinous O-linked glycosylated glycoprotein found in SF that is synthesized by superficial zone articular chondrocytes, as well as by cells in the meniscus, the synovial lining, and tendon (Figure 1.4.1.1) (Dunn et al., 2009) (Schmidt et al., 2009). PRG4 is encoded for by the PRG4 gene, which also encodes for lubricin and superficial zone protein (Ikegawa et al., 2000) (Schumacher et al., 1994) (Swann et al., 1981). PRG4 provides important cartilage-on-cartilage lubrication and the secreted forms of PRG4 are present both in SF and at the surface of tissues lining the joint (Young et al., 2006) (Zhang et al., 2011). A previous study demonstrated that PRG4 is present as a discrete layer covering the surface in a ruptured ACL or meniscus (Zhang et al., 2011).

Figure 1.4.1.1 Domain structure of PRG4 and the 12 exons of the PRG4 gene. The amino-terminus is on the left and the carboxyl-terminus on the right; Somatomedin B (SMB) domain, covalently linked chondroitin sulfate (CS) side chain, and hemopexin (PEX) domain (modified from Rhee et al., 2005).

A deficiency of PRG4 content reduces the chondroprotective properties that are usually provided to articular cartilage; therefore, PRG4 has shown to have a protective role in preventing cartilage degeneration (Young et al., 2006) (Elsaid et al., 2008). Loss of cellular mRNA PRG4 concentration has been demonstrated in an ovine meniscectomy
model at 3 months post-surgery (Young et al., 2006). Furthermore, a subsequent significant decrease in mRNA levels was shown (Young et al., 2006). Mutations in the PRG4 gene result in camptodactyly-arthropathy-coxa vara-pericarditis (CACP) syndrome, which is an autosomal recessive disorder in humans that is associated with synovial hyperplasia, cartilage degradation, and precocious joint failure (Marcelino et al., 1999).

PRG4 concentration has been shown to be altered with acute injury and OA. PRG4 concentrations in human SF from acutely injured ACL injury patients have been shown to be considerably lower than concentrations in uninjured contralateral joints (Elsaid et al., 2008) (Elsaid et al., 2009). Alterations in joint mechanics are associated with a decrease of PRG4 concentrations in mouse SF, as well as a decrease at the surface of the articular cartilage (Coles et al., 2010). In a rabbit knee injury model, PRG4 concentration in SF decreased from normal healthy values of 280 µg/ml to 20-100 µg/ml three weeks after injury (Elsaid et al., 2005). PRG4 concentrations in human SF have demonstrated an immediate decrease after acute injury and then return to normal at about one year after the injury (Elsaid et al., 2008). In late stages of OA, PRG4 concentrations have been reported to remain normal in a guinea pig model, while it can apparently increase or decrease in humans (Ludwig et al., 2010) (Teeple et al., 2008) (Temple-Wong et al., 2010). Thus, the natural history of PRG4 concentration during the onset or progression of OA is not clear. PRG4 concentration in human SF ranges from 30-300 µg/ml in healthy joints (Ballard et al., 2009) (Elsaid et al., 2008) (Hansen et al., 2010) (Ludwig et al., 2012) (Neu et al., 2010) (Temple-Wong et al., 2010) (Schmid et al., 2001). Cartilage degeneration is also associated with a decrease in SF and cartilage-
bound PRG4 after rat ACL transection (Elsaid et al., 2009). Furthermore, PRG4 expression is down regulated by proinflammatory cytokines (Elsaid et al., 2008).

Quantifying PRG4 concentration present in ovine SF (oSF) can be conducted by sandwich enzyme linked immunosorbent assay (ELISA). An ELISA is a biochemical technique used to detect the presence of a molecule of interest in a complex sample, such as SF, with the use of an antibody (Ludwig et al., 2012). In a sandwich ELISA, the plate is coated with a capture antibody and then the sample of interest, which in this case is PRG4, and any antigen present binds to the capture antibody. The detecting antibody is then added and binds to the antigen. Then the substrate is added, which binds to the detecting antibody, and then read by a spectrophotometer to measure the absorbance from the detectable signal. More detail of the ELISA will be given in Chapter 2.

1.4.2 Hyaluronan (HA)

HA is another boundary lubricant that is present in the SF (Neu et al., 2010) (Schmidt et al., 2007). It is a linear polymer of repeating disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine (Fam et al., 2007). The SF lining cells secrete HA into the SF (Walker & Helewa, 2004). HA is responsible for the high viscosity of SF which reduces friction between the synovial folds, and is necessary for hydrodynamic lubrication of articular cartilage (Levangie & Norkin, 2001) (Teeple et al., 2010) (Walker & Helewa, 2004). HA is also critical in boundary lubrication mechanisms, specifically when working synergistically with PRG4 to enhance boundary lubrication (Schmidt et al., 2007).
HA composition is apparently compromised with acute ACL injury and OA. With acute ACL injury, HA concentration has been reported to decrease in an ovine model; SF HA concentration decreased from 1.65 to 0.68 µg/mL (Pitsillides et al., 1999). HA concentration in human SF ranges from 1-4 mg/mL in healthy joints and decreases after injury or OA to 0.1-1.3 mg/mL (Balaz, 1974) (Chmiel & Walitza, 1980) (Dahl et al., 1985) (Mazzucco et al., 2004) (Watterson & Esdaile, 2000). HA concentration in human SF has been reported to remain normal in advanced OA and demonstrate a lower shift in HA molecular weight (MW) distribution (Dunn & Marino, 2009) (Temple-Wong et al., 2010).

Intra-articular HA injections are an approved treatment for knee OA pain to rejuvenate the lubricating properties of SF. However, it is unknown if the reduction of pain correlates to long-term chondroprotective properties (Teeple et al., 2010). Although the mechanism is not completely understood, several clinical trials and meta-analysis have shown HA injections to provide some pain relief and enhance joint function (Waddell, 2007). Commercially available HA ranges from 0.5-10MDa and 8-15mg/mL (Waddell, 2007) (Watterson & Esdaile, 2000). While PRG4 as an intra-articular treatment has not been approved for human use, animal models are encouraging. Interestingly, PRG4 or PRG4+HA injections decreased radiographic and histologic scores of cartilage damage in a rat model (Teeple et al., 2010). Also, there was a reduction in cartilage damage following ACL transection surgery with PRG4 supplementation from various sources in a rat model: human synoviocyte PRG4, recombinant human PRG4, or human SF lubricin (Jay et al., 2010). Moreover, there was significantly lower cartilage degradation scores reported in the PRG4 injected animals in comparison to the non-
treated or PBS injected animals (Jay et al., 2010). Similarly, PRG4 supplementation in ACL-transected rat knee joints demonstrated chondroprotective effects on the progression of OA (Flannery et al., 2009). Although future research is needed, PRG4 injections with OA or after acute ACL injury may protect against articular cartilage degradation and structural damage.

Similar to PRG4, HA concentration can be determined by a sandwich ELISA. HA MW distribution is determined by agarose gel electrophoresis, which is a biochemical method used to separate protein molecules by charge density using an electric field applied to a gel matrix. Assuming the molecules have the same charge, the shorter molecules migrate further than larger molecules, which is useful in determining the MW of the HA in the SF sample as it is compared to a pre-made MW marker with known MWs of HA. More detail of the ELISA and HA MW distribution will be given in Chapter 2.

1.5 Knee Injury and Cartilage Damage

1.5.1 Anterior Cruciate Ligament Injury

The knee is the most prevalent location of joint OA. Knee OA can be caused by joint trauma to the ligaments or meniscus. Originating on the anterior aspect of the tibia, the ACL inserts on the medial aspect of the lateral femoral condyle. The ACL is comprised of two distinct functional bundles: an anterior medial bundle and posterior lateral bundle (Junkin et al., 2009). The ACL provides stability to the knee joint by restraining anterior translation of the tibia in relation to the femur, while also acting as a secondary stabilizer against valgus stress (Levangie & Norkin, 2001).
Severe injury to the cartilage surface, menisci, ligaments, or subchondral bone of the knee can lead to the development of secondary OA (Klug & Weseloh, 2000) (Thornton et al., 2009). There is a ten-fold increase risk of OA in individuals that have previously suffered knee ligament or meniscal injuries (Gillquist & Messner, 1999) (Roos et al., 1998). Injury to the ACL commonly results in functional disability and is more severe when the ACL injury is combined with other damage to the MCL or meniscus (Tapper et al., 2008). There is an association between ACL injury and onset of secondary OA in the joint, which has been demonstrated consistently in long-term follow up with patients (Minas, 2011). Sports are a common cause of ACL injury, and, unfortunately, ACL tears are becoming more common in young athletes (Junkin et al., 2009) (McTimoney, 2007). Female athletes are at a higher risk of a non-contact ACL injury than male athletes (Junkin et al., 2009).

The ACL has poor healing capacity when it is ruptured. As the ACL does not heal without operative treatment and are often symptomatically unstable, patients often favour ACL reconstruction surgery over an ACL deficient knee (Feagin & Curl, 1976). There are approximately 400,000 ACL reconstructions annually in the United States (Junkin et al., 2009). However, in most cases, OA tends to develop over time in spite of a successful reconstructive surgery. Radiographic evidence of OA has been found in 80-90% of ACL reconstructed patients as few as 7 years after knee surgery (Arnoczky & McDevitt, 2000). This suggests that following knee injury some important biochemical process is likely irreparably altered in that joint. Non-operative management of ACL deficient knees leaves the patient with an unstable knee and has shown an increased risk of articular cartilage injury, and even bracing has shown that ACL deficient patients may not prevent
changes in articular cartilage degradation in the knee (Junkin et al., 2009) (McTimoney, 2007) (Roos et al., 1995).

1.5.2 Meniscus Injury

The menisci are fibrocartilaginous structures in the knee joint located between the tibial plateau and femoral condyles. The principal function of the menisci is load distribution (Aagaard & Frankel, 1999) (Andrews et al., 2011) (Minas, 2011). In addition to the ACL, the meniscus acts as a secondary stabilizer of the knee, especially when the knee is ACL deficient.

Significant meniscal loss is a strong contributor to the development of OA in the knee (Beveridge et al., 2011) (Minas, 2011). Radiographic changes in underlying bone have been observed after meniscectomy (MEN); OA changes in the articular cartilage develop in the lateral compartment in 2-5 years and in the medial compartment in 10-15 years (Minas, 2011). Without the lateral meniscus, there is increased translation and shear loading on the femoral condyle and softening of the lateral tibial plateau (Minas, 2011).

Meniscal damage often requires surgical intervention (McTimoney, 2007). Operative interventions can include partial meniscal MEN or meniscal repair. With MEN, the magnitude of average load per unit area on the tibial plateau increases by 6-7 times and doubles on the femoral condyle (Radin et al., 1984). In a previous ovine lateral MEN model that tested kinematics, there was more cartilage damage in all MEN sheep in comparison to the sham surgery (SHAM), occurring at the anterior and posterior regions of the lateral plateau and condyle (Beveridge et al., 2011). Thus, the lateral compartment
is exceptionally meniscal dependent and a lateral MEN is associated with poorer outcomes (Chatain et al., 2003).

1.6 Ovine Animal Model

The use of an animal model provides the opportunity to investigate the progression of a diseased state, as OA progression is difficult to study in humans. OA progression can dependent on many genetic and environmental factors (hormones, exercise, occupation, lifestyle, body mass index) (Little & Smith, 2008). Surgical induced destabilization is the most common induction method of OA development and ACL transection surgery is an established technique for inducing OA in animal models (Roos et al., 1998). An advantage of this model is predictable disease onset and progression (Little & Smith, 2008). Furthermore, it is common to use animal models to detect OA at earlier stages (Little & Smith, 2008). Due to technical and ethical limitations, an ovine model was utilized to investigate the properties of joints because the procedure is extremely invasive. An ideal animal model should be reproducible, allow the definition various stages in the pathophysiology of OA, large enough to allow multiple analyses, and recapitulate the human pathology (Little & Smith, 2008).

Advantages of large animal models, specifically ovine, include animal size, regional tissue analysis, and clinical outcome measures are known. The criterion for the animal model selection for this study was as follows: trainable, slow progression of cartilage damage, and large joint. The ovine model also provided a slow progression of cartilage damage, thus potentially increasing the window of time to study the mechanisms of OA and interventions to treat it (Tapper et al., 2008).
1.7 Thesis Objectives

After an ACL injury, the articular surface frequently becomes damaged and leads to OA. This OA development can be attributed to many factors, one of which may be failure of key lubrication mechanisms. Such failure may be due to changes in SF lubricant composition and lubricating function (as discussed in Chapter 2). We hypothesized that SF boundary lubricant composition is altered in post-traumatic OA in a post-knee injury ovine model. The objective of this thesis was to define the composition-function relationship between SF lubricant molecules, PRG4 and HA, using biochemical methods to characterize lubricant composition in SF from a post-knee injury ovine model and to use biomechanical methods to characterize the lubricating functionality of these same joints.

Therefore, the specific aims of this investigation were:

1. to determine the PRG4 and HA concentration of oSF;
2. to quantify HA MW distribution of oSF;
3. to determine cartilage lubricating ability of oSF; and
4. to determine the isoelectric point (pl) of PRG4 in oSF.
CHAPTER 2: CHARACTERIZATION OF BOUNDARY LUBRICANT COMPOSITION IN A POST-KNEE INJURY OVINE MODEL

2.1 Abstract

**Purpose:** Determine changes in 1) PRG4 and HA concentration, 2) HA MW distribution, and 3) cartilage lubricating ability of SF from SHAM, ACL/MCL transection, and lateral MEN in a post-knee injury ovine model at 20 weeks.

**Methods:** oSF was collected at euthanization 20 weeks after surgery, with the contralateral joint serving as the non-operative control (CTRL). PRG4 and HA concentration in oSF was measured by sandwich ELISA, and HA MW distribution by agarose gel electrophoresis. Cartilage boundary lubricating ability of oSF was measured by a cartilage-cartilage friction test.

**Results:** PRG4 and HA concentration in SHAM, ACL/MCL, and MEN oSF was similar in comparison to the contralateral CTRL oSF. The HA MW distribution in the operated oSF for all ranges were similar to the respective CTRL oSF. The kinetic coefficient of friction ($\mu_{\text{kinetic,Neq}}$) in operated and CTRL oSF were similar in all groups, and were significantly lower than those in phosphate buffered saline. Lubricant composition and function of oSF at 20 weeks post-knee surgery is similar to that of the contralateral CTRL.

**Conclusion:** Earlier time points may need to be examined in future studies to identify potential alterations, and assess the potential efficacy of lubricant biotherapeutic intervention.
2.2 Introduction

OA is a degenerative joint disease involving the breakdown of the articular cartilage that covers the ends of articulating bones of articular joints (Gelber et al., 2000). OA is a multifactorial disease that may include a number of contributing factors: genetics, biomechanics, metabolic changes, or developmental insults (Coles et al., 2010) (van de Velde et al., 2009). Severe injury to the cartilage, menisci, or the ACL of the knee can also lead to the development of secondary OA at later stages of life. After an injury and with aging, the articular surface frequently becomes eroded and roughened, which can lead to tissue degeneration and OA. OA can significantly affect the quality of life, as there is no cure, and the disease severely restricts the mobility of millions of patients globally.

Boundary lubrication is an important and operative lubrication mechanism for the protection and maintenance of cartilage and joint health (Elsaid et al., 2008). Cartilage tissue, located at the articulating surfaces of bones within diarthrodial joints such as the knee joint, bears load and slides relative to an opposing tissue surface in a fluid-filled synovial cavity with low-friction and low-wear properties (Schmidt et al., 2007). SF provides boundary lubrication function at the surface of articular cartilage, however boundary lubricating ability of SF may be altered in OA and acute joint injury due to decreased and/or modifications in lubricant constituents such as PRG4 and HA concentrations (Schmidt et al., 2007).

PRG4 and HA are molecules that are present in SF, both of which adsorb to the articular cartilage, and play a vital role in the boundary lubricating ability (Schmidt et al., 2007). PRG4 is a mucinous glycoprotein found in SF that is synthesized by superficial
zone articular chondrocytes and synoviocytes (Jay et al., 2000) (Schumacher et al., 1994). PRG4 forms a friction-reducing and boundary lubricating anti-adhesive coating on the surface of articular cartilage that protects the joint from tissue wear and breakdown (Rhee et al., 2005). The concentration of PRG4 in human SF from injured ACL injury patients has been shown to be considerably lower than that of SF concentrations in the uninjured contralateral joints, returning to normal approximately one year after the injury (Figure 2.2.1) (Elsaid et al., 2008). In later stages of OA, PRG4 concentrations have been reported to remain normal in a guinea pig model, increase in a human model, and decrease in a human model (Figure 2.2.1) (Ludwig et al., 2012) (Neu et al., 2010) (Teeple et al., 2008) (Temple-Wong et al., 2010). Cartilage degeneration is also associated with a decrease of PRG4 in SF and at the articular cartilage surface of rat ACL transection surgeries (Elsaid et al., 2009). Additionally, HA contributes to hydrodynamic and boundary lubrication mechanisms in the knee joint, acting synergistically with PRG4 to enhance boundary lubrication (Schmidt et al., 2007). The concentration of HA has been reported to decrease with acute ACL injury in an ovine model but appears to remain normal in advanced OA (Figure 2.2.1) (Pitsillides et al., 1999). Furthermore, HA has demonstrated a shift to lower HA MW distribution in humans with injury and in advanced OA, which can affect the SF’s cartilage boundary lubricating ability (Figure 2.2.1) (Dunn & Marino, 2009) (Hansen et al., 2010) (Kwiecinski et al., 2011).
### Figure 2.2.1
Summary of lubricant molecules of interest with ACL injury and OA (Dunn & Marino, 2009) (Elsaid et al., 2008) (Jay et al., 2004) (Neu et al., 2010) (Pitsillides et al., 1999) (Teeple et al., 2008) (Temple-Wong et al., 2010).

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<th>Acute Anterior Cruciate Ligament (ACL) Injury</th>
<th>OA</th>
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<td>[PRG4]</td>
<td>![Arrow Down] Returns to normal</td>
<td>Normal</td>
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<td>HA</td>
<td>![Arrow Down] Concentration</td>
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<td>Boundary Lubricating Ability</td>
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Alterations in boundary lubricating ability of SF due to changes in lubricant composition after various ACL/MCL transections surgeries and MEN surgeries may contribute to increased wear at the articular surface in a small animal model (Jay et al., 2007). However, PRG4 lubricant changes have yet to be investigated in a large animal model, such as ovine. In an ovine OA model, significant cartilage damage has been shown in MEN surgery compared to SHAM surgery, occurring at the anterior and posterior regions of the lateral plateau and condyle at 20 weeks after knee surgery (Beveridge et al., 2011). Furthermore, the lubricant composition and function in the oSF collected during these studies remains to be examined. The objectives of this study were therefore to determine 1) PRG4 and HA concentration, 2) HA MW distribution, and 3) cartilage lubricating ability in 20 week post-knee injury and contralateral CTRL oSF.
2.3 Methods

2.3.1 Subjects and Surgical Approach

In previous studies, sixteen skeletally mature, 3-4 year-old female Suffolk-cross sheep were allocated randomly into one of three groups: surgical sham (SHAM) \( n=5 \), ACL/MCL transection surgery \( n=6 \), or lateral MEN surgery \( n=5 \) (Beveridge et al., 2011) (Tapper et al., 2008). Surgeries were performed under general halothane anaesthesia and were accomplished via arthrotomy to the right stifle joint and the left joint served as the respective CTRL, as described previously (Beveridge et al., 2011) (Tapper et al., 2008). ACL/MCL transection was performed by a 10 cm incision along the midline of the patellar tendon, skin was medially retracted, and layers of fat and fascia were dissected to expose the MCL (Tapper et al., 2008). The MCL was cut midsubstance by transection at the joint line, which is perpendicular to its long axis, and the lateral retinaculum was incised from the patella to the tibia to allow the patella to be dislocated medially with the joint in extension (Tapper et al., 2008). The joint was flexed to expose the joint space and ACL (Figure 2.3.1.1). The ACL was hooked and transected perpendicular to the long axis through the full thickness of both bands (Figure 2.3.1.2) (Tapper et al., 2008).

MEN surgery was performed by making an incision along the midline of the patellar tendon, retracting the skin, fat, and fascia. Then, the lateral joint capsule was dissected to access both the anterior and posterior horns of the lateral meniscus (Beveridge et al., 2011). The meniscal horns and meniscofemoral ligament were transected and the meniscus was removed (Figure 2.3.1.3) (Beveridge et al., 2011).
The SHAM surgery, which is the open arthrotomy, involved the same approaches as the experimental groups, involving a controlled medial dislocation of the patella and dissection of the medial joint capsule. After the surgeries were completed, before closure, the surgical site was flushed with saline and Penicillin G Sodium (Novopharm Limited, Toronto, ON) (Beveridge et al., 2011). Deep layers were closed with 2-0 Vicryl sutures (Ethicon Inc., Markham, ON), the skin was closed in two layers with 2-0 PDS II (Ethicon Inc., Markham, ON), and sprayed with both Gentocin (Schering-Plough Animal Health Corp, Union, NJ) and Op-site (Smith & Nephew, Hull, UK) (Beveridge et al., 2011). The animals were sacrificed 20 weeks after surgery, and oSF was collected and stored at -80°C. All procedures were reviewed and approved by the University of Calgary Animal Care Committee and comply with the Canadian Council on Animal Care guidelines.
Figure 2.3.1.1 Ovine joint with the ACL exposed.

Figure 2.3.1.2 Right ovine joint. ACL (A), posterior lateral (PL) band of the ACL (B), and anterior medial (AM) band of the ACL (C) in an ovine knee.
Figure 2.3.1.3 Right tibial plateau in an ovine joint with (A) and without (B) the lateral meniscus.

2.3.2 PRG4 Composition

PRG4 was prepared from cartilage discs that were harvested from bovine stifle joints, as described previously (Schmidt et al., 2007). Briefly, cartilage discs were incubated and cultured for 28 days in Dulbecco’s modified Eagle’s media with 0.01% bovine serum albumin, 25 µg/mL ascorbic acid, and 10 ng/mL recombinant human transforming growth factor β1 (Schmidt et al., 2007). Cell culture media was changed every three days, conditioned media was collected for processing and stored at -20°C. The conditioned culture medium was pooled and then purified by anion-exchange chromatography (DEAE-Sepharose) (equilibrated with 0.15M NaCl, 0.005M EDTA, and 0.05M sodium acetate, pH 6.0) (Schmidt et al., 2007). The 0.3-0.6M NaCl eluate was collected, concentrated with a Centricon Plus 30kDa molecular weight cutoff filter, and then quantified by a bicinchoninic acid protein assay. PRG4 purity was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), protein stain,
and western blot using anti-PRG4 polyclonal antibody (LPN) antibody (data not shown), as described previously (Ludwig et al., 2012).

A custom ELISA was used to determine PRG4 concentration in CTRL and post-knee injury oSF using anti-PRG4 antibody LPN for capture and peanut agglutinin lectin (PNA) (Sigma-Aldrich, St. Louis, MO) for detection (Ludwig et al., 2012). The oSF samples were treated with S. Hyaluronidase (MJS Biolynx Inc. Brockville, ON) (3 hr at 37°C) and Sialidase S-66 (Prozyme, Hayward, CA) overnight. High-binding 96-well-plates were coated overnight with capture antibody LPN (2 µg/mL) in 50mM sodium bicarbonate buffer, pH 9.5. After coating, the plates were washed by filling the wells with phosphate buffered saline (PBS) and 0.1% Tween-20 (PBST) (Ludwig et al., 2012). Sample wells were blocked with 5% non-fat dry milk in PBS, and incubated for one hour at 37°C prior to removal of the blocking solution. The oSF samples and PRG4 controls were added by 2X serial dilution and incubated with nutating/shaking at 37°C for one hour, and then washed with PBST. Peroxidase-coupled PNA (5 µg/mL) was added to the plate for one hour and incubated at 37°C, followed by a washing of PBST. Lastly, 3,3′,5,5′-tetramethylbenzidine substrate (Sigma-Aldrich, St. Louis, MO) was added to each well, incubated for 30 minutes at room temperature with shaking, followed by the addition of 2M sulfuric acid (Ludwig et al., 2012). Absorbance was measured by spectrophotometry and a standard control curve was determined from the purified PRG4 samples and the concentration of PRG4 in the oSF samples was calculated.
2.3.3 HA Composition

A commercially available sandwich ELISA (R&D Systems, Minneapolis, MN) was used to determine HA concentration levels in CTRL and post-knee injury oSF. The high binding 96-well-plate was coated with 100µl dilute capture reagent (0.5 µg/mL in PBS) and incubated overnight at room temperature. The plate was washed by filling the wells with PBST. The plate was blocked with blocking solution (5% Tween20 in PBS with 0.05% NaN₃), incubated for an hour at room temperature, and washed with PBST. The diluted oSF samples (120000X) and standards (0.5 µg/mL) were loaded in triplicate, and 4X serial dilutions were conducted. The plate was incubated for 2 hours at room temperature and washed with PBST. The detection reagent (0.3 µg/mL) was added to each well, incubated at room temperature for 2 hours, and then washed. Streptavidin-Horse Radish Peroxidase was loaded into each well, incubated at room temperature for 20 minutes while avoiding direct light, and washed. Finally, the substrate solution (1:1 mixture of Color Reagent A hydrogen peroxide and Color Reagent B TMB) was added to each well for 20 minutes, avoiding direct light, and 50µL 2M sulfuric acid was added as the stop solution. Absorbance was measured via spectrophotometry. A standard curve was determined from the HA controls and the concentration of HA in the oSF samples was calculated.

Agarose gel electrophoresis (1%) was used to quantify HA MW distribution for CTRL and post-knee injury oSF, in duplicate (Kwiecinski et al., 2011) (Lee & Cowman, 1994). The oSF samples were diluted to 0.5mg/mL HA and treated with Proteinase K (Roche, Laval, QC) overnight. The oSF samples were diluted in distilled water and added to 0.06% bromophenol blue solution (2M sucrose in TAE), then 18µl of the oSF samples
were loaded into the gel into respective wells. Hi-Ladder (0.5-1.5 MDa) and Mega-Ladder (1.5-6.1 MDa) (Oklahoma City, OK, USA) were used as MW markers. The oSF samples and MW markers were loaded into a horizontal 1.0% (w/v) agarose slab gel (1cm thick, 15cm long, 10cm wide with 20 wells) prepared in Tris-acetate-EDTA buffer (0.4M Tris, 0.05M sodium acetate, 0.009M EDTA, pH 7.9) (Kwiecinski et al., 2011) (Lee & Cowman, 1994). Electrophoresis was performed for 3 hours at 50V in a horizontal gel apparatus (Bio-Rad, Mississauga, ON) submerged in TAE buffer. The gel was then stained with Stains-All (0.005% in 50% ethanol) and then de-stained the following day for 24 hours with 10% ethanol. The bands were then visualized using the Chemigenius Bio Imaging System (Lee & Cowman, 1994). The migration of HA was assessed by densitometric analysis with Image J (NIH, Bethesda, MD).

2.3.4 Cartilage Lubricating Ability

Cartilage lubricating ability of equally pooled oSF from each experimental group was assessed using normal bovine osteochondral cores with a previously described in vitro cartilage-cartilage friction test under boundary lubrication conditions (Schmidt et al., 2007). Briefly, fresh osteochondral samples (n=12) were prepared from the patellofemoral groove of skeletally mature bovine stifle joints, as described previously (Figure 2.3.4.1) (Schmidt et al., 2007). Samples were rinsed vigorously over night in PBS at 4°C to rid the articular surface of residual SF (Kwiecinski et al., 2011). Samples were bathed in ~0.3mL subsequent test lubricants (core bathed in ~0.2mL, annulus bathed in ~0.1mL) at 4°C overnight prior to boundary lubrication testing (Figure 2.3.4.1). The following test sequence was used over 4 consecutive days with n=4 for each
oSF surgical group: PBS, operated oSF, CTRL oSF, bovine SF (bSF) (Figure 2.3.4.2). Cartilage boundary lubrication tests were performed using a Bose ELF 3200 (Bose EnduraTEC, Minnetonka, MN), as described previously (Schmidt et al., 2007). Cartilage surfaces were compressed at a constant rate of 0.002 mm/s to 18% of the total cartilage thickness, allowed to stress relax for 40 minutes, then rotated against each other at an effective velocity of 0.3 mm/s (+2 revolutions and then -2 revolutions) with pre-sliding durations (Tps) of 1200, 120, 12, and 1.2 seconds without removing compression. The test sequences were then repeated in the opposite direction of rotation. Static (µ\textsubscript{static,Neq}) and kinetic (µ\textsubscript{kinetic,Neq}) friction coefficients were calculated for each lubricant.
Figure 2.3.4.1 Boundary lubrication test set up and parameters (Schmidt & Sah, 2007).

Figure 2.3.4.2 Friction testing lubricant sequence.
2.3.5 **Statistical Analysis**

Independent t-tests were used to assess changes in PRG4 and HA composition and HA MW distribution within each surgical ovine model. A repeated measures ANOVA was used to determine the effects of lubricant and Tps, as a repeated factor, on $\mu_{\text{static,Neq}}$ and $<\mu_{\text{kinetic,Neq}>}$, with Tukey post-hoc testing on $<\mu_{\text{kinetic,Neq}>}$ at Tps = 1.2s. For all statistical tests, the differences between SHAM, MEN, and ACL/MCL transection outcome measures were considered significant if $p<0.05$. Statistical analysis was performed with Systat 12 (Systat, Richmond, CA). Data are presented as mean±SEM.

2.4 **Results**

2.4.1 **PRG4 Composition**

PRG4 concentration was similar between the operated and un-operated contralateral CTRL knees in all cases (Figure 2.4.2.1). PRG4 concentration in the SHAM oSF was $131±26$ µg/mL in comparison to the respective contralateral CTRL oSF of $193±49$ µg/mL ($n=5$, $p=0.30$). PRG4 concentration in the ACL/MCL transection oSF was $225±7$ µg/mL in comparison to the respective contralateral CTRL oSF of $296±58$ µg/mL ($n=6$, $p=0.25$). PRG4 concentration in the MEN oSF was $244±62$ µg/mL in comparison to the respective contralateral CTRL oSF of $173±54$ µg/mL ($n=5$, $p=0.41$).

2.4.2 **HA Composition**

HA concentration was similar between the operated and un-operated contralateral CTRL knees in all cases (Figure 2.4.2.1). HA concentration in the SHAM oSF was $0.82±0.11$ mg/mL in comparison to the respective contralateral CTRL oSF of $0.86±0.18$
mg/mL \((n=5, \ p=0.86)\). HA concentration in the ACL/MCL transection oSF was 0.83±0.29 mg/mL in comparison to the respective contralateral CTRL oSF of 0.95±0.05 mg/mL \((n=6, \ p=0.69)\). HA concentration in the MEN oSF was 1.02±0.34 mg/mL in comparison to the respective contralateral CTRL SF of 0.97±0.15 mg/mL \((n=5, \ p=0.90)\).

HA MW distribution was similar in all experimental groups when compared to un-operated contralateral CTRL oSF (Figure 2.4.2.2). The relative HA concentration (as a % of total concentration) in the SHAM oSF for >6.1 MDa (1% vs. 0%), 3.1-6.1 MDa (38% vs. 33%), 1.1-3.1 MDa (35% vs. 35%), 0.5-1.1 MDa (16% vs. 18%), and <0.5 MDa (11% vs. 13%) ranges were similar with respect to the CTRL oSF (all \(p>0.05\)) (Figure 2.4.2.2A). The HA concentration in the ACL/MCL transection oSF for >6.1 MDa (1% vs. 0%), 3.1-6.1 MDa (42% vs. 39%), 1.1-3.1 MDa (30% vs. 37%), 0.5-1.1 MDa (19% vs. 18%), and <0.5 MDa (8% vs. 5%) ranges were similar with respect to the CTRL oSF (all \(p>0.05\)) (Figure 2.4.2.2B). The HA concentration in the MEN oSF for >6.1 MDa (0% vs. 0%), 3.1-6.1 MDa (35% vs. 30%), 1.1-3.1 MDa (37% vs. 39%), 0.5-1.1 MDa (19% vs. 22%), and <0.5 MDa (9% vs. 8%) ranges were similar with respect to the CTRL oSF (all \(p>0.05\)) (Figure 2.4.2.2C).
Figure 2.4.2.1 PRG4 and HA concentration for SHAM (n=5), ACL/MCL transection (n=6), and MEN (n=5) at 20 weeks post-surgery in oSF with respective CTRL oSF. Data is presented as mean±SEM.
**Figure 2.4.2.2** HA MW distribution for SHAM (A) \((n=5)\), ACL/MCL transection (B) \((n=6)\), and MEN (C) \((n=5)\) at 20 weeks post-surgery in oSF with respective CTRL oSF. Data is presented as mean±SEM.
2.4.3 Cartilage Lubricating Ability

All oSF functioned as effective friction-reducing cartilage boundary lubricants. No significant differences were observed between friction coefficient values obtained for ACL/MCL, MEN and SHAM and their respective contralateral CTRLs in all of the three-test sequences. Lubricants and Tps modulated friction. $\mu_{\text{static,Neq}}$ increased with increasing Tps for all lubricants, with an interaction (all p<0.001) (Figure 2.4.3.1A, Figure 2.4.3.2A, Figure 2.4.3.3A). Values of $\mu_{\text{static,Neq}}$ were greatest in PBS, while operated oSF, respective CTRL oSF, and bSF were all similar and lower than PBS (Figure 2.4.3.1A, Figure 2.4.3.2A, Figure 2.4.3.3A). $<\mu_{\text{kinetic,Neq}}>$ also varied with lubricant, only slightly with Tps (values at 1.2s were within 15±8% of those at 1200s), with an interaction (all p<0.001). Therefore for brevity and clarity, and as previously, values of $<\mu_{\text{kinetic,Neq}}>$ are presented only at Tps = 1.2s (Schmidt et al., 2007). $<\mu_{\text{kinetic,Neq}}>$ at Tps=1.2s in PBS was significantly higher than both operated, CTRL oSF, and bSF in all cases (all p<0.05) (Figure 2.4.3.1B, Figure 2.4.3.2B, Figure 2.4.3.3B). $<\mu_{\text{kinetic,Neq}}>$ at Tps=1.2s in operated oSF (SHAM=0.045±0.008 Figure 2.4.3.1B, ACL/MCL=0.045±0.011 Figure 2.4.3.2B, MEN=0.039±0.004 Figure 2.4.3.3C) were similar to CTRL oSF (0.040±0.013, 0.034±0.004, 0.041±0.005, respectively, p=0.93-1.0) and bSF (0.031±0.004, 0.035±0.002, 0.037±0.005, respectively p=0.85-1.0) in all experimental groups. In all cases, CTRL oSF was also not statistically different than bSF (p=0.85-1.0), indicating bovine cartilage is an acceptable substrate for evaluating the cartilage lubricating ability of oSF.
Figure 2.4.3.1 Static ($\mu_{\text{static},\text{Neq}}$) (A) and kinetic $<\mu_{\text{kinetic},\text{Neq}}>$ at $T_{ps}=1.2s$ (B) friction coefficients of SHAM oSF ($n=4$). Data is presented as mean±SEM.
Figure 2.4.3.2 Static ($\mu_{\text{static},\text{Neq}}$) (A) and kinetic $<\mu_{\text{kinetic},\text{Neq}}>$ at $T_{ps}=1.2s$ (B) friction coefficients of ACL/MCL transection oSF ($n=4$). Data is presented as mean±SEM.
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Figure 2.4.3.3 Static ($\mu_{\text{static,Neq}}$) (A) and kinetic $<$\mu_{\text{kinetic,Neq}}$> at $T_{ps}=1.2\text{s}$ (B) friction coefficients of MEN oSF ($n=4$). Data is presented as mean±SEM.
2.4 Discussion

This study provides insight into the oSF lubricant composition-function relationship following knee injury in an ovine model. There was no difference in PRG4 and HA composition and cartilage lubricating ability in oSF 20 weeks post knee surgery. These results suggest that if alterations in oSF lubricant composition and/or function following knee injury exist, such changes occur earlier, and are potentially restored by 20 weeks. These results emphasize that earlier time points may be the key in defining the composition-function relationship in oSF lubricant molecules.

While the sample size in this study was small, sufficient oSF was obtained to quantify lubricant composition and biomechanical function, and describe the similarities related to the CTRL oSF 20 weeks post-knee surgery. Previously identified limitations include the invasive nature of the surgeries and kinematic testing (Beveridge et al., 2011) (Tapper et al., 2008). The kinematic testing and surgical methods demonstrated good reproducibility of un-injured sheep (Tapper et al., 2008). A potential bias that could have been introduced during the recovery after knee surgery was the weight distribution between both limbs, as it was assumed that the animals did not favor their uninjured limb and equal weight was distributed. This is a potential limitation to the OA progression model, thus affecting the loading of the joint and weight distributed between limbs. The oSF samples were pooled equally from each animal within groups for friction testing, and the oSF samples were tested on a bovine cartilage substrate in order of increasing anticipated lubricating ability to minimize the carryover effects. In all cases, CTRL oSF was also not statistically different than bSF (Figure 2.4.3.1, Figure 2.4.3.2, Figure
2.4.3.3), indicating bovine cartilage is an acceptable substrate for evaluating the lubricating ability of oSF.

Following knee surgery in the ovine model, the PRG4 concentration remained normal in OA SF in all operated groups: SHAM, ACL/MCL transection, and the MEN (Figure 2.4.2.1). These results agree with previous findings that PRG4 concentration is not altered in OA SF in an animal model, however other human studies have shown either an increase and decrease (Ludwig et al., 2012) (Neu et al., 2010) (Teeple et al., 2008) (Temple-Wong et al., 2010). While SF lubricant composition can be significantly altered immediately after an acute injury, levels can return to normal at later stages, though still potentially at a ‘semi-acute’ stage (Elsaid et al., 2008) (Pitsillides et al., 1999). This suggests that the key in determining the PRG4 composition-function relationship may be time dependent, and should be targeted at an earlier stage of OA model or following acute injury. The decrease in PRG4 concentration after acute knee injury, previously reported in humans, may be due a decrease in protein expression/synthesis by synoviocytes and chondrocytes, loss of cells, or an increase in the catabolism of PRG4 (Elsaid et al., 2008). However, the mechanism still remains unknown. The specific time course of such alterations, if any, remains to be fully elucidated in these models, as PRG4 expression and localization has been shown to be decreased as late as 12 weeks post operatively in a similar ovine MEN model (Young et al., 2006). Future studies should evaluate earlier time points of OA degeneration, potentially after acute injury, as these time points can be seen as windows of opportunity for potential biotherapeutic intervention implication to aid in joint preservation.
In this study, HA levels in the operated knee oSF were similar to the HA levels in the CTRL knee oSF for all three surgical procedures (Figure 2.4.2.1), which are similar to previous human studies (Dunn & Marino, 2009) (Temple-Wong et al., 2010). HA concentration from oSF from the knee joint has been previous reported in the range of 0.68-1.65mg/mL, which is similar to the 0.82-1.02 mg/mL ranges that were reported for both the CTRL oSF and operated oSF in this study (Pitsillides et al., 1999). Also, these results show no alterations in the MW distribution between any of the oSF from the ACL/MCL transection, MEN, or SHAM with the respective CTRL oSF (Figure 2.4.2.2), which agree with the cartilage lubricating ability results and suggest that there is no detectable difference in oSF function.

Cartilage lubricating ability has been reported to remain normal in human OA SF, which agrees with the results of this study (Figure 2.4.3.1, Figure 2.4.3.2, Figure 2.4.3.3), even though some OA samples have shown to be deficient in lubricating ability (Jay et al., 2004) (Ludwig et al., 2012). The relationship between SF lubricant composition-function and cartilage surface integrity at various time points remains to be elucidated as well, such as 2 weeks post operatively, since MEN at 20 weeks has been shown to result in significant cartilage damage compared to CTRL (Young et al., 2006). This suggests that both pristine SF and cartilage are essential for suitable joint function.

In a previous lateral MEN study, there was more cartilage damage than SHAM surgery in all sheep occurring at the anterior and posterior regions of the lateral plateau and condyle (Beveridge et al., 2011). Future studies could look at the PRG4 and HA composition-function relationships at earlier time points (2-10 weeks) to gain understanding of potential lubricant alterations. If earlier time points are examined and
suggest that PRG4 composition is compromised, then the stages at which biotherapeutic injections are given may need to be further evaluated. This new knowledge could be used to provide an understanding of PRG4, and possibly PRG4+HA, as an earlier stage biotherapeutic application. Also, testing varying levels of cartilage surface degradation with normal SF versus injury post-knee SF and a normal surface may provide further insight into cartilage function.
CHAPTER 3: CHARACTERIZATION OF PRG4 IN OVINE SYNOVIAL FLUID USING TWO DIMENSIONAL GEL ELECTROPHORESIS

3.1 Abstract

**Purpose:** Determine the isoelectric point (pI) of PRG4 in ACL/MCL transection and lateral MEN oSF using 2D gel electrophoresis as an initial assessment of PRG4 glycosylations.

**Methods:** 2D electrophoresis with western blotting was used to determine protein pI and protein MW. The first dimension was isoelectric focusing (IEF), which was used to determine protein pI, and the second dimension was SDS-PAGE, which was used to determine protein MW. Samples analyzed using 2D electrophoresis include native PRG4, normal bSF, operated ACL/MCL oSF, operated MEN oSF, and contralateral CTRL oSF samples. J108N antibody and PNA-Horse Radish Peroxidase (PNA-HRP) were used for western blotting.

**Results:** PRG4 pI is ~4.0-4.4. PRG4 is present in the high MW region for ACL/MCL oSF and MEN oSF, and the respective contralateral CTRL oSF. It appears that there is a stronger J108N immunoreactive band in the contralateral control oSF than the operated oSF, for both ACL/MCL and MEN oSF. In addition, the pI of PRG4 of the high MW PRG4 species (~460kDa) appears to shift from ~4.0-4.4 to ~6.0-6.2 in MEN oSF.

**Conclusion:** The results of these preliminary studies using 2D electrophoresis suggest that PRG4 pI is ~4.0-4.4 in oSF, and is altered in MEN oSF at 20 weeks in an post-knee injury ovine model. Future studies are required to determine the significance of this apparent shift in pI, which may be due to variations in the PRG4 glycosylation.
3.2 Introduction

2D polyacrylamide gel electrophoresis (henceforth referred to as 2D electrophoresis) is a powerful tool used in proteomics and is an established technique for high-resolution profiling of low abundance proteins (Mahon et al., 2001). Significant advances have been made to 2D electrophoresis to improve methodology, and it is now a widely used method (Smith et al., 2001) (Garfin, 2003). 2D electrophoresis is used to analyze protein content and separate proteins by their pI and MW (Gorg et al., 2004). Proteins are first separated on the basis of their net charge by isoelectric focusing (IEF) and then secondly on the basis of molecular mass by SDS-PAGE (Garfin, 2003).

In IEF, protein focuses or accumulates at the point where the protein carries no net electric charge, also referred to as the pI (Garfin, 2003). IEF separates different molecules or proteins by electric charge differences; a protein in a pH region below the pI will migrate towards the cathodes (negative region), and a protein with a pH region above the pI will migrate towards the anode (positive region). An immobilized pH gradient (IPG) strip is used to separate the protein during IEF. The protein migrates through the pH gradient and individual proteins are immobilized in the pH gradient when they reach their pI (Garfin, 2003).

The 2D separation of proteins uses SDS-PAGE to separate proteins according to their electrophoretic mobility, which can be the length of polypeptide chain or molecular weight. The small proteins will migrate further to the bottom of the gel and the large molecules will be concentrated at the top of the gel. The proteins are detected by staining techniques (Simply Blue Protein Stain, Coomassie Brilliant Blue, SYPRO Ruby, etc.) to visualize the separated proteins on the gel. The protein bands can be excised from the
SDS-PAGE gel for further analysis such as mass spectrometry or chemical microsequencing to facilitate protein identification (Garfin, 2003). Or, the proteins in the gel can also be transferred, or blotted, onto membranes, and probed with specific antibodies (i.e. western blotting).

Low abundance proteins, such as PRG4 in SF, can be studied with 2D electrophoresis. The disease progression of rheumatoid arthritis patients treated with antibody CD4 has been previously investigated with 2D electrophoresis, and has been found to be a feasible method of analysis (Smith et al., 2001). SF from rheumatoid arthritis patients in affected joints was analyzed and changes in acute-phase proteins measured by 2D gel electrophoresis were correlated with clinical improvement and clinical chemistry measurements (Smith et al., 2001). Analysis of cartilage has been conducted via 2D electrophoresis and has demonstrated that 59 proteins were expressed differently in OA patients compared to normal cartilage donors (Wu et al., 2007). Also, synovium from OA and rheumatoid arthritis patients has been analysed via 2D electrophoresis and demonstrated five regions that were significantly different (Tilleman et al., 2005). Thus, the 2D gel electrophoresis can be used for quantitative analysis of protein expression in SF. Lastly, a previous study has shown that there were significantly different protein spots expressed between patients with juvenile idiopathic arthritis from controls groups (Rosenkranz et al., 2010). Optimization of 2D electrophoresis techniques has been recently conducted to try to study human joint proteomics to analyze joint cartilage synovium, chondrocytes, synoviocytes, and SF to gain knowledge about disease mechanisms (Ruiz-Romero et al., 2010). Significant advances in 2D electrophoresis
methods have been made within the last decade, which has led to high resolution and detection of proteins in complex mixtures.

Glycosylations are an enzymatic process that attaches glycans to proteins, lipids, or organic molecules, and is a form of post-translational modification. Glycosylation properties include sialylation, sulfation, and fucosylation, and patterns of glycosylation can change in both disease and inflammation. One type of glycosylation of interest is O-linked glycosylations, specially O-linked β(1-3)Gal-GalNAc oligosaccharides, because they are abundant in the mucin-like region of exon 6 in PRG4 (Estrella et al., 2010). A previous detailed analysis of human PRG4 revealed that core 1 O-linked oligosaccharides NeuAcα2–3Galβ1–3GalNAc and NeuAcα2–3Galβ1–3(NeuAcα2–6)-GalNAc were the most prominent structures in PRG4, and core 2 proteins were also found in low amounts (Estrella et al., 2010). Analysis of PRG4 glycosylation and structure could be a potential tool for detecting OA progression by detecting alterations in SF and synovium. The addition of O-linked glycosylations increases the number of oligosaccharide complexes to proteins, therefore resulting in a more acidic pI. This occurs because there are more negative charges on the sugar in the O-linked glycosylations, thus making the pI more acidic to counteract the negative charge. PRG4 boundary lubrication is mediated by the O-linked β(1-3)Gal-GalNAc oligosaccharides and deglycosylation has shown to reduce lubricating ability (Jay et al., 2001). Specially, the removal of sialic acid and core 1 oligosaccharides demonstrates a loss in boundary lubrication (Jay et al., 2001). Also, deglycosylation decreased the apparent MW from 280kDa to 120kDa on a SDS-PAGE (Jay et al., 2000).
2D electrophoresis has been used with rheumatoid arthritis and juvenile idiopathic arthritis patients, but, to the best of our knowledge, has not been used to quantify changes in the joint composition from oSF in a post-knee injury model. The objective of the 2D electrophoresis development was to gain insight into PRG4 glycosylations by determining the pI of PRG4 in ACL/MCL and MEN oSF as an initial assessment of PRG4 glycosylations.

3.3 Methods

3.3.1 First Dimension – IEF

SF samples (n=4) were prepared via enzymatic digestion: the SF samples were pre-treated with S. Hyaluronidase (HA’se; used to reduce the binding of PRG4 to HA by digesting any HA in the SF) (MJS Biolynx Inc. Brockville, ON) (overnight at 37°C) and the PRG4 samples were pre-treated with Sialidase S-66 neuraminidase (cleave the glycosidic linkages of neuraminic acid to remove sialic acid caps) (Prozyme, Hayward, CA) (overnight at 37°C) or, in some cases, O-Glycanase (cleaves disaccharide unit from serine or threonine residues of glycoproteins). After the sample was prepared, it was reconstituted in 200µl rehydration/sample buffer made up of 10ml 8M urea, 2% CHAPS, 50mM dithiothreitol, 0.2% (w/v) Bio-Lyte 3/10 ampholytes, bromophenol blue (ReadyPrep 2-D Starter Kit; Bio-Rad, Mississauga, ON). 190 µl of the reconstituted sample was loaded into the horizontal focusing tray and the 11cm ReadyStrip IPG strip (Non-linear pH 3-10; Bio-Rad, Mississauga, ON) on top of the sample in the horizontal well. The IPG strip was covered with mineral oil in the focusing tray and then placed into the PROTEAN® IEF System (Bio-Rad, Mississauga, ON). The IPG strips were passively
rehydrated for 12 hours at 20°C. IEF was conducted with the pre-set, linear method as follows: 250 V for 15 minutes, 8000 V for 2.5 hours, 8000 V for 35000 vhours, followed by a 500 V hold, according to manufacturer’s instructions (Bio-Rad Laboratories, 2012). After IEF was completed, either SDS-PAGE was conducted or the IPG strips were stored in a rehydration/equilibration tray at -80°C. Equilibration Buffer I of 20mL 6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol, 2% (w/v) dithiothreitol (ReadyPrep 2-D Starter Kit; Bio-Rad, Mississauga, ON) and Equilibration Buffer II of 20mL 6M urea, 2% SDS, 0.375M Tris-HCl (pH 8.8), 20% glycerol (ReadyPrep 2-D Starter Kit; Bio-Rad, Mississauga, ON) were prepared (Bio-Rad Laboratories, 2012). If the IPG strips were frozen, the IPG strips were thawed and equilibrated. 2mL of Equilibration Buffer I was added to the rehydration/equilibration tray for 10 minutes and mixed on a plate rocker, decanted, followed by the addition of 2mL of Equilibration Buffer II for 10 minutes and mixed on a plate rocker.

3.3.2 Second Dimension – SDS-PAGE

Criterion Precase Tris-HCl (4-20%) gels were used with the Criterion Cell (Bio-Rad, Mississauga, ON) chamber to conduct SDS-PAGE. The IPG strip, previously equilibrated in buffer, was placed in the gel and 1mL agarose gel overlay was used to secure the IPG strip. 1X Tris/Glycine/SDS gel running buffer (Bio-Rad, Mississauga, ON) was used to fill the reservoir and 10µl MW marker (Bio-Rad, Mississauga, ON) was used to quantify MW. The Criterion Cell chamber was plugged into the PowerPac™ HC High-Current Power Supply (Bio-Rad, Mississauga, ON) and electrophoresis was
conducted at a constant 200V for 65 minutes. After SDS-PAGE was terminated, western blotting and/or protein staining was conducted.

3.3.3 Western Blotting

The Criterion™ Blotter (Bio-Rad, Mississauga, ON) was used to transfer the gel to the polyvinylidene fluoride (PVDF) blotting membrane. The transfer buffer of 1X Tris/Glycine transfer buffer (Bio-Rad, Mississauga, ON) was prepared previously and stored at 4°C to improve heat dissipation during the transfer. The PVDF blotting membrane was pre-wet in 100% methanol for 10s, rinsed in distilled water, and then soaked in transfer buffer for 10 minutes. The membrane sandwich setup is diagramed below (Figure 3.3.3.1) and was placed into the Criterion™ Blotter (Figure 3.3.3.2). Transfer buffer was used to fill the reservoir, the Criterion™ Blotter was plugged into the PowerPac™ HC High-Current Power Supply (Bio-Rad, Mississauga, ON), and the transfer was conducted at a constant 100V for 2 hours. Additionally, an ice pack and stir bar were placed into the bottom of the Criterion™ Blotter, and the transfer buffer was changed out at 1 hour to help improve heat dissipation.
Figure 3.3.3.1 Western blotting transfer sandwich set up (Bio-Rad Laboratories, 2012).

Figure 3.3.3.2 Criterion Blotter set up (Bio-Rad Laboratories, 2012).
After the transfer was terminated, immuno-staining was conducted. The membrane was soaked in 5% non-fat dry milk in 0.1% Tween in tris buffered saline (TBST) for 60 min at room temperature on a plate rocker. The membrane was soaked in primary antibody J108N (Dr. Matt Warman, Harvard University) with 3% non-fat dry milk in TBST at 1:1000 overnight at 4°C, while rocking. The next day, the membrane was washed 3 x 10 minutes in TBST, while rocking, and then soaked in secondary antibody goat anti-rabbit (Chemicon) at 1:2000 for 1 hour, while rocking (Schmidt et al., 2009). Again, the membrane was washed 3 x 10 minutes in TBST, and then developed with ECL Prime (GE Healthcare, Baie d’Urfe, QC) developing solution. The membrane was imaged via the Chemigenius² Bio Imaging System and GeneSnap (SynGene, Frederick, MD) software program. If the membrane was re-probed, the membrane was washed in 3 x 10 minutes in TBST then soaked in PNA-Horse Radish Peroxidase (PNA-HRP) (Sigma-Aldrich, St. Louis, MO) at 1:250 for 1 hour, while rocking. After the incubation, the membrane was developed with FEMTO (Thermo Scientific, Nepean, ON) and imaged as described above.

3.3.4 Protein Staining

After SDS-PAGE or western blotting, gel protein staining was conducted to visualize protein content. The gel was washed 3 x 5 minutes in distilled water, while rocking. The gel was then submerged in Simply Blue Protein Stain (Invitrogen, Carlsbad, CA) for 1 hour at room temperature, while rocking. The gel was subsequently washed 3 x 5 minutes in distilled water, while rocking. The gel was imaged via the Bio-Rad GS-800
Calibrated Densitometer (Bio-Rad, Mississauga, ON) and QuantityOne 1-D Analysis software program.

3.4 Results

3.4.1 One-Dimensional (1D) Electrophoresis and Western Blotting to Determine Antibody Reactivity

Preliminary blotting studies with SDS-PAGE shows immunoreactivity to anti-PRG4 antibody J108N and PNA in a banding pattern on the top of the membrane, or high MW region. Western blotting with a one-dimensional gel (for MW only) showed that PRG4 is present at the ~460kDa range in both reduced (R; disulphide bonds are broken in/between PRG4 molecule(s)) and non-reduced (NR) samples (Figure 3.4.1.1). There is a more prominent band in the NR sample, in comparison to the R sample (Figure 3.4.1.1). When PRG4 was pre-treated with neuraminidase, there was a strong immunoreactive band in the ~460kDa range. When PRG4 was pre-treated with both neuraminidase and O-Glycanase, there was a weaker band present and a lower shift in molecular weight (Figure 3.4.1.1). Therefore, PRG4 was present in bSF at the ~460kDa range (Figure 3.4.1.1).

Western blotting with a 1D electrophoresis also showed that PRG4 is present in the ~460kDa range in oSF, with (+) and without (-) neuraminidase (Figure 3.4.1.2). When the oSF sample was pre-treated with neuraminidase, there was a stronger signal with the PNA. However, when the oSF sample was pre-treated with neuraminidase and J108N antibody was used, there was a weaker signal (Figure 3.4.1.2). There was immunoreactive PRG4 species with PNA at the ~460kDa range, but there was not a band
present when the J108N antibody was used (Figure 3.4.1.2). Although the PRG4 species was not present in the ~460kDa range with the J108N antibody, there was smaller molecular weight species in the ~75kDa and ~50kDa range (Figure 3.4.1.2). These lower molecular weight PRG4 species are consistent with the oSF samples in both the PNA western blot and the J108N western blot. Lastly, when no primary was added to the membrane during the western blotting, there was very minimal signal in the membrane to show no reactivity with the secondary antibody (Figure 3.4.1.2). However, there is some non-specific signal at the ~40-50kDa range (Figure 3.4.1.2). Based on these findings, the J108N antibody and PNA-HRP were used to evaluate the pI of PRG4 and oSF in 2D electrophoresis.
Figure 3.4.1.1 Western blot using PNA-HRP with a 1D 4-20% Criterion Tris-HCl gel with 2μg purified PRG4 and 100μl bSF. PRG4 samples were either reduced (R) or non-reduced (NR). Neuraminidase was added (+) or not added (-). O-Glycanase was added (+). The bSF was treated with HA’se. Red box indicates high MW PRG4 band of interest.
**Figure 3.4.1.2** Western blot using PNA-HRP and the J108N antibody (goat anti-rabbit secondary antibody) with a 1D 4-20% Criterion Tris-HCl 12 well gel with 2μg purified PRG4 and 100μl oSF samples. PRG4 samples were either reduced (R) or non-reduced (NR). Neuraminidase was added (+) or not added (-). All SF samples were treated with HA’se. Red box indicates high MW PRG4 band of interest and blue box indicates other possible bands of interest.
3.4.2 Two-Dimensional Electrophoresis with PRG4 and Western Blotting

Preliminary protein staining studies with SDS-PAGE show that PRG4 is present in the 2D gels in the ~460kDa range at a pI ~4.0-4.4 (Figure 3.4.2.1). Of possible interest, are the horizontal protein band at ~100kDa and the vertical protein band at the pI of ~7.5 (Figure 3.4.2.1).

Western blotting with 2D electrophoresis demonstrated minimal immunoreactive species detected in the membrane from the J108N western blot (Figure 3.4.2.2 A). There was one broad range of blots, but no precise banding patterns. There may be a possible horizontal immunoreactive band at pI ~3-5 and 25kDa, but it is not clear (Figure 3.4.2.2 A). Western blotting with 2D electrophoresis showed PRG4 immunoreactive species detected in the membrane from the re-probed PNA western blot; the band was at the ~460kDa range at a pI ~4.0-4.4 (Figure 3.4.2.2 B). Similar to the J108N western blot, the re-probed PNA membrane demonstrated that there may be a possible horizontal immunoreactive band at pI ~3-5 at 25kDa (Figure 3.4.2.2 B).
**Figure 3.4.2.1** Protein stain of 50μg purified PRG4 with Simply Blue Protein Stain with a 2D 4-20% Criterion Tris-HCl IPG strip well gel. PRG4 sample was reduced. Red box indicates high MW PRG4 band of interest and blue box indicates other possible bands of interest.
**Figure 3.4.2.2** Western blot using J108N primary antibody with goat anti-rabbit secondary antibody (A) and re-probed western blot using PNA-HRP (B) with a 2D 4-20% Criterion Tris-HCl IPG strip gel with 50μg purified PRG4. PRG4 sample was reduced and treated with neuraminidase. Red box indicates high MW PRG4 band of interest.
3.4.3 Two-Dimensional Electrophoresis with oSF and Western Blotting

Western blotting with 2D electrophoresis showed that PRG4 is present at the \(~460\)kDa range in oSF (Figures 3.4.3.1, 3.4.3.2, 3.4.3.3, 3.4.3.4) at a pI \(~4.0-4.4\), which is consistent with the preliminary 1D electrophoresis results. There was strong J108N immunoreactivity at the 100kDa-460kDa range in the ACL/MCL CTRL oSF (Figure 3.4.3.1 A) and there was slight J108N immunoreactivity banding at the 150kDa and 460kDa range in the ACL/MCL transection oSF (Figure 3.4.3.1 B) at a pI \(~4.0-4.4\). There is also horizontal banding at 50kDa and 75kDa in the ACL/MCL CTRL oSF and horizontal banding at 25kDa, 50kDa, and 75kDa in the ACL/MCL transection oSF (Figure 3.4.3.1). There may be a possible difference in immunoreactive signal strength when comparing the ACL/MCL CTRL oSF (Figure 3.4.1.1 A) to the ACL/MCL oSF (Figure 3.4.1.1 B); the stronger signal in the ACL/MCL CTRL oSF and weaker signal in the ACL/MCL oSF. Similarly, when the membranes were re-probed with PNA, there was anti-PNA immunoreactivity at the 100kDa-460kDa range in the ACL/MCL CTRL oSF (Figure 3.4.3.2 A). Also, there was darker PNA immunoreactivity banding at the 150kDa and 460kDa ranges in the ACL/MCL transection oSF (Figure 3.4.3.2 B) at a pI \(~4.0-4.4\) with the PNA antibody in comparison to the J108N antibody. Again, there was slight horizontal banding throughout the membranes, with the ACL/MCL CTRL oSF having a more dominant signal (Figure 3.4.3.2).

There was J108N immunoreactivity banding at the 75kDa, 150kDa, and 460kDa range in the MEN CTRL oSF (Figure 3.4.3.3 A) and there was slight J108N immunoreactivity banding at the 150-250kDa range in the MEN oSF (Figure 3.4.3.3 B) at a pI \(~4.0-4.4\). Of interest, is the possible shift in higher pI at the high MW species.
(~460kDa), as there was a J108N immunoreactive band at a pI ~6.0-6.2 in the MEN oSF and minimal immunoreactive species at pI ~4.0-4.4 (Figure 3.4.3.3 B). Also, there was horizontal banding at ~40kDa and pI 5.0-7.5 (Figure 3.4.3.3 B). Similarly to the J108N immunoreactivity banding, when the membranes were re-probed with PNA, there was a stronger PNA immunoreactivity at the 75kDa, 150kDa, and 460kDa range in the MEN CTRL oSF (Figure 3.4.3.4 A). Again, there is a strong high MW (~460kDa) immunoreactive species at a pH ~6.0-6.2 in the MEN oSF (Figure 3.4.3.4 B) when compared to the high MW band at pH ~4.0-4.4 in the MEN CTRL oSF (Figure 3.4.3.4 A). In contrast to the J108N antibody, there was much darker horizontal banding at ~40-100kDa and pI 5.0-7.5 with the PNA (Figure 3.4.3.4 B).
Figure 3.4.3.1 Western blot using J108N primary antibody with goat anti-rabbit secondary antibody with a 2D 4-20% Criterion Tris-HCl IPG strip gel with 80μl ACL/MCL CTRL (A) or ACL/MCL (B) transection oSF. oSF was treated with HA’se. Red box indicates high MW PRG4 band of interest.
Figure 3.4.3.2 Re-probed western blot using PNA-HRP with a 2D 4-20% Criterion Tris-HCl IPG strip gel with 80μl ACL/MCL CTRL (A) or ACL/MCL (B) transection oSF. oSF was treated with HA’se. Red box indicates high MW PRG4 band of interest.
Figure 3.4.3.3 Western blot using J108N primary antibody with goat anti-rabbit secondary antibody with a 2D 4-20% Criterion Tris-HCl IPG strip gel with 100μl MEN CTRL (A) or MEN (B) oSF. oSF was treated with HA’se. Red box indicates high MW PRG4 band of interest and blue box indicates other possible bands of interest.
Figure 3.4.3.4 Re-probed western blot using PNA-HRP with a 2D 4-20% Criterion Tris-HCl IPG strip gel with 100μl MEN CTRL (A) or MEN (B) oSF. oSF was treated with HA’se. Red box indicates high MW PRG4 band of interest and blue box indicates other possible bands of interest.
3.5 Discussion

The pI of PRG4 in post-knee injury oSF was determined with 2D electrophoresis, and, what changes, if any, in pI exist between both ACL/MCL and MEN oSF and contralateral CTRL oSF. The analyses described demonstrated the presence of PRG4 in oSF. These preliminary results may provide insight into the possible changes PRG4 structure in oSF and the results suggest that PRG4 structure may be altered in post-knee injury oSF at 20 weeks.

While the sample size in these preliminary studies was small (n=4), enough oSF was obtained to determine the pI and MW of PRG4 in oSF at 20 weeks post-knee surgery. The oSF was pooled equally into respective surgical or contralateral CTRL groups for the 2D electrophoresis. Quantification of immunoreactive bands and proteins spots, matching spots between gels, and detecting protein can be difficult as each sample is run on one gel. Thus, gels are usually run in duplicate or even triplicate to reduce inter-gel variability. However, this was not possible in this study as minimal oSF remained from the biochemical and biomechanical testing. Lastly, computer software for densitometry was not used to compare the protein spots.

Preliminary blotting studies with 1D SDS-PAGE indicated that PNA immunoreactivity was present in a banding pattern in the high MW region on the membrane, which is consistent with previous work (Schmidt et al., 2009). The reduced PRG4 that was pre-treated with neuraminidase demonstrated the strongest PNA immunoreactivity (Figure 3.4.1.1), which occurs because PNA binds to the galactose portion of the oligosaccharides. This neuraminidase digests the sialic acid caps that lie on the terminal of the glycosylated portion of the mucin region, which is demonstrated with
an expected lower shift in molecular weight as the protein is now smaller without capping (Figure 3.4.1.1) (Ludwig et al., 2012). Also, when the PRG4 sample was treated with neuraminidase and O-Glycanase, there is an expected further combined lower shift in MW (Figure 3.4.1.1). Furthermore, 1D SDS-PAGE demonstrated that PRG4 is present in oSF in the high MW (~460kDa) region, serving as a control for comparison to the 2D electrophoresis blots (Figure 3.4.1.2). No J108N immunoreactive bands were observed in the PRG4, but high MW PNA immunoreactive bands were observed (Figure 3.4.1.2). Lastly, there was minimal signal with the no primary blot, suggesting that the PRG4 present is specific (Figure 3.4.1.2). These preliminary data provide an indication of what would be present in oSF before the use of 2D electrophoresis.

The 2D methodology extends upon other PRG4 characterization methods. Initially, protein staining using reduced PRG4 was conducted to determine where the PRG4 was in the large 2D Tris-HCl gel. Various amounts of PRG4 were loaded (10 µg, 50 µg, and 100 µg) into the IPG strips to determine which quantity of PRG4 would yield quantifiable results (data not shown). Using 50 µg yielded the clearest protein stain with the most identifiable signal, thus 50 µg was used for the subsequent experiments (Figure 3.4.2.1). For the SDS-PAGE, 200V for 65 minutes was found to be the most optimal for separation in the gel electrophoresis. As recommended by the Bio-Rad instruction manual, transfer conditions were 100 V for 0.5 hours at 4°C (Bio-Rad Laboratories, 2012). However, as the PRG4 protein is large (~460kDa), the transfer conditions were not optimal. A range of durations (0.5 hours-2.5 hours) was evaluated, and 100 V for 2
hours 4°C was found to be the most optimal for transfer of high MW protein to the PVDF membrane.

The results from the western blotting and protein staining from the 2D electrophoresis suggest that PRG4 has a pI ∼4.0-4.4 (Figure 3.4.2.1 and Figure 3.4.2.2), which is not consistent with previous literature of pH 7.9-8.1 (Jay, 1990). If PRG4 did have a pI of ∼8.0, it would be slightly positively charged at the physiological pH of 7.2. Thus, with a slightly more acidic pI (pI ∼4.0-4.4), PRG4 would be negatively charged at the physiological pH of 7.2, which has been postulated to be important for its lubricating function. Although there are a large number of negative changes that are associated with the carbohydrate groups, there may be more positive amino acids in the protein chain, specifically lysine. There is minimal research conducted on the pI of PRG4, however, more research could be conducted to verify the pI.

The strength of the immunoreactive PRG4 band appears to be slightly weaker in the ACL/MCL oSF compared to the contralateral ACL/MCL CTRL oSF, in both the J108N and PNA immunoreactive bands (Figure 3.4.3.1 and Figure 3.4.3.2). Similarly, the strength of the immunoreactive PRG4 band appears to be slightly weaker in the MEN oSF in comparison to the contralateral MEN CTRL oSF (Figure 3.4.3.3 and Figure 3.4.3.4). Therefore, such results suggest that the PRG4 is present and protein structure may be altered in ACL/MCL transected and MEN joints in comparison to control joints, however, no quantitative assessment was completed. Of particular interest, there may be a shift in pI in the MEN oSF from pI ∼4.0-4.4 to pI ~6.0-6.2 (Figure 3.4.3.3 and Figure 3.4.3.4). This suggests that PRG4 structure may be altered in a MEN ovine oSF 20 weeks after surgery, as there is a pI shift from more acidic (pI 4.0-4.4) to less acidic (pI 6.0-6.2).
This is interesting and functionally significant as decreased glycosylation results in decreased lubrication function (Jay et al., 2001). Furthermore, if there are fewer PRG4 glycosylations in OA oSF, and potentially acute knee injury oSF, there may be decreased lubrication function leading to further OA progression and cartilage damage. Even though lubrication function was similar at 20 weeks in the MEN oSF in this study (Chapter 2), this initial preliminary data suggests that using 2D electrophoresis may provide insight into the changes of PRG4 structure.

Future studies should run 2D electrophoresis on oSF in duplicates or triplicates to increase the reproducibility and confirm findings of the results, as the method with SF is novel. Further studies could also examine the pI of recombinant human PRG4 (rh-PRG4) compared to the native PRG4 using 2D electrophoresis. This would provide insight into the composition and structure of rh-PRG4 to see if there are differences between native PRG4 and rh-PRG4. This method could be essential prior to the development of PRG4 in biotherapeutics during the rh-PRG4 scaling up process. Computer tools, such as PDQuest and Progenesis, were not utilized to quantify the protein spots, but it should be something that could be used in further studies, as it has been used previously (Ruiz-Romero et al., 2010). In-gel differentiation analysis co-detection, background subtraction, normalization, and quantification of spots should be used in the future (Rosenkranz et al., 2011). Also, biological variation analysis that compare multiple gels and the statistical analysis of protein abundance could be used (Rosenkranz et al., 2011). Lastly, future studies could examine synovium and SF from humans and other animal models to gain insight into other structural changes in other proteins or stages of OA development.
In summary, these findings indicate 2D electrophoresis may be a useful tool in the pI and MW analysis of PRG4 glycosylations. As an initial assessment, PRG4 pI is ~4.0-4.4 and there appears to be more PRG4 immunoreactivity signal in contralateral CTRL groups than the operated oSF groups. Lastly, the pI of PRG4 may have been shifted from pI of ~4.0-4.4 to ~6.0-6.2 in MEN oSF. These results suggest that PRG4 pI may be altered in MEN surgery at 20 weeks in an ovine model, which may have potential implications in lubrication function with altered protein glycosylations. This technique of characterizing PRG4 glycosylations could provide another dimension to quantifying PRG4 in OA or acute injury SF, in addition to concentration.
CHAPTER 4: BACKGROUND SUMMARY, SUMMARY OF FINDINGS, AND FUTURE WORK

4.1 Background Summary

OA is a debilitating disease that involves the breakdown of articular cartilage that covers articulating bones and severely restricts millions of patients globally. Cartilage lubrication is an essential mechanism for the protection and maintenance of joints. PRG4 and HA are SF lubricants that contribute to the boundary lubrication of cartilage and maintenance of the joint. PRG4 composition in SF is unclear with respect to OA; PRG4 concentration has been variably reported to remain normal, increase, or decrease with OA. Also, HA concentration has reported to remain normal with OA, however it has demonstrated a lower shift in MW. Lastly, boundary lubricating ability has been reported to remain normal or decrease in OA, depending on the model and state of OA.

The hypothesis of the current work was that SF boundary lubricant composition is altered in post-traumatic OA in a post-knee injury ovine model. Thus, the objective of this thesis was to attempt to define the composition-function relationship between SF lubricant molecules, PRG4 and HA, by determining 1) PRG4 and HA concentration, 2) HA MW distribution, 3) cartilage lubricating ability, and 4) the pI of PRG4 in SF from a post-knee injury ovine model at 20 weeks.

To address this hypothesis, mature female Suffolk-cross sheep were subject to one of three surgical procedures: SHAM, ACL/MCL transection, or MEN, with the left joint of each individual being used as an unoperated contralateral CTRL. SF was collected at euthanization at 20 weeks post surgery. An ELISA was used to measure PRG4 and HA
concentration and agarose gel electrophoresis was used to quantify HA molecular weight distribution for oSF. Cartilage lubricating ability of oSF was assessed using an in vitro cartilage-cartilage friction test. Lastly, 2D electrophoresis was used to determine PRG4 pl and MW as an initial assessment of PRG4 glycosylations.

4.2 Summary of Findings

The overall motivation of this thesis work was to define the composition-function relationship between SF lubricant molecules, PRG4 and HA. The major findings were:

1. There was no detectable difference in PRG4 concentration between operated oSF and respective contralateral CTRL oSF in all experimental groups at 20 weeks post-knee surgery (SHAM, ACL/MCL transection, or MEN).
   a. SHAM and SHAM CTRL PRG4 concentration: 105-242 µg/mL.
   b. ACL/MCL and ACL/MCL CTRL PRG4 concentration: 218-354 µg/mL.
   c. MEN and MEN CTRL PRG4 concentration: 119-306 µg/mL.

2. There was no detectable difference in HA concentration between operated oSF and respective contralateral CTRL oSF in all experimental groups at 20 weeks post-knee surgery (SHAM, ACL/MCL transection, or MEN).
   a. SHAM and SHAM CTRL HA concentration: 0.68-1.04 mg/mL.
   b. ACL/MCL and ACL/MCL CTRL HA concentration: 0.54-1.12 mg/mL.
   c. MEN and MEN CTRL HA concentration: 0.68-1.36 mg/mL.

3. HA MW distribution was similar between operated oSF and respective contralateral CTRL oSF in all experimental groups at 20 weeks post-knee surgery (SHAM, ACL/MCL transection, or MEN).
4. Cartilage lubricating ability similar between operated oSF and respective contralateral CTRL oSF in all experimental groups at 20 weeks post-knee surgery (SHAM, ACL/MCL transection, or MEN).

   a. $\mu_{\text{static,Neq}}$ increased with increasing pre-sliding durations for all lubricants.
   
   b. $\mu_{\text{static,Neq}}$ was greatest in PBS, while operated oSF, respective CTRL oSF, and bSF were all similar and lower than PBS.
   
   c. $<\mu_{\text{kinetic,Neq}}>$ at $T_{ps}=1.2s$ in PBS was significantly higher than operated oSF, CTRL oSF, and bSF, all of which were similar and lower in all experimental groups.

5. Initial assessment of the pI of PRG4 in oSF indicated the pI was ~4.0-4.4.

4.3 Future Work

The work presented in this thesis can be expanded upon in many ways. While sixteen mature sheep were used for the OA development model (SHAM surgery $n=5$, ACL/MCL transection $n=6$, or MEN surgery $n=5$), using more subjects would increase the power of the study to detect differences.

The framework in Chapter 2 provides insight into the composition-function of lubricant molecules, specifically PRG4 and HA, at 20 weeks after knee injury. As PRG4 and HA concentration have been shown by others to be altered immediately after acute injury, it would be potentially interesting to explore composition-function of lubricant molecules at earlier time points in this ovine model to determine how they are altered in acute injury in a large animal model (Elsaid et al., 2005) (Elsaid et al., 2008) (Elsaid et al., 2009) (Pitsillides et al., 1999). This would provide some understanding of what time
points the lubrication mechanism is altered and indicate potential windows of opportunity that biotherapeutic injections could be beneficial. As many OA patients and post-knee injury patients usually receive HA intra-articular injections several months or years after injury or surgery, investigating the lubrication mechanism may lead to injections at earlier time points to potentially preserve the boundary lubrication mechanism. Possible time points of interest would be 2-10 weeks after injury to determine how PRG4 concentration is altered. Lastly, as 20 weeks in an ovine model may still be considered to be a “semi-acute” stage, potentially looking at the composition-function of oSF at 30-40 weeks, or potentially longer time frames, may provide insight into changes in lubrication composition in severe OA development.

Biotherapeutic strategies could heavily influence OA patients by preserving or regenerating the boundary lubrication mechanism, thus protecting damage to articular cartilage. PRG4 injections have shown to decrease radiographic and histologic scores of cartilage damage, all in small animal models (Flannery et al., 2009) (Jay et al., 2010) (Teeple et al., 2010). This previous work is encouraging, and the potential use of a large animal model in the future could examine PRG4 and PRG4+HA injections at both early and later time points. Future studies should use an ovine model to explore the option of intra-articular injections with PRG4 and/or PRG4+HA to examine cartilage damage, cartilage lubricating ability, and lubricant composition to further understand the changes in lubricant composition and affects of injections. Furthermore, the use of PRG4 could potentially be used in the future as a biotherapeutic injectable and could be tested in a large animal model. Lastly, the mechanism of which PRG4 and HA are attached to the articular surface is still unknown, so insight into the attachment of the lubricant
molecules to the surface would aid in the understanding of boundary lubrication and potentially intra-articular injections.

Additional biochemical and biomechanical testing may assist in understanding the joint composition and lubrication. It would be of interest to further examine the metabolic changes via inflammatory markers (i.e. interleukin-1B, tumor necrosis factor-α), cytokines, and the activity of articular cartilage degrading enzymes (i.e. collagenases, aggrecanases) in oSF at various time points in an knee injury or OA model. Testing the lubricating ability of varying levels of cartilage degradation or varying stages of OA progression may provide understanding of the cartilage or SF alterations, or both, in the knee joint with injury and OA. Furthermore, testing normal SF with OA diseased cartilage and multiple time points or testing normal cartilage with varying post-knee injury or OA SF at multiple time points may assist in the determination of changes occur in lubricating ability in both the SF and cartilage.

The results in Chapter 3 indicate that 2D gel electrophoresis could be a valuable tool in assessing protein glycosylations, specifically alterations in PRG4 structure. Further biochemical characterization of PRG4 glycosylation would expand upon the initial work of using 2D electrophoresis to assess oSF. Examining the pI of rh-PRG4 compared to the native PRG4 would provide insight into possible differences in rh-PRG4 structure. Also, it is essential that computer tools, such as PDQuest and Progenesis, be used to accurately quantify protein spots and to define a statistical analysis of protein abundance (Ruiz-Romero et al., 2010). Lastly, multiple gels could be analyzed to ensure adequate reproducibility and analyzing SF from other injury or OA models at different time points to see if there are alterations in PRG4 glycosylation.
Understanding the role PRG4 and/or HA play in altered lubricating properties of injured SF will contribute to the fundamental joint lubrication mechanism and potentially to alternative biotherapeutic strategies for improved SF substitutes for OA treatment or maintenance of healthy joints. This project aimed to address the importance of ACL injury and to establish an ovine model of OA development in which we can study the potential impact of lubrication abnormalities in OA progression. While we found no definite abnormalities at 20 weeks in PRG4/HA compositon or lubricating function of oSF, these results does not preclude potential changes at earlier in points. We did find some potential changes related to PRG4 glycosylation with 2D electrophoresis that warrant further investigation as well.
REFERENCES


