THE ROLE OF LUBRICIN AND GALECTINS IN OSTEOARTHRITIS AND CARTILAGE LUBRICATION

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THE ROLE OF LUBRICIN AND GALECTINS IN OSTEOARTHRITIS AND CARTILAGE LUBRICATION

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The aim of this thesis was to investigate how lubricin and galectins function within the synovial environment to maintain joint integrity and enhance articular cartilage lubrication. The specific aims of the research were to 1) evaluate how lubricin gene and glycoprotein expression were altered in equine carpal osteoarthritis (OA); 2) investigate the interactions between synovial lubricin and galectins-1 and -3 to determine if lubricin and galectins could synergistically enhance articular cartilage boundary lubrication and; 3) evaluate equine mesenchymal stem cell (MSC) galectin expression and the role of galectins-1 and -3 on MSC properties.

Lubricin gene expression was quantified in joint tissues obtained from horses with experimental and naturally occurring carpal OA. Experimental OA was induced by creating a 6mm osteochondral chip fracture within the middle carpal joint (MCJ) of horses followed by high-speed treadmill exercise, and joints were assessed 70 days post-fragmentation using gross, histological and immunohistochemical parameters. Synovial fluid lubricin concentrations were serially assessed in experimental horses and at arthroscopy in clinical cases. Lubricin gene expression decreased in OA cartilage but increased in synovial membrane. In both experimental and naturally occurring OA, synovial fluid lubricin levels increased, peaking at 21 days post-fragmentation in experimental horses. Increased lubricin immunolocalization was

observed at sites of cartilage damage, including fibrillation, clefts, and at sites of fibrocartilaginous repair tissue.

Glycan phenotyping of equine lubricin and fluorescent lectin staining of cartilage revealed the presence of core-1 O-linked oligosaccharides on lubricin capable of binding to galectins. Biochemical assays demonstrated strong binding between lubricin and galectin-3, suggesting a potential role for galectin-3 in stabilization of the articular cartilage lubricin boundary layer. Boundary lubrication of cartilage was measured using a custom friction testing apparatus in the presence of recombinant galectins. Galectin-3 enhanced boundary lubrication, but only in the presence of lubricin. Galectin-1 and Galectin-3C, an oligomerization-incompetent mutant, did not enhance lubrication.

Equine galectins-1 and -3 were cloned and recombinantly produced, and gene expression was evaluated in equine MSCs. MSCs endogenously expressed high levels of galectins and elevated galectin-1: galectin-3 ratios as compared to other synovial cell types. Galectin-1 and galectin-3 mRNA expression decreased in the presence of the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor α (TNF-α). Galectins facilitated MSC adhesion, and mature focal adhesion complexes were abrogated in the presence of the pan-galectin inhibitor β-lactose. Galectins promoted cell spreading and motility in 2D culture, resulting in cells with greater surface areas and more protrusions.

BIOGRAPHICAL SKETCH

Heidi Lynn Reesink was born in Iowa City, Iowa on August 6th, 1981 to Marcia Jane Reesink and William George Reesink. Heidi attended Iowa State University where she obtained her Bachelor of Science degree in Zoology in 2003. After receiving her Veterinariae Medicinae Doctoris degree from the University of Pennsylvania School of Veterinary Medicine in 2007, Heidi completed an internship in Large Animal Surgery and Critical Care at the University of Pennsylvania's New Bolton Center in Kennett Square, PA. Heidi completed large animal surgical residency training at Cornell University from 2008-2011 and attained diplomate status in the American College of Veterinary Surgeons in 2012. Following residency training, Heidi began her Ph.D. studies and was awarded a National Institutes of Health, Institutional National Research Service Award from Cornell University and a Grayson-Jockey Club Research Foundation, Storm Cat Career Development Award for her Ph.D. training. From 2011-2015, Heidi completed the dissertation research submitted herein.

This dissertation is dedicated to my parents who have loved and supported me unconditionally, and to my fiancée, Matt, who has been my source of constant encouragement.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ACJ	Antebrachialcarpal joint
ACL	Anterior cruciate ligament
AGEs	Advanced glycation end products
AIA	Antigen induced arthritis
ASF	Asialofetuin
CACP	Camptodactyly-arthropathy-coxa vara-pericarditis syndrome
CD	Cluster of differentiation
CIA	Collagen induced arthritis
COF	Coefficient of friction
CRD	Carbohydrate recognition domain
COMP	Cartilage oligomeric matrix protein
DPBS	Dulbecco's phosphate-buffered saline
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-related kinase
FA	Focal adhesion
FAK	Focal adhesion kinase
FRAP	Fluorescence recovery after photobleaching
GalNAc	N-Acetylgalactosamine

HA	Hyaluronic acid
HCAM	Homing cell adhesion molecule (CD44)
IA	Intra articular
ICAM-1	Intracellular adhesion molecule 1 (CD54)
IFN-ϒ	Interferon gamma
IL-1β	Interleukin-1 beta
IL-10	Interleukin 10
JIA	Juvenile idiopathic arthritis
LC-MS	Liquid chromatography-mass spectrometry
LPS	Lipopolysaccharide
MALDI/TOF-MS	Matrix assisted laser desorption ionization / time of flight mass spectrometry
MAL II	*Maackia amurensis* lectin
MCJ	Middle carpal joint
MPM	Multi-photon microscopy
MSC	Mesenchymal stromal cell
MSF	Megakaryocyte stimulating factor
MW	Molecular weight
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OA	Osteoarthritis
PBMCs	Peripheral blood mononuclear cells
PGE_2	Prostaglandin E_2

PHA-L	Leucoagglutinin
PNA	Peanut agglutinin
PRG4	Proteoglycan-4
PTOA	Post traumatic osteoarthritis
RA	Rheumatoid arthritis
SAPL	Surface active phospholipid
SF	Synovial fluid
SHG	Second harmonic generation
siRNA	Small interfering ribonucleic acid (RNA)
SNA	*Sambucus nigra* lectin
SZP	Superficial zone protein
S-WGA	Succinylated wheat germ agglutinin
TNF-α	Tumor necrosis factor alpha
UCB-derived MSC	Umbilical cord blood-derived mesenchymal stromal cell

CHAPTER 1

GENERAL INTRODUCTION

Overarching Goal of Dissertation Research

The overarching goal of this thesis project was to investigate the role of lubricin and galectins in joint homeostasis. In this dissertation research, lubricin was evaluated in an equine model of osteoarthritis (OA); the effects of lubricin-galectin binding on cartilage lubrication were interrogated; and galectin expression and function were investigated in equine mesenchymal stromal cells (MSCs) as a potential mechanism for therapeutic effects observed in OA.

This introductory chapter provides relevant background information and discussion of lubricin, galectins and articular cartilage lubrication. The first aim of this thesis is presented in chapter 2, which investigates how lubricin is altered in a clinically relevant equine model of OA. Lubricin, the primary boundary lubricant in synovial fluid, increases in both naturally occurring carpal OA and in a carpal osteochondral fragmentation model of OA. Lubricin gene expression is upregulated in synovial tissues, lubricin glycoprotein levels are increased in synovial fluid, and lubricin staining is most intense at sites of cartilage damage and repair.

The second aim, presented in chapter 3, examines the interaction between lubricin and two members of the galectin family of carbohydrate binding proteins,

galectin-1 and galectin-3. Galectin-3 is demonstrated to bind to lubricin with high affinity in a carbohydrate-dependent fashion, thereby stabilizing the articular cartilage boundary layer and enhancing cartilage lubrication. Furthermore, this interaction is affected by the glycan profile of lubricin, with increased levels of sialylation decreasing the affinity of galectin-3 for lubricin. Importantly, lubrication is only enhanced in the presence of fully functional, pentavalent galectin-3 and not an oligomerization-incompetent mutant, suggesting that the crosslinking behavior is critical for facilitating lubrication. This chapter provides evidence for a novel biomechanical role for galectin-3 in synovial joint biology.

Because galectins hold promise for OA therapy and MSCs are currently used intra-articularly to quell joint inflammation in hopes of preventing OA progression, galectin expression and function in equine MSCs were investigated. Equine MSCs express high levels of both galectin-1 and galectin-3, with elevated ratios of galectin-1: galectin-3 as compared to other synovial cell types. MSC galectin expression is diminished upon treatment with the pro-inflammatory cytokines IL-1β and TNF-α. Galectins enhance equine MSC adhesion, cell spreading and motility in an *in vitro* wound healing model. These properties suggest that galectins may play an important role in MSC adhesion and trafficking, as well as in joint homeostasis.

Overall conclusions of this research and future directions are presented together in the final chapter.

Anatomy and Function of Articular Cartilage

Articular cartilage is a specific type of hyaline cartilage, characterized by a low-friction gliding surface and high compressive strength. Hyaline cartilage is aneural, avascular and alymphatic and composed mostly of extracellular matrix with chondrocytes making up only 1-5% of the volume of articular cartilage (1). The cartilage matrix is critical in providing the physiologic properties that allow cartilage to resist compression, absorb shock and facilitate low-friction motion. By wet weight, water makes up the largest portion of cartilage matrix, accounting for 65% of cartilage weight, followed by predominantly type II collagen (10-20%) and proteoglycans (10-20%) (2).

Zonal Structure of Articular Cartilage

Cartilage is a highly organized structure that can be anatomically divided into four zones: the superficial (tangential) zone, the middle (transitional) zone, the deep (radial) zone, and the zone of calcified cartilage that marks the boundary between subchondral bone and cartilage **(Figure 1.1)**. In addition, the thin surface film present on the articular surface of the superficial cartilage zone is referred to as the lamina splendens, or boundary layer of articular cartilage (3). Although the thickness of the lamina splendens layer (estimated at somewhere between 150nm and 2μm) (3, 4) is nearly negligible compared to the deeper zones, this is the zone that will be discussed

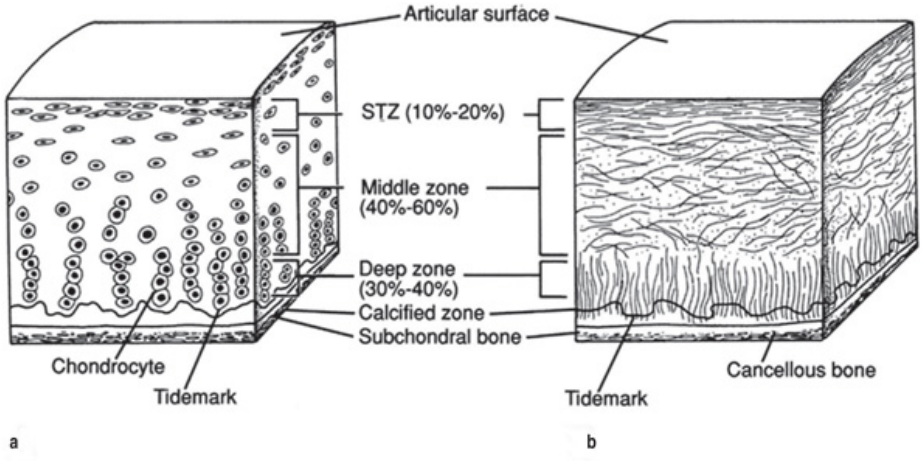


Figure 1.1. Structure of articular cartilage. A, Schematic diagram of chondrocyte organization in the three main zones of the uncalcified cartilage (STZ = superficial tangential zone), the tidemark, and the subchondral bone. B, Sagittal, cross-sectional diagram of collagen fiber architecture shows the three salient zones of articular cartilage. Reprinted from the Journal of the American Academy of Orthopaedic Surgeons, Vol. 2, No. 4, Buckwalter JA, Mow, VC and Ratcliffe A, Restoration of Injured or Degenerated Articular Cartilage, pp. 192-201, © 1994, with permission from Wolters Kluwer Health.

in chapter 3, as the lamina splendens is critical in protecting cartilage from damage due to shear forces and enabling near frictionless joint motion (4).

Superficial Zone

The superficial zone of cartilage makes up approximately 10-20% of the total volume of articular cartilage, has the highest collagen content and lowest proteoglycan content, and collagen fibrils are densely packed and oriented parallel to the articular surface to provide a smooth gliding surface (5). Chondrocytes are elongated in this region, expressing high levels of lubricin or superficial zone protein (SZP), a proteoglycan splice variant produced by chondrocytes from the gene proteoglycan 4 (PRG4), that confers boundary lubricating ability (6, 7).

Middle and Deep Zones

The middle zone of cartilage, encompassing 40-60% of articular cartilage volume, has a higher compressive modulus and less organized collagen arrangement as compared to the superficial zone, with larger, more rounded chondrocytes. The deep zone, which makes up 30% of the volume, consists of large diameter collagen fibrils oriented perpendicular to the articular surface, with the highest proteoglycan content, lowest water content and highest compressive modulus (5). The tidemark separates the deep zone from the calcified cartilage.

Osteoarthritis

Definition of OA

OA is the most common type of arthritis; however, it is difficult to precisely define. OA results from a combination of mechanical and biological events that disrupt the normal balance of degradation and synthesis of articular cartilage, extracellular matrix (ECM) and subchondral bone, resulting in catabolic processes overwhelming anabolic processes. The relationships between these mechanical and biological risk factors for OA can be summarized by two distinct mechanisms: 1) adverse effects of "abnormal" loading on normal cartilage or 2) adverse effects of "normal" loading on abnormal cartilage (8). Whereas OA used to be considered a disease of excessive 'wear-and-tear 'on joints, OA is now considered to be a complex, multi-factorial disease due to a more complete understanding of the molecular events underlying OA progression (9).

Etiology and Pathophysiology of OA

Intra-articular fractures and ligamentous, meniscal and other soft tissue injuries often lead to the development of post-traumatic osteoarthritis (PTOA), one of the most common causes of altered load distribution across a joint, due to loss of articular surface and ligamentous instability. Obesity and supraphysiologic loading due to either repetitive loading or single excessive loading events can also result in

"abnormal" loading. Other risk factors for OA include age, sex, ethnicity, inflammation and metabolic disease. Interestingly, metabolic OA has recently been characterized as a subtype of OA (10–12), with both obesity-related loading and obesity-related metabolic factors (13) playing a role in the development and progression of OA. As such, OA can be currently classified as a systemic disorder rather than a focal musculoskeletal disorder of synovial joints (14).

OA has traditionally been viewed as a disease of articular cartilage; however, all joint structures are affected during the development of OA, including articular cartilage, calcified cartilage, subchondral cortical and trabecular bone, synovium and the joint capsule (8). A current topic of debate is which structure(s) is(are) affected first in the course of OA, and subchondral bone is attracting increasing attention as a major player in the pathogenesis of OA (15). Whether OA begins with articular cartilage changes, synovitis, and/or subchondral bone may depend upon the initial insult, and there may be numerous possible courses by which the disease may progress, with resultant OA being a common clinical manifestation of distinct biochemical and mechanical pathways (16).

Rheumatoid Arthritis and Other Rheumatic Diseases

There are more than 100 rheumatic diseases, or diseases that affects joints and connective tissues, currently recognized by the American College of Rheumatology, which encompass degenerative conditions such as OA, inflammatory conditions such as rheumatoid arthritis (RA), and autoimmune diseases such as system lupus

erythematosus (SLE) (17). Although the American College of Rheumatology still defines OA as a degenerative condition and RA as an inflammatory condition, OA and RA share many features in common, and the dichotomy between inflammatory and degenerative arthritis is becoming less clear (16). For example, inflammation is a key finding in OA, due to the production of catabolic cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα), and immune-mediated inflammation due to macrophage, T cell, and B cell invasion into OA synovial tissue (14).

Clinical Features and Current Treatment Strategies for OA

Clinical features of OA are similar across species and include pain, stiffness, decreased range of motion, and synovial effusion. Radiographic findings include osteophytosis, enthesiophytosis, joint space narrowing, subchondral bone sclerosis and subchondral bone cyst formation. There are no treatments that markedly alter the progression of OA, and most therapies have focused on symptom-modifying treatments, including analgesics and physical therapy, with end-stage disease typically resulting in arthrodesis or joint replacement. Therefore, identifying and understanding novel potential strategies for arthritis prevention and treatment, focusing on both mechanical and biological features, will be of immense importance to the field of arthritis research.

Lubricin

Discovery of Lubricin

Lubricin, also referred to as superficial zone protein (SZP), proteoglycan 4 and megakaryocyte stimulating factor (MSF), is encoded by the proteoglycan 4 (PRG4) gene **(Figure 1.2)**. In humans, lubricin is an approximately 227kD core protein, consisting of a repeating degenerate sequence of EPAPTTK residues that comprise the mucin domain and undergo extensive glycosylation (30-50% w/w basis) with *O*-linked oligosaccharide chains (18, 19). The entire central mucin-rich domain of lubricin is encoded by a single exon, exon 6 **(Figure 1.2)**. The carboxy-terminus of lubricin, containing the hemopexin domain, is involved in binding to articular cartilage (20), most likely to type II collagen (21, 22), hyaluronan (22, 23), fibronectin or other extracellular matrix components. Effective boundary lubrication by lubricin is dependent upon both its ability to adsorb to articular cartilage (24) and the presence of *O*-linked β(1-3)Gal-GalNAc oligosaccharides (25). In addition to boundary lubrication, lubricin has also been demonstrated to possess both chondroprotective (26, 27) and anti-adhesive properties, decreasing synovial overgrowth and pannus formation on articular cartilage (28).

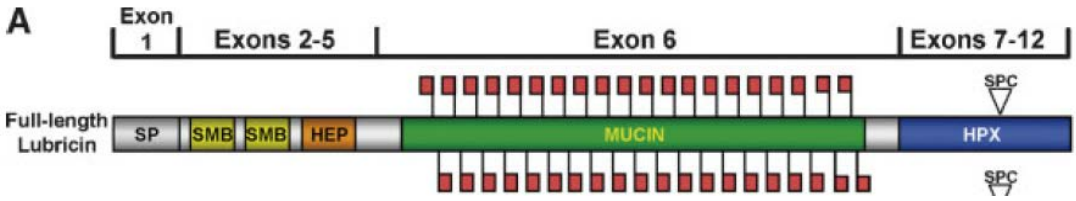


Figure 1.2. Structure of lubricin. (A) Schematic representation of full-length lubricin. Domains indicated are: SP, signal peptide; SMB, somatomedin B-like; HEP, heparin-binding; MUCIN, mucin-like; HPX, hemopexin-like. Lubricating/cytoprotective (NeuAc)Gal-GalNAc oligosaccharides are represented by red flags. Reprinted from the Journal of Orthopaedic Research, Vol. 25, Jones ARC, Gleghorn JP, Hughes CE, *et al.*, Binding and Localization of Recombinant Lubricin to Articular Cartilage Surfaces, pp. 283-292, © 2007, with permission from John Wiley & Sons, Inc.

Species Similarities and Tissue Distribution of Lubricin

There is substantial sequence similarity across human, bovine and equine lubricin orthologues, especially in the highly conserved mucin-rich domain, which is rich in serine and threonine residues and reacts with the peanut agglutinin (PNA) lectin. The number of EPAPTTK repeats appears to scale with the size of the animal, with the *Prg4* PCR product increasing in size from mouse to rat to monkey to human (29). Lubricin was first purified from bovine synovial fluid more than three decades ago (30). Since that time, lubricin or alternative splice variants of the *Prg4* gene have been identified in diverse tissues, including kidney, lung, liver, heart, brain, muscle, small intestine, bone, and cartilage (29). Tissue-specific expression of lubricin splice variants has led authors to propose that lubricin possesses alternative functions besides boundary lubrication (31). Lubricin has been detected in a variety of musculoskeletal tissues, including articular cartilage, synovium, meniscus (32), cruciate ligaments (33), intervertebral disc (34) and tendon (31, 35), where it likely functions as a boundary lubricant. Recently, lubricin has also been characterized as an ocular surface boundary lubricant (36, 37).

Functions of Lubricin

Lubrication. Lubricin is essential to synovial boundary lubrication, but exactly how it is oriented on the articular cartilage surface or what molecular partners it is interacting with are not fully understood (21). Hyaluronic acid (HA), lubricin and

surface-active phospholipids are all synovial fluid components thought to be important in mediating boundary lubrication. Lubricin may be interacting with HA at the articular cartilage surface, synergistically enhancing lubrication (23, 38–40); however, in some testing regimes, no synergism between lubricin and HA has been documented (41). Comparison of HA, lubricin and phospholipids in healthy donors vs. patients with early-stage OA, late-stage OA and RA revealed positive correlations between concentrations of lubricin and HA, lubricin levels and HA molecular weight (MW) distribution, and concentrations of HA and phospholipids, supporting a model involving close interactions between these molecules (42).

Chondroprotection. Although most of the lubricin literature has focused on its biophysical role as a boundary lubricant in synovial fluid, there is also evidence that lubricin protects cartilage through mechanisms distinct from is lubricating activity. Indeed, lubricin may protect cartilage and prevent chondrocyte apoptosis through its function as a boundary lubricant (27). However, the presence of tissue-specific lubricin isoforms in other tissues, including lung, liver, heart and bone suggest that lubricin has other potential biological activities besides lubrication (31). Homologues of lubricin stimulate megakaryocyte growth and platelet maturation in bone (43), in addition to hematopoietic and endothelial cell proliferation (44). Overexpression of *Prg4* in a mouse model decreased activity of transcription factors that mediate chondrocyte hypertrophy and terminal differentiation and increased activity of hypoxia-inducible factor 1 α *(Hif-1α)*, suggesting that influencing

chondrocyte transcriptional networks may be another mechanism whereby *Prg4* may mediate its protective effects in OA (26).

Anti-adhesion. Lubricin is anti-adhesive, limiting adhesion of synoviocytes and other adhesion-dependent cell lines *in vitro*, with subsequent anti-proliferative effects (28). This anti-adhesive property of lubricin is thought to prevent the synovial cell hyperproliferation and pannus formation seen in *Prg4-/-* mice (45). Although the anti-adhesive properties of lubricin appear to be beneficial in protecting against the development of OA, anti-adhesion may also be associated with some undesirable effects. For example, use of a carbodiimide derivatized HA lubricin construct in canine flexor tendon reconstruction eliminated adhesions and improved functional repair; however, the construct appeared to adversely affect tendon healing (46). Lubricin gene expression was upregulated 20- to 300-fold in hip prostheses affected by aseptic loosening (47), and lubricin has been localized to pseudomembranes around loose hip and knee prostheses (48), suggesting that lubricin may contribute to orthopaedic implant loosening.

Lubricin in OA

Considerable evidence supports the hypothesis that lubricin is critical to maintaining joint health and preventing wear-associated cartilage damage and ensuing arthritis. Human patients with an autosomal recessive genetic disorder affecting the PRG4 gene, called camptodactyly-arthropathy-coxa vara-pericarditis (CACP)

syndrome, develop juvenile-onset precocious joint failure affecting multiple joints (49). Several different mutations have been described in the PRG4 gene, but most are nonsense mutations, frameshift deletions, or splicing defects that lead to premature termination and complete lack of lubricin synthesis, which leads to CACP syndrome (50). Interestingly, 14 out of 20 of the mutations described in PRG4 occur in exon 6, the exon that codes for the entire central, mucin-rich region of the gene (50). Likewise, *Prg4-/-* mice develop many of the same features of the syndrome, including joint contracture, synovial hyperproliferation and pannus formation, and early-onset polyarthropathy (28, 45, 51). Additionally, synovial fluid from lubricin null mice fails to reduce friction in boundary mode lubrication regimes (52).

Lubricin synovial fluid concentrations are decreased in experimental rodent models of PTOA, including the ACL-deficient guinea pig stifle (53) and the ACL-deficient rat stifle (54). Lubricin mRNA expression is decreased and coefficients of friction (COF) are increased in an antigen-induced arthritis (AIA) rat model, suggesting that lubricin may also contribute to wear and precocious joint failure in inflammatory arthritis (55). Furthermore, replenishing lubricin, either through gene therapy (26) or administration of recombinant lubricin (56–59) delays the development and progression of arthritis in rodent PTOA models. Notably, lubricin was more effective than HA in improving radiographic and histological OA scores in the ACL-deficient rat knee (59), and lubricin supplementation also attenuated OA in ovariectomized rats (60).

Although lubricin gene and/or glycoprotein expression is decreased in several models of PTOA in rodents, the picture is less clear for clinically relevant, large animal models of OA. *Prg4* gene expression was downregulated in a sheep meniscectomy model 3 months post-operatively (61), whereas lubricin synovial fluid (SF) concentrations increased 2-4 weeks post-ACL transection in a separate ovine study (62). Despite the increased lubricin concentrations, boundary lubrication was significantly impaired in synovial fluid obtained from ACL-deficient ovine knees 2-4 weeks post-injury, possibly due to diminished concentrations of high MW HA (62). Lubricin synovial fluid concentrations were increased in a small cohort of horses undergoing carpal or fetlock arthroscopy for acute injury, and COF were elevated in these synovial fluid samples despite the elevations in lubricin concentrations (63). Similarly, in human synovial fluid obtained from patients with acute tibial plateau fractures, lubricin synovial fluid concentrations increased 2-fold (64). Another study demonstrated increased lubricin synovial fluid concentrations and cartilage immunostaining in human patients with advanced OA, indicating that lubricin may be ineffective at lubricating joints with advanced disease (65). However, in human patients presenting to the emergency room due to ACL injury, lubricin synovial fluid concentrations were decreased, in conjunction with elevations in pro-inflammatory cytokines (66).

Lubricin Therapy

There is considerable interest in the use of lubricin supplementation as a potential therapeutic for OA, and data in rodent models strongly supports this course of action. It is possible that *Prg4* expression and lubricin synovial fluid concentration are elevated in acute injuries to protect the joint from OA, but that lubricin alone is not sufficient to restore boundary lubrication in acute instability or fragmentation models. However, it is critical to understand how lubricin and other components of synovial fluid are altered in clinically relevant, large animal models of OA and to determine whether lubricin replacement therapy is effective in delaying the severity and progression of OA in these models before this therapy can be translated to humans.

Glycobiology of Lubricin

Protein Glycosylation and Function

Protein glycosylation, or the synthesis of sugars on a protein backbone, occurs post-translationally, primarily in the endoplasmic reticulum and Golgi apparatus of the cell. Glycosylation departs from the general theme of synthesis for protein, DNA, and RNA in that it is not governed by a pre-determined template. This means that glycosylation is highly complex, heterogeneous and difficult to decipher, but also more flexible in that sugar synthesis is able to respond to local environmental conditions, such as temperature, pH, and substrate availability.

O-linked Glycans in Boundary Lubrication

Extensive *O*-linked glycosylation within lubricin's mucin-rich domain is critical for its boundary lubricating function. The central, mucin-rich region of lubricin encoded by exon 6 is rich in serine and threonine, and lubricin is approximately 50% (w/w) glycosylated with O-linked β-(1-3)-Gal-GalNAc residues (30, 67, 68). These anionic sugars play an important role in boundary layer lubrication, with the penultimate galactose of utmost importance (69).

Glycobiology is Altered in OA

Changes in glycosylation are a hallmark of certain disease states, such as cancer and chronic inflammation (70). Altered glycosylation is present in diseases ranging from cancer (71) to diabetes (72) to colitis (73). Strong correlations between serum protein glycosylation and RA have been established (74), and serum levels of total and free sialic acids correlate with both RA and systemic sclerosis (SSc) (75). Advanced glycation end products (AGEs), formed *in vivo* by non-enzymatic reactions between proteins and carbohydrates (the Malliard reaction), accumulate with age and in metabolic conditions characterized by hyperglycemia (76), and AGEs are thought to contribute to the pathogenesis of both OA (77) and RA (78). Given the importance of lubricin's *O*-linked β(1-3)Gal-GalNAc oligosaccharides (25) in mediating cartilage lubrication, inflammation-induced changes in the glycosylation of lubricin could potentially have a major impact on boundary lubricating ability (79).

Lubricin Glycosylation in Arthritis

Significant variations in the concentration of sialic acid residues on lubricin have been measured in clinical samples (80), with increased sialylation of lubricin in synovial fluid samples from patients with RA as compared to OA (79) and increased sialylation and fucosylation of synovial fluid fibronectin in patients with established RA as opposed to acute RA (81). Additionally, increases in sulfated core 2 O-glycans have been detected in RA patients (82), leading authors to propose that altered glycosylation may facilitate inflammation through immune cell adhesion molecules such as selectins or sialyl Lewis x epitopes. Altered glycosylation of proteins, especially increased glycan branching and sialylation, have been correlated with diverse inflammatory disease states (83), including arthritis and other rheumatic diseases (74, 75, 84, 85), Crohn's disease (83, 86) and cancer (87). Recent *O*-linked glycomapping of human synovial fluid lubricin from RA and OA patients revealed 168 O-linked glycan sites (primarily core 1 and core 1 sialylated structures), of which the majority were mono- rather than disialylated (19). It is still unknown; however, how the glycosylation status of lubricin from clinically normal patients compares with lubricin from OA or RA patients.

Limitations of Current Lubricin Glycan Analysis

Limitations of lubricin glycosylation analysis to date include those issues related to the interpretation of mass spectrometry and the availability of suitable

samples. Mass spectrometry of carbohydrates is rapidly advancing; however, a limitation of LC-MS to identify glycan structures, as it has been employed in previous studies, is that nonsialylated β(1-3)Gal-GalNAc is not retained by the graphitized carbon column of the LC-MS (79). Samples of synovial fluid from normal, healthy human patients are difficult to access. Therefore, the majority of the studies that have been performed have evaluated synovial fluid lubricin from a small number of patients with either OA or RA, and lack healthy, control lubricin for comparison.

Cartilage Lubrication

Cartilage Biomechanics

Articular cartilage is repeatedly subjected to both compressive and shear forces. As discussed above, the anatomical structure of cartilage is designed to resist these forces, with the deep zone of cartilage being stiffer with a greater modulus and lower permeability than the superficial zone, which has a higher hydraulic permeability and functions to mitigate shear stresses. The biphasic structure of cartilage, composed of a solid matrix phase and an interstitial fluid phase, accounts for the ability of cartilage to respond to biomechanical forces by dissipating more than 90% of the load to pressurized fluid, thereby minimizing stresses on the solid phase (88). This fluid phase is thought to be important in mediating cartilage lubrication.

Modes of Lubrication and the Stribeck Curve

In order to demonstrate how friction varies with sliding speed as a separating hydrodynamic or elastohydrodynamic film is developed, a graph referred to as a 'Stribeck curve' is typically employed (89). A 'Stribeck curve' describes friction as a function of the viscosity of the lubricant and the entraining velocity, divided by contact pressure (90) **(Figure 1.3)**. Friction is the force resisting the relative motion of solid surfaces or fluid layers sliding against each other, and the coefficient of friction is a dimensionless term, describing the ratio of the shear force relative to the normal force. Healthy articular cartilage has an extremely low coefficient of friction, ranging from 0.001 to 0.03 (91).

Fluid-film lubrication involves separation of the two interfaces by a viscous fluid that prevents contact of surface asperities. In hydrodynamic lubrication, which occurs at higher velocities, fluid film forces are generated by the sliding speed of the articulating surfaces. Under conditions not conducive for fluid film lubrication, including slow sliding speeds, high loads and low viscosity, opposing surfaces are separated by a molecular film or boundary lubrication (92). At conditions intermediate between these extremes, mixed lubrication occurs, which possesses features of both fluid film and boundary lubrication.

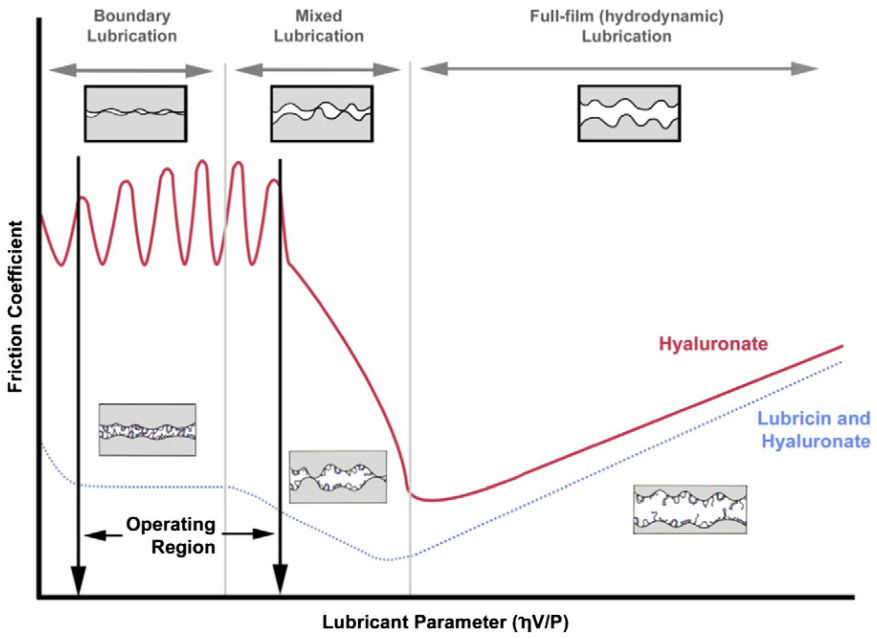


Figure 1.3. Stribeck curve in synovial tribology. Boundary lubrication occurs in the presence of lubricin at very low sliding speeds when the lubricating film is the same thickness as the surface roughness manifested by flattened high points (asperities) on the apposing cartilage surfaces. In the absence of lubricin, adhesion occurs and friction is higher producing a stick-slip phenomenon. At higher entraining speeds, in the hydrodynamic regime, the intervening fluid adjoining the surface will move at the same speed as the joint surface during locomotion. The two cartilage surfaces move relative to each other and will drag fluid into the interface. Synovial fluid (SF) that enters a converging gap in this manner will see a pressure increase as the gap converges, which creates hydrodynamic lift, and forces the surfaces apart like a wedge. Mixed lubrication occurs between boundary and hydrodynamic lubrication, as the name would suggest. The fluid film thickness is slightly greater than the surface roughness, so that there is very little asperity contact. The friction coefficient is the ratio of sliding to normal force, and the lubrication parameter is the product of SF viscosity (η) and entraining velocity (v), divided by the contact pressure (P). Reprinted from Matrix Biology, Vol. 39, Jay GD and Waller KA, The biology of Lubricin: Near frictionless joint motion, pp. 17-24, © 2014, with permission from Elsevier.

Cartilage Lubrication Theories and Boundary Lubrication

A variety of theories have been proposed to describe the lubrication mechanism of articular cartilage (93). Within the joint, a complex combination of mechanical, physical, chemical and biological processes is taking place, and no single lubrication theory is completely able to describe frictional behavior in biological joints. Although data argues against hydrodynamic or full fluid film modes of lubrication in cartilage (94), the exact lubrication mechanism is not fully clear. Synovial joints most likely operate under a mixed lubrication regime, where cartilage is subjected to both fluid film and boundary lubrication. Boundary lubrication, also called boundary film lubrication, can be defined as: 1) a condition of lubrication in which friction is controlled by a chemical or physical interaction between rubbing surfaces that results in a surface film with physical properties different from both the rubbing surfaces and the bulk lubricant (89) and 2) a condition of lubrication in which the load is carried by the surface asperities rather than the lubricant. A critical feature of boundary lubrication is the adsorption of the lubricant to the articular cartilage surfaces.

There are several theories regarding the predominant molecular species involved in boundary lubrication in synovial joints, and many authors suggest that a supramolecular complex or interactions of several molecular species are important in mediating articular cartilage lubrication (42). Lubricin/SZP/PRG4, HA, and surface active phospholipids (SAPL) are all proposed to play a major role in boundary

lubrication, but the extent to which each species mediates lubrication and how each species does so is still contested. For example, following hyaluronidase digestion, synovial fluid still lubricates nearly as well as native synovial fluid (95) and HA alone does not possess boundary lubricating abilities *in vitro* (58, 69). Some authors propose that bulky HA molecules mechanically trap lubricin at the surface of articular cartilage, thereby facilitating lubrication (23). Others suggest that lubricin's only role is to adsorb to cartilage and trap SAPL, and that it is this phospholipid bilayer that mediates lubrication (96, 97). Evidence in support of lubricin as the primary boundary lubricant in synovial fluid has been mentioned above. Boundary lubrication corresponds to the regime of loading at low sliding speeds, where friction is relatively high. Because the fluid film is thin or negligible in this regime and cartilage asperities are in direct contact with one another, this is the lubrication regime where cartilage is thought to be most susceptible to wear (52, 98). For this reason, there is considerable interest in understanding and recapitulating effective boundary lubrication for the prevention and treatment of OA.

Galectins

General Overview of Galectins

Galectins comprise a family of more than 15 β-galactoside binding proteins, and abundant evidence suggests that galectins are master regulators of immune cell homeostasis (99, 100). Galectins share significant sequence similarity in their

carbohydrate-recognition domains (CRDs) and, based upon this CRD organization, are divided into 3 subfamilies. "Proto-type" galectins contain only one CRD and include galectin-1, -2, -5, -7, -10, -11 and -13; "tandem-repeat type" galectins possess two CRDs connected by non-conserved amino acid sequences and include galectin-4, -6, -8, -9 and -12; and "chimeric type" galectins contain one CRD and one non-lectin N-terminal domain that gives the only member of this family, galectin-3, the ability to form multimers (101) **(Figure 1.4)**. Galectin-3 is unique in that it is functionally pentavalent, capable of aggregating immune cells and cross-linking cells or matrix components. Galectins function distinctly through intracellular and extracellular pathways, with intracellular signaling resulting from N-terminal protein-protein interactions and extracellular functions arising primarily from carbohydrate-dependent interactions on the cell surface or within extracellular matrices (99).

Galectins in Inflammation and Chronic Disease States

Galectin-3 is prototypically associated with amplification of the inflammatory cascade, whereas galectin-1 exhibits predominantly anti-inflammatory and immunosuppressive effects (99); however, these effects are cell and context-dependent (102). While galectin-1 modulates T cell function and apoptosis, ameliorating autoimmune diseases such as graft vs. host disease (103), lupus (104), and autoimmune diabetes (105) in experimental models, galectin-1 also leads to cancer evasion, tumor escape, and metastasis (106). Galectin-3 is a central regulator of chronic inflammation and tissue fibrosis, with elevations in galectin-3 found in

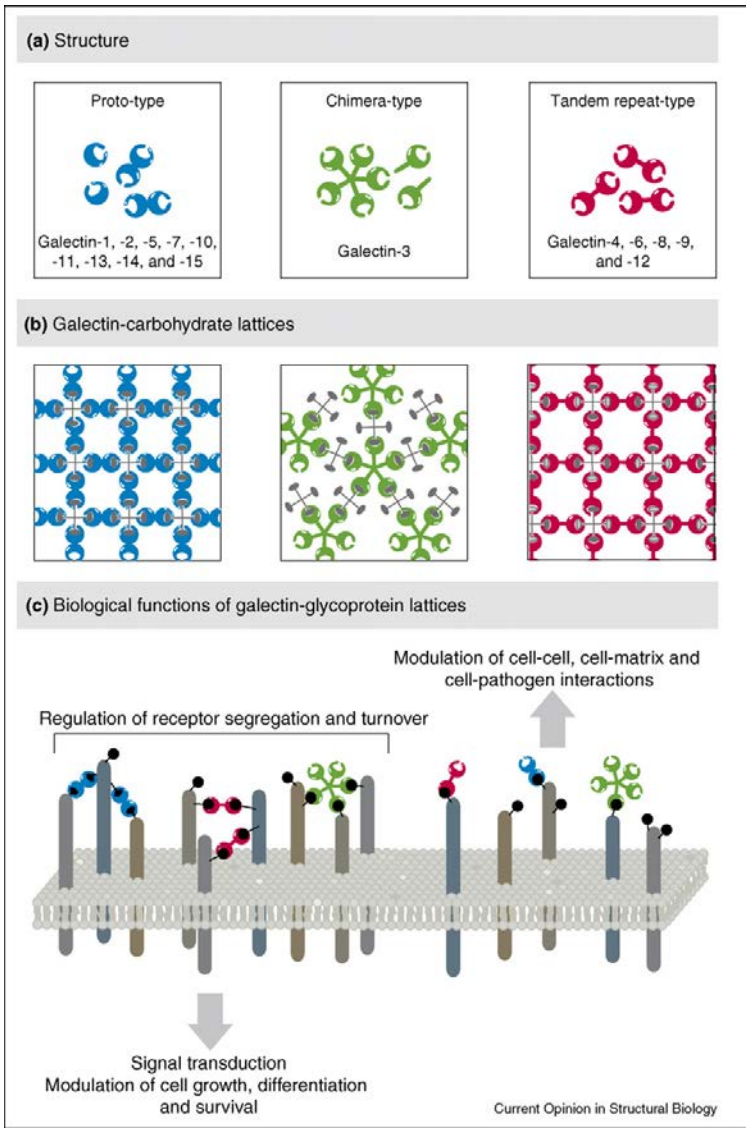


Figure 1.4. Biochemistry and functional relevance of galectin-glycoprotein lattices. **(a)** Schematic representation of the structure of different monomeric and oligomeric members of the galectin family. Proto-type galectins contain one CRD and exist in solution as homodimers. Chimera-type galectins are thought to undergo a conformational change following carbohydrate ligand binding, which enables their oligomerization as pentamers. Tandem-repeat type galectins contain two distinct CRDs in tandem, connected by a linker of up to 70 amino acids, and are thus inherently dimeric. **(b)** Schematic representation of lattice formation between multivalent galectins and multivalent carbohydrate ligands. **(c)** Biological relevance of galectin-glycoprotein lattices. Reprinted from Current Opinion in Structural Biology, Vol. 17, Rabinovich GA, Toscano MA, Jackson SS and Vasta GR, Functions of cell surface galectin-glycoprotein lattices, pp. 513-520, © 2007, with permission from Elsevier.

hepatic, renal and pulmonary fibrosis, in addition to atherosclerosis (107). Conversely, galectin-3 plays a protective role in the development of type 2 diabetes and related metabolic disorders (108), dampens endotoxin-mediated inflammatory responses (109), and promotes wound healing (110, 111).

Galectins in Arthritis

Galectins have been implicated in multiple arthritic conditions, with decreased galectin-1 and/or increased galectin-3 expression found in RA (112, 113), juvenile idiopathic arthritis (JIA) (114) and OA (115). However, the exact role of galectins and how they contribute to the pathogenesis of disease is not fully understood. Galectin-3 expression is increased in OA chondrocytes (116), and increased galectin-3 staining has been demonstrated at sites of synovial and cartilage invasion by immune cells in both RA and during inflammatory phases of OA (113), leading authors to hypothesize that galectin-3 is both involved in inflammation and a novel marker of disease activity in RA. Mechanistically, most efforts have focused on intracellular roles of galectins in mouse galectin knockout models or in models of experimental immune-mediated arthritis.

Galectin-1 in Arthritis

Galectin-1 expression is downregulated in the synovial fluid of patients with RA and JIA (112, 114, 117). Galectin-1 knockout mice are at increased risk of

developing collagen induced arthritis (CIA) (118), and recombinant galectin-1 or gene-delivered galectin-1 suppressed CIA by enhancing T cell apoptosis and inhibiting IL-2 secretion (119). Galectin-1-nanogold therapy (120) and lentiviral-mediated delivery of both galectin-3 shRNA and galectin-1 ameliorated CIA in rats (121). Thus, the majority of evidence supports the hypothesis that galectin-1 plays a protective role in immune-mediated arthritis. On the other hand, galectin-1 immunostaining is increased, especially within areas of severe degeneration (Mankin score ≥ 9), in human OA cartilage samples (122).

Galectin-3 in Arthritis

Elevations in galectin-3 have been associated with arthritides, as both serum and synovial fluid concentrations of galectin-3 are increased in RA as compared to OA (113), and also in OA as compared to healthy controls (115). Galectin-3 is also increased in JIA (114) and correlates with disease activity, severity and progression (123). Furthermore, a mutation in the LGALS3 gene, which codes for galectin-3, predisposes to the development of RA (124). Proteomic analysis of synoviocyte lesions microdissected from RA synovial tissues revealed elevated galectin-3 levels as compared to OA synovial tissues (125).

Galectin-3 gene expression was upregulated in peripheral blood mononuclear cells (PBMCs) from rats with CIA (126), and combined IA lentiviral-mediated delivery of galectin-3 shRNA and galectin-1 ameliorated collagen induced arthritis in

rats (121). Antigen induced arthritis-associated joint inflammation and bone erosion was reduced in galectin-3 knockout mice, and exogenous administration of recombinant galectin-3 partially restored the arthritic phenotype and inflammatory cytokine expression profile (127). The role of galectin-3 has been studied in immune-mediated arthritis, but data about how galectin-3 is altered in OA is lacking. One group suggests that intracellular galectin-3 is chondroprotective (116, 128), whereas extracellular galectin-3 has deleterious roles in synovial joints (129). Galectin-3 is highly expressed intracellularly in hypertrophic chondrocytes, whereas studies suggest that OA chondrocytes express higher levels of cell surface galectin-3 (116). Proposed roles for galectin-3 in synovial joints include functions as a receptor for advanced glycation end products (AGEs), as an inhibitor of apoptosis in chondrocytes, and as a chemoattractant for leukocytes (130).

Other Galectins in Arthritis

Several galectins may play a role in RA, including galectins-1, -2, -3 , -8 and -9 (131). Galectin-8 is produced by human RA synovial fibroblasts (132), and autoantibodies against galectin-8 and other galectins have been detected in the sera of RA patients (133). Moreover, a galectin-8 gene variant has recently been associated with RA (134). Galectin-9 suppressed CIA by regulating T cell immune responses (135, 136) and regulating Fc gamma R expression on macrophages (137). Galectin-9-induced apoptosis of hyperproliferative RA fibroblast-like synoviocytes (FLS) has also been proposed as a mechanism to suppress RA (136).

Galectins and Adhesion, Chemotaxis and Motility

Galectins are master regulators of the immune response; however, galectins also modulate fundamental cellular processes such as adhesion, spreading, chemotaxis and motility (138). For example, galectin-3 regulates integrin-mediated adhesion to collagen-I and IV (139). Galectin-1 promotes human neutrophil migration (140), and both galectin-1 and galectin-3 are chemoattractive to monocytes (141, 142). In addition, galectin-3 promotes keratinocyte migration during re-epithelialization of corneal, intestinal and skin wounds (110, 111), and galectin-3-laminin binding results in β_4-EGFR crosslinking and ERK activation in keratinocytes, resulting in adhesion and motility (143).

Mesenchymal Stem Cells in Osteoarthritis Therapy

Definition of MSC

Mesenchymal stem cells (MSCs), also referred to as mesenchymal stromal cells, are plastic-adherent, non-hematopoietic, multipotent progenitor cells capable of differentiating into mesenchymal lineages, including cartilage, bone, adipose and muscle (144).

MSC Immunomodulatory Properties and Secretome

Mesenchymal stem cells (MSCs) were initially appealing as a cell source for the repair of articular cartilage and other musculoskeletal injuries due to their multi-lineage potential (145); however, low rates of long-term survival, engraftment and differentiation in musculoskeletal tissues (146, 147) diminished some of this initial appeal. More recently, the paradigm for the use of MSCs in musculoskeletal tissue injuries has shifted from the expectation that MSCs will contribute directly towards tissue repair to the assumption that MSCs will provide a microenvironment conducive to tissue repair through expression of trophic and immunomodulatory factors (148, 149). MSCs have been used intra-articularly to decrease joint inflammation and promote cartilage healing in both experimental animal models of OA (147, 150, 151) and in human clinical trials (152, 153); however, the mechanisms by which MSCs perform these actions are not fully understood.

The regenerative potential of MSC therapy appears to be due, at least in part, to paracrine functions of MSCs. The repertoire of trophic and immunomodulatory factors produced by MSCs has been coined the MSC secretome, and there has been much interest in recent years in harnessing this secretome to promote tissue repair and inhibit inflammation. Lipopolysaccharide (LPS), tumor necrosis factor-α (TNFα) and other pro-inflammatory cytokines and damage-associated molecular patterns (DAMPs) from injured tissues and macrophages are thought to activate MSCs to secrete anti-inflammatory molecules, such as PGE_2, IL-10 and several others (148).

MSCs and Galectins

Human MSCs constitutively express galectins, especially galectins-1 and -3, at both gene (154) and protein levels (155, 156), and galectin-1 has been detected in MSC-derived microvesicles (157). Evidence is accumulating that both galectin-1 (158–160) and galectin-3 play a significant role in the immunomodulatory properties of MSCs (154, 155, 161). For example, siRNA-mediated knockdown of both gal-1 and gal-3 abrogated MSC immunosuppressive properties (155), and addition of exogenous galectin-3 restored immunomodulatory properties of MSCs in *in vitro* mixed lymphocyte cultures (154).

Inhibition of T cell proliferation appears to be mediated via different mechanisms in equine MSCs, depending upon whether cells are derived from bone marrow, adipose tissue, cord blood or umbilical cord tissues (162). PGE_2 inhibition reversed the immunomodulatory effects of equine MSCs on T cells *in vitro* by restoring secretion of TNFα and IFN-ϒ, suggesting that PGE_2 is a critical factor in the equine MSC secretome (162). The role of galectins in equine MSCs has not been studied.

MSCs in Musculoskeletal Tissue Repair

Cell based therapy for OA and focal cartilage defects has been employed for nearly two decades. However, few randomized controlled clinical trials have

demonstrated meaningful clinical benefit for the application of MSCs in rheumatic diseases, including systemic lupus erythematosus, RA and OA (153). In the one published randomized, controlled study investigating the use of expanded, bone marrow-derived MSCs in patients with knee OA, the MSC treated group had superior MRI scores at 12 months and superior clinical scores at 2 years (152).

Although there is still promise for the application of MSCs in the treatment of OA, future efforts should be directed at understanding some of the fundamental processes that govern MSC biology and fate in the synovial joint. By understanding how MSCs home to damage tissues, adhere to damaged cartilage or synovium, migrate to sites of tissue injury and respond to inflammation, we may be able to manipulate the cells or environment to maximize the survival, engraftment and secretory activities of these cells.

Conclusion

OA is a debilitating degenerative joint disease, often precipitated by articular trauma, which leads to inflammation, chondrocyte loss and matrix degradation. Lubricin appears to be critical for maintaining normal joint homeostasis and protecting articular cartilage, conferring these benefits through a combination of boundary lubrication, chondroprotection and anti-adhesive properties. However, little is known about the disposition of lubricin in clinically relevant large animal models, such as the

horse, including how the glycoprotein is modified in disease and what other molecules lubricin is interacting with at the cartilage surface.

Galectins-1 and -3 are carbohydrate-binding proteins altered in rheumatic diseases, including OA, RA and JIA. Although both galectins appear to be increased in human OA cartilage, their functions in the synovial fluid milieu and articular cartilage matrix are not fully understood. While the general consensus is that galectin-1 plays a protective role in autoimmune and inflammatory arthritis, the verdict is still out about galectin-3. Correlation is not equivalent to causation, and the presence of increased galectin-3 concentrations in synovial fluid or increased galectin-3 immunostaining in OA cartilage does not mean that galectin-3 necessarily precedes the development of or promotes the progression of arthritis. On the contrary, galectin-3 may be elevated as a protective response during the development of arthritis.

There are currently no effective disease-modifying treatments for OA and; although MSC injections appear to provide some benefit, rates of cell survival and engraftment are low, and clinical gains have been modest. A more thorough understanding of fundamental MSC properties, including adhesion, spreading and motility are critical for improving success rates for cell-based gene therapy or direct cell injection approaches to treating OA. Furthermore, a mechanistic understanding of the role of lubricin and galectins in the pathogenesis of OA is warranted for the development of novel pharmacologic therapies for OA.

References

1. Bhosale AM, Richardson JB (2008) Articular cartilage: structure, injuries and review of management. *Br Med Bull* 87(1):77–95.

2. Buckwalter JA, Mankin H. J (1997) Instructional Course Lectures, The American Academy of Orthopaedic Surgeons - Articular Cartilage. Part I: Tissue Design and Chondrocyte-Matrix Interactions. *J Bone Jt Surg* 79(4):600–11.

3. Orford CR, Gardner DL (1985) Ultrastructural histochemistry of the surface lamina of normal articular cartilage. *Histochem J* 17(2):223–233.

4. Kumar P, et al. (2001) Role of uppermost superficial surface layer of articular cartilage in the lubrication mechanism of joints. *J Anat* 199(3):241–250.

5. Pearle AD, Warren RF, Rodeo S a (2005) Basic science of articular cartilage and osteoarthritis. *Clin Sports Med* 24(1):1–12.

6. Schumacher BL, Hughes CE, Kuettner KE, Caterson B, Aydelotte MB (1999) Immunodetection and partial cDNA sequence of the proteoglycan, superficial zone protein, synthesized by cells lining synovial joints. *J Orthop Res* 17(1):110–120.

7. Schumacher, et al. (1994) A novel proteoglycan synthesized and secreted by chondrocytes of the superficial zone of articular cartilage. *Arch Biochem Biophys* 311(1):144–152.

8. Goldring MB, Goldring SR (2010) Articular cartilage and subchondral bone in the pathogenesis of osteoarthritis. *Ann N Y Acad Sci* 1192:230–7.

9. Musumeci G, et al. (2015) Osteoarthritis in the XXIst Century: Risk Factors and Behaviours that Influence Disease Onset and Progression. *Int J Mol Sci* 16(3):6093–6112.

10. Zhuo Q, Yang W, Chen J, Wang Y (2012) Metabolic syndrome meets osteoarthritis. *Nat Rev Rheumatol* 8(12):729–37.

11. Katz JD, Agrawal S, Velasquez M (2010) Getting to the heart of the matter: osteoarthritis takes its place as part of the metabolic syndrome. *Curr Opin Rheumatol* 22(5):512–519.

12. Šalamon L, et al. (2015) Differences in the prevalence and characteristics of metabolic syndrome in rheumatoid arthritis and osteoarthritis: a multicentric study. *Rheumatol Int*. doi:10.1007/s00296-015-3307-0.

13. Velasquez MT, Katz JD (2010) Osteoarthritis: another component of metabolic syndrome? *Metab Syndr Relat Disord* 8(4):295–305.

14. Malemud CJ (2015) Biologic basis of osteoarthritis. *Curr Opin Rheumatol* 27(3):289–294.

15. Li G, et al. (2013) Subchondral bone in osteoarthritis: insight into risk factors and microstructural changes. *Arthritis Res Ther* 15(6):223.

16. Sokolove J, Lepus CM (2013) Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Ther Adv Musculoskelet Dis* 5(2):77–94.

17. American College of Rheumatology: Diseases and Conditions (2015) Available at: http://www.rheumatology.org/I-Am-A/Patient-Caregiver/Diseases-Conditions [Accessed August 9, 2015].

18. Swann DA, Sotman S, Dixon M, Brooks C (1977) The isolation and partial characterization of the major glycoprotein (LGP-I) from the articular lubricating fraction from bovine synovial fluid. *Biochem J* 161(3):473–85.

19. Ali L, et al. (2014) The O-glycomap of lubricin, a novel mucin responsible for joint lubrication, identified by site-specific glycopeptide analysis. *Mol Cell proteomics*. doi:10.1074/mcp.M114.040865.

20. Jones ARC, et al. (2007) Binding and Localization of Recombinant Lubricin to Articular Cartilage Surfaces. *J Orthop Res* 12(March):10–12.

21. Chang DP, Guilak F, Jay GD, Zauscher S (2014) Interaction of lubricin with

type II collagen surfaces: adsorption, friction, and normal forces. *J Biomech* 47(3):659–66.

22. Majd SE, et al. (2014) Both hyaluronan and collagen type II keep proteoglycan 4 (lubricin) at the cartilage surface in a condition that provides low friction during boundary lubrication. *Langmuir* 30(48):14566–72.

23. Greene GW, et al. (2011) Adaptive mechanically controlled lubrication mechanism found in articular joints. *Proc Natl Acad Sci U S A* 108(13):5255–9.

24. Gleghorn JP, Jones ARC, Flannery CR, Bonassar LJ (2009) Boundary mode lubrication of articular cartilage by recombinant human lubricin. *J Orthop Res* 27(6):771–7.

25. Jay GD, Harris D a, Cha CJ (2001) Boundary lubrication by lubricin is mediated by O-linked beta(1-3)Gal-GalNAc oligosaccharides. *Glycoconj J* 18(10):807–15.

26. Ruan MZC, et al. (2013) Proteoglycan 4 expression protects against the development of osteoarthritis. *Sci Transl Med* 5(176):176ra34.

27. Waller K a, et al. (2013) Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. *Proc Natl Acad Sci U S A* 110(15):5852–7.

28. Rhee DK, et al. (2005) The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest* 115(3):622–31.

29. Ikegawa S, Sano M (2000) Isolation, characterization and mapping of the mouse and human PRG4 (proteoglycan 4) genes. *Cytogenet Cell Genet* 90(3-4):291–297.

30. Swann DA, Slayter HS, Silver FH (1981) The molecular structure of lubricating glycoprotein-I, the boundary lubricant for articular cartilage. *J Biol Chem* 256(11):5921–5.

31. Sun Y, et al. (2006) Mapping lubricin in canine musculoskeletal tissues. *Connect Tissue Res* 47(4):215–221.

32. Schumacher BL, Schmidt TA, Voegtline MS, Chen AC, Sah RL (2005) Proteoglycan 4 (PRG4) synthesis and immunolocalization in bovine meniscus. *J Orthop Res* 23(3):562–8.

33. Lee SY, Niikura T, Reddi a H (2008) Superficial zone protein (lubricin) in the different tissue compartments of the knee joint: modulation by transforming growth factor beta 1 and interleukin-1 beta. *Tissue Eng Part A* 14(11):1799–808.

34. Shine KM, Spector M (2008) The presence and distribution of lubricin in the caprine intervertebral disc. *J Orthop Res* 26(10):1398–1406.

35. Funakoshi T (2008) Lubricin Distribution in the Goat Infraspinatus Tendon: A Basis for Interfascicular Lubrication. *J Bone Jt Surg* 90(4):803.

36. Samsom ML, et al. (2014) Characterization of full-length recombinant human Proteoglycan 4 as an ocular surface boundary lubricant. *Exp Eye Res* 127C:14–19.

37. Schmidt T a, et al. (2013) Transcription, translation, and function of lubricin, a boundary lubricant, at the ocular surface. *JAMA Ophthalmol* 131(6):766–76.

38. Das S, et al. (2013) Synergistic interactions between grafted hyaluronic acid and lubricin provide enhanced wear protection and lubrication. *Biomacromolecules* 14(5):1669–1677.

39. Schmidt T a, Gastelum NS, Nguyen QT, Schumacher BL, Sah RL (2007) Boundary lubrication of articular cartilage: role of synovial fluid constituents. *Arthritis Rheum* 56(3):882–91.

40. Lee DW, et al. (2014) Effects of molecular weight of grafted hyaluronic acid on wear initiation. *Acta Biomater* 10(5):1817–1823.

41. Chang DP, et al. (2009) Friction Force Microscopy of Lubricin and Hyaluronic Acid between Hydrophobic and Hydrophilic Surfaces. *Soft Matter* 5(18):3438–3445.

42. Kosinska MK, et al. (2015) Articular Joint Lubricants during Osteoarthritis and Rheumatoid Arthritis Display Altered Levels and Molecular Species. *PLoS One* 10(5):e0125192.

43. Merberg DM, et al. (1993) Comparison of vitronectin and megakaryocyte stimulating factor. *Biology of Vitronectins and Their Receptors*, eds Preissner KT, Rosenblatt C, Kost J, Wegerhoff J, Mosher DF (Elsevier Science, Amsterdam), pp 49–52.

44. Liu YJ, et al. (2004) Hemangiopoietin, a novel human growth factor for the primitive cells of both hematopoietic and endothelial cell lineages. *Blood* 103(12):4449–56.

45. Rhee DK, et al. (2005) Consequences of disease-causing mutations on lubricin protein synthesis, secretion, and post-translational processing. *J Biol Chem* 280(35):31325–32.

46. Zhao C, et al. (2013) Resurfacing with chemically modified hyaluronic acid and lubricin for flexor tendon reconstruction. *J Orthop Res*:1–7.

47. Morawietz L, et al. (2003) Differential gene expression in the periprosthetic membrane: lubricin as a new possible pathogenetic factor in prosthesis loosening. *Virchows Arch* 443(1):57–66.

48. Cheriyan T, et al. (2013) Lubricin and smooth muscle α-actin-containing myofibroblasts in the pseudomembranes around loose hip and knee prostheses. *Acta Biomater* 9(3):5751–8.

49. Marcelino J, et al. (1999) CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet* 23(3):319–322.

50. Ciullini Mannurita S, et al. (2014) CACP syndrome: identification of five novel mutations and of the first case of UPD in the largest European cohort. *Eur J Hum Genet* 22(2):197–201.

51. Coles JM, et al. (2010) Loss of cartilage structure, stiffness, and frictional properties in mice lacking PRG4. *Arthritis Rheum* 62(6):1666–74.

52. Jay GD, et al. (2007) Association between friction and wear in diarthrodial joints lacking lubricin. *Arthritis Rheum* 56(11):3662–9.

53. Teeple E, et al. (2008) Coefficients of Friction, Lubricin, and Cartilage Damage in the Anterior Cruciate Ligament-Deficient Guinea Pig Knee. 26(2):231–237.

54. Elsaid KA, Machan JT, Waller K, Fleming BC, Jay GD (2009) The impact of anterior cruciate ligament injury on lubricin metabolism and the effect of inhibiting tumor necrosis factor alpha on chondroprotection in an animal model. *Arthritis Rheum* 60(10):2997–3006.

55. Elsaid KA, Jay GD, Chichester CO (2007) Reduced expression and proteolytic susceptibility of lubricin/superficial zone protein may explain early elevation in the coefficient of friction in the joints of rats with antigen-induced arthritis. *Arthritis Rheum* 56(1):108–16.

56. Flannery CR, et al. (2009) Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin. *Arthritis Rheum* 60(3):840–7.

57. Jay GD, et al. (2010) Prevention of Cartilage Degeneration and Restoration of Chondroprotection by Lubricin Tribosupplementation in the Rat Following ACL Transection. *Arthritis Rheum* 62(8):2382–2391.

58. Jay GD, et al. (2012) Prevention of cartilage degeneration and gait asymmetry by lubricin tribosupplementation in the rat following anterior cruciate ligament transection. *Arthritis Rheum* 64(4):1162–71.

59. Teeple E, et al. (2011) The Effects of Supplemental Intra-Articular Lubricin and Hyaluronic Acid on the Progression of Post-Traumatic Arthritis in the Anterior Cruciate Ligament Deficient Rat Knee. *Am J Sports Med* 39(1):164–172.

60. Cui Z, Xu C, Li X, Song J, Yu B (2015) Treatment with recombinant lubricin attenuates osteoarthritis by positive feedback loop between articular cartilage and subchondral bone in ovariectomized rats. *Bone* 74:37–47.

61. Young AA, et al. (2006) Proteoglycan 4 downregulation in a sheep meniscectomy model of early osteoarthritis. *Arthritis Res Ther* 8(2):R41.

62. Atarod M, Ludwig TE, Frank CB, Schmidt T a., Shrive NG (2015) Cartilage boundary lubrication of ovine synovial fluid following anterior cruciate ligament transection: a longitudinal study. *Osteoarthr Cartil* 23(4):640–647.

63. Antonacci JM, et al. (2012) Effects of equine joint injury on boundary lubrication of articular cartilage by synovial fluid: role of hyaluronan. *Arthritis Rheum* 64(9):2917–26.

64. Ballard BL, et al. (2012) Effect of Tibial Plateau Fracture on Lubrication Function and Composition of Synovial Fluid. *J Bone Jt Surg* 94(10):e64 1.

65. Neu CP, Reddi AH, Komvopoulos K, Schmid TM, Di Cesare PE (2010) Increased friction coefficient and superficial zone protein expression in patients with advanced arthritis. *Arthritis Rheum* 62(9):2680–2687.

66. Elsaid K, et al. (2008) Decreased Lubricin Concentrations and Markers of Joint Inflammation in Synovial Fluids from Patients with Anterior Cruciate Ligament Injury. *Arthritis Rheum* 58(6):1707–1715.

67. Jay GD, Tantravahi U, Britt DE, Barrach HJ, Cha CJ (2001) Homology of lubricin and superficial zone protein (SZP): Products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25. *J Orthop Res* 19(4):677–687.

68. Swann DA, Silver FH, Slayter HS, Stafford W, Shore E (1985) The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem J* 225(1):195–201.

69. Jay GD (1992) Characterization of a bovine synovial fluid lubricating factor.I.Chemical, surface activity and lubricating properties. *Connect Tissue Res* 28(1-2):71–88.

70. Dube DH, Bertozzi CR (2005) Glycans in cancer and inflammation--potential for therapeutics and diagnostics. *Nat Rev Drug Discov* 4(6):477–88.

71. Varki A, Kannagi R, Toole BP (2009) Glycosylation Changes in Cancer. *Essentials of Glycobiology*, eds Varki A, Cummings RD, Esko JD (Cold Spring Harbor Laboratory Press). 2nd Ed. Available at:

http://www.ncbi.nlm.nih.gov/books/NBK1963/ [Accessed September 13, 2015].

72. Brownlee M (1995) Advanced protein glycosylation in diabetes and aging. *Annu Rev Med* 46:223–234.

73. Hayes C a, Nemes S, Karlsson NG (2012) Statistical analysis of glycosylation profiles to compare tissue type and inflammatory disease state. *Bioinformatics* 28(13):1669–76.

74. Albrecht S, Unwin L, Muniyappa M, Rudd PMP (2014) Glycosylation as a marker for inflammatory arthritis. *Cancer Biomarkers* 14(1):17–28.

75. Chrostek L, et al. (2014) Sialic acid level reflects the disturbances of glycosylation and acute-phase reaction in rheumatic diseases. *Rheumatol Int* 34(3):393–9.

76. Simm A (2013) Protein glycation during aging and in cardiovascular disease. *J Proteomics* 92(Dm):248–59.

77. Verzijl N, Bank RA, TeKoppele JM, DeGroot J (2003) AGEing and osteoarthritis: a different perspective. *Curr Opin Rheumatol* 15(5):616–22.

78. Miyata T, et al. (1998) Increased pentosidine, an advanced glycation end product, in plasma and synovial fluid from patients with rheumatoid arthritis and its relation with inflammatory markers. *Biochem Biophys Res Commun* 244(1):45–9.

79. Estrella RP, Whitelock JM, Packer NH, Karlsson NG (2010) The glycosylation of human synovial lubricin: implications for its role in inflammation. *Biochem J* 429(2):359–67.

80. Swann DA, Bloch KJ, Swindell D, Shore E (1984) The lubricating activity of human synovial fluids. *Arthritis Rheum* 27(5):552–6.

81. Przybysz M, Maszczak D, Borysewicz K, Szechiński J, Katnik-Prastowska I (2007) Relative sialylation and fucosylation of synovial and plasma fibronectins in relation to the progression and activity of rheumatoid arthritis. *Glycoconj J*

24(9):543–50.

82. Jin C, et al. (2012) Human synovial lubricin expresses sialyl Lewis x determinant and has L-selectin ligand activity. *J Biol Chem* 287(43):35922–33.

83. Goodarzi MT, Turner GA (1998) Reproducible and sensitive determination of charged oligosaccharides from haptoglobin by PNGase F digestion and HPAEC/PAD analysis: Glycan composition varies with disease. *Glycoconj J* 15:469–475.

84. Goodarzi MT, et al. (1998) Sialyl Lewis(x) expression on IgG in rheumatoid arthritis and other arthritic conditions: a preliminary study. *Glycoconj J* 15(12):1149–54.

85. Collins ES, et al. (2013) Glycosylation status of serum in inflammatory arthritis in response to anti-TNF treatment. *Rheumatology (Oxford)* 52(9):1572–82.

86. Rhodes JM (1996) Unifying hypothesis for inflammatory bowel disease and associated colon cancer: Sticking the pieces together with sugar. *Lancet* 347(8993):40–44.

87. Saldova R, et al. (2013) Increase in sialylation and branching in the mouse serum N-glycome correlates with inflammation and ovarian tumour progression. *PLoS One* 8(8):e71159.

88. Chubinskaya S, Malfait A, Wimmer M (2013) Form and Function of Articular Cartilage. *Orthopaedic Basic Science Foundations of Clinical Practice*, eds O'Keefe RJ, Jacobs JJ, Chu CR, Einhorn TA (American Academy of Orthopaedic Surgeons, Rosemont, IL), pp 183–197. 4th Ed.

89. Spikes H (2012) Boundary Lubrication and Boundary Lubricating Films. *Handbook of Lubrication and Tribology, Vol. II Theory an Design*, ed Bruce RW (Taylor & Francis Group), pp 1–16.

90. Jay GD, Waller K a. (2014) The biology of Lubricin: Near frictionless joint motion. *Matrix Biol* 39:17–24.

91. Forster H, Fisher J (1996) The influence of loading time and lubricant on the

friction of articular cartilage. *Proc Inst Mech Eng H* 210(2):109–19.

92. Mcnary SM, Athanasiou KA, Ph D, Reddi AH (2012) Engineering Lubrication in Articular Cartilage. *Tissue Eng Part B Rev* 18(2):88–100.

93. Scherge M, Gorb S (2013) *Biological Micro- and Nanotribology: Nature's Solutions* (Springer Science & Business Media) Available at: https://books.google.com/books?id=2L7oCAAAQBAJ&pgis=1 [Accessed September 11, 2015].

94. Gleghorn JP, Bonassar LJ (2008) Lubrication mode analysis of articular cartilage using Stribeck surfaces. *J Biomech* 41(9):1910–8.

95. Radin EL, Swann DA, Weisser PA (1970) Separation of a hyaluronate-free lubricating fraction from synovial fluid. *Nature* 228(5269):377–8.

96. Seror J, Zhu L, Goldberg R, Day AJ, Klein J (2015) Supramolecular synergy in the boundary lubrication of synovial joints. *Nat Commun* 6:6497.

97. Hills BA (1989) Oligolamellar lubrication of joints by surface active phospholipid. *J Rheumatol* 16(1):82–91.

98. Lee DW, Banquy X, Israelachvili JN (2013) Stick-slip friction and wear of articular joints. *Proc Natl Acad Sci U S A* 110(7):E567–74.

99. Rabinovich GA, et al. (2002) Galectins and their ligands: Amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol* 23(6):313–320.

100. Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. *Biochim Biophys Acta - Gen Subj* 1572(2-3):274–284.

101. Cooper DNW, Barondes SH (1999) God must love galectins; he made so many of them. *Glycobiology* 9(10):979–984.

102. Smetana K, André S, Kaltner H, Kopitz J, Gabius H-J (2013) Context-dependent multifunctionality of galectin-1: a challenge for defining the lectin as therapeutic target. *Expert Opin Ther Targets* 17(4):379–92.

103. Baum LG, et al. (2003) Amelioration of graft versus host disease by galectin-1. *Clin Immunol* 109(3):295–307.

104. Liu SD, et al. (2011) Galectin-1-induced down-regulation of T lymphocyte activation protects (NZB x NZW) F1 mice from lupus-like disease. *Lupus* 20(5):473–84.

105. Perone MJ, et al. (2006) Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. *J Immunol* 177(8):5278–89.

106. Camby I, Le Mercier M, Lefranc F, Kiss R (2006) Galectin-1: a small protein with major functions. *Glycobiology* 16(11):137R–157R.

107. Henderson NC, Mackinnon AC, Rooney C, Sethi T (2012) Galectin-3 : A Central Regulator of Chronic Inflammation and Tissue Fibrosis.

108. Pugliese G, Iacobini C, Pesce CM, Menini S (2014) Galectin-3: an emerging all-out player in metabolic disorders and their complications. *Glycobiology* 25(2):136–150.

109. Li Y, et al. (2008) Galectin-3 is a negative regulator of lipopolysaccharide-mediated inflammation. *J Immunol* 181(4):2781–9.

110. Yabuta C, Yano F, Fujii a, Shearer TR, Azuma M (2014) Galectin-3 enhances epithelial cell adhesion and wound healing in rat cornea. *Ophthalmic Res* 51(2):96–103.

111. Panjwani N (2014) Role of galectins in re-epithelialization of wounds. *Ann Transl Med* 2(9):89.

112. Xibillé-Friedmann D, Bustos Rivera-Bahena C, Rojas-Serrano J, Burgos-Vargas R, Montiel-Hernández J-L (2013) A decrease in galectin-1 (Gal-1) levels correlates with an increase in anti-Gal-1 antibodies at the synovial level in patients with rheumatoid arthritis. *Scand J Rheumatol* 42(2):102–7.

113. Ohshima S, et al. (2003) Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis Rheum* 48(10):2788–95.

114. Harjacek M, et al. (2001) Expression of galectins-1 and -3 correlates with defective mononuclear cell apoptosis in patients with juvenile idiopathic arthritis. *J Rheumatol* 28(8):1914–1922.

115. Alturfan a A, Eralp L, Emekli N (2008) Investigation of inflammatory and hemostatic parameters in female patients undergoing total knee arthroplasty surgery. *Inflammation* 31(6):414–21.

116. Guévremont M, et al. (2004) Galectin-3 surface expression on human adult chondrocytes: a potential substrate for collagenase-3. *Ann Rheum Dis* 63(6):636–43.

117. Dasuri K, et al. (2004) The synovial proteome: analysis of fibroblast-like synoviocytes. *Arthritis Res Ther* 6(2):R161–8.

118. Iqbal AJ, et al. (2013) Endogenous galectin-1 exerts tonic inhibition on experimental arthritis. *J Immunol* 191(1):171–7.

119. Rabinovich GA, et al. (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190(3):385–398.

120. Huang Y-J, et al. (2012) Multivalent structure of galectin-1-nanogold complex serves as potential therapeutics for rheumatoid arthritis by enhancing receptor clustering. *Eur Cell Mater* 23:170–81; discussion 181.

121. Wang C-R, et al. (2010) Intra-articular lentivirus-mediated delivery of galectin-3 shRNA and galectin-1 gene ameliorates collagen-induced arthritis. *Gene Ther* 17(10):1225–33.

122. Toegel S, et al. (2014) Human osteoarthritic knee cartilage: fingerprinting of adhesion/growth-regulatory galectins in vitro and in situ indicates differential upregulation in severe degeneration. *Histochem Cell Biol* 142(4):373–88.

123. Ezzat MHM, El-Gammasy TMA, Shaheen KYA, Osman AOY (2011) Elevated production of galectin-3 is correlated with juvenile idiopathic arthritis disease activity, severity, and progression. *Int J Rheum Dis* 14(4):345–52.

124. Hu C-Y, Chang S-K, Wu C-S, Tsai W-I, Hsu P-N (2011) Galectin-3 gene (LGALS3) +292C allele is a genetic predisposition factor for rheumatoid arthritis in Taiwan. *Clin Rheumatol* 30(9):1227–33.

125. Hayashi J, Kihara M, Kato H, Nishimura T (2015) A proteomic profile of synoviocyte lesions microdissected from formalin-fixed paraffin-embedded synovial tissues of rheumatoid arthritis. *Clin Proteomics* 12(1):20.

126. Shou J, et al. (2006) Identification of blood biomarkers of rheumatoid arthritis by transcript profiling of peripheral blood mononuclear cells from the rat collagen-induced arthritis model. *Arthritis Res Ther* 8(1):R28.

127. Forsman H, et al. (2011) Galectin 3 aggravates joint inflammation and destruction in antigen-induced arthritis. *Arthritis Rheum* 63(2):445–54.

128. Boileau C, et al. (2008) Intracellular localisation of galectin-3 has a protective role in chondrocyte survival. *Ann Rheum Dis* 67(2):175–81.

129. Janelle-Montcalm A, et al. (2007) Extracellular localization of galectin-3 has a deleterious role in joint tissues. *Arthritis Res Ther* 9(1):R20.

130. Reboul P, Martel-Pelletier J, Pelletier J-P (2004) Galectin-3 in osteoarthritis: when the fountain of youth doesn't deliver its promises. *Curr Opin Rheumatol* 16(5):595–8.

131. Li S, Yu Y, Koehn CD, Zhang Z, Su K (2013) Galectins in the Pathogenesis of Rheumatoid Arthritis. *J Clin Cell Immunol* 4(5). doi:10.4172/2155-9899.1000164.

132. Eshkar Sebban L, et al. (2007) The involvement of CD44 and its novel ligand galectin-8 in apoptotic regulation of autoimmune inflammation. *J Immunol* 179(2):1225–35.

133. Sarter K, et al. (2013) Autoantibodies against galectins are associated with antiphospholipid syndrome in patients with systemic lupus erythematosus. *Glycobiology* 23(1):12–22.

134. Pál Z, et al. (2012) Non-synonymous single nucleotide polymorphisms in genes for immunoregulatory galectins: association of galectin-8 (F19Y) occurrence with autoimmune diseases in a Caucasian population. *Biochim Biophys Acta* 1820(10):1512–8.

135. Seki M, et al. (2008) Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 127(1):78–88.

136. Seki M, et al. (2007) Beneficial effect of galectin 9 on rheumatoid arthritis by induction of apoptosis of synovial fibroblasts. *Arthritis Rheum* 56(12):3968–76.

137. Arikawa T, et al. (2009) Galectin-9 ameliorates immune complex-induced arthritis by regulating Fc gamma R expression on macrophages. *Clin Immunol* 133(3):382–92.

138. Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich G a (2007) Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci* 64(13):1679–700.

139. Friedrichs J, Manninen A, Muller DJ, Helenius J (2008) Galectin-3 regulates integrin alpha2beta1-mediated adhesion to collagen-I and -IV. *J Biol Chem* 283(47):32264–72.

140. Auvynet C, et al. (2012) Galectin-1 promotes human neutrophil migration. *Glycobiology* 23(1):32–42.

141. Sano H, et al. (2000) Human Galectin-3 Is a Novel Chemoattractant for Monocytes and Macrophages. *J Immunol* 165(4):2156–2164.

142. Malik RKJ, Ghurye RR, Lawrence-Watt DJ, Stewart HJS (2009) Galectin-1 stimulates monocyte chemotaxis via the p44/42 MAP kinase pathway and a pertussis toxin-sensitive pathway. *Glycobiology* 19(12):1402–7.

143. Kariya Y, Kawamura C, Tabei T, Gu J (2010) Bisecting GlcNAc residues on laminin-332 down-regulate galectin-3-dependent keratinocyte motility. *J Biol Chem* 285(5):3330–40.

144. Ben-Ami E, Berrih-Aknin S, Miller A (2011) Mesenchymal stem cells as an immunomodulatory therapeutic strategy for autoimmune diseases. *Autoimmun Rev* 10(7):410–5.

145. Pittenger MF, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143–147.

146. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM (2010) Mesenchymal stem cells and osteoarthritis: remedy or accomplice? *Hum Gene Ther* 21(10):1239–50.

147. Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 48(12):3464–74.

148. Prockop DJ, Youn Oh J (2011) Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Mol Ther* 20(1):14–20.

149. Caplan AI (2009) Why are MSCs therapeutic? New data: new insight. *J Pathol* 217(2):318–24.

150. McIlwraith CW, et al. (2011) Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. *Arthroscopy* 27(11):1552–61.

151. Mokbel AN, et al. (2011) Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model. *BMC Musculoskelet Disord* 12(1):259.

152. Wong KL, et al. (2013) Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up. *Arthroscopy* 29(12):2020–8.

153. Tyndall A (2015) Mesenchymal stromal cells and rheumatic disorders. *Immunol Lett*:1–7.

154. Liu GY, et al. (2013) Secreted galectin-3 as a possible biomarker for the immunomodulatory potential of human umbilical cord mesenchymal stromal

cells. *Cytotherapy* 15(10):1208–1217.

155. Sioud M, Mobergslien A, Boudabous A, Fløisand Y (2011) Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. *Int J Oncol* 38(2):385–90.

156. Sioud M (2011) New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. *Scand J Immunol* 73(2):79–84.

157. Mokarizadeh A, et al. (2012) Microvesicles derived from mesenchymal stem cells: potent organelles for induction of tolerogenic signaling. *Immunol Lett* 147(1-2):47–54.

158. Gieseke F, et al. (2010) Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. *Blood* 116(19):3770–9.

159. Najar M, et al. (2010) Modulated expression of adhesion molecules and galectin-1: role during mesenchymal stromal cell immunoregulatory functions. *Exp Hematol* 38(10):922–32.

160. Lepelletier Y, et al. (2010) Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. *Stem Cells Dev* 19(7):1075–9.

161. Sioud M, Mobergslien a, Boudabous a, Fløisand Y (2010) Evidence for the involvement of galectin-3 in mesenchymal stem cell suppression of allogeneic T-cell proliferation. *Scand J Immunol* 71(4):267–74.

162. Carrade Holt DD, et al. (2014) Equine mesenchymal stem cells inhibit T cell proliferation through different mechanisms depending on tissue source. *Stem Cells Dev* 23(11):1258–65.

CHAPTER 2

INCREASED LUBRICIN GENE EXPRESSION, SYNOVIAL FLUID CONCENTRATIONS AND IMMUNOSTAINING IN AN EQUINE OSTEOARTHRITIS MODEL

Abstract

Objective. The goals of this study were 1) to quantify proteoglycan 4 (*Prg4*) gene expression; 2) to assess lubricin immunostaining in cartilage and synovial membrane; and 3) to measure synovial fluid lubricin concentrations in both clinical and experimental models of equine carpal osteoarthritis (OA).

Methods. Lubricin synovial fluid concentrations and *Prg4* expression were analyzed in research horses undergoing experimental OA induction (n=8) and in equine clinical patients with carpal OA (n=58). Lubricin concentrations were measured using a custom sandwich enzyme-linked immunosorbent assay (ELISA), and *Prg4* expression was quantified using qRT-PCR. Lubricin immunostaining was assessed in the experimental model.

Results. Lubricin concentrations increased in synovial fluid immediately following induction of OA (p=0.0003), peaking at 21 days post-operatively in OA joints vs. sham-operated controls (330.9 ± 69.2 ug/mL vs. 110 ± 19.4 ug/mL, p=0.0153). Lubricin concentrations also increased in horses with naturally occurring OA as compared to control joints (151.7 ± 32.2 ug/mL vs. 67.6 ± 4.3 ug/mL, p=0.0157). Synovial membrane *Prg4* expression increased nearly 2-fold in naturally occurring OA (p=0.0025); whereas cartilage *Prg4* expression decreased 2.5-fold (p=0.0248). Lubricin immunostaining was more pronounced in synovial membrane from OA joints as compared to controls, with intense lubricin localization to sites of cartilage damage in OA joints.

Conclusions. Although PRG4 gene expression is decreased in OA cartilage, synovial membrane PRG4 expression, synovial fluid lubricin concentrations and lubricin immunostaining all increase in an equine model of OA. Lubricin may be elevated to protect joints from post-traumatic OA.

Introduction

Lubricin, a mucinous glycoprotein encoded by the proteoglycan 4 (PRG4) gene, functions as both a boundary lubricant and chondroprotective agent in synovial joints. Patients with camptodactyly-arthropathy-coxa vara-pericarditis (CACP) fail to express PRG4 and subsequently develop early-onset polyarthropathy (1, 2). Studies have revealed that PRG4-deficient synovial fluid in a subset of patients with osteoarthritis (OA) fails to lubricate cartilage and that boundary lubrication can be restored by the addition of exogenous recombinant lubricin (3). In human patients with anterior cruciate ligament (ACL) injury (4) and in rodent models of ACL injury (4–6), synovial fluid lubricin concentrations are decreased in injured joints as compared to controls. Synovial fluid lubricin concentrations were also decreased in a population of human patients with late-stage OA and RA (7). Mounting evidence suggests that lubricin supplementation, either through genetic overexpression (8) or recombinant lubricin supplementation (9–12) delays the progression of OA in rodent models. Accordingly, there is considerable interest in the use of lubricin supplementation as a potential therapeutic for OA, but information about lubricin

expression and tissue localization in large animal naturally occurring OA and translational large animal experimental models of OA is limited.

Large animal models such as the horse more closely recapitulate OA in humans than small animal models because cartilage thickness and joint volume more closely approximate human cartilage (13–15), and cartilage is subject to loading forces of similar or greater magnitude than human cartilage (15). Moreover, the equine carpal fragment model has the added benefits of allowing repeated synovial fluid sampling and controlled, athletic exercise (15, 16). However, there is substantial controversy about how lubricin is altered in large animal models of arthritis. In one report, *Prg4* mRNA levels were significantly decreased in a sheep meniscectomy model three months post-operatively, and gene expression correlated with decreased lubricin immunostaining in degenerative articular cartilage (17). Conversely, lubricin synovial fluid concentrations and boundary lubrication properties were similar between operated and contralateral control limbs, leading investigators to conclude that lubricin should be evaluated at earlier time points in the development of OA (18). In dogs undergoing unilateral cranial cruciate ligament transection, lubricin immunohistochemical staining did not differ between OA and control limbs 13 weeks post-operatively (19), and quantitation of lubricin using Western blot analysis revealed an 83% increase in lubricin in acutely injured (≤ 3 weeks) equine joints (20). Thus, although several studies in rodent models suggest that lubricin decreases in experimental arthritis, it remains unclear whether lubricin levels are increased,

decreased or unchanged in large animal models of OA and over what time course these changes occur.

Furthermore, some investigations report increased synovial fluid lubricin concentrations in human patients with intra-articular fracture (21) or late-stage OA (22), bringing into question whether it is appropriate to extrapolate from rodent models to humans. A primary limitation of studies to date evaluating lubricin synovial fluid concentrations in human patients (3, 4) and in most experimental animal models of OA is that synovial fluid lubricin concentrations are only evaluated at a single time point (6, 18, 20). A recent longitudinal analysis of synovial fluid lubricin concentrations in an ovine anterior cruciate ligament transection model revealed increased lubricin concentrations at 2 and 4 weeks post injury as compared to 20 weeks; however, synovial fluid samples were not evaluated prior to injury (23). Because serial lubricin quantitation has been limited, it is difficult to make inferences as to how lubricin concentrations change over the course of OA, whether altered lubricin concentrations precede the development of OA, and whether or not intra-articular lubricin supplementation may be indicated in clinically relevant large animal models or humans. To our knowledge, no studies have evaluated *Prg4* expression, serial synovial fluid lubricin concentrations and immunostaining in the same model.

Our objective, therefore, was to assess changes in synovial fluid lubricin concentrations at serial intervals both before and after osteochondral fragmentation in an equine OA model (16), and to assess *Prg4* expression and immunostaining from

articular cartilage and synovium at study termination 70 days post-OA induction. In addition, we sought to quantify *Prg4* and lubricin glycoprotein expression in cartilage, synovial membrane and synovial fluid samples from horses with naturally occurring carpal OA injuries similar to the carpal osteochondral fragment experimental model. We hypothesized that *Prg4* expression, lubricin synovial fluid concentrations and lubricin synovial membrane and cartilage immunostaining would decrease in both experimental and naturally occurring OA in horses.

Materials and Methods

Samples

Prg4 expression and lubricin synovial fluid concentrations were analyzed in samples from 2 equine cohorts: equine clinical patients with carpal OA (n=36) or healthy carpal joints (n=22), and research horses undergoing carpal osteochondral fragmentation for OA induction (n=8). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee, and all synovial fluid and tissue samples were collected with informed owner consent.

Naturally Occurring OA

Healthy and OA synovial fluid, synovial membrane and cartilage tissues were harvested from the antebrachialcarpal (ACJ) and middle carpal (MCJ) joints of horses presenting to the Cornell University Equine Hospital for arthroscopy or from horses donated to the hospital for research purposes. Cytokine and catabolic enzyme expression from this cohort of primarily Thoroughbred horses ranging in age from 2-11 years has been previously described (24). Radiographic evaluation of all OA joints was performed prior to surgery or euthanasia, and joints were assigned a score of normal, mild, moderate or severe OA according to the radiographic presence of osteophytes, enthesiophytes, osteoproliferation, joint space narrowing, or chronic fracture lines, with additional arthroscopic/gross scoring used to corroborate the radiographic score. Thirty-six horses with carpal OA underwent radiography prior to surgery or euthanasia, and 22 horses with normal joints were included in the study.

Experimental OA

The arthroscopically created equine carpal osteochondral fragment-exercise model of OA has been previously described (16, 25). Briefly, an 8mm osteochondral fragment was created at the distal dorsal aspect of the radial carpal bone of one randomly assigned forelimb. The contralateral forelimb was sham-operated without creation of a fragment. Synovial fluid samples were obtained from both limbs at the time of initial arthroscopy and at weekly intervals post-operatively. Horses were

exercised on a high-speed treadmill 5 times weekly for the study duration. On day 70 post-induction, horses were euthanized, and synovial membrane biopsies obtained for RNA expression. Synovial fluid was collected, and synovial membrane and osteochondral blocks, including the radial carpal bone fragment and parent bone and the opposite third carpal bone, were harvested for histological processing and immunohistochemistry. Eight Thoroughbred horses, aged 2-6 years old, were used for the study.

Isolation of RNA and Real-Time Quantitative PCR Lubricin Assay

Synovial membrane tissue and cartilage was snap-frozen in liquid nitrogen, pulverized and stored at -80°C prior to isolation of RNA using the PerfectPure RNA Tissue Kit (5Prime, Gaithersburg, MD), and RNA purity and concentration were assessed with UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA). PRG4 gene expression was quantified by real-time PCR using the Taqman One-Step RT-PCR technique (Absolute Quantitative PCR; ABI PRISM 7900 HT Sequence Detection System, Applied Biosystems, Foster City, CA). Primer Express Software Version 2.0 (Applied Biosystems, Foster City, CA) was used to design primers and dual-labeled fluorescent probes (6-carboxyfluorescein (FAM) as the 5' reporter dye and Iowa Black® FQ as the 3' quenching dye) for quantification of equine PRG4. Primers were designed as follows:
Fwd: 5' – TGCGGTGCTTCCCCATAC – 3';
Rev: 5' – AAACAGGAACCCATCAGAAAGTG – 3';

Probe: 5' – /56FAM/ATAGCAGGCCGCCTTCCCGG/3IABkFQ/ - 3'. The total copy number of mRNA was determined using a validated standard curve of equine PRG4 C-terminal cDNA, and these values were normalized to the housekeeping gene 18S.

Purification of Equine Synovial Fluid Lubricin

Methods for purification of synovial fluid lubricin have been previously described in detail (10). Briefly, synovial fluid was collected from two healthy 4 to 6-year-old horses, clarified by high-speed centrifugation at 10,000xg for 1 h and stored at -80°C. Synovial fluid was digested with 1U/mL Streptomyces hyaluronidase (Calbiochem®, EMD Chemicals, San Diego, CA) in 50mM NaAc buffer, pH 5.5 at 4°C for 16 h on an end-over-end rocker and purified using a HiTrap™ diethylaminoethyl (DEAE) fast flow sepharose FPLC column (GE Healthcare Life Sciences, Little Chalfont, UK). Lubricin was confirmed by Coomassie staining (Teknova, Hollister, CA) and immunoblotting with a C-terminal lubricin polyclonal antibody (ab28484, Lot: GR116636-3, Abcam®, Cambridge, UK). The final protein concentration of lubricin fractions was quantitated using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL).

Synovial Fluid Lubricin (Sandwich ELISA)

A sandwich ELISA using peanut agglutinin (PNA) (Sigma-Aldrich, St. Louis, MO) and anti-lubricin monoclonal antibody 9G3 (MABT401; EMD Millipore, Darmstadt, Germany) has been previously described (5) and was used for quantification of equine synovial fluid lubricin. 12 hours after coating high binding 96-well plates (Corning Inc., Corning, NY) with 10 μg/mL of PNA in 50mM sodium bicarbonate buffer, pH 9.5, plates were blocked with phosphate buffered saline (PBS)+3% EIA-grade BSA (Sigma-Aldrich, St. Louis, MO) for 1 hour. Serial dilutions of purified equine lubricin and diluted equine SF samples were incubated for 1 hour at room temperature, followed by washing in PBS+0.1% Tween20. mAb 9G3 was added to the plate at a dilution of 1:10,000 for 1 hour at room temperature and washed with PBS+0.1% Tween20. Chicken anti-mouse IgG-horseradish peroxidase (EMD Millipore, Darmstadt, Germany) was added at 1:2,000 dilution for 1 hour, followed by washing three times in PBS+0.1% Tween20 with a final rinse in PBS alone. TMB reagent was added (Pierce, Rockford, IL), the reaction was stopped with 2N H2SO4, and absorbance was measured at 450 nm with 540nm background subtraction.

Histological Processing

Paraffin-embedded sagittal sections were obtained from the weight-bearing regions of the fragmented radial carpal bone and parent bone, in addition to the

articulating third carpal bone opposite the fragment in the experimental animals described above. Histological staining using lubricin-specific monoclonal antibody 6a8 (courtesy of G. Jay) at a 1:1,000 dilution was performed, in addition to Safranin O/Fast Green staining for assessment of cartilage sGAG content. Synovial membrane sections from the middle carpal joint tissue adjacent to the fracture site were obtained for mAb 6a8 lubricin staining and hematoxylin and eosin staining. Representative synovial membrane and osteochondral sections were imaged using an Aperio ScanScope slide scanner (Leica, Wetzlar, Germany) at 20X magnification, exported as .tif files with ImageScope software (Leica, Wetzlar, Germany) and compiled using Adobe Illustrator CS5.1 software (Adobe Systems Inc., San Jose, CA).

Statistical Analysis

Naturally occurring OA. ELISA and qRT-PCR data were analyzed using a 1-way ANOVA with Tukey's HSD *post hoc* tests. Comparisons between normal and OA joints (1, 2, and 3 grouped together) were made using a 2-sample t-test. Log values were used to normalize cartilage gene expression data in order to satisfy model assumptions, and summary statistics were performed on the untransformed data. A p-value < 0.05 was considered significant. *Experimental OA.* Lubricin ELISA data were analyzed via repeated measures analysis using a mixed model in which fixed effects included treatment (control vs. OA), day and a treatment*day interaction term, and random effects included horse and individual limb nested within horse to account for pairing of data. This analysis was followed with post hoc comparisons of specific

contrasts for each time point to assess the difference between control vs. OA joints, with a Bonferroni correction applied to account for multiple comparisons. Synovial membrane qRT-PCR data were analyzed using a paired t-test. P-values < 0.05 were considered significant. Statistical analysis was performed using JMP Pro 11.0 software (SAS Institute Inc., Cary, NC).

Results

Naturally occurring OA

Synovial membrane PRG4 gene expression increased in naturally occurring equine carpal OA **(Figure 2.1*A*)**; whereas cartilage PRG4 transcription levels decreased **(Figure 2.1*B*)**. Differences in PRG4 gene expression were statistically significant between control and moderate OA cases for both synovial membrane ($40.3x10^4 \pm 0.38x10^4$ copies/ng vs. $77.9x10^4 \pm 0.99x10^4$ copies/ng, p=0.0025) and cartilage ($45.5x10^4 \pm 1.36x10^4$ copies/ng vs. $18.8x10^4 \pm 0.82x10^4$ copies/ng, p=0.0248). Synovial fluid lubricin concentrations in horses with naturally occurring OA (n=47) demonstrated a trend for increased lubricin levels in all OA grades as compared to controls. Because there were no significant differences in lubricin concentrations between mild, moderate and severe (1, 2 and 3) OA groups, all OA grades were combined for analysis. Lubricin concentrations were significantly elevated in OA vs. control joints (151.7 ± 32.2 ug/mL vs. 67.6 ± 4.3 ug/mL, p=0.0157) **(Figure 2.1*C*)**.

Experimental OA

Lubricin concentrations increased in synovial fluid following induction of OA. Lubricin concentrations were significantly elevated in OA joints vs. controls on days 21, 28, and 42 following induction of OA ($p<0.05$) **(Figure 2.2*B*)**. Twenty-one days post-operatively, synovial fluid lubricin concentrations were increased 3-fold in joints with carpal fragmentation as compared to sham-operated controls (330.9 ± 69.2 ug/mL vs. 110 ug/mL ± 19.4 ug/mL, $p=0.001$). The elevation in lubricin concentrations in synovial fluid did not correspond to an increase in synovial membrane PRG4 gene expression in horses with OA vs. controls ($1.85x10^5 \pm 0.21x10^5$ copies/ng vs. $1.77x10^5 \pm 0.19x10^5$ copies/ng) at day 70 post-carpal fragment induction **(Figure 2.2*A*)**.

The osteochondral fragment was apparent at gross examination in all horses **(Figure 2.3)**. Lubricin immunoreaction was increased in intensity and distribution within the synovial membrane of all OA limbs vs. controls (n=8), most prominently within the most superficial intimal layers and surrounding vasculature **(Figure 2.4)**. Lubricin immunostaining of the articular surface of the third carpal bone, which articulates with the osteochondral fragment, was variably affected in the OA limb as compared to the control, depending upon the degree of damage to the articular surface. The most dramatic lubricin localization to the third carpal bone was observed in articular surfaces with significant damage, including surface fissuring and fibrillation, proteoglycan loss, and replacement of hyaline cartilage with fibrocartilage **(Figure 2.5)**. Lubricin localization to the lamina splendens and superficial 1-3 layers of

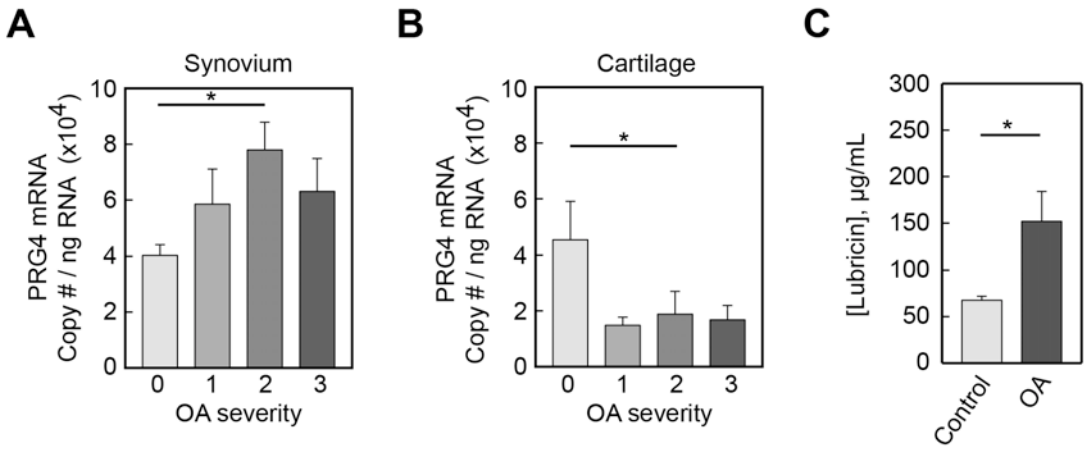


Figure 2.1. Proteoglycan 4 (PRG4) gene expression in cartilage **(A)** and synovial membrane **(B)** from normal equine carpal joints (n=22) and equine carpal joints with naturally occurring OA (n=36). Categories were numbered as normal (0), mild OA (1), moderate OA (2) and severe OA (3). Synovial membrane PRG4 expression was significantly increased in the moderate OA group as compared to the control group **(A)**, and cartilage PRG4 expression was significantly decreased in all OA groups as compared to controls **(B)**. **(C)** Synovial fluid lubricin concentrations in radiographically or grossly normal carpal joints vs. radiographically abnormal OA carpal joints. Lubricin concentrations were significantly elevated in OA joints as compared to controls. Data are presented as mean ± SEM. * = $P < 0.05$.

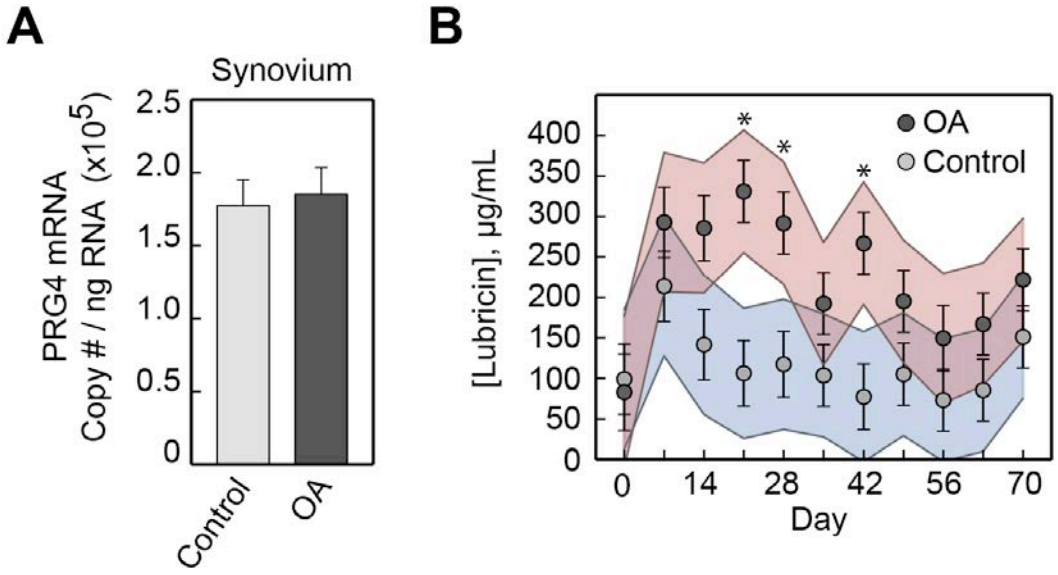


Figure 2.2. **(A)** Proteoglycan 4 (PRG4) gene expression in synovial membrane from sham-operated control and osteochondral fragment-induced OA joints in experimental horses 70 days post-fragmentation. **(B)** Serial lubricin synovial fluid concentrations from day 0 (immediately prior to fragment induction) to day 70 post-fragmentation in OA and control joints (n=8). Lubricin synovial fluid concentrations were increased in joints with carpal fragmentation vs. sham-operated controls on days 21, 28 and 42 following induction of OA ($p<0.05$). Data are presented as predicted means ± SEM with colored lines representing 95% confidence intervals. * = $P < 0.05$.

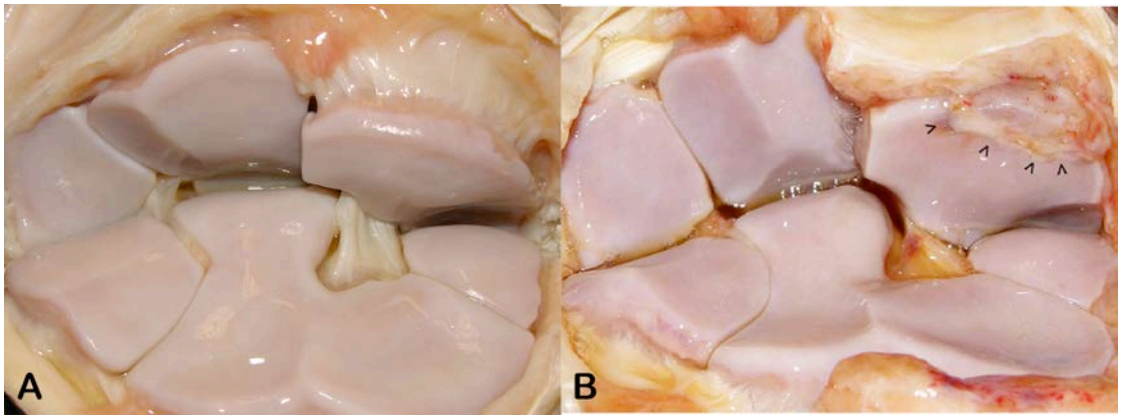


Figure 2.3. Photographs of the articular surface of the middle carpal joint at necropsy 70 days after osteochondral chip fracture of the radial carpal bone. **(A)** Right sham-operated joint, and **(B)** left OA joint, 70 days after chip fragmentation. ^ outline the osteochondral fragment of the dorsomedial radial carpal bone.

chondrocytes distant from the osteochondral fragment was similar to control cartilage **(Figure 2.5)**. Within the radial carpal bone fragment, lubricin immunostaining was significantly increased, particularly within the superficial 100-200 μm of the fibrocartilaginous repair tissue covering the articular margin of the fragment and at the transition to normal, hyaline cartilage of the parent bone **(Figure 2.6, ROI 1-2)**. Lubricin immunoreaction was also increased within the vascular lining cells and within the intraosseous vasculature, most notably within the fracture fragment and at the site of reintegration **(Figure 2.6, ROI 3)**.

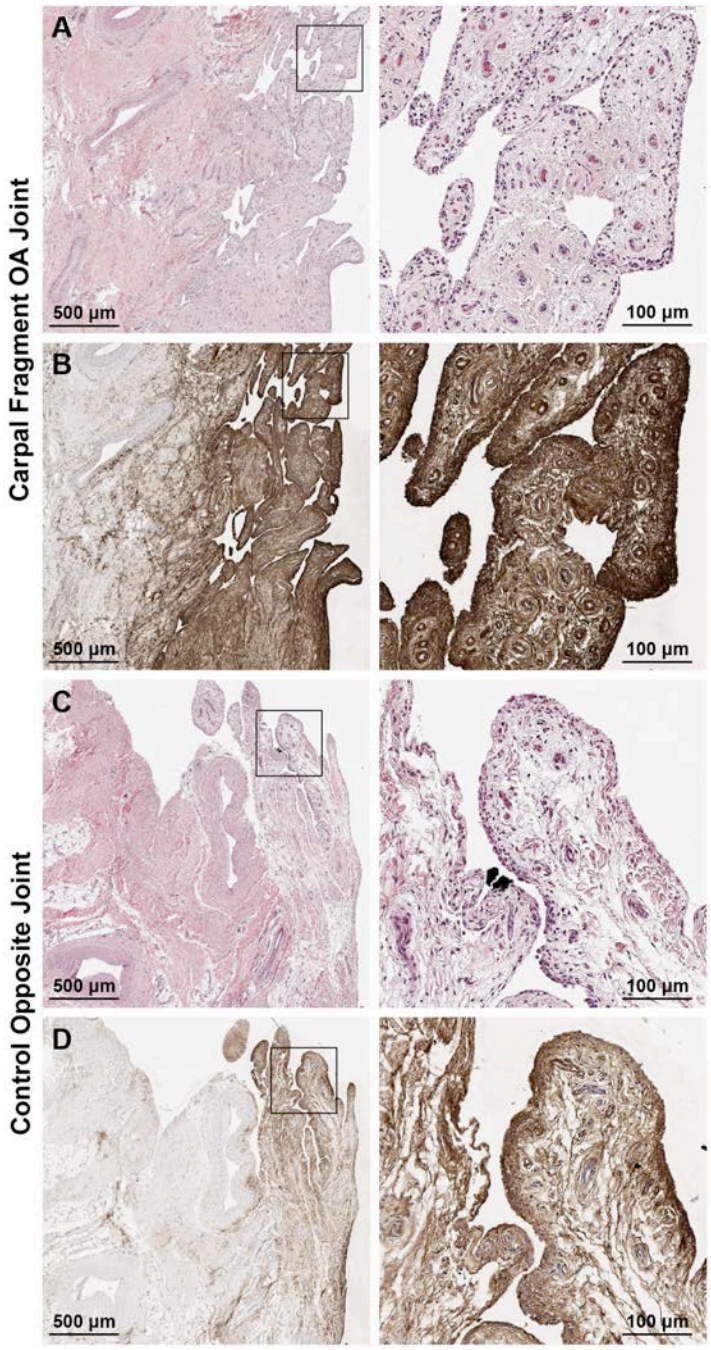


Figure 2.4. Hematoxylin and eosin **(A, C)** and lubricin immunostaining with monoclonal antibody 6a8 **(B, D)** of synovial tissues from the equine carpal osteochondral fragment model 70 days after fragment induction. H&E staining of the OA limb reveals synovial intimal hyperplasia, subintimal fibrosis, particularly adjacent to blood vessels, and increased vascularity **(A)** as compared to the opposite control limb from the same animal **(C)**. Lubricin immunostaining is increased throughout the entire synovial villus, most notably within the intimal layers and surrounding the vasculature **(B)** as compared to the control **(D)**.

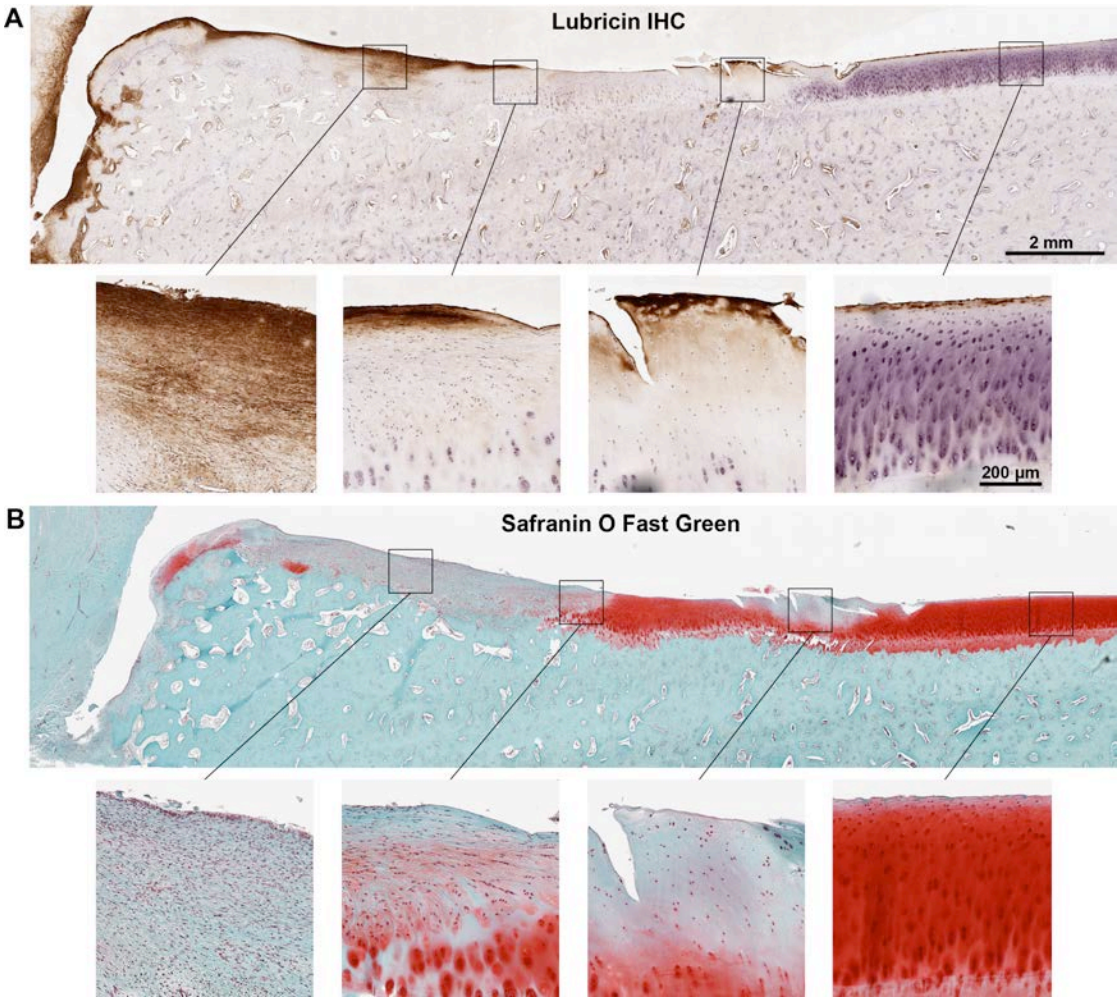
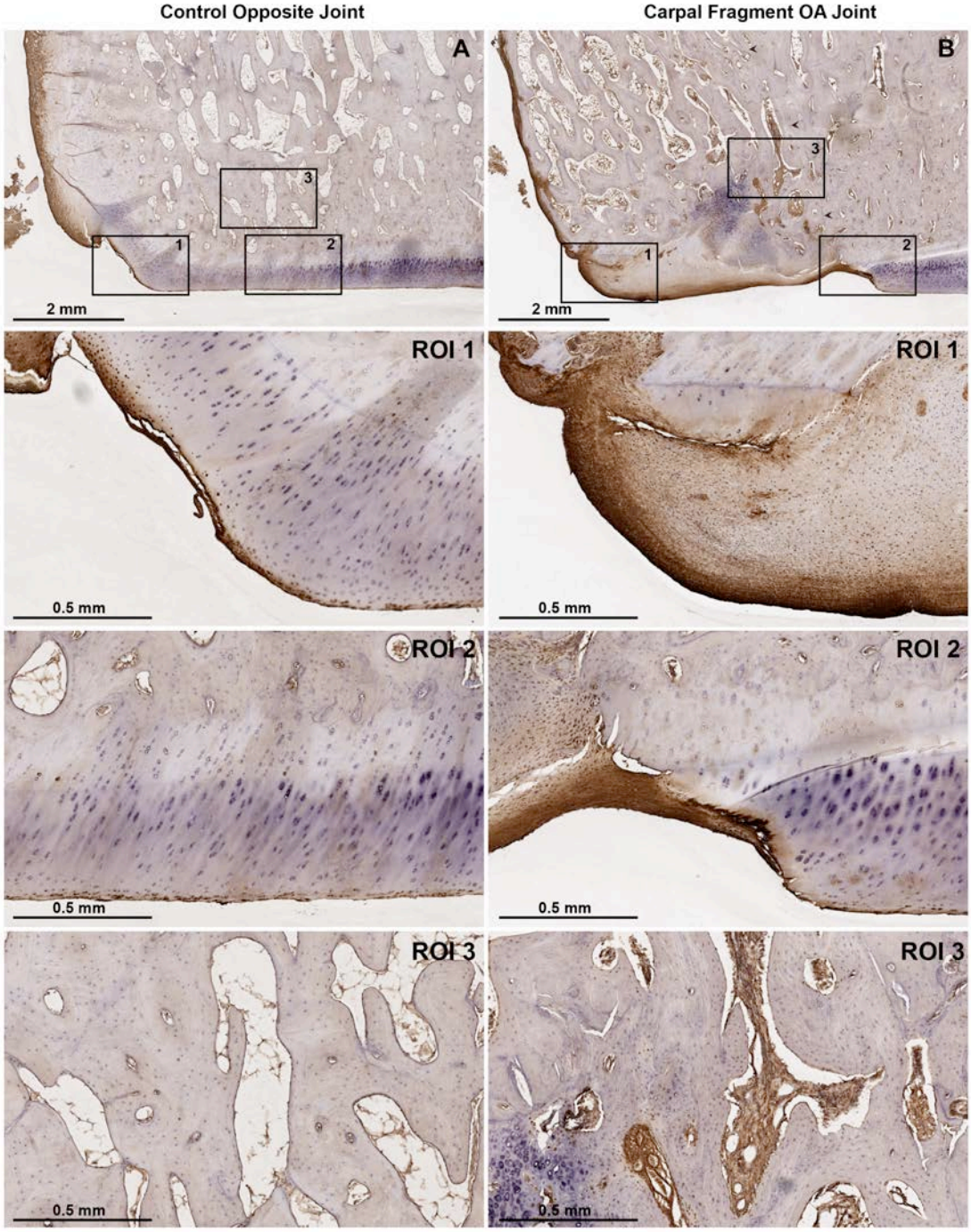


Figure 2.5. Sagittal osteochondral section obtained from the third carpal bone of the equine carpal osteochondral fragment model 70 days after fragment induction, stained with monoclonal antibody 6a8 for lubricin **(A)** and with Safranin O Fast Green (SFGO) for sulfated proteoglycans **(B)**. **(A)** Lubricin immunoreaction is significantly increased within the fibrocartilaginous repair tissue articulating with the radial carpal bone (RCB) fragment, both at the surface and extending more than 1mm deep within the tissue **(A, left two images)**. Lubricin localization is primarily confined to areas devoid of Safranin O staining **(A,B second image from the left)**. Lubricin intensely stains a region of fissured and fibrillated cartilage **(A, second image from right)** demonstrating significant cell loss and loss of proteoglycan **(B, second image from right)** as compared to staining of the superficial 2-3 cell layers in adjacent healthy cartilage distant from the RCB fragment **(A,B rightmost images)**.

Figure 2.6. Lubricin immunostaining of the equine carpal osteochondral fragment exercise model 70 days after fragment induction using monoclonal antibody 6a8. **(A)** Control and **(B)** fragmented articular surfaces of the distal radial carpal bone (RCB) at 1X magnification. Arrowheads in B delineate the site of (re-integration/reattachment) of the osteochondral fragment with the parent bone. Regions of interest (ROI) 1,2 and 3 represent 5X magnifications of the peripheral articular margin, the weight-bearing articular surface, and the bone within the fracture fragment **(B)**, respectively. Lubricin immunoreaction is localized to both the surface and deeper layers of fibrocartilage **(B, 1-2)** as compared to the superficial-most layers of healthy hyaline cartilage **(A, 1-2)**. Lubricin staining is also increased within the vascular channels of the RCB chip fracture fragment, particularly adjacent to the fracture site **(B, 3)** as compared to the healthy RCB **(A, 3)**.


Control Opposite Joint
Carpal Fragment OA Joint
A
B
1
2
3
2 mm
ROI 1
ROI 2
ROI 3
0.5 mm

Discussion

In the present study, we evaluated the time course of synovial fluid lubricin concentrations before and at weekly intervals after osteochondral fragment induction of OA in an equine treadmill exercise model. Contrary to prior studies in anterior cruciate ligament deficient small animal models (4–6) and to our experimental hypotheses, our results indicate that synovial fluid lubricin concentrations increase in response to osteochondral fragmentation in horses, peaking at approximately 21 days post-fragmentation, and gradually decreasing towards baseline over the subsequent 50 days **(Figure 2.3*B*)**. These findings in the experimental model were corroborated by increased synovial fluid lubricin concentrations in horses with naturally occurring carpal OA **(Figure 2.2*C*)**, in which abundant expression of TNFα in synovial membrane and cartilage and IL-1β in cartilage have been previously reported (24). Furthermore, lubricin immunostaining was most pronounced in the synovial tissues of OA as compared to control limbs of experimental horses **(Figure 2.4)**, with the most dramatic increases in lubricin expression in osteochondral tissues noted at sites of cartilage fibrillation, fissuring and chondrocyte apoptosis **(Figure 2.5)**, in addition to sites of osteochondral re-integration and repair tissue **(Figure 2.6)**. Taken together, these results suggest that lubricin is upregulated as part of the endogenous repair response in post-traumatic OA in horses. In addition to protecting cartilage from wear by functioning as a boundary lubricant, lubricin may be exerting anabolic effects in fracture healing and at sites of hyaline cartilage repair.

In some animals, mild traumatic changes were observed in the sham-operated joint, although degenerative changes were most pronounced in the OA joint. One explanation for these findings is that horses undergo compensatory overloading of the sham-operated limb during exercise due to lameness associated with the osteochondral fracture. Although there may be significant advantages to pairing limbs within the same horse in terms of reducing the number of experimental animals and reducing statistical variability, sham-operated joints may not be truly representative of healthy joints due to supraphysiologic loading associated with the rigorous high-speed treadmill exercise (26). Recent investigations have revealed that controlled, low intensity treadmill exercise correlates with increased lubricin cartilage immunolocalization in exercised rats (27). However, after anterior cruciate ligament transection, forced joint exercise decreases cartilage PRG4 expression (28), suggesting that exercise may promote lubricin production in healthy joints but not in the presence of joint instability. Intra-articular supplementation with lubricin increased lubricin gene expression from cartilage in the anterior cruciate ligament transection rat model (28), demonstrating how auto-induction could be beneficial for sustaining lubricin expression following trauma.

The results of the present study reveal that lubricin concentrations are increased in synovial fluid and that lubricin immunolocalization is increased at sites of traumatic osteochondral injury in horses. Other authors have hypothesized that PRG4 may be upregulated in OA as a repair mechanism, but that the endogenous response may still be insufficient to prevent disease progression (8). To our knowledge, this is

the first study to evaluate serial synovial fluid lubricin concentrations before and after osteochondral injury, revealing that synovial fluid lubricin concentrations increase shortly after injury, followed by a gradual return to baseline **(Figure 2.3*B*)**.

An approximately two-fold increase in lubricin synovial fluid concentrations have been detected following tibial fractures in humans (21), paralleling the 124% and 200% increases in lubricin concentrations detected in our naturally occurring and experimental OA cases, respectively **(Figure 2.2*C* and Fig 2.3*B*)**. However, to our knowledge, lubricin cartilage and tissue localization at sites of osteochondral fracture has not been previously evaluated. Here, we demonstrate pronounced increases in lubricin immunostaining in fibrocartilaginous repair tissue and in the healing osteochondral fragments **(Figure 2.6)**, suggesting that lubricin may be an important mediator in intra-articular fracture repair and fibrocartilaginous healing of damaged hyaline cartilage. Lubricin's biological effects extend beyond its role as a mechanical lubricant, with known roles in chondroprotection through inhibition of chondrocyte apoptosis (29), inhibition of protein deposition and pannus formation (30) and normalization of subchondral bone remodeling (9). Furthermore, PRG4 expression has been shown to regulate transcriptional networks involved in chondrocyte hypertrophy and catabolism (8).

As has been previously reported in sheep models of OA (17), our studies demonstrated decreased PRG4 gene expression in naturally occurring equine OA cartilage. Interestingly, we observed significant elevations in PRG4 gene expression

from synovial tissues of horses with naturally occurring OA as compared to controls **(Figure 2.2*A*)**. These elevations in PRG4 gene expression are consistent with increased synovial membrane lubricin immunostaining in OA joints in our experimental fragment model of OA **(Figure 2.4,6)**. Although no significant differences were observed in PRG4 gene expression from synovial tissues in the experimental model **(Figure 2.3*A*)**, it is possible that 70-days after injury is too late to detect any change. The equine osteochondral fragment model of OA induces synovitis and progressive OA changes; however, it is a self-limiting model of OA without major instability. Based upon peak synovial fluid lubricin concentrations at approximately 21 days post-injury, we would predict synovial membrane PRG4 gene expression to peak prior to 21 days.

Limitations of the naturally occurring OA cohort are the qualitative nature of the OA grading criteria and limited information on duration of injury prior to presentation. In order to address this constraint and because there were no statistically significant differences in the lubricin ELISA data between the three severity grades, all OA grades (1,2, and 3) were combined together and compared to normal joints. OA grading criteria and duration of injury were not limitations in the experimental OA study, and lubricin ELISA results revealed similar, more than 2-fold increases in lubricin concentrations in arthritic joints as compared to healthy joints. Another limitation of this study is the lack of information about the molecular degradation of lubricin within the joint. Monoclonal antibody 9G3 detects a combined peptide and O-linked glycosylated epitope within lubricin's KEPAPTTTT mucin-rich domain

which is relatively insensitive to the presence of sialic acids but sensitive to the presence of *O*-linked glycans (31). The peanut agglutinin lectin used in the ELISA analysis also binds to lubricin's mucin-rich domain. Recent glycosylation analysis of equine synovial fluid lubricin suggests that equine joints with OA or osteochondral fragmentation have elevated levels of non-sialylated *O*-linked glycans as compared to normal joints (12.5 ± 5.1 and 11.1 ± 4.5 vs. 10.1 ± 1.9, respectively) (32). Although it is possible that differences in sialylation may contribute in part to differences detected using the mAb 9G3 PNA ELISA, the 2% differences in non-sialylated variants between normal joints and OA joints were quite modest compared to the 3-fold increases observed in synovial fluid lubricin concentrations in our study. Another limitation of this study is the lack of information about the molecular degradation of lubricin within the joint. Although it is clear that an *O*-linked glycosylated epitope of lubricin is increased in both experimental and naturally occurring OA in horses, it is not obvious whether this epitope is still attached to functional N- and C-terminal domains. A C-terminal proteolytic cleavage site has been detected in equine synovial fluid lubricin; however, no differences were observed in the abundance of endogenously cleaved peptides in healthy joints vs. those with osteochondral fragmentation (32). We cannot rule out the possibility that the elevated lubricin detected in synovial fluid has been sheared off of the articular cartilage and does not reflect increased lubricin synthesis in response to injury. Nonetheless, the ELISA data suggesting that lubricin is increased in arthritis, coupled with the increase in PRG4 synovial membrane gene expression and the increased lubricin immunostaining in OA joints provides supporting evidence that lubricin expression may be upregulated in

post-traumatic arthritis. Likewise, increased lubricin immunostaining does not necessarily represent increased synthesis from the cartilage and synovial membrane but could represent increased affinity of lubricin binding to injured tissues in disease.

Lubricin tissue localization was most pronounced in repair tissue at sites of re-integration of the osteochondral fragment with the parent radial carpal bone **(Figure 2.6*B*)**. Not only was lubricin surface staining more intense, but lubricin localized to repair tissue several cells layers deep within the fibrocartilage, in some cases extending more than 1μm deep to the tissue surface. Lubricin immunostaining was also increased within the vascular lining cells of the bone of the osteochondral fragment. These findings suggest that lubricin, in addition to enhancing lubrication of immature fibrocartilaginous repair tissue after intra-articular fracture, may possess anabolic functions distinct from its lubricating properties in joint trauma. Lubricin has been localized to multiple fibrocartilaginous structures, including meniscus (33, 34), intervertebral disc (35, 36) and the fibrocartilaginous disc of the temporomandibular joint (37–39), and it has been shown to protect temporomandibular joint surfaces from degradation (40). In injured meniscal fibrocartilage, lubricin expression is decreased as compared to in intact menisci (41). Lubricin has also been found in a variety of other tissues, including tendon, ligament, muscle and skin, and variations in distribution and splicing have led authors to purport tissue-specific functions, ranging from lubrication to anti-adhesion to regulation of cell growth (42).

Lubricin is an important boundary lubricant with diverse biological roles within the synovial environment. Lubricin supplementation has been proposed as a

novel OA therapy in humans, with mounting evidence for its clinical application. Prior to translating lubricin supplementation therapy into humans, further investigation is warranted in clinically relevant large animal models such as the horse and in distinct types of joint injury, including ligamentous instability and intra-articular fracture. The majority of evidence indicating that lubricin is depleted in joint injury has been derived from rodent models of knee instability (4–6) or in clinical cases of cruciate injury and late-stage knee OA in humans (4, 7), whereas data in human patients with intra-articular tibial plateau fracture (21) and in our equine osteochondral fragmentation model, lubricin is increased. One possibility that remains to be investigated is whether lubricin tribosupplementation may be indicated in instability injuries but not osteochondral fragmentation or intra-articular fracture. In addition, further mechanistic work is required to understand what role lubricin has following intra-articular fracture and fibrocartilaginous repair of damaged hyaline cartilage.

References

1. Bahabri S, et al. (1998) The camptodactyly-arthropathy-coxa vara-pericarditis syndrome: clinical features and genetic mapping to human chromosome 1. *Arthritis Rheum* 41(4):730–735.

2. Marcelino J, et al. (1999) CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet* 23(3):319–322.

3. Ludwig TE, McAllister JR, Lun V, Wiley JP, Schmidt T a (2012) Diminished cartilage-lubricating ability of human osteoarthritic synovial fluid deficient in proteoglycan 4: Restoration through proteoglycan 4 supplementation. *Arthritis Rheum* 64(12):3963–71.

4. Elsaid K, et al. (2008) Decreased Lubricin Concentrations and Markers of Joint Inflammation in Synovial Fluids from Patients with Anterior Cruciate Ligament Injury. *Arthritis Rheum* 58(6):1707–1715.

5. Elsaid KA, Machan JT, Waller K, Fleming BC, Jay GD (2009) The impact of anterior cruciate ligament injury on lubricin metabolism and the effect of inhibiting tumor necrosis factor alpha on chondroprotection in an animal model. *Arthritis Rheum* 60(10):2997–3006.

6. Teeple E, et al. (2008) Coefficients of Friction, Lubricin, and Cartilage Damage in the Anterior Cruciate Ligament-Deficient Guinea Pig Knee. 26(2):231–237.

7. Kosinska MK, et al. (2015) Articular Joint Lubricants during Osteoarthritis and Rheumatoid Arthritis Display Altered Levels and Molecular Species. *PLoS One* 10(5):e0125192.

8. Ruan MZC, et al. (2013) Proteoglycan 4 expression protects against the development of osteoarthritis. *Sci Transl Med* 5(176):176ra34.

9. Cui Z, Xu C, Li X, Song J, Yu B (2015) Treatment with recombinant lubricin attenuates osteoarthritis by positive feedback loop between articular cartilage and subchondral bone in ovariectomized rats. *Bone* 74:37–47.

10. Teeple E, et al. (2011) The Effects of Supplemental Intra-Articular Lubricin and Hyaluronic Acid on the Progression of Post-Traumatic Arthritis in the Anterior Cruciate Ligament Deficient Rat Knee. *Am J Sports Med* 39(1):164–172.

11. Jay GD, et al. (2010) Prevention of Cartilage Degeneration and Restoration of Chondroprotection by Lubricin Tribosupplementation in the Rat Following ACL Transection. *Arthritis Rheum* 62(8):2382–2391.

12. Flannery CR, et al. (2009) Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin. *Arthritis Rheum* 60(3):840–7.

13. Frisbie DD, Cross MW, McIlwraith CW (2006) A comparative study of articular cartilage thickness in the stifle of animal species used in human pre-clinical studies compared to articular cartilage thickness in the human knee. *Vet Comp Orthop Traumatol* 19(3):142–6.

14. McIlwraith CW, Fortier LA, Frisbie DD, Nixon AJ (2011) Equine Models of Articular Cartilage Repair. *Cartilage* 2(4):317–326.

15. Chu CR, Szczodry M, Bruno S (2010) Animal models for cartilage regeneration and repair. *Tissue Eng Part B Rev* 16(1):105–15.

16. McIlwraith CW, Frisbie DD, Kawcak CE (2012) The horse as a model of naturally occurring osteoarthritis. *Bone Joint Res* 1(11):297–309.

17. Young AA, et al. (2006) Proteoglycan 4 downregulation in a sheep meniscectomy model of early osteoarthritis. *Arthritis Res Ther* 8(2):R41.

18. Barton KI, et al. (2013) Characterization of proteoglycan 4 and hyaluronan composition and lubrication function of ovine synovial fluid following knee surgery. *J Orthop Res* 31(10):1549–54.

19. Desrochers J, Amrein MW, Matyas JR (2013) Microscale surface friction of articular cartilage in early osteoarthritis. *J Mech Behav Biomed Mater* 25:11–22.

20. Antonacci JM, et al. (2012) Effects of equine joint injury on boundary

lubrication of articular cartilage by synovial fluid: role of hyaluronan. *Arthritis Rheum* 64(9):2917–26.

21. Ballard BL, et al. (2012) Effect of Tibial Plateau Fracture on Lubrication Function and Composition of Synovial Fluid. *J Bone Jt Surg* 94(10):e64 1.

22. Neu CP, Reddi AH, Komvopoulos K, Schmid TM, Di Cesare PE (2010) Increased friction coefficient and superficial zone protein expression in patients with advanced arthritis. *Arthritis Rheum* 62(9):2680–2687.

23. Atarod M, Ludwig TE, Frank CB, Schmidt T a., Shrive NG (2015) Cartilage boundary lubrication of ovine synovial fluid following anterior cruciate ligament transection: a longitudinal study. *Osteoarthr Cartil* 23(4):640–647.

24. Kamm JL, Nixon a J, Witte TH (2010) Cytokine and catabolic enzyme expression in synovium, synovial fluid and articular cartilage of naturally osteoarthritic equine carpi. *Equine Vet J* 42(8):693–9.

25. Frisbie DD, Ghivizzani SC, Robbins PD, Evans CH, McIlwraith CW (2002) Treatment of experimental equine osteoarthritis by in vivo delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 9(1):12–20.

26. Teeple E, Jay GD, Elsaid K a, Fleming BC (2013) Animal models of osteoarthritis: challenges of model selection and analysis. *AAPS J* 15(2):438–46.

27. Musumeci G, et al. (2014) Physical activity ameliorates cartilage degeneration in a rat model of aging: A study on lubricin expression. *Scand J Med Sci Sport*:222–230.

28. Elsaid K a., et al. (2012) The impact of forced joint exercise on lubricin biosynthesis from articular cartilage following ACL transection and intra-articular lubricin's effect in exercised joints following ACL transection. *Osteoarthr Cartil* 20(8):940–948.

29. Waller K a, et al. (2013) Role of lubricin and boundary lubrication in the prevention of chondrocyte apoptosis. *Proc Natl Acad Sci U S A* 110(15):5852–7.

30. Rhee DK, et al. (2005) The secreted glycoprotein lubricin protects cartilage surfaces and inhibits synovial cell overgrowth. *J Clin Invest* 115(3):622–31.

31. Ai M, et al. (2015) Anti-Lubricin Monoclonal Antibodies Created Using Lubricin-Knockout Mice Immunodetect Lubricin in Several Species and in Patients with Healthy and Diseased Joints. *PLoS One* 10(2):e0116237.

32. Svala E, et al. (2015) Characterisation of lubricin in synovial fluid from horses with osteoarthritis. *Equine Vet J*. doi:10.1111/evj.12521.

33. Musumeci G, et al. (2014) Lubricin expression in human osteoarthritic knee meniscus and synovial fluid: a morphological, immunohistochemical and biochemical study. *Acta Histochem* 116(5):965–72.

34. Zhang D, et al. (2011) Lubricin distribution in the torn human anterior cruciate ligament and meniscus. *J Orthop Res* 29(12):1916–1922.

35. Shine KM, Spector M (2008) The presence and distribution of lubricin in the caprine intervertebral disc. *J Orthop Res* 26(10):1398–1406.

36. Shine KM, Simson J a, Spector M (2009) Lubricin distribution in the human intervertebral disc. *J Bone Joint Surg Am* 91(9):2205–2212.

37. Leonardi R, Almeida LE, Loreto C (2011) Lubricin immunohistochemical expression in human temporomandibular joint disc with internal derangement. *J Oral Pathol Med* 40(7):587–592.

38. Leonardi R, Rusu MC, Loreto F, Loreto C, Musumeci G (2012) Immunolocalization and expression of lubricin in the bilaminar zone of the human temporomandibular joint disc. *Acta Histochem* 114(1):1–5.

39. Wei L, Xiong H, Li B, Cheng Y, Long X (2010) Boundary-lubricating ability and lubricin in synovial fluid of patients with temporomandibular joint disorders. *J Oral Maxillofac Surg* 68(10):2478–2483.

40. Hill A, Duran J, Purcell P (2014) Lubricin Protects the Temporomandibular Joint Surfaces from Degeneration. *PLoS One* 9(9):e106497.

41. Musumeci G, Loreto C, Carnazza ML, Cardile V, Leonardi R (2013) Acute injury affects lubricin expression in knee menisci: An immunohistochemical study. *Ann Anat* 195(2):151–158.

42. Sun Y, et al. (2006) Mapping lubricin in canine musculoskeletal tissues. *Connect Tissue Res* 47(4):215–221.

CHAPTER 3

GALECTIN-3 REINFORCES THE LUBRICATING BOUNDARY LAYER OF ARTICULAR CARTILAGE

Abstract

Lubricin is a mucinous, synovial fluid glycoprotein that enables near frictionless joint motion via adsorption to the surface of articular cartilage and its lubricating properties in solution. Extensive *O*-linked glycosylation within lubricin's mucin-rich domain is critical for its boundary lubricating function; however, it is unknown exactly how glycosylation facilitates cartilage lubrication. Here, we find that the lubricin glycome is enriched with terminal β-galactosides, known binding partners for a family of multivalent lectins called galectins. Of the galectin family members present in synovial fluid, we find that galectin-3 is a specific, high-affinity binding partner for lubricin. Considering the known ability of galectin-3 to crosslink glycoproteins, we hypothesized that galectins could augment lubrication via biomechanical stabilization of the lubricin boundary layer. We find that competitive inhibition of galectin binding results in lubricin loss from the cartilage surface, and addition of multimeric galectin-3 enhances cartilage lubrication. We also find that galectin-3 has low affinity for the surface layer of osteoarthritic cartilage and has reduced affinity for sialylated O-glycans, a glycophenotype associated with inflammatory conditions. Together, our results suggest that galectin-3 reinforces the lubricin boundary layer; which, in turn, enhances cartilage lubrication and may delay the onset and progression of arthritis.

Introduction

Lubricin is a large, mucinous glycoprotein critical for boundary lubrication of articular cartilage. Lubricin protects against cartilage damage, with intra-articular lubricin supplementation mitigating the severity and progression of post-traumatic arthritis in animal models (1–5), and patients with a genetic deficiency in lubricin production developing precocious multiple joint failure (6, 7). Originally isolated from synovial fluid (8) and expressed by synovial fibroblasts (9) or as the variant superficial zone protein by superficial zone chondrocytes (9), lubricin is a semi-flexible rod with an extensive mucin-rich central domain. The mucin-rich domain of lubricin is composed of a repeating degenerate sequence of EPAPTTK residues that undergo extensive glycosylation (30-50% w/w basis) with *O*-linked oligosaccharide chains (10, 11). Effective boundary lubrication by lubricin is dependent upon both its ability to adsorb to articular cartilage (12, 13) and the presence of *O*-linked β(1-3)Gal-GalNAc oligosaccharides (14); however, it is still not fully understood how lubricin interacts with other cartilage matrix or synovial fluid components and how the boundary layer is either stabilized or replenished during the lubrication cycle.

Glycans are in large part responsible for the boundary lubricating properties of lubricin. Mucinous glycoproteins, like lubricin, are defined by their densely clustered pendant glycans that initiate with N-acetylgalactosamine saccharides covalently linked to the polypeptide through the oxygen molecules of serine and threonine side chains (*O*-linked). Site-specific glycan analysis of lubricin identified 168 independent *O*-

glycosylation sites (11), of which galactosamine, galactose and N-acetylneuraminic acid comprise 98% of the carbohydrate residues (10). Although the specific mechanisms whereby lubricin mediates boundary lubrication are still debated, several authors propose that repulsive hydration forces (15, 16) or charge repulsion (16), mediated by the negatively charged sialic acids of lubricin's glycans, are primary forces governing lubrication. However, removal of sialic acids alone only decreased boundary lubrication by 19.3% (14) whereas partial removal of β(1-3)Gal-GalNAc oligosaccharides resulted in a 77.2% reduction in lubricating ability (14). These findings suggest that glycans may have functions in lubrication beyond their accepted roles in hydration and charge repulsion.

Changes in glycosylation are a hallmark of disease states characterized by chronic inflammation (17). Site-specific glycopeptide analysis of lubricin has revealed heterogeneity in glycan structure at specific sites within lubricin's mucin-rich domain (11), and significant variations in the concentration of sialic acid residues on lubricin have been measured in clinical samples (18). Recent studies characterizing synovial fluid lubricin from patients with osteoarthritis (OA) and rheumatoid arthritis (RA) (19) and synovial fluid lubricin from horses with normal joints, OA joints and osteochondral fragmentation (20) have revealed changes in the glycosylation of lubricin in disease. Given the importance of lubricin's *O*-linked β(1-3)Gal-GalNAc oligosaccharides (G D Jay, Harris, & Cha, 2001) in mediating cartilage lubrication, inflammation-induced changes in the glycosylation of lubricin could potentially have a

major impact on its interaction with other synovial fluid constituents and boundary lubricating ability (11, 19).

A considerable body of evidence is accumulating to suggest that lubricin may be an effective therapy for the treatment of osteoarthritis (OA). Lubricin synovial fluid concentrations are decreased in experimental rodent models of post-traumatic OA, including the anterior cruciate ligament (ACL)-deficient guinea pig stifle (21) and the ACL-deficient rat stifle (22). Furthermore, replenishing lubricin, either through gene therapy (2) or administration of recombinant lubricin (3–5, 23) delays the development and progression of arthritis in rodent OA models. However, despite the critical role that *O*-linked glycosylation plays in mediating boundary lubrication, little effort has been devoted to characterizing the glycosylation of administered recombinant lubricin or the glycoprotein product of lubricin gene therapy approaches. Thus, it is unknown what glycan composition of lubricin could be used clinically to derive the optimal therapeutic benefit.

In biology, a major function of glycans is to mediate biomolecular interactions; however, little attention has been focused on elucidating mechanisms by which glycans may facilitate lubrication distinct from repulsive or hydration mechanisms. Glycans mediate protein-protein interactions through lectins such as siglecs, selectins and galectins. Lectin glycan interactions play a critical role in several biological contexts, ranging from lattice formation to the receptor signaling and glycoprotein

clustering and assembly (24–26).

Therefore, our objective was to examine the lubricin glycome in order to identify potential synovial fluid binding partners for lubricin. Based upon the enrichment of terminal β-galactoside residues and the high density of β(1-3)Gal-GalNAc T-antigens in normal equine synovial fluid lubricin, we hypothesized that synovial fluid galectins may be binding partners for lubricin. Here, we find that galectin-3 is a specific, high-affinity binding partner for lubricin, capable of enhancing boundary lubrication by cross-linking oligosaccharide moieties on lubricin and stabilizing the lubricin lattice.

Materials and Methods (Abbreviated)

Synovial fluid purification and glycophenotyping

Synovial fluid was obtained from healthy joints of two young horses and purified using diethylaminoethyl (DEAE) affinity chromatography using previously described methodology (3), with some modifications. The O-linked glycans from equine synovial fluid lubricin from healthy carpal joints were characterized with mass spectrometry. Lubricin and galectin immunohistochemistry was performed on osteochondral sections from healthy equine middle carpal joints, and confocal and multi-photon imaging of equine femoral condyle cartilage explants was performed to

assess fluorescent lectin binding and lubricin and galectin binding.

Galectin cloning and recombinant production

RNA was purified from equine kidney tissue, and gene-specific primers were designed against the NCBI predicted sequences of equine galectin-1 and galectin-3. Recombinant human galectin-1 and galectin-3 constructs were obtained from C. Bertozzi and, along with the galectin-3C mutant and equine galectins, were recombinantly expressed and purified using β-lactosyl sepharose affinity chromatography similar to previously described methods (27).

Lubricin galectin binding kinetics

Lubricin purified from normal equine synovial fluid or bovine asialofetuin was coated to high-binding, 96-well ELISA plates. Recombinant, biotinylated equine galectin-1 or galectin-3 was added to each well at serial concentrations, and affinities were measured using streptavidin-HRP, TMB and a monochromator.

Lubricin deglycosylation

FPLC-purified equine synovial fluid lubricin was digested with the following combinations of deglycosylation enzymes: i) N-glycanase® PNGase F, ii) Sialidase A™, or iii) O-glycanase® (Endo-α-N-Acetylgalactosaminidase) + prO-LINK

Extender™ (β(1-4) Galactosidase + β-N-Acetylglucosaminidase) (PROzyme®, Glyko®, Hayward, CA) listed in **Table 3.1.**

Cartilage tribometry

Articular cartilage explants were aseptically harvested from the femoropatellar groove of young bovine stifles and incubated in PBS or recombinant human galectin-1, galectin-3, or galectin-3C. Tribological testing was performed using a previously described custom friction apparatus (28).

Supplemental Methods

Ethics Statement

Tissue harvesting protocols for this study were approved by the Cornell University Institutional Animal Care and Use Committee (Protocol Number: 2011-027). All sample collection was performed following humane euthanatization of horses using sodium pentobarbital. For mechanical testing, cartilage from calf knees was obtained from a local abattoir. The proper euthanasia methodology for the abattoir was approved by the USDA.

Table 3.1 Enzymes used to deglycosylate equine synovial fluid lubricin

Enzyme Name	**Function**
N-Glycanase® (PNGase F)	Removes all N-glycans
Sialidase A™	Removes sialic acids
O-Glycanase®	Removes O-glycans after removal of extended structures
β(1-4)-Galactosidase	Removes glycans (e.g. polylactosamines)
β-N-Acetylglucosaminidase	Removes glycans (e.g. polylactosamines)

Purification of Equine Synovial Fluid Lubricin

Following euthanasia via overdose of sodium pentobarbital, the elbow, carpal, fetlock, tarsal and stifle joints of two young (4 and 6-year-old) horses free from clinical signs of joint disease were clipped and aseptically prepared with povidone iodine scrub (Betadine, Purdue Pharma L.P., Stamford, CT) and alcohol. Synovial fluid was aspirated from each joint using an 18 gauge, 3.5-inch spinal needle (BD Medical, Franklin Lakes, NJ) and placed in 15mL polypropylene conical tubes for clarification via high-speed centrifugation at 10,000xg for 1 h. The synovial fluid supernatants were transferred to fresh polypropylene tubes and stored at -80°C. Clarified synovial fluid was thawed, pooled and purified using previously described methodology, (3) with some modifications. Equine synovial fluid was concentrated five-fold using 100kD molecular weight cut off centrifugal filter devices (Amicon® Ultra, Millipore, Tullagreen, Carrigtwohill, IRL) by centrifugation at 4,000xg, 4°C. The mucinous synovial fluid retentates were re-suspended in 50mM NaAc buffer, pH 5.5, with proteolytic inhibitors, to the original synovial fluid volume. Hyaluronic acid digestion was performed using Streptomyces hyaluronidase (Calbiochem®, EMD Chemicals, San Diego, CA) at a final concentration of 1U/mL of re-suspended synovial fluid at 4°C for 16 h on an end-over-end rocker. The hyaluronidase-digested synovial fluid samples were centrifuged at 4,000xg, 4°C for 5 minutes to pellet debris, and the supernatant was purified using a HiTrap™ diethylaminoethyl (DEAE) fast flow sepharose column (GE Healthcare Life Sciences, Little Chalfont, UK) for fast protein liquid chromatography (FPLC). Lubricin was eluted from the DEAE column

after rinsing with 50mM NaAc using a 200mM step-wise elution gradient from 200mM to 1M NaCl. Each 200mM fraction was loaded onto a 4-20% tris-glycine gradient gel (Bio-Rad, Hercules, CA) for reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie staining (Teknova, Hollister, CA). The 400-600mM fraction with a single prominent band at >250kD was dialyzed against phosphate buffered saline (PBS) with proteolytic inhibitors at 4°C for 36 h, with 3 exchanges of PBS. Immunoblotting of all FPLC-purified fractions was performed using a polyclonal antibody raised in rabbit and directed against the synthetic peptide CLPNIRKQPDGYDYYAFSKDQ corresponding to amino acids 1356-1374 of the C-terminus of human lubricin (ab28484, Lot: GR116636-3, Abcam®, Cambridge, UK), in addition to the monoclonal antibody 9G3 raised in mouse and directed against the mucin domain of human lubricin (MABT401; EMD Millipore), which is highly conserved across species. Both antibodies were predicted to cross-react with equine lubricin based on sequence homology of the C-terminus and mucin-rich region of lubricin, and antibodies were validated in equine using synovial fluid as a positive control and serum as a negative control. The final protein concentration of lubricin fractions was quantitated using a bicinchoninic acid protein assay (Thermo Scientific, Rockford, IL) using bovine serum albumin as the standard and read out on a microplate reader (Tecan Safire, Mannedorf, Switzerland) at 562nm absorbance.

MALDI/TOF-MS Glycosylation Analysis of Synovial Fluid Lubricin

For lubricin glycosylation analysis, synovial fluid was aspirated in similar fashion from the carpal joints of a healthy 5-year old horse and clarified by centrifugation. The >100kD synovial fluid retentate was digested at with 1U/mL of Streptomyces hyaluronidase in 50mM NaAc buffer, pH 5.5 with proteolytic inhibitors overnight at 4°C. Lubricin was purified using DEAE anion-exchange resin, rinsing with 350mM NaCl and eluting in 1M NaCl. The purified lubricin was de-salted overnight using a 50kD tube dialyzer (G-Biosciences, St. Louis, MO), followed by removal of fibronectin via gelatin separopore® 4B purification (Bioworld, Dublin, OH) and elution in ultrapure water. For mass spectrometry, the lubricin samples were dried and subjected to beta-elimination to cleave O-linked glycans. O-linked glycans were cleaved with NaOH and serine-linked GalNAc was reduced with $NaBH_4$. The reaction was neutralized with acetic acid, desalted, and cleaned of borates prior to permethylation. O-linked glycans were permethylated for structural characterization by mass spectrometry, dissolved with methanol and crystallized with α-dihydroxybenzoic acid, 20mg/mL, in 50% methanol: water. Glycan analysis was performed in the positive ion mode by MALDI-TOF/TOF-MS using AB SCIEX TOF/TOF 5800 (Applied Biosystems, Thermo-Scientific, Waltham, MA).

Lubricin and Galectin Cartilage Immunohistochemistry

Osteochondral blocks were harvested from the carpal bones of the middle carpal joint of a healthy 4-year old horse, fixed in 4% paraformaldehyde, de-calcified in 10% EDTA for three weeks and embedded in paraffin. Thin (5 μm) sagittal sections were obtained for immunohistochemical staining of lubricin and galectins. Sections were deparaffinized, re-hydrated in three changes of xylene and serial alcohol, followed by treatment with 1% hyaluronidase (Sigma-Aldrich, St. Louis, MO) solution in 20mM sodium acetate at 37°C for 30 min. Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 30 min. After blocking in normal goat or rabbit serum, a mouse monoclonal antibody against lubricin (MABT401; EMD Millipore) or a goat polyclonal antibody against galectin-3 (sc-19280; Santa Cruz) was added at 1:200 or 1:100 dilutions and incubated at room temperature for 1 hr. After three washes in PBS-T (0.1%), the sections were incubated with a biotinylated goat anti-mouse IgG (Vectastain, Vector Labs) or a biotinylated rabbit anti-goat IgG (Vectastain, Vector Labs). After 3 rinses in PBS, immunodetection was performed using the Vectastain ABC Kit and ImmPACT DAB reagent (Vector Laboratories). The sections were rinsed in PBS, counterstained using hematoxylin (Fisher) and imaged with a 20x objective using an Olympus DP80 (Olympus) camera and an Olympus IX73 microscope (Olympus).

Confocal and Multi-photon Imaging of Cartilage Explants

Articular cartilage biopsies were harvested from the medial femoral condyle of adult equine stifle joints using a 6mm biopsy punch (Miltex, York, PA) and immediately frozen at -80°C. For lectin imaging, cartilage explants were thawed to room temperature, rinsed in PBS and incubated for 1 hour at 4°C in 10ug/mL FITC-conjugated lectin (Vector Labs, Burlingame, CA), or Atto488-conjugated MAL-II lectin. MAL-II (Vector Labs) was conjugated with Atto488 in the presence of 150mM β-lactose. Explants were rinsed in PBS and stained with 1ug/mL Hoechst 33342 (Life, Thermo Scientific, Waltham, MA) for 20 minutes to stain chondrocyte nuclei, followed by several rinses in PBS. Explants were imaged in glass dishes (MatTek, Ashland, MA) using a Zeiss inverted laser scanning microscope with a 40X water immersion objective. For fluorescent galectin and/or lubricin staining, explants were first incubated for 1 hour at 4°C in 100mM β-lactose to extract endogenous galectins. Following re-equilibration in PBS, each explant was incubated in 10ug/mL of A647-labeled galectin-1 or galectin-3 in PBS for 1 hour at room temperature. As a control for non-specific binding, explants were also incubated with A647-labeled galectin-1 or galectin-3 in the presence of 300mM β-lactose. For lubricin staining, explants were incubated in 10ug/mL of A568-labeled anti-lubricin mAb (MABT401, EMD Millipore). To compare lubricin staining following β-lactose removal of galectins, explants were incubated in either PBS or PBS + 100mM β-lactose at 4°C for 12 hours, followed by 3 rinses in PBS, incubation in 10ug/mL of A568-labeled anti-lubricin mAb for 1 hour, and Hoechst staining. Explants were rinsed in PBS, and z-stacks

were obtained for four regions of interest on five separate explants for quantitation. All experiments were performed in triplicate.

Cloning, Expression and Purification of Galectins

Human galectin-1, galectin-3 and galectin-3C. The N-terminal truncation mutant, Gal-3C, was made through PCR amplification of a human Gal3 template with forward (5'- GGCAGGATCCTACCCTGGAGCACCTGGAGCTTATC -3') and reverse (5'-GGCAGCGGCCGCTTATATCATGGTATATGAAGCACTG-3') primers that introduced 5' BamHI and 3' NotI restriction sites for cloning into pET21a. Recombinant human galectin-1 and galectin-3 constructs were obtained from C. Bertozzi and, along with the galectin-3C mutant, were expressed in XL1-Blue competent *E. coli*. Recombinant galectins were purified using β-lactosyl sepharose affinity chromatography similar to previously described methods (27). Briefly, Sepharose® 6B (Sigma-Aldrich, St. Louis, MO) was activated with 40% divinyl sulfone prepared in 0.5M Na_2CO_3, pH 11.0, followed by washing with distilled, deionized water and 0.5 M Na_2CO_3, pH 10.0, and conjugation with 40% β-lactose (Santa Cruz Biotechnologies, Santa Cruz, CA) in 0.5 M Na_2CO_3, pH 10.0. The conjugated lactosyl sepharose was incubated with 0.5M Na_2CO_3, pH 8.8 plus β-mercaptoethanol and equilibrated in PBS, pH 7.5 for storage at 4°C prior to use. Bacterial lysates were incubated with 20mL of β-lactosyl sepharose slurry per liter volume of bacterial culture in a 50mL conical tube for 3 h at 4°C. The slurry-lysate mixture was loaded onto a 100mL gravity column, and washed 3 times with 3 column

volumes of PBS + 8mM dithiothreitol (DTT). Galectins were eluted from the lactosyl sepharose resin with 1.5 column volumes of 0.1M β-lactose + 8mM DTT, followed by concentration with 3K (galectin-1) or 15K (galectin-3 and -3C) molecular weight cut off centrifugal filter devices (Amicon® Ultra, Millipore, Tullagreen, Carrigtwohill, IRL) to 3mL final volume. Galectins were centrifuged at 13,000rpm for 10 minutes, and the supernatant was loaded onto a HiLoad® 16/60 Superdex® 200 gel filtration column (GE Healthcare Life Sciences, Little Chalfont, UK) for FPLC. Fractions were analyzed by SDS-PAGE, followed by Coomassie staining (Teknova, Hollister, CA), and pure fractions yielding a 15kD band for galectin-1 or a 31kD band for galectin-3 were pooled and concentrated to 8mg/mL based on 280nm absorbance (0.602 for galectin-1 and 1.372 for galectin-3) for storage in 0.1M β-lactose + 8mM DTT at -80°C.

Equine galectin-1 and galectin-3. cDNA was isolated from kidney tissue harvested from a 19-year-old Thoroughbred mare following euthanatization. RNA was purified following renal tissue homogenization and lysis with Proteinase K (PerfectPure, 5PRIME, Gaithersburg, MD), followed by cDNA synthesis (SuperScript® First-Strand Synthesis, Invitrogen, Carlsbad, CA).

Gene-specific primers were designed against the predicted sequences of equine galectin-1 and galectin-3 from the National Center for Biotechnology Information (NCBI), with the equine galectin-1 forward primer designed to begin at nucleotide (nt) 292 of the predicted equine sequence based upon sequence homology between the

human homologue, translated as MACGLVASNLNKPGECL and the fact that the predicted equine galectin-1 mRNA sequence was 292bp longer than the human homologue. Thus, the galectin primers were designed as follows:
(Galectin-1 Fwd: 5'- ATGGCTTGTGGTCTGGTCGCCAGCA –'3,
Galectin-1 Rev: 5'- TCACTCAAAGGCCACACACTTGATCT –3',
Galectin-3 Fwd: 5'- ATGTCAGACGGTTTTTCGCTTA –3',
Galectin-3 Rev: 5'- TTATATCATAGTGTGCGAAGCACTG –3').

Bands corresponding to 408bp for galectin-1 and 718bp for galectin-3 were gel purified (QIAquick, QIAGEN GmbH, Hilden, Germany) and sub cloned from the pCR™2.1-TOPO® vector into a pET21a bacterial expression vector, followed by sequencing by the Cornell University Biotechnology Resource Center. Following sequencing, equine galectins-1 and -3 were recombinantly expressed in XL1-Blue competent *E. coli* and purified using lactosyl sepharose chromatography and FPLC gel filtration as previously described for the purification of human galectins-1, -3 and -3C. Equine galectins were concentrated to 8mg/mL based on 280nm absorbance, using extinction coefficients of 0.600 for galectin-1 and 1.336 for galectin-3 predicted from the equine galectin sequences on Expasy's ProtParam online tool. Following concentration, equine galectins were stored in 0.1M β-lactose + 8mM DTT and snap frozen at -80°C.

Measurement of Lubricin Galectin Binding Kinetics

Biotinylation of equine galectins. Recombinant equine galectin-1 and -3 were biotinylated in the presence of 200mM β-lactose + 8mM DTT to preserve the carbohydrate recognition domain binding capacity of galectins using an EZ-LINK™ NHS-PEG_4-Biotinylation Kit according to manufacturer instructions (Thermo Scientific, Rockford, IL). Following desalting, a small aliquot of galectins were saved in PBS for quantification of biotin incorporation by measuring the change in 500 nm absorbance before and after the addition of biotinylated galectins to HABA/Avidin Solution. The remaining biotinylated galectins were re-suspended in 200mM β-lactose + 8mM DTT and snap frozen at -80°C prior to performing binding assays. A HABA microplate assay was used to quantify equine galectin biotin incorporation, measured as 1.42 biotin molecules per molecule of galectin-1 and 2.63 biotin molecules per molecule of galectin-3.

Colorimetric plate assay. Lubricin purified from normal equine synovial fluid or bovine asialofetuin (Sigma-Aldrich, St. Louis, MO) was coated to high-binding, 96-well ELISA plates (Corning Inc., Corning, NY) in sodium carbonate buffer, pH 9.6, at 4°C and at a final concentration of 4ug/mL. After incubation for 16 hours, wells were aspirated and rinsed in PBS plus 0.1% Tween, followed by blocking with Pierce® Protein-Free (PBS) Blocking Buffer (Thermo Scientific, Rockford, IL). Recombinant, biotinylated equine galectin-1 or galectin-3 was added to each well in duplicate at concentrations ranging from 0.078ug/mL to 5ug/mL in PBS-T (0.1%) for 1hr at room

temperature, with and without β-lactose (Santa Cruz Biotechnologies, Santa Cruz, CA) at concentrations of 50, 150 or 500mM. After aspiration and rinsing in PBS-T (0.1%), high-sensitivity streptavidin-HRP (Thermo Scientific, Rockford, IL) was added at a 1:10,000 dilution in PBS-T (0.1%) for 30 minutes, followed by a final aspiration and rinse in PBS-T (0.1%). Finally, trimethylbenzidine reagent (Pierce, Rockford, IL) was added for 10 minutes, the reaction was stopped with 2N H_2SO_4, and absorbance was measured at 450nm with 540nm subtraction. Binding assays were performed similarly for FPLC-purified equine synovial fluid lubricin digested with Sialidase A™ (Glyko).

Lubricin Deglycosylation

Enzymatic reactions. Buffer exchange of FPLC-purified equine synovial fluid lubricin into ultrapure water was performed using spin desalting columns (Zeba™, Thermo Scientific, Rockford, IL), followed by concentration to 1mg/mL with 100K molecular weight cut off centrifugal filter devices (Amicon® Ultra, Millipore, Tullagreen, Carrigtwohill, IRL). Concentrated, buffer-exchanged purified lubricin was digested with the following combinations of deglycosylation enzymes: i) N-glycanase® PNGase F, ii) Sialidase A™, or iii) O-glycanase® (Endo-α-N-Acetylgalactosaminidase) + prO-LINK Extender™ (β(1-4) Galactosidase + β-N-Acetylglucosaminidase) (PROzyme®, Glyko®, Hayward, CA) listed in **Table 1**. Deglycosylation reactions were carried out according to the non-denaturing protocol in the manufacturer's instructions, using 50μg of lubricin per reaction in the presence

of 0.25M sodium phosphate, pH 7.0 at 37°C for 16 hrs.

Electrophoresis and SYPRO staining. Following deglycosylation, 5ug (10uL) of native lubricin and each lubricin reaction were resolved on a NuPAGE® Novex® 3-8% Tris-Acetate gel under denaturing conditions. Bands were detected using fluorescent SYPRO® Ruby gel stain and compared to HiMark™ pre-stained high molecular weight standards (Invitrogen, Carlsbad, CA) to assess the extent of deglycosylation.

Cartilage Tribometry

Articular cartilage explants were aseptically harvested from the femoropatellar groove of young bovine stifles and stored in PBS at -20°C prior to tribological testing. After thawing to room temperature, cylindrical samples (6 mm diameter by 2 mm high) were incubated in sterile PBS or recombinant human galectin-1, galectin-3, or galectin-3C at a concentration of 50μg/mL for 1 h. Cartilage samples were submerged in a bath of PBS or galectin for tribological testing using a custom friction apparatus. This testing method has been previously described (28). Briefly, the friction apparatus linearly oscillated each sample against a glass counterface three times at a controlled speed of 0.32mm/sec after allowing the cartilage sample to relax for 1 hour under an applied normal strain of 30%. Custom Matlab code (The Mathworks, Natick, MA) was used to calculate the mean equilibrium frictional coefficient (μ_{eq}) based on the final two oscillations. Tribometry data were compared using 2-sample student's t-

tests with $p < 0.05$ considered significant, and all values are reported as mean ± SEM.

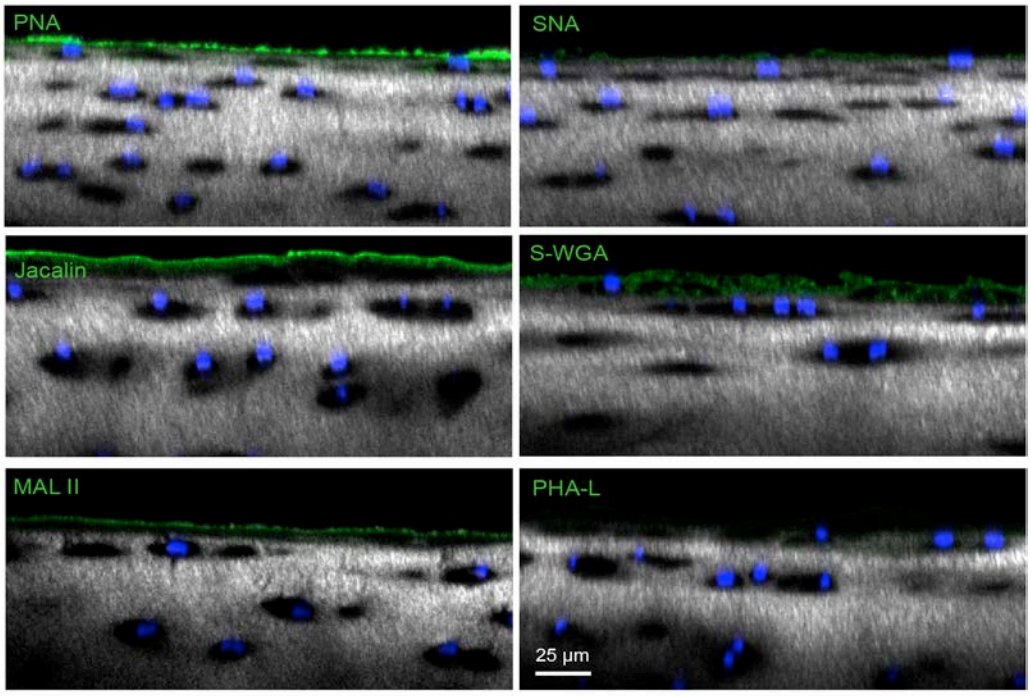
Results

Glycophenotype of the Articular Cartilage Boundary Layer

In order to determine what glycans were present on healthy cartilage, adult equine articular cartilage explants were incubated with fluorophore-conjugated lectins followed by nuclear staining with Hoechst 33342 and combined confocal and multiphoton microscopy **(Figure 3.1*A*)**. Peanut agglutinin (PNA), which binds to nonsialylated core-1 *O*-glycans, intensely stained the cartilage boundary layer. *Maackia amurensis* lectin II (MALII), which preferentially binds to α2-3 sialylated core-1 *O*-glycans, and jacalin, which binds to both sialylated and nonsialylated core-1 *O*-glycans, also labeled the surface layer of articular cartilage. Although there was faint staining of the superficial zone interterritorial matrix with succinylated wheat germ agglutinin (S-WGA) and phytohemagluttinin-leucoagglutinin (PHA-L), no appreciable boundary staining was present for either of these lectins, indicating that core-2 *O*-glycans and complex, branched *N*-glycans do not contribute substantially to the oligosaccharide population of the cartilage boundary layer. Weak staining of the cartilage surface by *Sambucus nigra* (SNA) was detected, indicating the presence of low quantities of α2-6 linked sialic acids. Taken together, these results suggest that the articular cartilage boundary layer is composed of a significant proportion of nonsialylated core-1 *O*-glycans.

Figure 3.1. The glycophenotype of lubricin. A) Healthy equine articular cartilage imaged with confocal and multiphoton microscopy (40X) following incubation with fluorophore-conjugated lectins. Chondrocyte nuclei are stained with Hoechst 33342 (blue), and collagen is imaged using second harmonic generation microscopy (gray). PNA boundary layer staining indicates the presence of nonsialylated core-1 O-glycans (Galβ(1-3)GalNAc). Both MAL II, which preferentially binds to α2-3 sialylated core-1 *O*-glycans, and jacalin, which binds to both sialylated and nonsialylated core-1 *O*-glycans, labeled the boundary layer of articular cartilage. Faint staining of the superficial zone interterritorial matrix with S-WGA was present, whereas no appreciable boundary staining was present for either S-WGA or PHA-L, demonstrating that core-2 *O*-glycans and complex, branched *N*-glycans do not contribute substantially to the boundary layer oligosaccharide layer. Lectins: PNA, peanut agglutinin; jacalin; MAL II, *Maackia amurensis* lectin II; SNA, *Sambucus nigra;* S-WGA, S-wheat germ agglutinin; PHA-L,leucoagglutinin. **B)** Relative ion intensity of O-linked oligosaccharides detected by MALDI/TOF-mass spectrometry in two healthy equine synovial fluid samples. Monosialylated structures predominate, followed by nearly equal distributions of disialylated and nonsialylated core-1 O-glycans.

A



B

Percentage of O-linked glycans detected from two lubricin samples with MALDI/TOF-MS.

Predicted glycan structure	Permethylated mass (mass+Na), m/z[1]	Lubricin ACJ Percent[2]	Lubricin MCJ Percent[2]
Core-1 O-glycan (T-Antigen)	534	22.8	22.8
Sialyl Core-1 O-glycan (Sialyl T-Antigen)	895	54.1	60.3
Core-2 O-glycan	983	0.7	1.4
Disialyl Core-1 O-glycan (Disialyl T-Antigen)	1256	22.4	15.5

[1]All masses are single charge.

[2]Calculated by dividing the area units of each O-linked glycan by the total area units of all O-linked glycans detected within a sample; average of 3 spectra.

Lubricin is the predominant carrier of O-linked glycans within the cartilage boundary layer. The glycosylation profiles of lubricin from human patients with OA and RA have been described (11, 19, 29). To further characterize lubricin's O-linked glycans present on lubricin in healthy joints, equine synovial fluid lubricin obtained from the healthy carpal joints of a 5-year old horse was analyzed for *O*-linked glycans using mass spectrometry **(Figure 3.1*B*)**. Monosialylated core-1 *O*-glycans predominated (57.2%), followed by approximately similar concentrations of nonsialylated (22.8%) and disialylated (19%) structures. Core-2 *O*-glycans were detected as <2% of the population of *O*-glycans.

Lubricin and Galectin Colocalization within the Cartilage Boundary Layer

Core 2 O-glycans are high affinity ligands for galectin-1 (30), and nonsialylated core 1 O-glycans are high affinity ligands for galectin-3 (31). Based on the observed glycophenotype of lubricin, we predicted that galectin-3 would strongly associate with the cartilage boundary layer. In order to investigate whether synovial fluid galectins may bind to the *O*-linked glycans present within the cartilage lamina splendens, we performed immunohistochemical staining for both lubricin and galectins. Immunostaining of osteochondral sections obtained from healthy equine carpal joints demonstrate intense staining of both lubricin and galectin-3 on the surface of articular cartilage **(Figure 3.2*A,B,E,F*)** whereas control sections incubated without primary antibodies demonstrate the absence of antigen-independent staining **(Figure 3.2*C,D*)**. Lubricin was immunodetected within superficial zone chondrocytes,

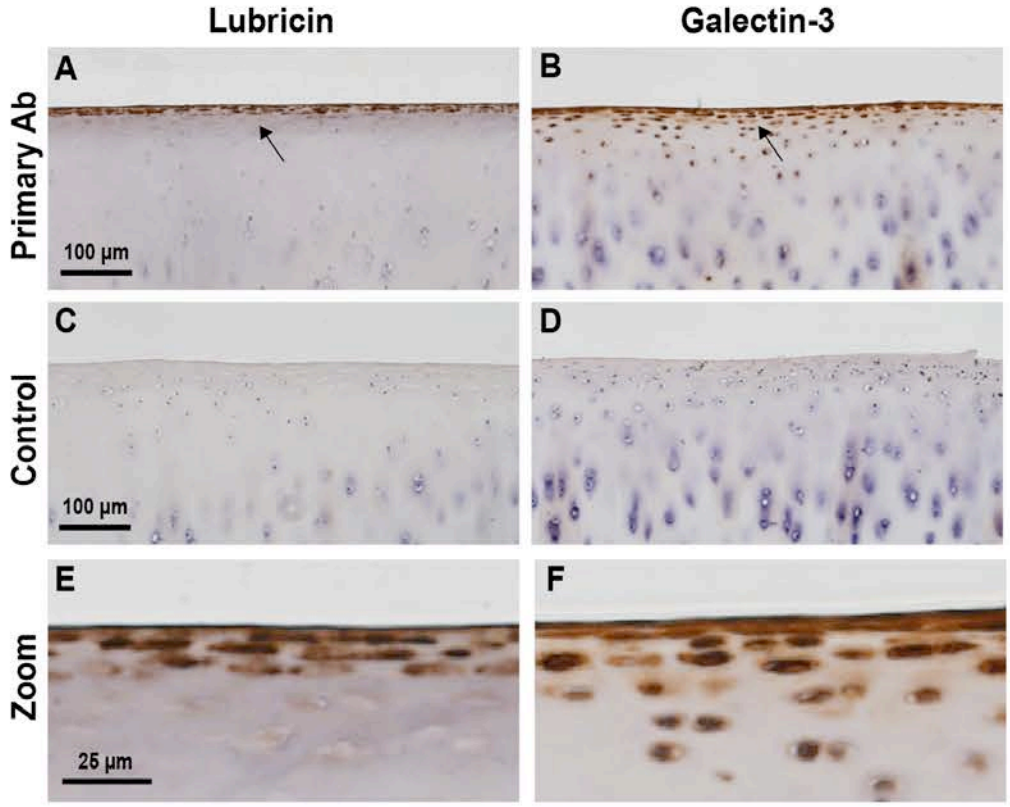


Figure 3.2. Lubricin and galectin-3 both localize to the cartilage surface. Immunohistochemical detection of lubricin and galectin-3 in photomicrographs of equine articular cartilage imaged at 20X **(A-D)** and magnified 5X **(E-F)**. **A,E)** Lubricin and **B,F)** galectin-3 are both detected on the boundary layer of articular cartilage. Lubricin immunoreaction is observed in superficial zone chondrocytes and as a distinct layer along the lamina splendens **(E)**, and galectin-3 immunoreaction is present within both superficial and middle zone chondrocytes and the lamina splendens **(F)**. **C-D)** Negative controls. Omission of primary antibody incubation confirms the absence of antigen-independent staining.

as has previously been reported in a variety of species (32–34), with intense staining localized primarily to a three-cell layer thick zone of flattened superficial zone chondrocytes. Lubricin staining was also present in deeper superficial zone chondrocytes, but chondrocytes from the middle and deep zones of cartilage were negative. Galectin-3 was immunodetected in both superficial and middle zone chondrocytes, with sporadic positive cells in deeper layers.

To demonstrate more definitively the colocalization of both lubricin and galectin-3 within the lamina splendens, articular cartilage explants from the femoral condyles of healthy and severe OA equine knee joints were incubated in the presence of Alexa647-conjugated recombinant equine galectins-1 and -3 and/or Alexa568-labeled anti-lubricin antibody. In healthy articular cartilage, lubricin and galectin-3 both intensely stained the boundary layer **(Figure 3.3*A*)**, whereas galectin-1 was nearly undetectable. Carbohydrate-specificity of galectin staining was demonstrated by incubation with a competitive inhibitor, β-lactose, which abolished galectin-3 surface binding. Galectin-3 localization to the lamina splendens was significantly diminished in severely degenerated OA cartilage **(Figure 3.3*A*)**. In severe OA cartilage explants, the second harmonic generation signal within the cartilage matrix was significantly diminished, presumably due to collagen loss. In healthy cartilage explants, lubricin and galectin-3 colocalized to the articular cartilage boundary layer **(Figure 3.3*B*)**, with focal, intense staining confined primarily to the lamina splendens **(Figure 3.3*D*)**. With its ability to form pentamers, galectin-3 has the potential to link

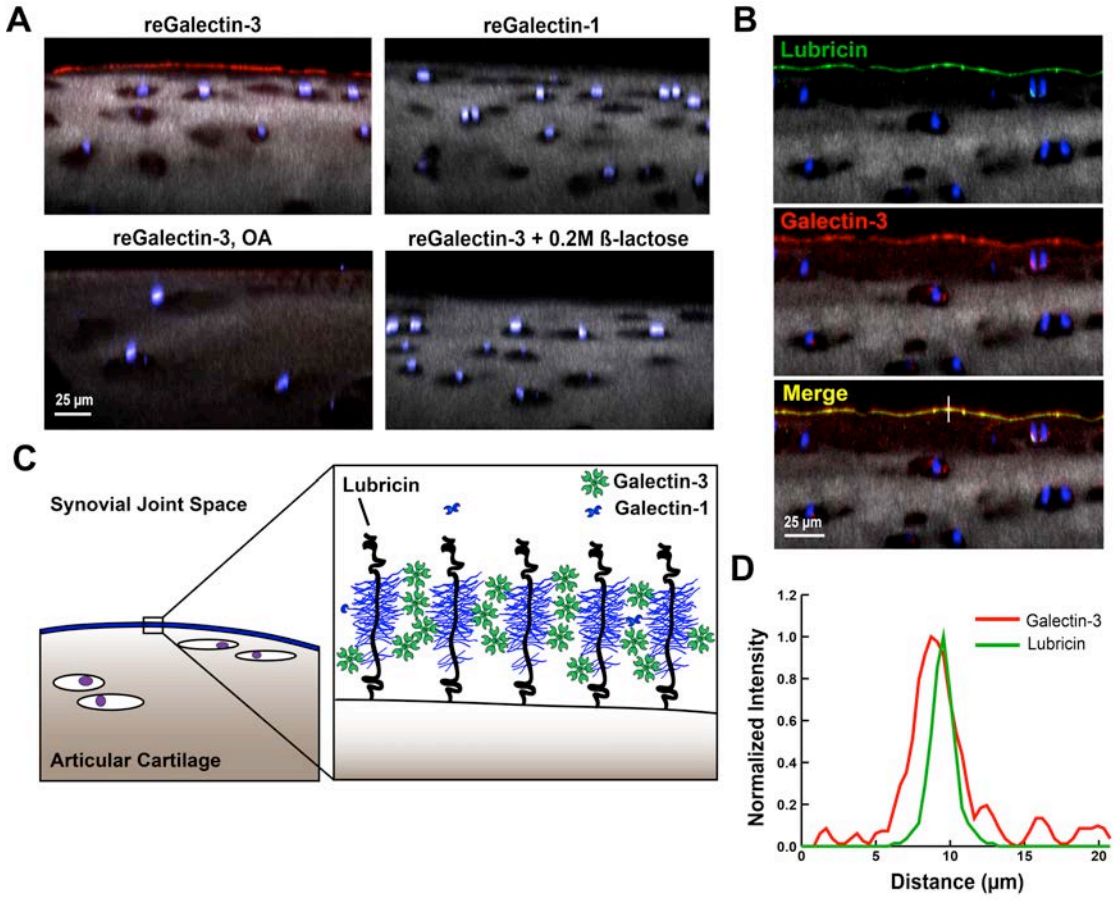


Figure 3.3. Galectin-3 binds to articular cartilage and colocalizes with lubricin. **A)** Equine articular cartilage explants imaged using confocal and multiphoton microscopy (40X) following incubation with Alexa647-conjugated galectins. Chondrocyte nuclei are stained with DAPI (blue), and collagen is imaged using second harmonic generation microscopy (gray). Galectin-3 prominently localizes to the boundary layer of healthy cartilage whereas galectin-1 does not. Galectin-3 staining is significantly decreased in joints with severe osteoarthritis (OA) and in the presence of 0.2M β-lactose, suggesting carbohydrate-specific binding. **B)** Lubricin stained with anti-lubricin mAb MABT401 and A647-conjugated galectin-3 binding to the surface of articular cartilage, demonstrating colocalization of lubricin and galectin-3. **C)** Proposed role of galectin-3 in stabilizing the articular cartilage lubricin boundary layer. Pentavalent galectin-3 binds to glycans on adjacent lubricin polymer brushes, providing mechanical stabilization to the boundary layer through lubricin crosslinking. **D)** Line scan from the white line in B demonstrating colocalization of lubricin and galectin-3 at the level of the articular cartilage boundary layer.

multiple lubricin monomers, thereby reinforcing the lubricin-galectin lattice **(Figure 3.3*C*)**.

Purification of Synovial Fluid Lubricin

Coomassie staining of equine diethlyaminoethyl (DEAE) FPLC-purified synovial fluid fractions **(Figure 3.4*A*)** subjected to SDS-PAGE on a 4-20% tris-glycine gel revealed a single, prominent protein band at >250kD for the 400-600mM fraction, consistent with equine lubricin **(Figure 3.4*B,C*)**. Faint bands were present at ~70kD and 25kD, and immunoblotting with a C-terminal lubricin antibody (ab28484, Abcam) shows reactivity with the >250kD, 70kD and 25kD bands. Similar 70kD bands, consistent with a minimally glycosylated PRG4 fraction containing both N- and C-terminal fragments, have been detected following 1.0 NaCl DEAE affinity purification of bovine synovial fluid upon silver staining and immunoblotting (35). Lower molecular weight bands in the 37-75 kD range have been detected in the synovial fluid of human OA and RA patients (19), suggesting that lubricin is subject to cleavage. SyPRO Ruby® staining of DEAE FPLC-purified equine synovial fluid on a 3-8% tris-acetate gel performed under denaturing conditions yielded two major >460kD bands, including a higher molecular weight smear, consistent with a species with variable glycosylation **(Figure 3.5).** Heavily glycosylated proteins are known to run as smears during gel electrophoresis due to the heterogeneity of glycosylation and imperfect charge-to-mass ratio that occurs with bulky, charged terminal sugars (36).

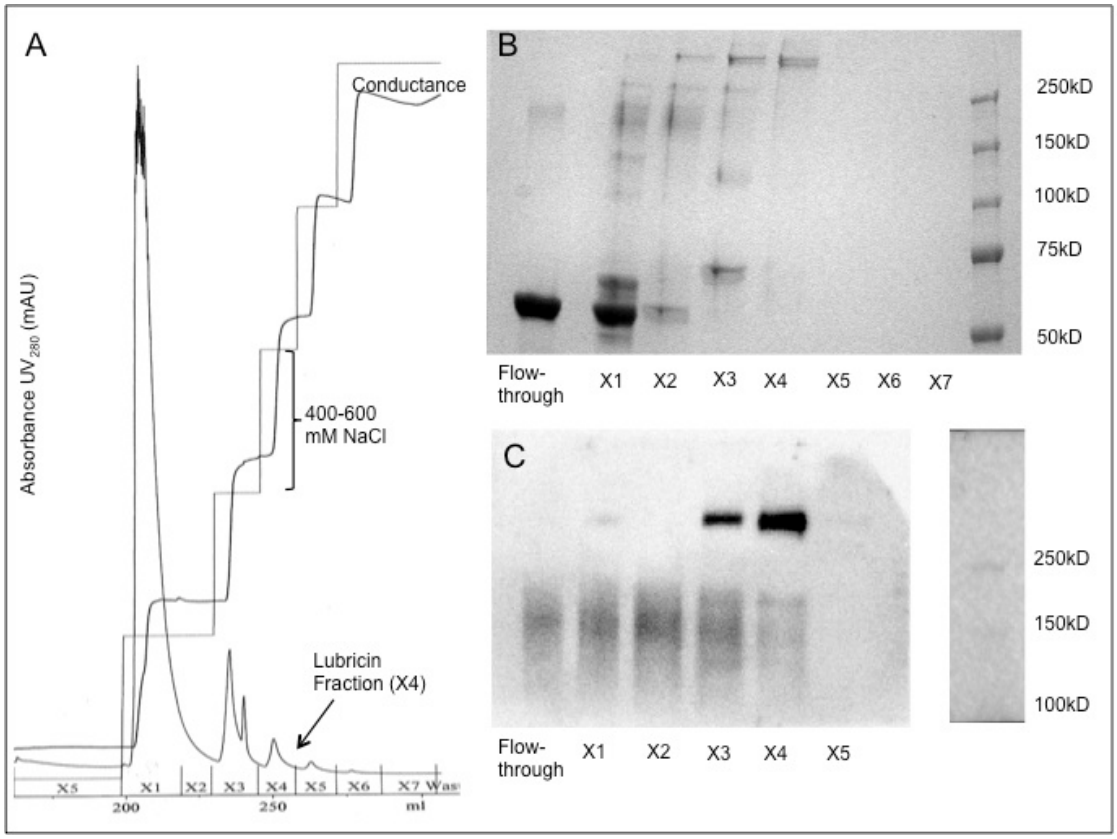


Figure 3.4. Purification of equine synovial fluid lubricin. A) FPLC chromatogram and **B)** Coomassie and **C)** immunoblot analyses of equine lubricin in FPLC DEAE anion exchange fractions. SDS-PAGE was performed using 4-20% tris-glycine gradient gels. Immunoblotting was performed with an anti-lubricin antibody (ab28484; Abcam) under non-reducing conditions.

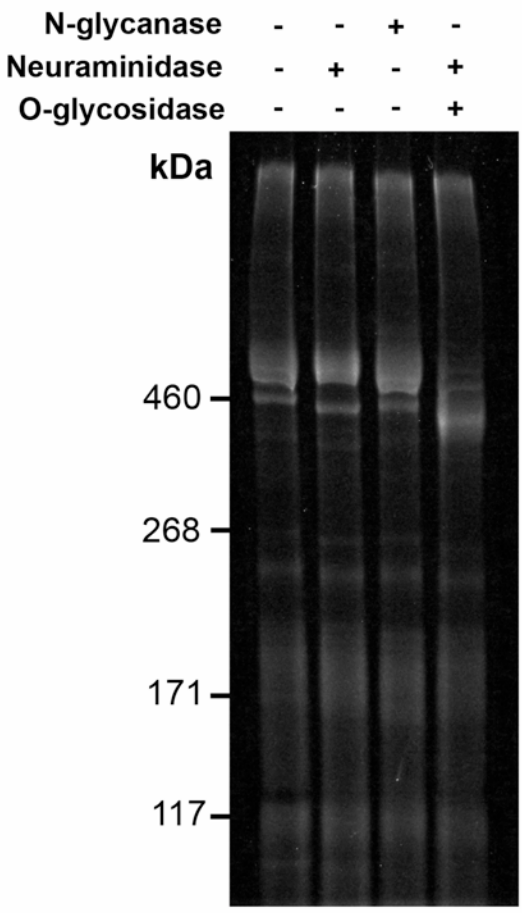
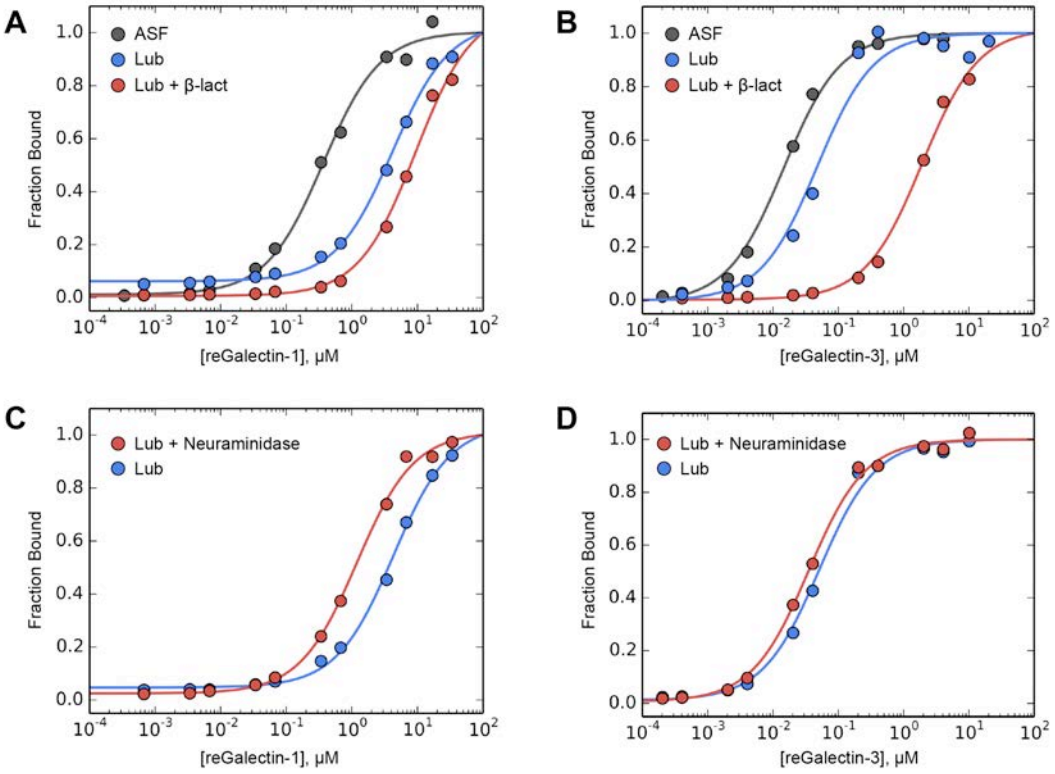


Figure 3.5. Deglycosylation of equine synovial fluid lubricin. SYPRO Ruby®-stained 3-8% tris-acetate gel with FPLC-purified equine lubricin and lubricin treated with sialidase, N-glycanase and O-glycosidase + sialidase.

Sialidase treatment results in a slight reduction in molecular size of lubricin, with a more dramatic reduction in the presence of both O-glycosidase and sialidase.

Measurement of Lubricin Galectin Binding Kinetics

In order to assess whether synovial fluid galectins may be contributing to the stability and function of the lubricin cartilage boundary layer, we employed a colorimetric binding assay using biotinylated galectins and synovial fluid-purified lubricin. Recombinant equine galectin-3 bound to lubricin with high-affinity, with a measured dissociation constant (K_d) of 51 nM **(Figure 3.6*B,E*)** as compared to 4.3 μM for galectin-1 **(Figure 3.6*A,E*)**. In comparison, asialofetuin, a known high-affinity galectin-binding protein (37), yielded a 15 nM K_d for galectin-3 binding (**Figure 3.6*B,E*)** and a 0.36 μM K_d for galectin-1 binding **(Figure 3.6*A,E*)**. Lubricin-galectin binding was carbohydrate-specific, as demonstrated by the approximately 37-fold increase in K_d for galectin-3 in the presence of 0.1M β-lactose. Removing terminal sialic acid residues from lubricin via incubation in Sialidase A™ increased the affinity of both galectin-1 (**Figure 3.6*C,E*)** and galectin-3 **(Figure 3.6*D,E*)** for synovial fluid lubricin, consistent with experimental observations of galectin binding to nonsialylated vs. sialylated variants of biantennary type *N*-glycans (38). These data suggest that galectin-3, but not galectin-1, can bind to intra-articular lubricin with high affinity and that lubricin-galectin interactions are affected by the glycosylation status of lubricin, specifically terminal sialylation. Given that lubricin sialylation is increased in RA as compared to OA (19) and that sialylation is increased in OA



E

Dissociation constants for galectin-1 and galectin-3.		
	reGalectin-1	reGalectin-3
Glycoprotein	Kd, μM[1]	
Asialofetuin (ASF)	0.36628 ± 0.042	0.01467 ± 0.001
Lubricin (Lub)	4.29699 ± 0.350	0.05107 ± 0.005
Lubricin + 0.1M β-lactose	9.42522 ± 1.299	1.91299 ± 0.276
Lubricin + Neuraminidase	1.19338 ± 0.105	0.03524 ± 0.003

[1]Calculated using non-linear least squares regression.

Figure 3.6. Galectin-3 binds to lubricin with high affinity. Experimental binding curves for **A)** galectin-1 and **B)** galectin-3 to asialofetuin (ASF), equine synovial fluid FPLC-purified lubricin, and equine synovial fluid FPLC-purified lubricin + 0.1M β-lactose. Galectin-3 binds to lubricin with high affinity, and this interaction is inhibited by the addition of 0.1M β-lactose, indicative of carbohydrate-dependent binding. **C-D)** Binding curves for **C)** galectin-1 and **D)** galectin-3 to native lubricin vs. sialidase-treated lubricin. **E)** K_d values for galectin binding to ASF and lubricin. Calculations were performed by non-linear curve fitting with Python. The error of the fitted K_ds are the square root of variances returned by the fitting algorithm.

chondrocytes as compared to healthy chondrocytes (39), lubricin-galectin interactions may be adversely affected by glycosylation changes that occur in arthritis.

Galectin-3 Enhances Cartilage Boundary Lubrication

Mechanical functions of galectins in boundary lubrication have not been evaluated. We have previously described a method for measuring equilibrium coefficients of friction (COF) for full-thickness bovine cartilage explants using a cartilage-on-glass custom tribometer (28). As shown in the schematic **(Figure 3.7*A*)**, the tribometer linearly oscillates a cartilage explant against a polished glass counterface immersed in a PBS or synovial fluid solution. For these experiments, a solution of PBS alone or solutions of PBS with 50ug/mL of recombinant human galectin-1 and galectin-3 were tested. Galectin-3 decreased equilibrium friction coefficients for cartilage explants by 13% compared to PBS controls ($\mu = 0.231 \pm 0.017$ vs. $\mu = 0.265 \pm 0.020$, $p = 0.0422$), but only in the presence of endogenous articular lubricin **(Figure 3.7*B*).** When surface lubricin was extracted from cartilage explants using a 30-minute incubation in 1.5M NaCl (34), followed by re-equilibration in PBS, galectin-3 had no impact on COF ($p = 0.83$), suggesting that galectin-3 interaction with surface-adsorbed constituents was a prerequisite for cartilage lubrication. Galectin-1 had no effect on cartilage lubrication ($p = 0.96$).

Galectin-3 has been shown to rapidly precipitate as pentamers in the presence of multivalent ligands (40) and has been shown to crosslink cell surface receptors,

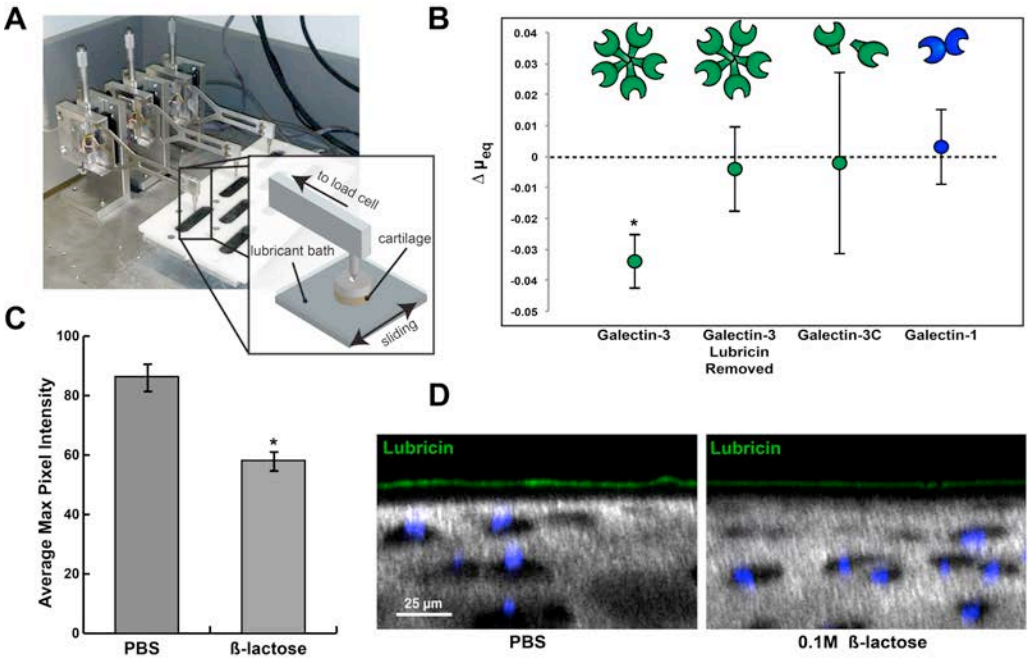


Figure 3.7. Galectin-3 enhances cartilage boundary lubrication. A) Schematic of custom tribometer used to measure boundary mode frictional coefficients for cartilage on glass in the presence of galectin solutions. **B)** Galectin-3 decreases equilibrium friction coefficients as compared to paired controls (PBS), but only in the presence of endogenous articular lubricin. When lubricin is extracted using a 30-minute incubation with 1.5M NaCl, galectin-3 no longer enhances lubrication. The galectin-3C mutant fails to enhance lubrication, suggesting that multimerization is critical for the ability of galectin-3 to facilitate boundary lubrication. Results are presented as mean ± standard deviation (SD) of n = 4. *, $p < 0.05$. **C)** Average maximum pixel intensity of boundary layer lubricin staining for cartilage explants incubated in either PBS or 0.2M β-lactose for 12hrs at 4°C. Values represent the mean ± standard error (SE) of five independent samples quantified in NIH ImageJ software. Lubricin staining is decreased in the 0.1M β-lactose treated explants, suggesting a role for galectins in stabilization of the lubricin boundary layer. **D)** Equine articular cartilage explants incubated in either PBS or 0.2M β-lactose for 12hrs at 4°C, followed by equilibration and incubation with α-lubricin mAb 9G3. Explants are imaged using confocal and multiphoton microscopy (40X). Chondrocyte nuclei are stained with DAPI (blue), and collagen is imaged using second harmonic generation microscopy (gray).

resulting in robust galectin-ligand lattices (41, 42). We next evaluated whether galectin-3 multimerization was necessary to facilitate cartilage lubrication. The galectin-3C truncation mutant, consisting of 143 amino acid residues from the carboxy-terminus of human galectin-3, retains the carbohydrate binding ability of galectin-3 but lacks the amino-terminal domain critical for multivalent behavior (43). As opposed to native galectin-3, the galectin-3C multimerization-incompetent mutant had no impact on boundary lubricating function ($p = 0.88$).

To assess whether multivalent galectin-3 could stabilize surface-adsorbed lubricin by increasing the residence time of adsorbed lubricin, we evaluated articular cartilage lubricin staining after a 12-hour incubation with 100mM β-lactose. In the presence of β-lactose to compete for galectin binding, lubricin surface staining was diminished by 32.6% (58.2 ± 3.1 vs. 86.4 ± 4.3, $n = 5$ explants, $p < 0.001$) **(Figure 3.7*C,D*)**, suggesting that removal of galectins leads to loss of articular lubricin. Taken together, our results suggest that multivalent interactions mediated by galectin-3 stabilize and reinforce the articular cartilage boundary layer, thereby facilitating boundary mode lubrication and protecting against cartilage damage.

Discussion

The glycosylation of lubricin is essential to its lubricating function. Besides contributing to the overall net negative charge and hydration of the articular cartilage surface layer (10, 11, 14), we demonstrate that lubricin's carbohydrates are also

critical for stabilizing and reinforcing the adsorbed lubricating layer through interactions with galectin-3. Here, we show that galectin-3 is a high-affinity, specific binding partner for lubricin capable of enhancing cartilage boundary lubrication. Furthermore, we demonstrate that boundary lubrication is only enhanced through multimerization-competent galectin-3, supporting the hypothesis that galectin-3 is reinforcing the lubricin boundary layer through formation of a stable lubricin-galectin lattice.

Galectins comprise a family of at least 15 multivalent lectins with specificity for β-galactoside sugars. Prototypical galectins, such as galectin-1, posses a single carbohydrate recognition domain and can form homodimers through N-terminal interactions. Galectin-3 is unique in that it can form pentamers, resulting in a functionally pentavalent carbohydrate-binding molecule. Through glycan binding, galectins can crosslink glycoproteins to form complex lattices on the cell surface or within the extracellular matrix (44, 45). Galectins are present in synovial fluid, and altered concentrations of galectins-1 and -3 have been found in juvenile idiopathic arthritis (46, 47) and RA (48, 49). Our studies suggested that equine synovial fluid lubricin is uniquely glycosylated, rich in terminal monosialylated and non-sialylated β-galactoside sugars. Therefore, we hypothesized that galectins could be crosslinking lubricin to facilitate lubrication in synovial joints.

Because lubricin and galectin-3 interact in a carbohydrate-dependent manner, glycosylation changes in arthritis may have significant effects on galectin-3

stabilization of the lubricin boundary layer. We are just beginning to understand how lubricin is glycosylated and how that glycosylation is altered in the inflammatory context of arthritis. Early studies revealed that 2/3 of the O-GalNAc-Gal oligosaccharide chains on lubricin were capped with terminal sialic acid residues (50). Mass spectrometry of human synovial fluid lubricin isolated from RA patients revealed more disialylated than monosialylated species as compared to OA patients, leading the authors to hypothesize that sialylation is unregulated with inflammatory disease severity (19). However, no studies to date have evaluated the glycosylation pattern of lubricin from healthy joints. In our lectin profiling of healthy equine cartilage, we demonstrate the presence of both sialylated and non-sialylated core 1 *O*-linked glycans, which are known binding partners for galectins. Our studies reveal that, in healthy equine synovial fluid, the *O*-linked glycophenotype of lubricin consists of primarily core 1 *O*-linked oligosaccharides that are either mono-, di- or nonsialylated, with monosialylated structures predominating. Glycomic analysis of synovial fluid from horses with normal articular cartilage, structural OA lesions, and osteochondral fragments have also revealed a predominance of monosialylated core 1 *O*-glycans (20).

The impact of galectins on cartilage lubrication and joint mechanics has neither been previously hypothesized nor investigated. Herein, we describe an extracellular role for galectins in promoting joint lubrication and cartilage protection in synovial joints. The implications of these findings in the understanding the role of galectins in joint health are important. Prior reports have primarily focused on galectins within the

synovial environment as mediators of inflammation in RA, juvenile idiopathic arthritis and antigen-induced arthritis in animal models (46, 49, 51–53). Galectin-3 expression is increased in OA chondrocytes (54), and increased galectin-3 immunohistochemical staining has been demonstrated at sites of synovial and cartilage invasion by immune cells in RA and during inflammatory phases of OA (49), leading authors to hypothesize that galectin-3 is both involved in inflammation and a novel marker of disease activity in RA. Recent lectin staining of human OA cartilage has revealed galectin-3 localization to OA chondrons and interterritorial matrix in severely degenerated cartilage (55). However, the exact role of galectins and how they contribute to the pathogenesis of disease is not well understood. Galectins function distinctly through intracellular and extracellular pathways, with intracellular signaling resulting from N-terminal protein-protein interactions and extracellular functions arising primarily from carbohydrate-dependent interactions on the cell surface or within extracellular matrices (56). One report indicates that intracellular localization of galectin-3 protects chondrocytes from apoptosis and that the absence of galectin-3 in knockout mice leads to the development of OA-like cartilage lesions (57). Our results describe a biomechanical role for galectin-3 in stabilizing the lubricin boundary layer, which suggests that galectin-3 may promote normal cartilage lubrication and joint homeostasis. It is possible that galectins have dual functions and/or concentration-dependent bipolar effects in synovial joints, with the potential to promote the development of arthritis through immune cell invasion and inflammation (49, 51, 58), but also to protect against the development of cartilage damage and ensuing arthritis by enhancing cartilage lubrication.

Our studies revealed that galectin-3 localized both to the boundary layer of healthy articular cartilage and within superficial and middle zone chondrocytes. Although galectin-3 staining has been previously demonstrated in synovial tissues (49) and in chondrocytes from human OA patients (54, 55), galectin-3 has not been previously documented to stain the surface layer of articular cartilage. Differences in antibody reactivity or tissue preparation may explain observed differences in galectin boundary layer immunohistochemical staining, with bulky molecules like hyaluronic acid potentially interfering with epitope exposure in the absence of hyaluronidase treatment. The surface layer is resistant to complete digestion by hyaluronidase (59), and hyaluronic acid is a bulky glycosaminoglycan known to interact with lubricin (60). Also, galectin-3 may not stain the boundary layer of mildly or severely degenerated OA cartilage due to disruption of the lamina splendens, including the surface-adsorbed lubricin layer, as is observed with lubricin immunolocalization in OA cartilage (22, 33). In fact, our imaging results suggest that galectin-3 staining of the articular cartilage surface in severe OA cartilage explants is almost completely abolished. These findings, coupled with the high binding affinities for lubricin and galectin-3 and the colocalization of fluorophore-labeled galectin-3 and lubricin mAb on the lamina splendens suggest that galectins are binding predominantly to lubricin. Although our results do not exclude the possibility that galectin-3 may be binding to glycans on other cartilage surface constituents, such as fibronectin, the sparse distribution of *N*-glycans on the cartilage surface as demonstrated by lectin staining suggests that *O*-linked glycans are the primary targets for galectin binding.

Herein, we provide a mechanism to explain how synovial fluid galectin-3 may enhance the biomechanics of cartilage lubrication and protect against the development of cartilage damage through stabilization of the lubricin boundary layer via lattice formation. Future investigation into how lubricin glycosylation is altered in arthritis and how these alterations impact galectin-3 binding and cartilage lubrication will be critical in fully understanding the role of galectins in cartilage biomechanics.

References

1. Cui Z, Xu C, Li X, Song J, Yu B (2015) Treatment with recombinant lubricin attenuates osteoarthritis by positive feedback loop between articular cartilage and subchondral bone in ovariectomized rats. *Bone* 74:37–47.

2. Ruan MZC, et al. (2013) Proteoglycan 4 expression protects against the development of osteoarthritis. *Sci Transl Med* 5(176):176ra34.

3. Teeple E, et al. (2011) The Effects of Supplemental Intra-Articular Lubricin and Hyaluronic Acid on the Progression of Post-Traumatic Arthritis in the Anterior Cruciate Ligament Deficient Rat Knee. *Am J Sports Med* 39(1):164–172.

4. Jay GD, et al. (2010) Prevention of Cartilage Degeneration and Restoration of Chondroprotection by Lubricin Tribosupplementation in the Rat Following ACL Transection. *Arthritis Rheum* 62(8):2382–2391.

5. Flannery CR, et al. (2009) Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin. *Arthritis Rheum* 60(3):840–7.

6. Marcelino J, et al. (1999) CACP, encoding a secreted proteoglycan, is mutated in camptodactyly-arthropathy-coxa vara-pericarditis syndrome. *Nat Genet* 23(3):319–322.

7. Bahabri S, et al. (1998) The camptodactyly-arthropathy-coxa vara-pericarditis syndrome: clinical features and genetic mapping to human chromosome 1. *Arthritis Rheum* 41(4):730–735.

8. Swann DA, Silver FH, Slayter HS, Stafford W, Shore E (1985) The molecular structure and lubricating activity of lubricin isolated from bovine and human synovial fluids. *Biochem J* 225(1):195–201.

9. Jay GD, Britt DE, Cha C-J (2000) Lubricin Is a Product of MSF Gene Expression by Human Synovial Fibroblasts. *J Rheumatol* 27(3):594–600.

10. Swann DA, Sotman S, Dixon M, Brooks C (1977) The isolation and partial

characterization of the major glycoprotein (LGP-I) from the articular lubricating fraction from bovine synovial fluid. *Biochem J* 161(3):473–85.

11. Ali L, et al. (2014) The O-glycomap of lubricin, a novel mucin responsible for joint lubrication, identified by site-specific glycopeptide analysis. *Mol Cell proteomics*. doi:10.1074/mcp.M114.040865.

12. Gleghorn JP, Jones ARC, Flannery CR, Bonassar LJ (2009) Boundary mode lubrication of articular cartilage by recombinant human lubricin. *J Orthop Res* 27(6):771–7.

13. Jay GD, Tantravahi U, Britt DE, Barrach HJ, Cha CJ (2001) Homology of lubricin and superficial zone protein (SZP): Products of megakaryocyte stimulating factor (MSF) gene expression by human synovial fibroblasts and articular chondrocytes localized to chromosome 1q25. *J Orthop Res* 19(4):677–687.

14. Jay GD, Harris D a, Cha CJ (2001) Boundary lubrication by lubricin is mediated by O-linked beta(1-3)Gal-GalNAc oligosaccharides. *Glycoconj J* 18(10):807–15.

15. Jay GD, Lane BP, Sokoloff L (1992) Characterization of a bovine synovial fluid lubricating factor III. The interaction with hyaluronic acid. *Connect Tissue Res* 28(4):245–255.

16. Benz M, Chen N, Jay G, Israelachvili J (2005) Static Forces, Structure and Flow Properties of Complex Fluids in Highly Confined Geometries. 33(1):39–51.

17. Dube DH, Bertozzi CR (2005) Glycans in cancer and inflammation--potential for therapeutics and diagnostics. *Nat Rev Drug Discov* 4(6):477–88.

18. Swann DA, Bloch KJ, Swindell D, Shore E (1984) The lubricating activity of human synovial fluids. *Arthritis Rheum* 27(5):552–6.

19. Estrella RP, Whitelock JM, Packer NH, Karlsson NG (2010) The glycosylation of human synovial lubricin: implications for its role in inflammation. *Biochem J* 429(2):359–67.

20. Svala E, et al. (2015) Characterisation of lubricin in synovial fluid from horses with osteoarthritis. *Equine Vet J*. doi:10.1111/evj.12521.

21. Teeple E, et al. (2008) Coefficients of Friction, Lubricin, and Cartilage Damage in the Anterior Cruciate Ligament-Deficient Guinea Pig Knee. 26(2):231–237.

22. Elsaid KA, Machan JT, Waller K, Fleming BC, Jay GD (2009) The impact of anterior cruciate ligament injury on lubricin metabolism and the effect of inhibiting tumor necrosis factor alpha on chondroprotection in an animal model. *Arthritis Rheum* 60(10):2997–3006.

23. Jay GD, et al. (2012) Prevention of cartilage degeneration and gait asymmetry by lubricin tribosupplementation in the rat following anterior cruciate ligament transection. *Arthritis Rheum* 64(4):1162–71.

24. Nagae M, Yamaguchi Y (2015) Sugar recognition and protein – protein interaction of mammalian lectins conferring diverse functions. *Curr Opin Struct Biol* 34:108–115.

25. Brewer CF, Miceli MC, Baum LG (2002) Clusters, bundles, arrays and lattices: Novel mechanisms for lectin-saccharide-mediated cellular interactions. *Curr Opin Struct Biol* 12(5):616–623.

26. van Kooyk Y, Rabinovich GA (2008) Protein-glycan interactions in the control of innate and adaptive immune responses. 9(6):593–602.

27. Pace KE, Hahn HP, Baum LG (2003) Preparation of recombinant human galectin-1 and use in T-cell death assays. *Methods Enzymol* 363(2001):499–518.

28. Gleghorn JP, Jones a RC, Flannery CR, Bonassar LJ (2007) Boundary mode frictional properties of engineered cartilaginous tissues. *Eur Cell Mater* 14:20–8; discussion 28–9.

29. Ali L, Jin C, Karlsson NG (2012) Glycoproteomics of Lubricin-Implication of Important Biological Glyco- and Peptide-Epitopes in Synovial Fluid. *Rheumatoid Arthritis - Etiology, Consequences and Co-Morbidities*, ed Lemmey A (InTech, Rijeka, Croatia), pp 131–152.

30. Earl LA, Bi S, Baum LG (2010) N- and O-Glycans modulate galectin-1 binding, CD45 signaling, and T cell death. *J Biol Chem* 285(4):2232–2244.

31. Krzeminski M, et al. (2011) Human galectin-3 (Mac-2 antigen): Defining molecular switches of affinity to natural glycoproteins, structural and dynamic aspects of glycan binding by flexible ligand docking and putative regulatory sequences in the proximal promoter region. *Biochim Biophys Acta - Gen Subj* 1810(2):150–161.

32. Young AA, et al. (2006) Proteoglycan 4 downregulation in a sheep meniscectomy model of early osteoarthritis. *Arthritis Res Ther* 8(2):R41.

33. Elsaid KA, Jay GD, Chichester CO (2007) Reduced expression and proteolytic susceptibility of lubricin/superficial zone protein may explain early elevation in the coefficient of friction in the joints of rats with antigen-induced arthritis. *Arthritis Rheum* 56(1):108–16.

34. Jones ARC, et al. (2007) Binding and Localization of Recombinant Lubricin to Articular Cartilage Surfaces. *J Orthop Res* 12(March):10–12.

35. Schmidt T a, Plaas AHK, Sandy JD (2009) Disulfide-bonded multimers of proteoglycan 4 PRG4 are present in normal synovial fluids. *Biochim Biophys Acta* 1790(5):375–84.

36. El-Rassi Z (2002) *Carbohydrate Analysis by Modern Chromatography and Electrophoresis* (Elsevier) Available at: https://books.google.com/books?id=xrHDySs1NZUC&pgis=1 [Accessed April 9, 2015].

37. Dam TK, et al. (2005) Galectins bind to the multivalent glycoprotein asialofetuin with enhanced affinities and a gradient of decreasing binding constants. *Biochemistry* 44(37):12564–71.

38. Hirabayashi J, et al. (2002) Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim Biophys Acta* 1572(2-3):232–54.

39. Toegel S, et al. (2014) Human osteoarthritic knee cartilage: fingerprinting of adhesion/growth-regulatory galectins in vitro and in situ indicates differential upregulation in severe degeneration. *Histochem Cell Biol* 142(4):373–88.

40. Ahmad N, et al. (2004) Galectin-3 Precipitates as a Pentamer with Synthetic Multivalent Carbohydrates and Forms Heterogeneous Cross-linked Complexes. *J Biol Chem* 279(12):10841–10847.

41. Nieminen J, Kuno A, Hirabayashi J, Sato S (2007) Visualization of galectin-3 oligomerization on the surface of neutrophils and endothelial cells using fluorescence resonance energy transfer. *J Biol Chem* 282(2):1374–1383.

42. Demetriou M, Granovsky M, Quaggin S, Dennis JW (2001) Negative regulation of T-cell activation and autoimmunity by Mgat5 N-glycosylation. *Nature* 409(6821):733–739.

43. Mirandola L, et al. (2011) Galectin-3C inhibits tumor growth and increases the anticancer activity of bortezomib in a murine model of human multiple myeloma. *PLoS One* 6(7):e21811.

44. Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich G a (2007) Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci* 64(13):1679–700.

45. Rabinovich GA, Toscano MA, Jackson SS, Vasta GR (2007) Functions of cell surface galectin-glycoprotein lattices. *Curr Opin Struct Biol* 17(5):513–20.

46. Harjacek M, et al. (2001) Expression of galectins-1 and -3 correlates with defective mononuclear cell apoptosis in patients with juvenile idiopathic arthritis. *J Rheumatol* 28(8):1914–1922.

47. Ezzat MHM, El-Gammasy TMA, Shaheen KYA, Osman AOY (2011) Elevated production of galectin-3 is correlated with juvenile idiopathic arthritis disease activity, severity, and progression. *Int J Rheum Dis* 14(4):345–52.

48. Xibillé-Friedmann D, Bustos Rivera-Bahena C, Rojas-Serrano J, Burgos-Vargas R, Montiel-Hernández J-L (2013) A decrease in galectin-1 (Gal-1) levels correlates with an increase in anti-Gal-1 antibodies at the synovial level in patients with rheumatoid arthritis. *Scand J Rheumatol* 42(2):102–7.

49. Ohshima S, et al. (2003) Galectin 3 and its binding protein in rheumatoid

arthritis. *Arthritis Rheum* 48(10):2788–95.

50. Swann DA, Mintz G (1979) The isolation and properties of a second glycoprotein (LGP-II) from the articular lubricating fraction from bovine synovial fluid. *Biochem J* 179(3):465–71.

51. Janelle-Montcalm A, et al. (2007) Extracellular localization of galectin-3 has a deleterious role in joint tissues. *Arthritis Res Ther* 9(1):R20.

52. Forsman H, et al. (2011) Galectin 3 aggravates joint inflammation and destruction in antigen-induced arthritis. *Arthritis Rheum* 63(2):445–54.

53. Li X, Zhao Q-Y, Yuan F-L (2011) Galectin 3 as a novel therapeutic target in rheumatoid arthritis: comment on the article by Forsman et al. *Arthritis Rheum* 63(10):3179–80.

54. Guévremont M, et al. (2004) Galectin-3 surface expression on human adult chondrocytes: a potential substrate for collagenase-3. *Ann Rheum Dis* 63(6):636–43.

55. Toegel S, et al. (2013) Glycophenotyping of osteoarthritic cartilage and chondrocytes by RT-qPCR, mass spectrometry, histochemistry with plant/human lectins and lectin localization with a glycoprotein. *Arthritis Res Ther* 15(5):R147.

56. Arthur CM, Baruffi MD, Cummings RD, Stowell SR (2015) Evolving mechanistic insights into galectin functions. *Methods Mol Biol* 1207:1–35.

57. Boileau C, et al. (2008) Intracellular localisation of galectin-3 has a protective role in chondrocyte survival. *Ann Rheum Dis* 67(2):175–81.

58. Reboul P, Martel-Pelletier J, Pelletier J-P (2004) Galectin-3 in osteoarthritis: when the fountain of youth doesn't deliver its promises. *Curr Opin Rheumatol* 16(5):595–8.

59. Kumar P, et al. (2001) Role of uppermost superficial surface layer of articular cartilage in the lubrication mechanism of joints. *J Anat* 199(3):241–250.

60. Das S, et al. (2013) Synergistic interactions between grafted hyaluronic acid and lubricin provide enhanced wear protection and lubrication. *Biomacromolecules* 14(5):1669–1677.

CHAPTER 4

GALECTIN-1 AND GALECTIN-3 ENHANCE EQUINE MESENCHYMAL STROMAL CELL ADHESION, SPREADING AND MOTILITY

Abstract

Objective. Mesenchymal stromal cells (MSCs) are used intra-articularly to quell inflammation and promote cartilage healing following joint trauma; however, mechanisms by which MSCs mitigate inflammation are still poorly understood. Galectins, a family of β-galactoside binding proteins, exhibit immunomodulatory properties, in addition to effects on cell adhesion and motility. Here, we asked whether equine bone marrow-derived MSCs express higher levels of galectins relative to other synovial cell types and if galectins mediate MSC adhesion and motility.

Methods. Equine galectin-1 and -3 gene expression were quantified in cultured MSCs, synoviocytes and chondrocytes, and both galectin gene and protein expression were measured in MSCs exposed to inflammatory cytokines (IL-1β, TNFα, or LPS). MSC adhesion, spreading and motility were studied on galectin-coated substrates in the presence and absence of the galectin inhibitor β-lactose. Adhesion complex formation was evaluated using confocal microscopy, and migration was quantified in MSCs following exposure to cytokines, galectins or β-lactose using a fluorescence-based microplate assay and a modified scratch assay.

Results. Equine MSCs expressed 3-fold higher galectin-1 mRNA levels as compared to cultured synoviocytes ($p=0.0005$) and 30-fold higher galectin-1 ($p<0.0001$) relative to cultured articular chondrocytes. The pro-inflammatory cytokines IL-1β and TNFα decreased galectin-1 and galectin-3 mRNA expression but had no effect on intracellular or secreted galectin protein levels. Galectin inhibition with β-lactose significantly impaired MSC cell spreading and reduced adhesion complex assembly,

decreasing MSC motility in a dose-dependent fashion. Recombinant galectins partially restored the effects of IL-1β and TNFα on MSC motility. Taken together, these results suggest that galectins may play an important role in MSC adhesion, spreading and motility.

Conclusions. Equine MSCs constitutively express high levels of galectin-1 relative to other synovial cell types, and MSC spreading and adhesion complex formation is enhanced in the presence of recombinant galectins-1 and-3 and inhibited by β-lactose. Further investigation into the role of intra-articular galectins is warranted, including how galectins may alter endogenous MSC migration, and how galectins may be leveraged to enhance MSC adhesion for cartilage and tissue repair.

Introduction

Joint trauma, inflammation and the subsequent development of osteoarthritis are common in both humans and horses. Joint inflammation is precipitated by the production of catabolic cytokines, such as IL-1β and TNFα, in addition to immune cell infiltration. Mesenchymal stem cells (MSCs) were initially appealing as a cell source for the repair of articular cartilage and other musculoskeletal injuries due to their multi-lineage potential (1) However, low rates of long-term survival, engraftment and differentiation in musculoskeletal tissues (2, 3) has led to a failure to realize these properties. Reasons for failure of MSCs to engraft are still elusive, and there is limited information about how MSCs adhere to damaged cartilage (4) or other synovial tissues. Alas, the paradigm has shifted from MSCs as tissue reparative cells to MSCs as immunomodulatory cells that provide a niche or microenvironment conducive to tissue repair by tissue progenitor cells (5, 6). MSCs have been used intra-articularly to decrease joint inflammation and promote cartilage healing in both experimental animal models of OA (3, 7, 8) and in human clinical trials (9, 10); however, the mechanisms by which MSCs perform these actions are not fully understood.

Various secreted molecules have been implicated in the anti-inflammatory and immunomodulatory properties of stem cells, including prostaglandin E_2 (PGE_2) (5, 11), indoleamine 2,3-dioxygenase (IDO) (12), interleukin-10 (IL-10) (5), tumor necrosis factor-inducible gene 6 (TSG-6) (13) and interleukin-1 receptor antagonist (IL-1Ra) (14). Recently, several studies have implicated soluble galectin-1 and galectin-3 as potent

mediators of MSC immunomodulatory properties (15–19). Galectins comprise a family of more than 15 β-galactoside binding proteins, and abundant evidence suggests that galectins are master regulators of immune cell homeostasis (20, 21). Galectin-1 possesses both immunosuppressive and anti-inflammatory effects in many chronic inflammatory and autoimmune disorders, including atherosclerosis (22), graft versus host disease (23) and systemic lupus erythematosus (24). In a collagen-induced arthritis model, gal-1 -/- mice displayed increased disease penetrance as compared to wild-type mice, with an accelerated disease onset and more severe arthritis (25). Both galectin-1 gene therapy and recombinant galectin-1 intra-articular administration abrogated clinical and histopathological signs of collagen-induced arthritis in a murine model (26).

Galectins are abundantly expressed in human umbilical cord blood-derived MSCs, which express high levels of galectin-1 and -3 and moderate expression of galectins-8 and -9 (17), and human MSCs constitutively express galectins-1, -3 and -8 at both gene and protein levels (16, 27). Compelling evidence suggests that both galectin-1 (18, 28, 29) and galectin-3 play a significant role in the ability of MSCs to downregulate immune responses (15–17). For example, siRNA-mediated knockdown of both gal-1 and gal-3 abrogated MSC immunosuppressive properties (16), and addition of exogenous galectin-3 restored immunomodulatory properties of MSCs in *in vitro* mixed lymphocyte cultures (17).

Beyond their role as immunomodulatory proteins, galectins also have diverse effects on cell adhesion, chemotaxis (30, 31) and migration in several cell types (21).

Galectins are expressed as intracellular, membrane-bound and secreted forms in MSCs (18), capable of effecting functions intracellularly through protein-protein interactions and extracellularly through protein-carbohydrate interactions (20, 32). For example, a recent elegant study used fluorescence recovery after photobleaching (FRAP) to demonstrate the role of galectin-3 and Mgat5, a glycosylation enzyme producing high affinity galectin ligands, in promoting integrin clustering, formation of focal adhesions (FA) and cell spreading (33). Focal adhesions are the major mechanical connections between the extracellular matrix and the contractile cytoskeleton of cells (34). FA integrin interactions lead to stable adhesion and spreading; but, in concert with FA, focal adhesion kinase (FAK) and adhesion structure turnover, can also enhance motility (33). Thus, galectins play a major role in adhesion and adhesion-dependent processes, such as motility, in diverse cell types by regulating both integrins and FA.

Interestingly, expression of adhesion molecules, such as CD54 (intracellular adhesion molecule 1) and CD58 (lymphocyte function-associated antigen 3) and secretion of galectin-1 by MSCs is tightly regulated (28). Galectins-1 and -3 both promote directional migration of monocytes (35), macrophages (35) and neutrophils (31). Galectin-1 enhances migration of human monocyte-derived dendritic cells through ECM (36) and stimulates motility of human umbilical cord-derived MSCs (37).

The efficiency of engraftment of MSCs to either intact or fibrillated cartilage is low (2, 3), and enhancing MSC adhesion to sites of articular cartilage damage is a challenge to achieving tissue repair. Mechanical forces are likely to impair adhesion of

MSCs to articular cartilage, and it has been postulated that one of the major benefits of knee joint distraction is that it enables MSCs to adhere more effectively to damaged cartilage (4). Improved MSC attachment to the articular cartilage surface is likely to enhance the benefits of MSCs in joint inflammation and tissue repair. Several groups have investigated the use of bioadhesives or other assistive materials to increase homing, retention and adhesion of MSCs at the cartilage surface (38). Because of their adhesive and chemotactic properties, in addition to their presence in synovial fluid (25, 39–41), galectins are an interesting potential target for manipulating MSC adhesion to injured cartilage.

Here, we asked whether equine MSCs express higher levels of galectins relative to other synovial cell types and if inflammatory cytokines would alter galectin-1 and galectin-3 expression in MSCs. Secondly, we investigated the role of galectins in MSC adhesion and motility in 2D culture and on the articular cartilage surface. Therefore, the objectives of our study were to: 1) quantify galectin-1 and -3 gene expression in equine mesenchymal stromal cells (MSCs), 2) evaluate the effect of inflammatory cytokines on MSC galectin gene and protein expression and 3) to determine the significance of galectins in MSC adhesion and motility on 2D substrates and adhesion to the surface of articular cartilage.

Materials and Methods

Ethics statement

All animal and tissue harvesting protocols were approved by the institutional animal care and use committee (Protocol Number: 2011-027).

Cell isolation and culture

Bone marrow was collected from the sternebrae of thirty-one young mature horses for primary MSC isolation as previously described (42). Briefly, bone marrow biopsy needles (Jamshidi, VWR Scientific, Bridgeport, NJ) were used to aspirate 60-180mL of sternebral bone marrow into one to three 60-mL syringes containing heparin (APP Pharmaceuticals, LLC; Schaumburg, IL) at a final concentration of 1,000 units/mL. MSCs were isolated via selective tissue culture plastic adherence after plating bone marrow 1:1 in Dulbecco's modified Eagles' media; 1,000mg/L glucose (Gibco-Life Technology, Grand Island, NY) supplemented with 1ng/mL bFGF (Gibco®, Invitrogen, Camarillo, CA), 25m*M* HEPES (Gibco-Life Technology, Grand Island, NY), 100units/mL penicillin-streptomycin, and 10% fetal calf serum. Non-adherent cells were removed via media changes every other day. Following colony formation, adherent cells were passaged and re-plated at 10-12,000 cells/cm^2.

Synovial membrane and articular cartilage tissues were aseptically harvested from the shoulder, stifle, carpal and fetlock joints of young mature horses (n=27 and 16, respectively), isolated and culture expanded as previously described for synoviocytes (43) and chondrocytes (44). Briefly, synovial lining was digested in 0.15% collagenase (Worthington Biochemical, Lakewood, NJ) and 0.015% DNAseI (Roche, Indianapolis, IN) for 3h at 37°C, followed by filtration and centrifugation at 250x *g* for 10 minutes. Synoviocytes were cultured in Dulbecco's modified Eagles' media; 4,500 mg/L glucose (Gibco-Life Technology, Grand Island, NY) supplemented with 25m*M* HEPES (Gibco-Life Technology, Grand Island, NY), 100units/mL penicillin-streptomycin, and 10% fetal calf serum. Articular cartilage was digested in 0.075% collagenase (Worthington Biochemical, Lakewood, NJ) overnight at 37°C, followed by filtration and centrifugation at 250x *g* for 10 minutes. Chondrocytes were cultured in Ham's F12 medium (Corning Inc., Corning, NY) supplemented with 50µg/mL ascorbic acid, 30µg/mL α-ketoglutarate, 300µg/mL L-glutamine, 25m*M* HEPES (Gibco-Life Technology, Grand Island, NY), 100units/mL penicillin/streptomycin and 10% fetal calf serum.

Cloning, sequencing and production of recombinant equine galectin-1 and galectin-3

To clone equine galectin-1 and galectin-3, the cDNA was isolated from the kidney of a 19-year-old Thoroughbred mare following euthanatization. RNA was purified following renal tissue homogenization and lysis with Proteinase K (PerfectPure, 5PRIME, Gaithersburg, MD), followed by cDNA synthesis (SuperScript® First-Strand Synthesis, Invitrogen, Carlsbad, CA). Gene-specific primers were designed against the

predicted sequences of equine galectin-1 and galectin-3 accessed from the National Center for Biotechnology Information, with the exception that the equine galectin-1 forward primer was designed to begin at nucleotide (nt) 292 of the predicted equine sequence based upon sequence homology with human galectin-1 and the conserved translational start site MACGLVASNLNKPGECL, beginning at nt 292 of the predicted equine galectin-1 sequence. Thus, the galectin cloning primers were designed as follows: (Galectin-1 Fwd: 5'- ATGGCTTGTGGTCTGGTCGCCAGCA –'3,
Galectin-1 Rev: 5'- TCACTCAAAGGCCACACACTTGATCT –3',
Galectin-3 Fwd: 5'- ATGTCAGACGGTTTTTCGCTTA –3',
Galectin-3 Rev: 5'- TTATATCATAGTGTGCGAAGCACTG –3').

Bands corresponding to 408bp for galectin-1 and 718bp for galectin-3 were gel purified (QIAquick, QIAGEN GmbH, Hilden, Germany) and sequenced by the Cornell University Biotechnology Resource Center. Following sequencing, galectins were subcloned from the pCR^{TM}2.1-TOPO® vector into a pET21a bacterial expression vector, sequenced and recombinantly expressed in XL1-Blue competent *E. coli* and purified using lactosyl sepharose chromatography and FPLC gel filtration as previously described for the purification of human galectins-1, -3 and -3C (45).

Equine galectin gene expression analysis

For constitutive galectin expression, passage 0 to 3 equine MSCs, synovial fibroblasts and chondrocytes were cultured for 72 hrs in appropriate growth media as

discussed above. Cells were collected in lysis buffer and frozen at -80°C for subsequent RNA isolation using a purification kit (5 Prime Perfect Pure RNA Purification Kit, Fischer Scientific). Synovial membrane tissue obtained from equine carpal joints was also snap frozen at -80°C for subsequent RNA isolation and purification. RNA purity and concentration was assessed using UV microspectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Waltham, MA). Gene expression was quantified through the use of quantitative real-time polymerase chain reaction (PCR), with all samples analyzed in duplicate (ABI PRISM 7900 Sequence Detection System, Applied Biosystems, Foster City, CA) using primers and a dual-labeled fluorescent probe [6-FAM™ as the 5' reporter label and Iowa Black® FQ as the 3' quenching label]. Primers and probes were generated using the equine galectin-1 and -3 sequences cloned from kidney tissue as described above and were designed using Primer Express Software version 2.0b8a (Applied Biosystems, Foster City, CA):
(Galectin-1 Fwd: 5'- CAAGGCAGACCTGACCATCA -'3,
Galectin-1 Rev: 5'- TCACGGCCTCCAGGTTGA -3',
Galectin-1 Probe: 5'- /56-FAM/ CTGCCGGAT/ZEN/GGCTACTCGTTCAAGTTC/ 3IABkFQ/ -3', Galectin-3 Fwd: 5'- TAAATTTCAACAGAGGGCATGATG -3',
Galectin-3 Rev: 5'- CAATGACTCTCCTGTTGTTCTCGTT -3'
Galectin-3 Probe: 5'- /56-FAM/ TGCCTTCCA/ZEN/CTTTAACCCGCGCTT/ 3IABkFQ/ -3'). Total copy number of mRNA was determined using a validated standard curve, and copy number was normalized to the housekeeping gene 18S.

Statistical analysis: One-way ANOVA with Tukey's post hoc tests were performed on log transformed gene expression data for galectin-1 and galectin-3 using GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA). Summary statistics were performed on the untransformed data, and significance was set at $P<0.05$.

Cytokine treatments

Cells were plated as passage 3 MSCs in 24-well plates at a concentration of 2 x 10^4 cells/cm^2. MSCs remained in serum-containing MSC growth media for 24 hrs prior to cytokine treatments. Serum-containing media was replaced with serum-free Opti-MEM (Invitrogen, Grand Island, NY) 4 hrs prior to stimulation. MSCs were stimulated with recombinant equine IL-1β (IBI Scientific, Peosta, IA) at 5ng/mL and 10ng/mL, recombinant equine TNF-α (IBI Scientific, Peosta, IA) at 25ng/ml and 50ng/ml or LPS from *E. coli* 055:B5 at 0.1 ug/mL, 1ug/mL, 10ug/mL and 50ug/mL (Sigma-Aldrich, St. Louis, MO). All experimentas were performed in duplicate wells. Control MSCs remained in serum-free Opti-MEM. Media supernatants were collected at 4, 8, 20 and 30 hrs post-treatment and immediately frozen and stored at -80°C. Cells were lysed at 4, 8, 20 and 30 hrs after treatment for RNA isolation, and gene expression was determined using qRT-PCR for galectin-1 and galectin-3 mRNA with 18S used as a housekeeping gene. Media supernatants were thawed for quantitation of galectin concentrations using custom ELISAs. Galectin-1 concentrations were quantified using a custom galectin-1 competition ELISA with goat α-mouse pAb AF1245 used as a capture antibody (R&D Systems, Inc., Minneapolis, MN, Lot #IRE0114051) and biotinylated recombinant equine

galectin-1, with recombinantly produced equine galectin-1 as a standard. Galectin-3 concentrations were quantified using a custom galectin-3 sandwich ELISA with the capture antibody pAb sc-19280 (Santa Cruz Biosciences, Santa Cruz, CA, Lot #K2514), the biotinylated detection antibody pAb BAF1197 (R&D Systems, Inc., Minneapolis, MN, Lot #JBB0214071) and with recombinantly produced equine galectin-3 as a standard. Following the addition of streptavidin-HRP (R&D Systems, Inc., Minneapolis, MN), TMB (Thermo Scientific, Rockford, IL) was used as colorimetric agents. Both ELISAs were validated for use with cell culture supernatants. All data are presented as means ± standard error. In parallel to the 24-well plates used for RNA isolation and supernatant collection for galectin ELISAs, MSCs were also plated in 6-well plates at a concentration of 2×10^4 cells/cm^2, receiving the same treatments as described above. At 4, 8, 20 and 30 hrs post-treatment, supernatants were aspirated, and cells were lysed with RIPA buffer containing protease inhibitors. A cell scraper was used to transfer cell lysates into Eppendorf tubes on ice, followed by shaking at 4°C for 30 min., high-speed centrifugation to pellet cell debris and storage of lysates at -80°C until further analysis. Cell lysates were thawed, denatured in reducing Laemmli buffer, and loaded onto tris-glycine 4-20% gradient gels (Bio-Rad Laboratories, Inc., Hercules, CA) prior to imunoblotting for actin (sc-1615, Santa Cruz Biosciences, Santa Cruz, CA, Lot# G0513), galectin-1 (AF1245, R&D Systems, Inc., Minneapolis, MN, Lot #IRE0114051) and galectin-3 (sc-19280, Santa Cruz Biosciences, Santa Cruz, CA, Lot #K2514). Blots were imaged using a gel doc station (ChemiDoc™ XRS, Bio-Rad Laboratories, Inc., Hercules, CA) and saved as .tif images. Bands were quantified in Fiji Image J, subtracting for background signal and normalizing by actin.

Statistical analysis: Galectin-1 and galectin-3 gene expression data was log transformed, and data were compared using a Friedman nonparametric test, matching data by primary cell line. Dunn's multiple comparisons test was performed using the cytokine-free Opti-MEM group as the untreated control. Galectin ELISA and lysate western blotting data were also compared using a Friedman nonparametric test, and differences between treatment groups were assessed using Dunn's multiple comparisons test in GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA).

MSC nucleofection and confocal imaging

Passage 3 MSCs were transfected with the fluorescent-protein tagged adhesion markers paxillin mEmerald and vinculin mApple using a human MSC nucleofector® electroporation kit (Lonza, Basel, Switzerland). Cells were passaged at 48 hrs post-nucleofection and plated onto 12-well fibronectin or galectin-coated glass plates at a concentration of 2x 10^3 cells/cm^2 in serum-free media (Dulbecco's modified Eagles' media, 1,000mg/L glucose; 1ng/mL bFGF; 25m*M* HEPES; 100units/mL penicillin-streptomycin). MSCs were plated onto fibronectin or galectin-coated plates in the presence and absence of 100 mM β-lactose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). At 8 hrs post-plating, glass plates were fixed in 4% paraformaldehyde for 10 minutes, rinsed in PBS, followed by actin staining with phalloidin Alexa647 and nuclear staining with DAPI for 15 minutes, followed by several PBS rinses. Cells were imaged

with a 60X objective on a spinning disk confocal microscope, and images were overlaid in Adobe Photoshop CS5 (Adobe Systems, Inc., San Jose, CA).

Migration assays

Ibidi® silicone insert migration assay. Passage 3 to 4 equine MSCs were plated onto 24-well tissue culture plates (Corning Inc., Corning, NY) within silicone inserts containing a defined, 500 μm cell-free gap for migration assays (Ibidi®, Martinsried, Germany). After 6 hrs, media was changed to either control media, media containing β-lactose (100mM, 200mM) or media supplemented with recombinant equine IL-1β (5ng/mL, 10ng/mL) or recombinant equine TNF-α (25ng/ml 50ng/ml). Twenty hours later, inserts were removed and media was replaced with control media, media containing β-lactose (100mM, 200mM) or media containing recombinant equine galectin-1 or galectin-3 (50ng/mL). Phase contrast images were obtained at 0, 3, 8, 12, 24 and 48 hours following insert removal, using 3 images at 10X magnification to image the entire cell-free gap. NIH Image J was used to define the x,y coordinates of the leading edges of cells migrating across the cell-free region. Custom Python software was designed to measure the mean linear cell-free distance for each image, which was normalized to the cell-free distance at time zero for each treatment.

Statistical analysis: Three independent experiments were performed using the Ibidi® cell migration inserts, and all treatment conditions were applied in duplicate. The normalized mean cell-free distances were analyzed using linear regression (JMP 11.0,

Cary, NC). Covariates, including time, time^2, treatment and MSC cell line were screened for significance at $p<0.10$. All covariates, except for MSC primary cell line, were significant and therefore included in the regression model.

Oris™ fluorescence-based microplate migration assay. Passage 3 to 4 equine MSCs were plated into 96-well Oris™ tissue culture treated cell migration plates (Platypus Technologies, LLC, Madison, WI) at a density of 16,000 cells/cm^2 in MSC growth media (Dulbecco's modified Eagles' media; 1,000mg/L glucose; 1ng/mL bFGF; 25m*M* HEPES; 100units/mL penicillin-streptomycin; 10% fetal calf serum) and allowed to adhere for 4 hours. At 4 hours, media was replaced with media containing: 200mM β-lactose, 50ng/mL recombinant equine galectin-1 or -3, 100ng/mL recombinant galectin-1 or -3, or control MSC growth media. Oris™ cell stoppers were removed at 24, 36 and 48 hrs, and fresh media was re-applied. At 48hrs, media was aspirated, 100μL of 1.2μM of calcein AM was used to label cells for 30 minutes at 37°C, and 3 PBS rinses were performed. The Platypus mask was applied to the 96-well plate, and fluorescence was measured using a microplate reader in bottom read mode (Tecan Safire, Tecan Group Ltd., Mannedorf, Switzerland).

Statistical analysis: Eight independent experiments were performed using the Platypus Oris™ cell migration assay, and all treatment conditions were applied in duplicate. Fluorescence intensity was normalized to the mean maximum fluorescence intensity of each control treatment, and normalized mean fluorescence intensity was analyzed as a percentage of the control. One-way ANOVA was performed for each time

point with differences in treatment conditions compared using Tukey's post hoc tests ($P<0.05$).

Results

MSC galectin expression

MSCs expressed approximately 3-fold higher galectin-1 as compared to cultured synoviocytes ($p=0.0005$) and 30-fold higher galectin-1 ($p<0.0001$) relative to cultured articular chondrocytes **(Figure 4.1)**. MSC galectin-3 expression was not significantly different from either synoviocytes or chondrocytes, though synoviocytes showed a trend for higher galectin-3 expression. As a result, MSCs had an elevated galectin-1: galectin-3 ratio (3.77) as compared to both synoviocytes and chondrocytes (0.62 and 0.34, respectively), consistent with an "anti-inflammatory" phenotype. Galectin-1 and -3 expression did not vary significantly with passage from P0 to P3 MSCs; however, galectin-1 and galectin-3 expression were significantly increased in cultured synoviocytes as compared to synovial membrane tissue directly harvested from equine carpal joints ($p<0.0001$ and $p<0.05$, respectively).

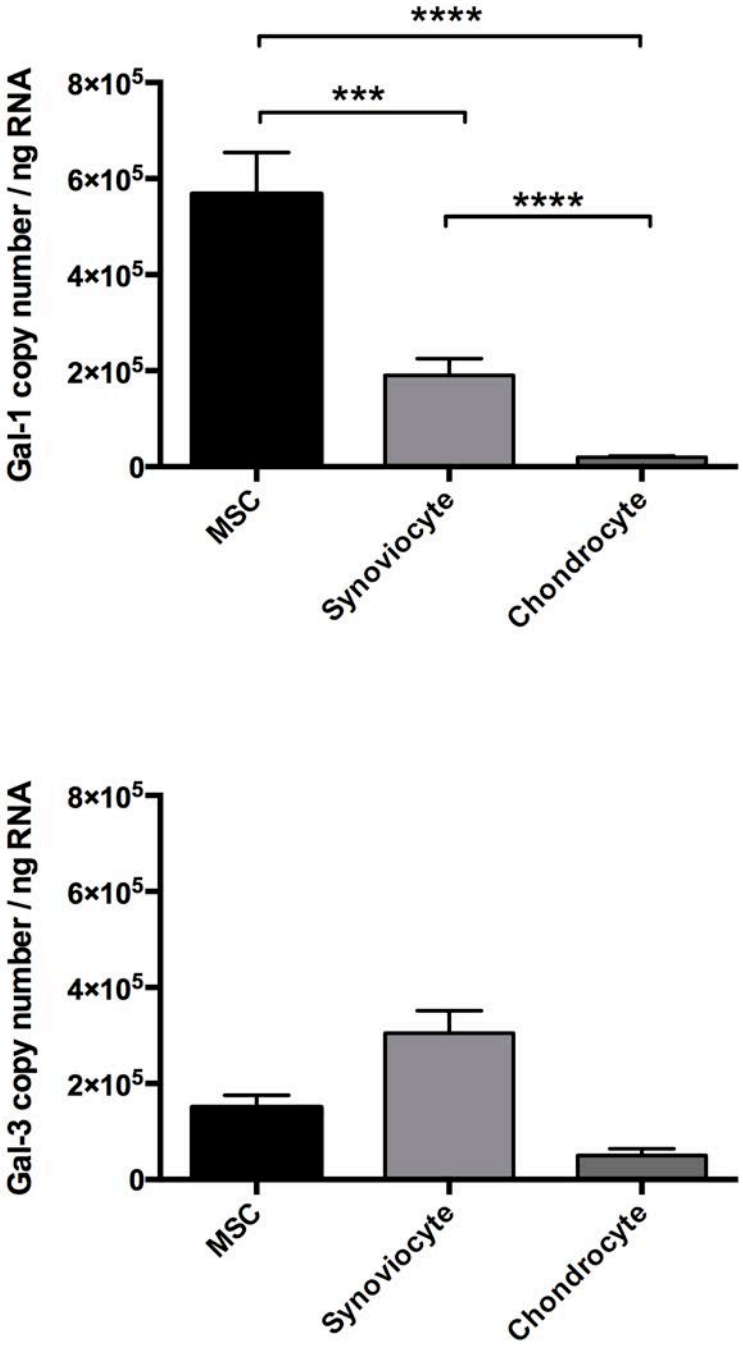


Figure 4.1. Galectin-1 and galectin-3 transcription levels (copy number/ng RNA) from cultured equine MSCs, synoviocytes and chondrocytes. Data are presented as mean ± s.e. Statistical analysis is performed on log transformed data (***$P < 0.001$, ****$P < 0.0001$, Tukey's post hoc tests).

Cytokine induced MSC galectin expression

The pro-inflammatory cytokines IL-1β and TNFα decreased both galectin-1 and galectin-3 mRNA expression in equine MSCs. IL-1β at 5-10ng/mL decreased MSC galectin-1 and galectin-3 mRNA expression approximately 3-fold at 20 and 30 hrs post-exposure **(Figures 4.2 and 4.3)**. Exposure to 50ng/mL of TNFα decreased galectin-1 mRNA expression at 20 and 30 hrs **(Figure 4.2)** and galectin-3 expression at 20 hrs **(Figure 4.3)**. The effect of LPS treatment was less pronounced. Exposure to 10μg/mL of LPS decreased galectin-1 mRNA expression at 30 hrs (P=0.05, **Figure 4.2**), and galectin-3 mRNA expression was decreased by most doses of LPS at 4 hrs and by 10μg/mL of LPS at 20 hrs **(Figure 4.3)**.

Although galectin mRNA expression decreased following exposure to both IL-1β and TNF-α, no changes were observed in galectin protein concentrations in media supernatants upon treatment with either cytokine **(Figure 4.4)**. Conversely, LPS induced a dose-dependent increase in galectin-1 expression that was significantly different from controls at 8 and 20 hrs post-exposure **(Figure 4.4)**. Galectin-3 concentrations in media supernatants did not significantly differ from controls, although high-dose LPS (50μg/mL) resulted in elevated galectin-3 media concentrations in one primary MSC cell line **(Figure 4.4)**. Galectins are present in intracellular, membrane-bound and secreted

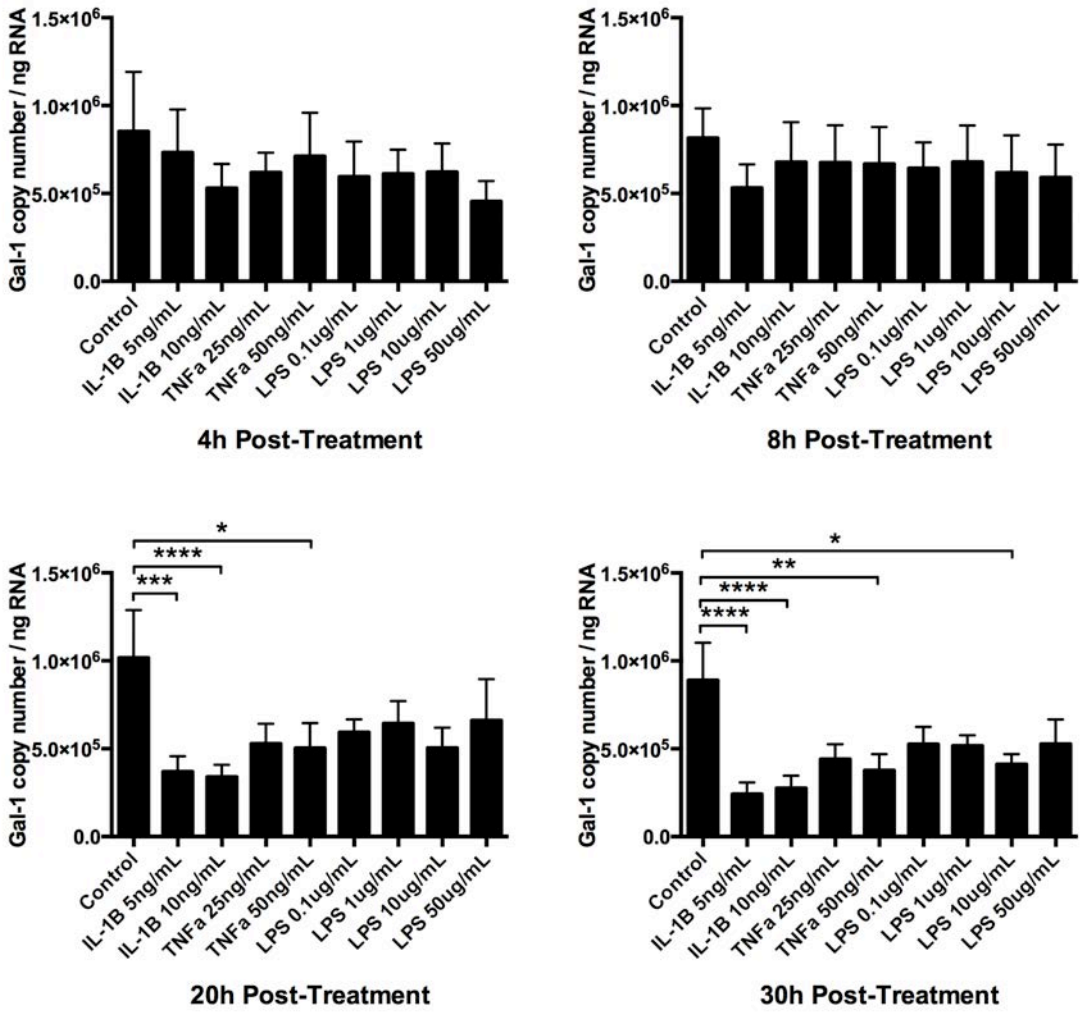


Figure 4.2. Galectin-1 transcription levels (copy number/ng RNA) in equine P3 MSCs 4, 8, 20 and 30 hours after stimulation with recombinant equine IL-1β, recombinant equine TNFα, or LPS. Data are presented as mean ± s.e. from three independent experiments performed in duplicate. Statistical analysis is performed on log transformed data (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, Friedman's test).

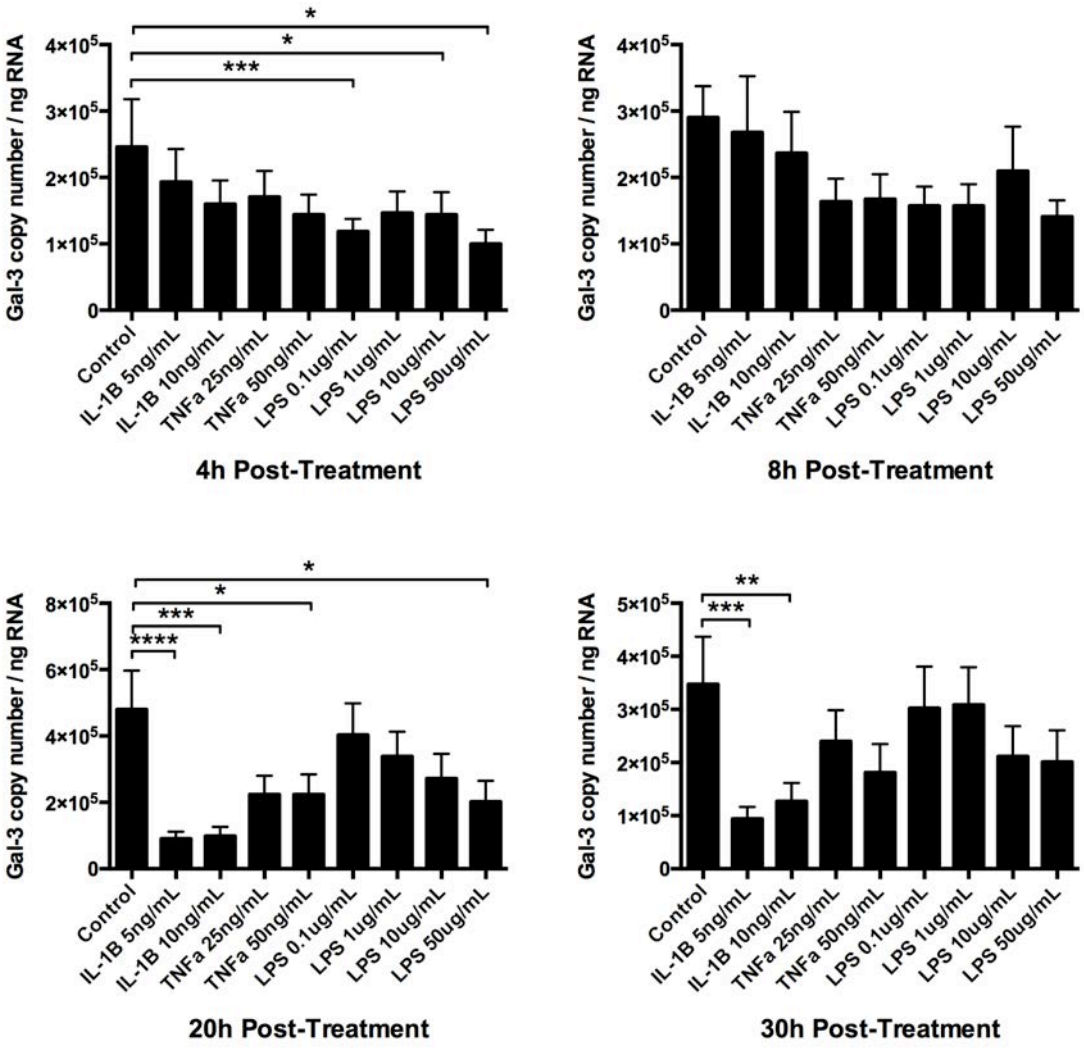
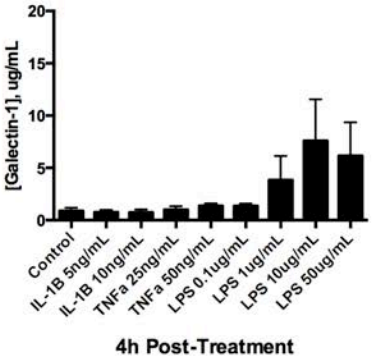


Figure 4.3. Galectin-3 transcription levels (copy number/ng RNA) in equine P3 MSCs 4, 8, 20 and 30 hours after stimulation with recombinant equine IL-1β, recombinant equine TNFα, or LPS. Data are presented as mean ± s.e. from three independent experiments performed in duplicate. Statistical analysis is performed on log transformed data (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001, Friedman's test).

Figure 4.4. Galectin-1 and galectin-3 secretion (μg/mL) measured in the supernatants of cytokine or LPS treated equine P3 MSCs at 4, 8, 20 and 30 hours following treatment. Data are presented as mean ± s.e. from three independent experiments performed in duplicate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Friedman's test).


[Galectin-1], ug/mL
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15
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Control
IL-1B 5ng/mL
IL-1B 10ng/mL
TNFa 25ng/mL
TNFa 50ng/mL
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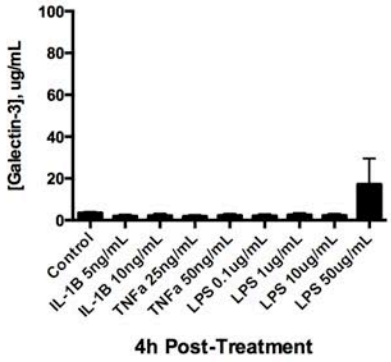
LPS 1ug/mL
LPS 10ug/mL
LPS 50ug/mL
4h Post-Treatment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LPS 50ug/mL
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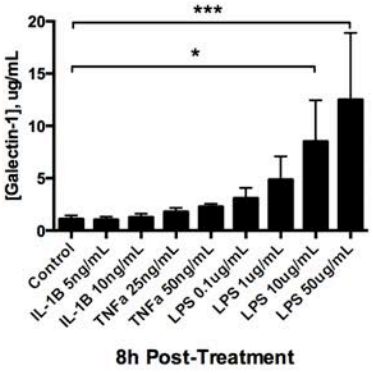
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IL-1B 5ng/mL
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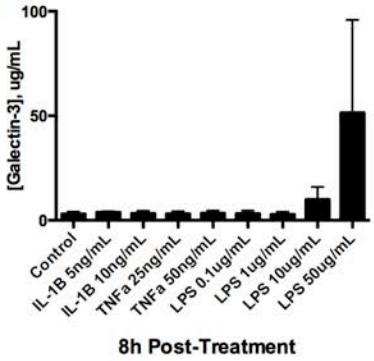
8h Post-Treatment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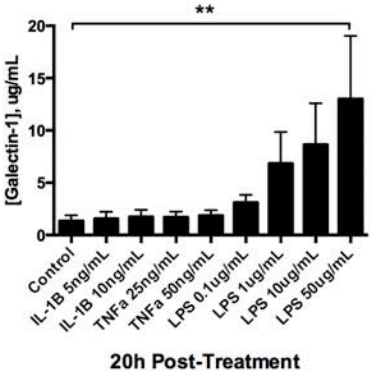
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TNFa 50ng/mL
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LPS 1ug/mL
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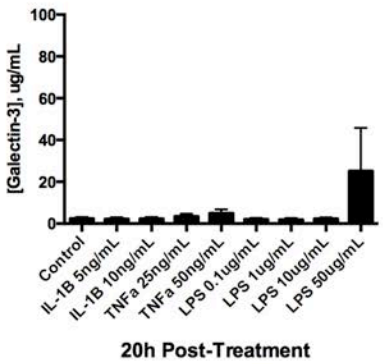
LPS 50ug/mL
20h Post-Treatment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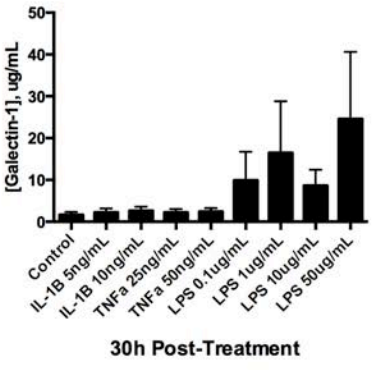
20h Post-Treatment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IL-1B 5ng/mL
IL-1B 10ng/mL
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LPS 0.1ug/mL
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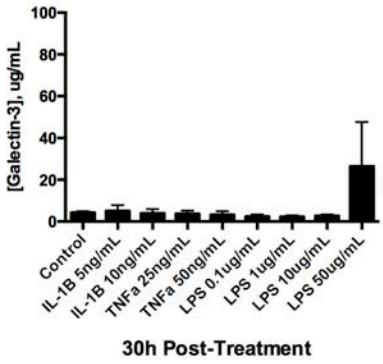
LPS 50ug/mL
30h Post-Treatment


[Galectin-3], ug/mL
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Control
IL-1B 5ng/mL
IL-1B 10ng/mL
TNFa 25ng/mL
TNFa 50ng/mL
LPS 0.1ug/mL
LPS 1ug/mL
LPS 10ug/mL
LPS 50ug/mL
30h Post-Treatment

forms (17, 32). Therefore, membrane bound and intracellular galectins were also quantified by immunoblotting for galectin-1 and galectin-3 in cell lysates. No statistically significant differences in either galectin-1 or galectin-3 concentrations were detected in cell lysates **(Figure 4.5)**.

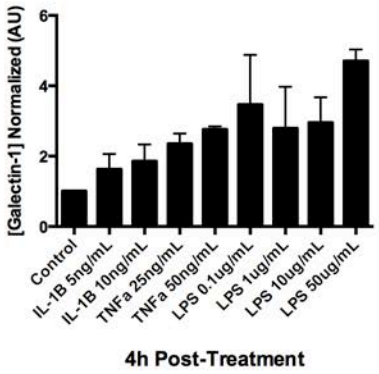
MSC adhesion with focal adhesion markers

Galectins have been shown to interact with integrins (46–49), and integrins play a significant role in MSC adhesion, spreading and motility (50, 51). When plated on fibronectin-coated glass substrates, equine P4 MSCs nucleofected with paxillin demonstrated distinct focal adhesions (FA) at the cell periphery **(Figure 4.6)**. In addition, FA were associated with actin stress fibers, indicating mature, mechanically stable adhesive structures. The addition of 100mM β-lactose inhibited adhesion complex assembly, suggesting that galectins play a role in MSC adhesion, at least in part through integrin-mediated mechanisms.

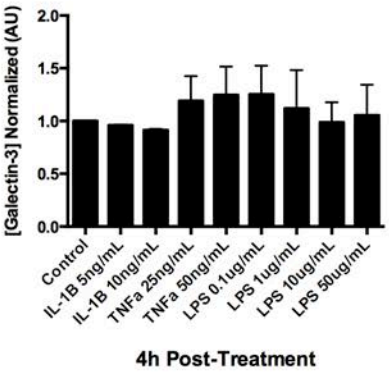
MSC cell spreading

In the presence of serum, 100mM β-lactose significantly decreased MSC cell spreading on BSA, galectin-1, and galectin-3-coated glass substrates and tissue culture

Figure 4.5. Galectin-1 and galectin-3 protein expression, expressed as absorbance units (AU) normalized to controls, in equine P3 MSC lysates at 4, 8, 20 and 30 hours following cytokine or LPS treatment. Data are presented as mean ± s.e. from three independent experiments. Differences are not significant.



4h Post-Treatment



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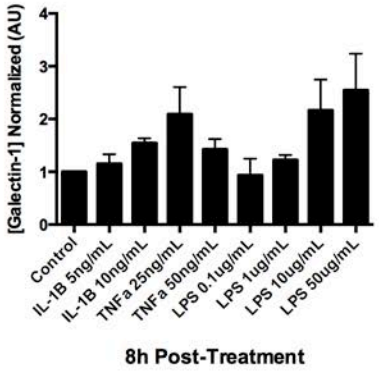
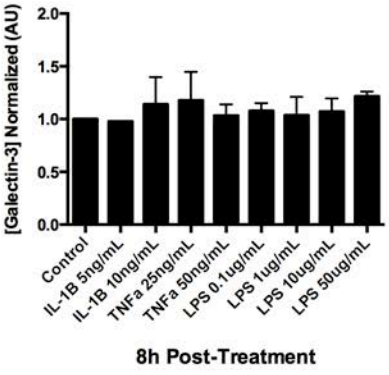


8h Post-Treatment



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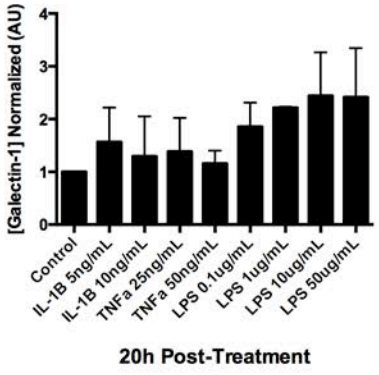

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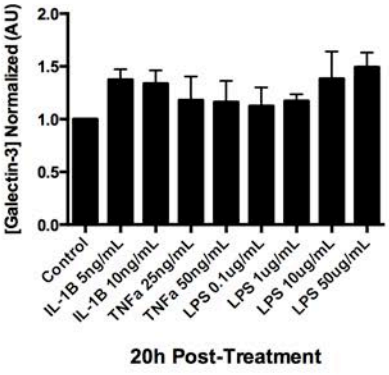

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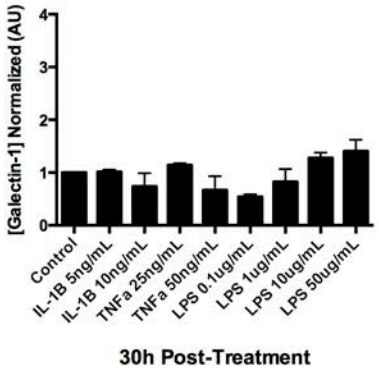

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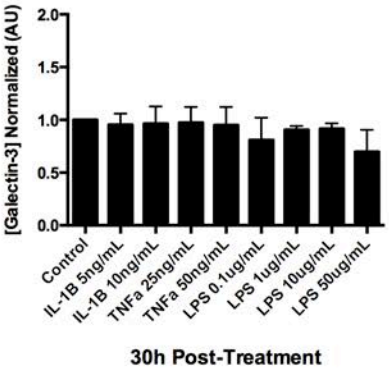

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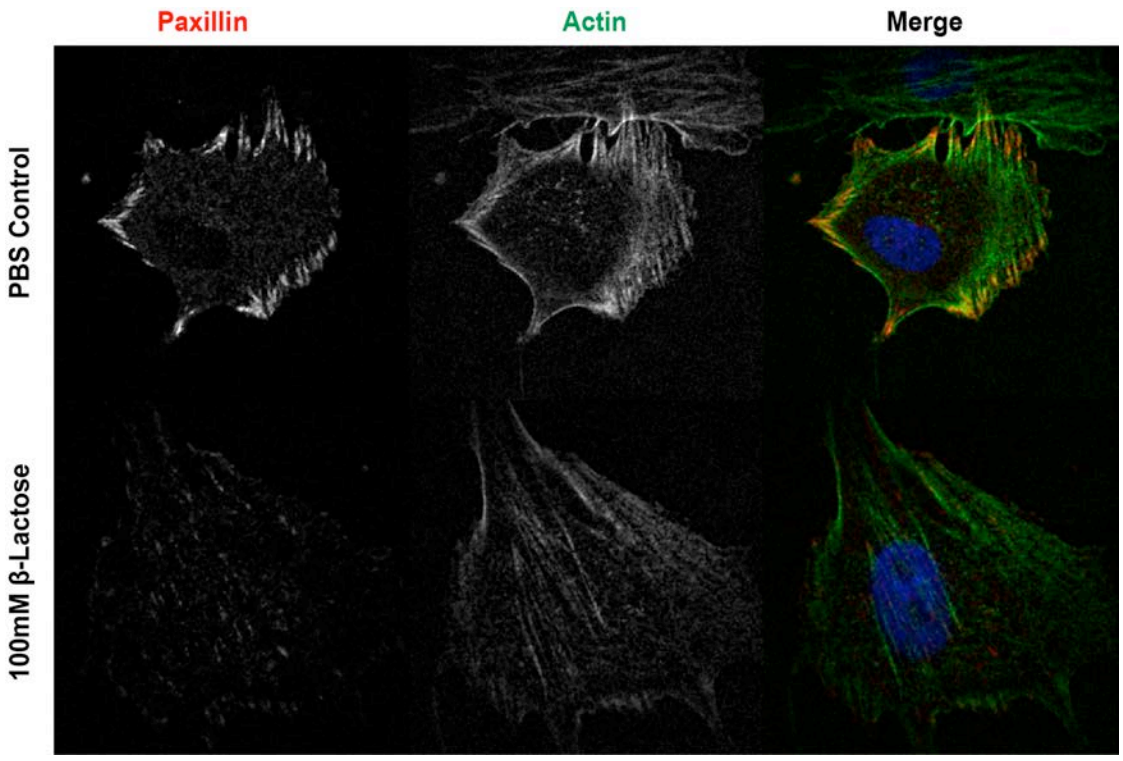

Figure 4.6. Equine P4 MSCs plated on fibronectin-coated glass substrates following nucleofection with the focal adhesion marker paxillin mEmerald and reacted with fluorescent probes for both actin (phalloidin Alexa647) and nuclei (DAPI). Distinct focal adhesion structures are present within MSCs plated on fibronectin substrates in the presence of control media but not in the presence of media with 100mM β-lactose.

plastic (P=0.05, **Figure 4.7**). In the absence of serum, 100mM β-lactose decreased cell spreading on BSA-coated substrates; however, there was a trend for decreased spreading on all substrates tested **(Figure 4.7)**. MSC cell spreading was increased on galectin-1-coated substrates as compared to tissue culture plastic when cultured in standard growth media **(Figure 4.7)**. These data suggest that galectins play a significant role in MSC adhesion and spreading.

MSC cell migration

Consistent with the adhesion phenotype, MSC migration rates were significantly decreased in the presence of 200mM β-lactose at 36 (P<0.01) and 48 hrs (P<0.0001) post-treatment using a fluorescence-based microplate assay **(Figure 4.8)**. Addition of recombinant equine galectin-1 or galectin-3 did not significantly alter MSC motility as compared to controls **(Figure 4.8)**.

Regression analysis of Ibidi® migration data similarly revealed a dose-dependent reduction in MSC motility in the presence of β-lactose and no effect of exogenous galectins on motility as compared to controls **(Figure 4.9)**. IL-1β (5ng/mL, 10ng/mL) and TNF-α (25ng/mL, 50ng/mL) both decreased MSC motility. The reduced motility induced by IL-1β exposure was partially rescued by treatment with 50ng/mL of galectin-1 or galectin-3 **(Figure 4.9)**; however, galectins were not effective in recovering motility after treatment with TNFα. These migration data suggest that galectins may play a role

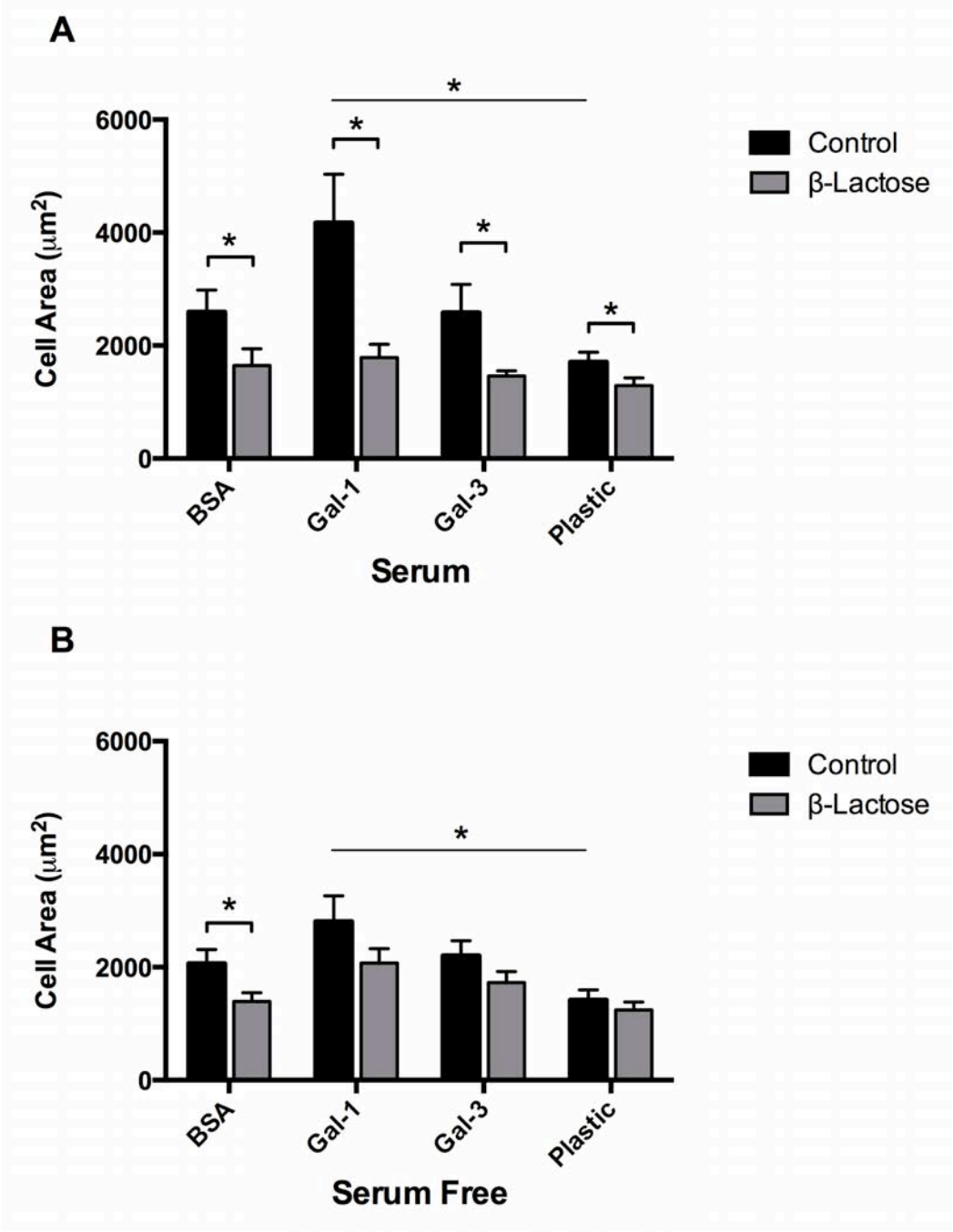

Figure 4.7. Cell spreading in equine P3 MSCs plated on glass substrates conjugated with BSA, recombinant equine galectin-1, recombinant equine galectin-3 or tissue-culture treated plastic 5 hrs post-plating. MSCs were cultured in growth media supplemented with or without 100mM β-lactose in the presence of serum **(A)** or in serum free media **(B)**. Data are presented as mean ± s.e. from two independent experiments. The effect of β-lactose treatment was compared within each substrate (*$P < 0.05$, t-tests), and between substrate comparisons were performed using a one-way ANOVA (*$P < 0.05$).

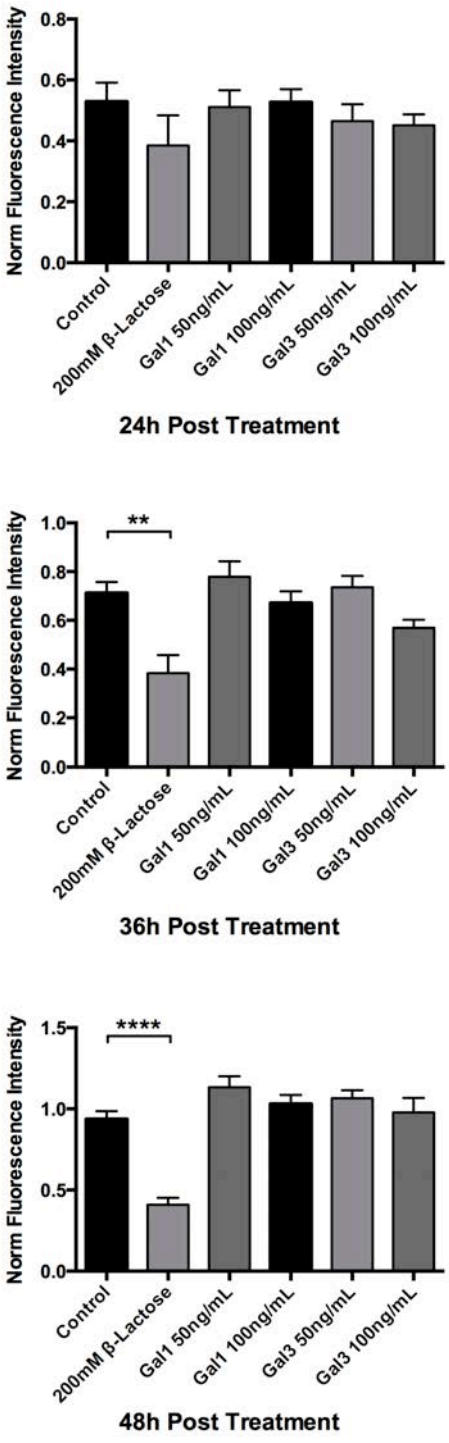

Figure 4.8. Equine P3-P4 MSC migration expressed as fluorescence intensity normalized to the mean maximum fluorescence intensity of the control. Treatment with 200mM β-lactose significantly abrogated MSC motility at 36 and 48 hrs post-treatment (n=16 replicates; $^{**}P < 0.01$, $^{****}P < 0.0001$, Dunnett's test).

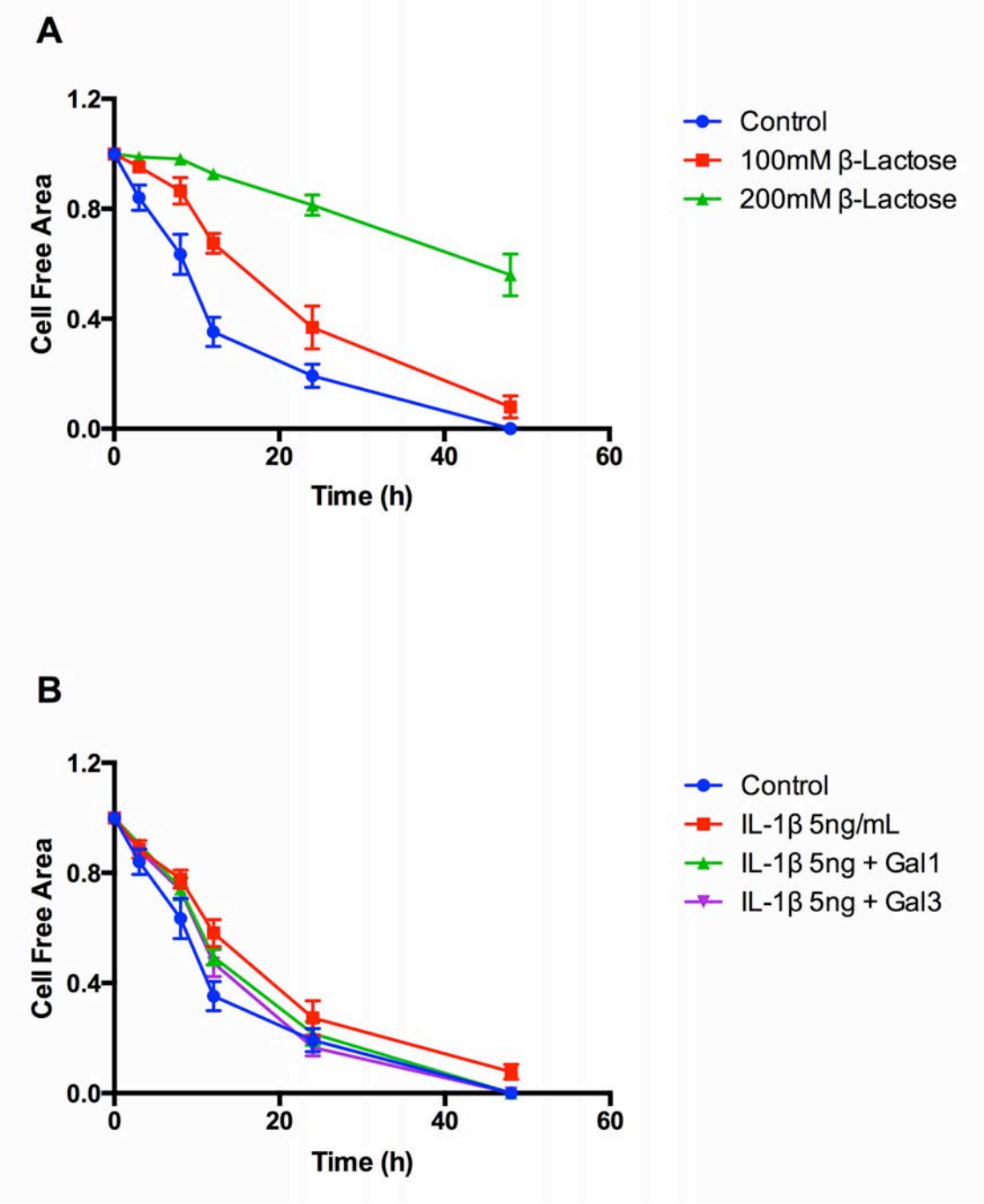

Figure 4.9. Equine P3 MSC migration expressed as cell free area within a 500µm gap, normalized to time 0h. Data are presented as mean ± s.e. from three independent experiments performed in duplicate. **(A)** Treatment with 100mM β-lactose and 200mM β-lactose significantly abrogated MSC motility (P<0.0001). **(B)** Pre-treatment with 5 ng/mL IL-1β significantly decreased MSC motility (P=0.0007), whereas pre-treatment with 5 ng/mL IL-1β followed by 50µg/mL recombinant equine galectin-1 or -3 did not significantly delay motility (Dunnett's test).

in MSC motility. Although exogenous galectins may increase MSC motility in the presence of inflammatory mediators such as IL-1β, there is no additional benefit to MSC motility under basal culture conditions.

Discussion

Equine MSCs constitutively express high levels of galectins relative to other synovial cell types **(Figure 4.1)**. Galectin-1 and galectin-3 have both been identified in the proteome of fibroblast-like synoviocytes (52); however, galectin protein expression was not quantified or compared to other cell types in this study. In RA synovial fibroblasts, galectin-3 expression is increased after adhesion to cartilage oligomeric matrix protein (COMP) (53). Galectins-1, -3 and -8 have been documented at both gene and protein levels in cultured OA chondrocytes, with elevation of galectin-1 in areas of severe cartilage degeneration (54) and galectin-3 in OA chondrons (55). Galectin-3 gene expression was increased 2.4-fold in OA cartilage as compared to normal cartilage, and galectin-3 was found in both the cytosol and membrane of chondrocytes, with a strong correlation between integrin β_1 and galectin-3 at the chondrocyte surface (56). Altered glycosylation in OA chondrocytes induced by IL-1β and TNFα, including increased sialylation and shifting sialic acid linkage types, has led authors to speculate that galectin function may be altered under inflammatory conditions (57).

In the presence of the *in vitro* inflammatory cytokines IL-1β and TNFα used to mimic osteoarthritic conditions *in vitro*, equine MSCs down-regulated expression of both

galectin-1 and galectin-3 mRNA **(Figures 4.2 and 4.3)**; however, no significant changes were observed in galectin concentrations in media supernatants **(Figure 4.4)**. In human MSC T-cell co-cultures, exposure to IL-1β, TNFα and IFN-ϒ resulted in increased galectin-1 expression (28); however, galectin-1 expression was only increased when MSCs were cultured in direct contact with T-cells (28). TNFα reduces galectin-3 expression within 18 hours in both OA and RA synovial fibroblasts (53).

Galectins are expressed intracellulary, on the cell surface and in secreted form (17, 32). Whereas exposure to IL-1β and TNFα decreased galectin-1 and -3 gene expression, no significant changes were observed in galectin concentrations in media supernatants **(Figure 4.4)** or cell lysates **(Figure 4.5)**. LPS exposure also resulted in decreased galectin-1 gene expression **(Figure 4.2)**; however, analysis of media supernatants revealed dose-dependent increases in secreted galectin-1 8-20h post-exposure **(Figure 4.4)**. There was a trend for increased galectin-1 in cell lysates in response to increasing concentrations of LPS; however, this trend was not statistically significant **(Figure 4.5)**. A limitation of quantifying galectins in cell lysates is the semi-quantitative nature of immunoblotting, which is not highly sensitive to changes in protein expression. Nonetheless, there appear to be discrepancies between galectin mRNA and protein expression in equine MSCs following treatment with IL-1β, TNFα and LPS. Further investigation may be required in order to interpret these discrepancies.

Confocal imaging of equine MSCs nucleofected with the focal adhesion protein paxillin-mEmerald revealed distinct adhesion complexes on fibronectin-coated glass

substrates **(Figure 4.6)**. Inhibition of MSC galectin binding with 100mM β-lactose nearly abolished FA structures **(Figure 4.6)** and decreased MSC adhesion and spreading **(Figure 4.7)**. MSCs spread more on galectin-1 coated substrates as compared to plastic, consistent with a study in which macrophages from gal3+/+ mice spread much more dramatically than macrophages that remained spindle-shaped from gal3-/- mice (58). Several adhesion-related molecules have been detected in human MSCs, including integrin subunits α4, α5, β1, αvβ3, αvβ5, ICAM-1 and CD44H (59). Cell area measurements of human MSCs were significantly increased on fibronectin substrates as compared to vitronectin or osteopontin, and fibronectin increased expression of α5 integrin (60). Galectins bind directly to integrins, promoting both integrin clustering (48) and signaling activity (49). Integrins play a major role in galectin-3-mediated regulation of cell adhesion, with intracellular galectin-3 upregulating α_4 and β_7 (46) and $\alpha_6\beta_1$ integrins (61) while exogenous galectin-3 upregulates β_2 integrin (62). Furthermore, galectin-3 can bind to integrins such as $\alpha_1\beta_1$ (63), and galectins can enhance the mechanical strength of adhesive bonds between integrins and extracellular matrix proteins, such as collagen-I and –IV (64).

MSC motility was markedly downregulated in the presence of the pan-galectin inhibitor β-lactose **(Figures 4.8 and 4.9)**. Pro-inflammatory cytokines (IL-1β and TNFα) also decreased MSC motility, and IL-1β induced reductions in MSC motility were partially reversed by the addition of recombinant galectins **(Figure 4.9)**, suggesting that galectins may play a role in stem cell properties such as adhesion, migration and homing. Galectin-1 has been shown to stimulate motility of human umbilical cord-derived MSCs

as a result of downregulation of smad2/3-dependent collagen 3/5 and upregulation of NF-kB-dependent fibronectin/ laminin 5 expression (37). In human monocyte-derived dendritic cells, galectin-1 upregulates a subset of genes related to cell migration through the extracellular matrix (ECM), with improved chemotactic migration through Matrigel (36). Although galectin-3-mediated motility has not been previously investigated in MSCs, galectin-3 promotes cell motility and invasion in a variety of cells, including monocytes and macrophages (35), neuroblasts (65), keratinocytes (66, 67), and several tumor cells (68).

MSCs delivered by systemic intravenous injection have been shown to migrate to sites of tissue injury, such as experimental cardiac infarcts (69). Mechanisms underlying migration and extravasation of MSCs are poorly defined. However, chemokine receptors and adhesion molecules play a critical role in tissue-specific homing of leukocytes, and leukocytes and MSCs share some common features in cellular adhesion and migration (70, 71). Leukocytes undergo a process of tethering, rolling, adhesion, and vascular transmigration via interactions with two separate classes of adhesion molecules, selectins and integrins, which are further modulated by chemokines (72). Galectin-1 functions as a ligand for P-selectin, suggesting that this interaction may play an important role in immunomodulatory targeting of human UCB-derived MSCs to sites of tissue inflammation (71).

In addition to their role as immunomodulatory cells, one of the goals in musculoskeletal MSC applications is the use of MSCs to repair cartilage, which has poor

intrinsic healing due to its hypocellular, avascular and aneural nature (73). MSCs have the potential to differentiate into chondrogenic lineages, and much effort has been focused in determining the optimal culture strategies to enhance chondrogenic differentiation in MSCs (74). Although there appear to be beneficial effects from direct injection of MSCs into OA joints even without substantial engraftment (2, 3), results have been underwhelming (10). A study evaluating the role of intra-articular MSC injection on the healing of microfractured cartilage defects in the equine stifle revealed increased aggrecan content and tissue stiffness, though there was no clinical or histological benefit, leading the authors to suggest that MSCs may be potentially beneficial in cartilage repair (7). Outcomes may be improved even more substantially by manipulating the synovial environment such that injected MSC survive and engraft in synovial tissue and cartilage. One potential method for increasing MSC adhesion and engraftment may be to overexpress galectins-1 and-3, which appear to play a role in processes such as adhesion, spreading and migration.

MSCs are capable of binding to articular cartilage both *in vitro* (75) and *in vivo* in canine joints undergoing knee joint distraction following induction of OA (4); however, little is known about the mechanisms that facilitate MSC adhesion to cartilage. Increased MSC adhesion to OA cartilage in a canine model treated with joint distraction has led investigators to propose that increased MSC adhesion may be a possible explanation for the remarkable cartilage repair results observed following joint distraction (4). Galectin-1 strongly immunolocalizes to superficial zone chondrocytes in porcine articular cartilage (76), and both galectin-1 and galectin-3 have been localized to the superficial regions of

human OA cartilage (54). Galectin-3 expression is increased in OA chondrocytes (56), and its localization to the chondrocyte surface is correlated with integrin-β1 expression (77). Therefore, it is possible that galectins serve as adhesive molecules to facilitate MSC adhesion to cartilage. Several groups have investigated the use of bioadhesives or other assistive materials to increase homing, retention and adhesion of MSCs at the cartilage surface (38). Galectins are promising candidates as cartilage-based MSC cytoadhesives because they are endogenous, non-immunogenic, and they possess anti-inflammatory and immunomodulatory benefits in addition to their adhesive properties.

Further investigation into the role of intra-articular galectins is warranted, including how MSCs contribute to synovial galectin expression and; conversely, how galectins affect MSC properties such as adhesion and motility. MSCs express high levels of galectin-1 relative to other synovial cell types. Galectin-1 has been shown to have potent immunomodulatory properties (78), and it is possible that some of the beneficial effects of intra-articular MSCs are mediated by galectins. Inflammatory cytokines downregulate galactin-1 and galectin-3 gene expression in equine MSCs; however, galectin protein expression remains relatively stable following cytokine exposure. MSC focal adhesion formation, cell spreading and migration are significantly inhibited by the pan-galectin inhibitor β-lactose, suggesting that this class of molecules may play an important role in these MSC properties. Future research efforts include investigating how galectins may enhance endogenous MSC migration and homing, and how galectins may be leveraged to enhance MSC adhesion for cartilage and tissue repair.

References

1. Pittenger MF, et al. (1999) Multilineage potential of adult human mesenchymal stem cells. *Science* 284(5411):143–147.
2. Coleman CM, Curtin C, Barry FP, O'Flatharta C, Murphy JM (2010) Mesenchymal stem cells and osteoarthritis: remedy or accomplice? *Hum Gene Ther* 21(10):1239–50.
3. Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 48(12):3464–74.
4. Baboolal TG, et al. (2015) Synovial fluid hyaluronan mediates MSC attachment to cartilage, a potential novel mechanism contributing to cartilage repair in osteoarthritis using knee joint distraction. *Ann Rheum Dis*:1–8.
5. Prockop DJ, Youn Oh J (2011) Mesenchymal Stem/Stromal Cells (MSCs): Role as Guardians of Inflammation. *Mol Ther* 20(1):14–20.
6. Caplan AI (2009) Why are MSCs therapeutic? New data: new insight. *J Pathol* 217(2):318–24.
7. McIlwraith CW, et al. (2011) Evaluation of intra-articular mesenchymal stem cells to augment healing of microfractured chondral defects. *Arthroscopy* 27(11):1552–61.
8. Mokbel AN, et al. (2011) Homing and reparative effect of intra-articular injection of autologus mesenchymal stem cells in osteoarthritic animal model. *BMC Musculoskelet Disord* 12(1):259.
9. Wong KL, et al. (2013) Injectable cultured bone marrow-derived mesenchymal stem cells in varus knees with cartilage defects undergoing high tibial osteotomy: a prospective, randomized controlled clinical trial with 2 years' follow-up. *Arthroscopy* 29(12):2020–8.
10. Tyndall A (2015) Mesenchymal stromal cells and rheumatic disorders. *Immunol Lett*:1–7.
11. Carrade Holt DD, et al. (2014) Equine mesenchymal stem cells inhibit T cell proliferation through different mechanisms depending on tissue source. *Stem Cells Dev* 23(11):1258–65.
12. Meisel R, et al. (2004) Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 103(12):4619–21.
13. Sala E, et al. (2015) Mesenchymal Stem Cells Reduce Colitis in Mice via Release of TSG6, Independently of Their Localization to the Intestine. *Gastroenterology* 149(1):163–176.e20.
14. Ortiz LA, et al. (2007) Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A* 104(26):11002–11007.

15. Sioud M, Mobergslien a, Boudabous a, Fløisand Y (2010) Evidence for the involvement of galectin-3 in mesenchymal stem cell suppression of allogeneic T-cell proliferation. *Scand J Immunol* 71(4):267–74.

16. Sioud M, Mobergslien A, Boudabous A, Fløisand Y (2011) Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. *Int J Oncol* 38(2):385–90.

17. Liu GY, et al. (2013) Secreted galectin-3 as a possible biomarker for the immunomodulatory potential of human umbilical cord mesenchymal stromal cells. *Cytotherapy* 15(10):1208–1217.

18. Gieseke F, et al. (2010) Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. *Blood* 116(19):3770–9.

19. Li C, et al. (2013) [Expression of Galectins in umbilical cord mesenchymal stem cells]. *Beijing Da Xue Xue Bao* 45(3):452–7.

20. Rabinovich GA, et al. (2002) Galectins and their ligands: Amplifiers, silencers or tuners of the inflammatory response? *Trends Immunol* 23(6):313–320.

21. Rabinovich GA, Rubinstein N, Toscano MA (2002) Role of galectins in inflammatory and immunomodulatory processes. *Biochim Biophys Acta - Gen Subj* 1572(2-3):274–284.

22. Nachtigal M, Al-Assaad Z, Mayer EP, Kim K, Monsigny M (1998) Galectin-3 expression in human atherosclerotic lesions. *Am J Pathol* 152(5):1199–1208.

23. Baum LG, et al. (2003) Amelioration of graft versus host disease by galectin-1. *Clin Immunol* 109(3):295–307.

24. Liu SD, et al. (2011) Galectin-1-induced down-regulation of T lymphocyte activation protects (NZB x NZW) F1 mice from lupus-like disease. *Lupus* 20(5):473–84.

25. Iqbal AJ, et al. (2013) Endogenous galectin-1 exerts tonic inhibition on experimental arthritis. *J Immunol* 191(1):171–7.

26. Rabinovich GA, et al. (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190(3):385–398.

27. Sioud M (2011) New insights into mesenchymal stromal cell-mediated T-cell suppression through galectins. *Scand J Immunol* 73(2):79–84.

28. Najar M, et al. (2010) Modulated expression of adhesion molecules and galectin-1: role during mesenchymal stromal cell immunoregulatory functions. *Exp Hematol* 38(10):922–32.

29. Lepelletier Y, et al. (2010) Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. *Stem Cells Dev* 19(7):1075–9.

30. Malik RKJ, Ghurye RR, Lawrence-Watt DJ, Stewart HJS (2009) Galectin-1

stimulates monocyte chemotaxis via the p44/42 MAP kinase pathway and a pertussis toxin-sensitive pathway. *Glycobiology* 19(12):1402–7.

31. Auvynet C, et al. (2012) Galectin-1 promotes human neutrophil migration. *Glycobiology* 23(1):32–42.

32. Liu F-T, Patterson RJ, Wang JL (2002) Intracellular functions of galectins. *Biochim Biophys Acta* 1572(2-3):263–73.

33. Goetz JG (2009) Bidirectional control of the inner dynamics of focal adhesions promotes cell migration. *Cell Adh Migr* 3(2):185–190.

34. Geiger B, Bershadsky A (2002) Exploring the neighborhood: adhesion-coupled cell mechanosensors. *Cell* 110(2):139–42.

35. Sano H, et al. (2000) Human Galectin-3 Is a Novel Chemoattractant for Monocytes and Macrophages. *J Immunol* 165(4):2156–2164.

36. Fulcher J a, et al. (2006) Galectin-1-Matured Human Monocyte-Derived Dendritic Cells Have Enhanced Migration through Extracellular Matrix. *J Immunol* 177:216–226.

37. Yun SP, Lee S-J, Jung YH, Han HJ (2014) Galectin-1 stimulates motility of human umbilical cord blood-derived mesenchymal stem cells by downregulation of smad2/3-dependent collagen 3/5 and upregulation of NF-κB-dependent fibronectin/laminin 5 expression. *Cell Death Dis* 5(2):e1049.

38. Shao Z, et al. (2012) Polycaprolactone electrospun mesh conjugated with an MSC affinity peptide for MSC homing in vivo. *Biomaterials* 33(12):3375–3387.

39. Xibillé-Friedmann D, Bustos Rivera-Bahena C, Rojas-Serrano J, Burgos-Vargas R, Montiel-Hernández J-L (2013) A decrease in galectin-1 (Gal-1) levels correlates with an increase in anti-Gal-1 antibodies at the synovial level in patients with rheumatoid arthritis. *Scand J Rheumatol* 42(2):102–7.

40. Alturfan a A, Eralp L, Emekli N (2008) Investigation of inflammatory and hemostatic parameters in female patients undergoing total knee arthroplasty surgery. *Inflammation* 31(6):414–21.

41. Ohshima S, et al. (2003) Galectin 3 and its binding protein in rheumatoid arthritis. *Arthritis Rheum* 48(10):2788–95.

42. Fortier LA, Nixon AJ, Williams J, Cable CS (1998) Isolation and chondrocytic differentiation of equine bone marrow-derived mesenchymal stem cells. *Am J Vet Res* 59(9):1182–1187.

43. Saxer R a., et al. (2001) Gene mediated insulin-like growth factor-I delivery to the synovium. *J Orthop Res* 19(5):759–767.

44. Nixon AJ, Lust G, Vernier-Singer M (1992) Isolation, propagation, and cryopreservation of equine articular chondrocytes. *Am J Vet Res* 53(12):2364–2370.

45. Pace KE, Hahn HP, Baum LG (2003) Preparation of recombinant human galectin-

1 and use in T-cell death assays. *Methods Enzymol* 363(2001):499–518.

46. Matarrese P, et al. (2000) Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. *Int J Cancer* 85(4):545–554.

47. Friedrichs J, Manninen A, Muller DJ, Helenius J (2008) Galectin-3 regulates integrin alpha2beta1-mediated adhesion to collagen-I and -IV. *J Biol Chem* 283(47):32264–72.

48. Priglinger CS, et al. (2013) Galectin-3 Induces Clustering of CD147 and Integrin-β1 Transmembrane Glycoprotein Receptors on the RPE Cell Surface. *PLoS One* 8(7):e70011.

49. Seguin L, et al. (2014) An integrin β3-KRAS-RalB complex drives tumour stemness and resistance to EGFR inhibition. *Nat Cell Biol* 16(5):457–68.

50. Chiu L-H, et al. (2012) Diverse effects of type II collagen on osteogenic and adipogenic differentiation of mesenchymal stem cells. *J Cell Physiol* 227(6):2412–20.

51. Krishna OD, Jha AK, Jia X, Kiick KL (2011) Integrin-mediated adhesion and proliferation of human MSCs elicited by a hydroxyproline-lacking, collagen-like peptide. *Biomaterials* 32(27):6412–6424.

52. Dasuri K, et al. (2004) The synovial proteome: analysis of fibroblast-like synoviocytes. *Arthritis Res Ther* 6(2):R161–8.

53. Neidhart M, et al. (2005) Galectin-3 is induced in rheumatoid arthritis synovial fibroblasts after adhesion to cartilage oligomeric matrix protein. *Ann Rheum Dis* 64(3):419–24.

54. Toegel S, et al. (2014) Human osteoarthritic knee cartilage: fingerprinting of adhesion/growth-regulatory galectins in vitro and in situ indicates differential upregulation in severe degeneration. *Histochem Cell Biol* 142(4):373–88.

55. Toegel S, et al. (2013) Glycophenotyping of osteoarthritic cartilage and chondrocytes by RT-qPCR, mass spectrometry, histochemistry with plant/human lectins and lectin localization with a glycoprotein. *Arthritis Res Ther* 15(5):R147.

56. Guévremont M, et al. (2004) Galectin-3 surface expression on human adult chondrocytes: a potential substrate for collagenase-3. *Ann Rheum Dis* 63(6):636–43.

57. Pabst M, et al. (2010) IL-1β and TNF-α alter the glycophenotype of primary human chondrocytes in vitro. *Carbohydr Res* 345(10):1389–1393.

58. Hsu DK, et al. (2000) Targeted disruption of the galectin-3 gene results in attenuated peritoneal inflammatory responses. *Am J Pathol* 156(3):1073–1083.

59. Conget P a, Minguell JJ (1999) Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 181(1):67–73.

60. Schwab EH, et al. (2013) Distinct effects of RGD-glycoproteins on Integrin-

mediated adhesion and osteogenic differentiation of human mesenchymal stem cells. *Int J Med Sci* 10(13):1846–59.

61. Raz A, et al. (1990) Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis. *Int J Cancer* 46(5):871–7.

62. Le Marer N, Hughes RC (1996) Effects of the carbohydrate-binding protein galectin-3 on the invasiveness of human breast carcinoma cells. *J Cell Physiol* 168(1):51–8.

63. Ochieng J, Warfield P, Green-Jarvis B, Fentie I (1999) Galectin-3 regulates the adhesive interaction between breast carcinoma cells and elastin. *J Cell Biochem* 75(3):505–14.

64. Friedrichs J, Manninen A, Muller DJ, Helenius J (2008) Galectin-3 regulates integrin alpha2beta1- mediated adhesion to collagen-I and -IV. *J Biol Chem* 283(47):32264–32272.

65. Comte I, et al. (2011) Galectin-3 maintains cell motility from the subventricular zone to the olfactory bulb. *J Cell Sci* 124(Pt 14):2438–2447.

66. Kariya Y, Kawamura C, Tabei T, Gu J (2010) Bisecting GlcNAc residues on laminin-332 down-regulate galectin-3-dependent keratinocyte motility. *J Biol Chem* 285(5):3330–40.

67. Panjwani N (2014) Role of galectins in re-epithelialization of wounds. *Ann Transl Med* 2(9):89.

68. Elola MT, Wolfenstein-Todel C, Troncoso MF, Vasta GR, Rabinovich G a (2007) Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci* 64(13):1679–700.

69. Pittenger MF, Martin BJ (2004) Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 95(1):9–20.

70. Chamberlain G, Fox J, Ashton B, Middleton J (2007) Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 25(11):2739–49.

71. Suila H, et al. (2014) Human umbilical cord blood-derived mesenchymal stromal cells display a novel interaction between P-selectin and galectin-1. *Scand J Immunol* 80(1):12–21.

72. Ebert LM, Schaerli P, Moser B (2005) Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. *Mol Immunol* 42(7):799–809.

73. Mankin HJ (1982) The response of articular cartilage to mechanical injury. *J Bone Joint Surg Am* 64(3):460–6.

74. Mackay AM, et al. (1998) Chondrogenic Differentiation of Cultured Human Mesenchymal Stem Cells from Marrow. *Tissue Eng* 4(4):415–428.

75. Hung BP, Babalola OM, Bonassar LJ (2013) Quantitative characterization of mesenchymal stem cell adhesion to the articular cartilage surface. *J Biomed Mater*

Res A 101(12):3592–8.

76. Marsich E, et al. (2008) Galectin-1 in cartilage: expression, influence on chondrocyte growth and interaction with ECM components. *Matrix Biol* 27(6):513–25.

77. Loeser RF, Carlson CS, McGee MP (1995) Expression of beta 1 integrins by cultured articular chondrocytes and in osteoarthritic cartilage. *Exp Cell Res* 217(2):248–57.

78. Cooper D, et al. (2010) Multiple functional targets of the immunoregulatory activity of galectin-1: Control of immune cell trafficking, dendritic cell physiology, and T-cell fate. *Methods Enzymol* 480(10):199–244.

CHAPTER 5

DISCUSSION

The overall goal of this thesis project was to investigate how lubricin and galectins function to maintain the integrity of the synovial joint. Specifically, the individual aims of the research were to 1) evaluate how lubricin gene and glycoprotein expression were altered in a clinically relevant equine model of OA and in naturally occurring equine carpal OA; 2) investigate the interactions between lubricin and galectins-1 and -3 and to determine if lubricin and galectins could synergistically enhance articular cartilage boundary lubrication and; 3) interrogate the role of galectins-1 and -3 on MSC properties, including adhesion, spreading and motility, and to assess how these properties and galectin expression were altered in the presence of pro-inflammatory cytokines.

Conclusions

Lubricin in equine OA

Proteoglycan 4 (PRG4) and its gene product, lubricin, play critical roles in maintaining cartilage health and protecting against OA in rodents (1–4). There is significant interest in the use of lubricin as a novel, therapeutic for OA; however, information about its disposition in clinically relevant, large animal models of joint

disease is limited. In chapter 2, we demonstrated that *Prg4* expression was decreased in cartilage obtained from horses with naturally occurring carpal OA. Contrary to our hypotheses, *Prg4* gene expression was increased in the synovial tissues of horses with naturally occurring carpal OA, and lubricin synovial fluid concentrations were increased in both naturally occurring OA and in an experimental chip fracture model of OA. Increases in synovial fluid lubricin concentrations were accompanied by increased lubricin localization to OA synovial tissues and sites of cartilage damage, including fibrillation, clefting and chondron formation. Overall, these data suggest that lubricin expression is increased in response to intra-articular injury in horses. However, we cannot rule out the possibility that the lubricin that we have measured in OA joints is not fully functional, either due to proteolytic degradation or alterations in post-translational modifications, such as glycosylation.

Lubricin and galectins in boundary lubrication

Lubricin enables nearly frictionless joint motion in boundary mode lubrication regimes, where cartilage is subjected to high load, low motion conditions. Lubricin mediates boundary lubrication by its properties in solution as well as adsorption to articular cartilage, within the lamina splendens. Although lubricin interacts with type II collagen (5, 6) and fibronectin (7), little is known about what other molecular interactions allow lubricin to bind to the articular cartilage surface and sustain a lubricating boundary layer. We hypothesized that lubricin might interact with the carbohydrate-binding proteins galectin-1 and galectin-3 via lubricin's mucin-rich O-

linked glycans. We found that galectin-3 co-localized with lubricin on the surface of articular cartilage. Galectin-3 bound to lubricin with high affinity in a carbohydrate-dependent fashion, whereas galectin-1 did not. Furthermore, galectin-3 but not the multimerization-incompetent galectin-3C mutant, enhanced cartilage boundary mode lubrication, suggesting that galectin-3 crosslinking reinforced the lubricin lattice. Hence, we concluded that lubricin and galectin-3 function synergistically to enhance joint lubrication. These data raise the possibility that the lubricin glycophenotype may be just as critical as lubricin concentrations for mediating boundary lubrication and also suggests that lubricin and galectin-3 might both be potential therapeutic agents for the treatment of OA.

MSC galectin expression and adhesion, spreading and motility

Human MSCs express high levels of galectins-1 and -3, and galectins modulate MSC anti-inflammatory and immunosuppressive effects *in vitro* (8, 9). Although galectins are known to mediate fundamental properties such as adhesion and migration in other cell types (10), little is known about how galectins influence basic MSC biology. Therefore, our objectives were to discern how galectin expression was altered in an *in vitro* inflammatory context mimicking OA, and to discover the role of galectins in equine MSC properties such as adhesion, spreading and motility. Equine MSCs expressed more galectin-1 as compared to other synovial cell types, and both galectin-1 and galectin-3 gene expression were downregulated by the pro-inflammatory cytokines IL-1β and TNF-α. Focal adhesion formation, actin

organization, cell spreading and motility were inhibited in equine MSCs by the pan galectin inhibitor β-lactose, indicating that galectins function in MSC adhesion and cytoskeletal organization. Taken together, these results suggest that galectins play a role in MSC properties such as adhesion, spreading and motility.

Summary

In summary, this dissertation research demonstrated that *Prg4* gene and lubricin glycoprotein expression are increased in both naturally occurring and experimental models of equine OA. High affinity binding between lubricin and galectin-3 enhances articular cartilage boundary lubrication, and galectins-1 and -3 facilitate equine MSC adhesion and spreading.

Future Directions

Future efforts include investigating whether the increased lubricin detected within OA joints is fully functional by correlating synovial fluid lubricin concentrations with boundary lubrication measurements. In addition, developing methods to assay for lubricin molecular degradation products and altered glycoforms will provide further insight into how lubricin is modified in joint disease and how glycosylation affects lubrication parameters. Lubricin glycosylation can be broadly assessed in both normal and OA joints using lectin microarrays, and more precisely quantified with MALDI/TOF-MS. Furthermore, analysis of glycosyltransferase

expression, including the *N*-acetylgalactosaminlytransferases and sialyltransferases St3Gal1 and St6Gal1 important for lubricin's *O*-glycosylation, will provide information about how glycosylation is changing in disease. One unanswered question is how lubricin composition and concentration vary between distinct classes of diarthrodial joints—for example, whether lubricin in the equine middle carpal joint (a complex hinge joint) differs from lubricin in the metacarpophalangeal/ metatarsophalangeal joints (uniaxial hinge joints), the stifle joint (large volume hinge joint with interposed meniscus) or the distal tarsal joints (planar joints). Furthermore, it is not understood how the type of trauma or joint injury affects lubricin composition. Future studies involve investigating how lubricin and hyaluronic acid vary in different types of injury, including osteochondral fragmentation, supraphysiologic single impact loading, joint instability due to loss of collateral ligament or meniscal stabilization and inflammatory or infectious etiologies. In addition, whether intra-articular lubricin supplementation or proteoglycan4 gene therapy is effective in ameliorating OA in clinically relevant, large animal species is currently unknown. Although the data in experimental rodent models is compelling, future efforts involve assessing the efficacy of lubricin tribosupplementation in the equine model.

Given the synergistic effects of lubricin and galectin-3 in cartilage lubrication, it will be interesting to unravel how galectin gene and protein expression change in OA and how alterations in lubricin glycoforms impact galectin crosslinking of the lubricin lattice. In addition to galectin-1 and galectin-3, other potentially relevant galectin family members, including galectins-2, -8, and -9, will be evaluated in

cartilage, synovial tissues and synovial fluid from horses with naturally occurring and experimental OA. It will be interesting to determine whether or not galectin-3 is the only galectin family member that binds specifically and with high-affinity to lubricin, and whether that binding is altered in the presence of distinct lubricin glycoforms. Kinetics of lubricin galectin multimerization can be performed using dynamic light scattering, isothermal titration calorimetry and/or surface plasmon resonance. Finally, it will be important to gain additional insight about the nanostructural orientation of the lubricin galectin lattice on the lamina splendens. This insight may be gained, in part, through a combination of high-resolution axial microscopy, such as cryo-electron microscopy (EM) in addition to studying the physicochemical interactions between lubricin, galectins and other synovial fluid constituents. Lubricin truncation mutants can be used to determine exactly how lubricin is oriented on and adsorbing to the surface of articular cartilage.

Although galectin-1 expression appears to protect against the development of antigen-induced arthritis in experimental rodent models, it is not understood how manipulation of galectins may be leveraged to treat arthritis. Specific small-molecule inhibitors of galectin-1 and galectin-3 are commercially available, and recombinant galectins can be produced for intra-articular administration in normal joints and in joints following induction of OA. These types of studies will provide the much-needed mechanistic insight about how galectins are involved in the development and progression of arthritis, including the role of galectins in cell adhesion, invasion and boundary lubrication.

MSCs might be leveraged as expression vectors for intra-articular galectin administration and; conversely, galectins may increase the efficacy of MSC-based therapies for cartilage repair by improving MSC adhesion, survival and engraftment within articular cartilage. MSCs are known to express high levels of galectins and, specifically, ratios of galectin-1:galectin-3; however, it is unknown how these expression profiles are maintained after intra-articular administration and whether there is any additional benefit to overexpression of galectins beyond endogenous expression levels. Studies to determine whether galectin gene expression is autoinductive and whether galectin overexpression interferes with IL-1β, TNF-α and other key catabolic cytokines that precipitate OA are still needed. Galectins might also be utilized as lectins for chondrocyte or progenitor cell adhesion to tissue-engineered scaffolds for cartilage repair with potential benefits in preventing chondrocyte apoptosis. Specific tissue engineering and regenerative medicine based applications of galectins remain to be investigated.

Finally, bringing together knowledge about lubricin glycosylation and galectin binding may enable us to optimize glycoengineered lubricin therapy or combined lubricin and galectin therapies for the treatment of OA. This information may be applied in the rational design of lubricin biomimetics with glycan functionality or in the production of recombinant lubricin with well-defined glycoforms. Lubricin and galectins both hold promise as potential therapeutic agents for the prevention and treatment of OA; however, additional studies are needed to precisely define the function of both lubricin and galectins in health and disease.

References

1. Flannery CR, et al. (2009) Prevention of cartilage degeneration in a rat model of osteoarthritis by intraarticular treatment with recombinant lubricin. *Arthritis Rheum* 60(3):840–7.

2. Coles JM, et al. (2010) Loss of cartilage structure, stiffness, and frictional properties in mice lacking PRG4. *Arthritis Rheum* 62(6):1666–74.

3. Teeple E, et al. (2011) The Effects of Supplemental Intra-Articular Lubricin and Hyaluronic Acid on the Progression of Post-Traumatic Arthritis in the Anterior Cruciate Ligament Deficient Rat Knee. *Am J Sports Med* 39(1):164–172.

4. Ruan MZC, et al. (2013) Proteoglycan 4 expression protects against the development of osteoarthritis. *Sci Transl Med* 5(176):176ra34.

5. Chang DP, Guilak F, Jay GD, Zauscher S (2014) Interaction of lubricin with type II collagen surfaces: adsorption, friction, and normal forces. *J Biomech* 47(3):659–66.

6. Majd SE, et al. (2014) Both hyaluronan and collagen type II keep proteoglycan 4 (lubricin) at the cartilage surface in a condition that provides low friction during boundary lubrication. *Langmuir* 30(48):14566–72.

7. Andresen Eguiluz RC, et al. (2015) Fibronectin mediates enhanced wear protection of lubricin during shear. *Biomacromolecules*. doi:10.1021/acs.biomac.5b00810.

8. Liu GY, et al. (2013) Secreted galectin-3 as a possible biomarker for the immunomodulatory potential of human umbilical cord mesenchymal stromal cells. *Cytotherapy* 15(10):1208–1217.

9. Sioud M, Mobergslien A, Boudabous A, Fløisand Y (2011) Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. *Int J Oncol* 38(2):385–90.

10. Di Lella S, et al. (2011) When galectins recognize glycans: from biochemistry to physiology and back again. *Biochemistry* 50(37):7842–57.